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The Role of Matrix Proteins in Eggshell Formation

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In this article we review the results of recent proteomic, transcriptomic and genomic analyses of the eggshell constituents and draw attention to the impact of this data on current understanding of eggshell mineralization. The most abundant constituents of the chicken eggshell have been identified. An exciting new approach is to determine which genes are upregulated during the onset of mineralization. New information from studies with purified native or recombinant eggshell proteins are necessary for *in vitro* tests to gain insight into the role of each isolated matrix component, and eventually to learn how they may function synergistically. One important goal will be to determine the impact and importance of post-translational modification of matrix components (glycosylation, glycanation, phosphorylation, etc.), which could greatly alter their properties and interactions. These investigations will continue to provide new insights into function of integrated defense strategies that operate at biomineralized barriers. Genes involved in the physical or chemical defense of the egg are functional candidates for marker assisted selection to improve egg and eggshell quality.

Key words: calcite, eggshell, osteopontin, Ovocalyxin, Ovocleidin

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1. Introduction

The avian egg is a reproductive structure that protects the embryo from external physical and microbial attacks; at the same time this complex structure regulates the exchange of metabolic gases and water, and provides calcium to the developing embryo. The unfertilized egg is a nutritious foodstuff for human consumption and shell quality is of paramount importance. Alterations in eggshell properties are directly related to increasing risk of egg contamination and food-borne outbreaks for the consumer. Many studies have been conducted on avian eggs and most have centered on the egg of the domestic chicken (*Gallus gallus*). This considerable body of work has provided insight into the structure and formation of the eggshell (Hincke *et al.,* 2008a). In this article we review the results of recent proteomic, transcriptomic and genomic analyses of the eggshell constituents and draw attention to the impact of this data on current understanding of eggshell mineralization.

2. Eggshell Biosynthesis/Formation

Calcified matrices in vertebrate biology are biphasic composites that usually contain collagenous and non-collagenous elements in intimate contact with mineral (Robey, 1996). While the avian eggshell is a complex and highly structured calcitic bioceramic with extensive intermingling of both its organic and inorganic phases, it also demonstrates a spatial separation between its organic framework and mineralized components, with a modest overlap between the eggshell membrane and calcified eggshell (Arias *et al.,* 1993; Dennis *et al.,* 1996; Nys *et al.,* 1999; Nys *et al.,* 2004) (Fig. 1). During avian egg formation, it sequentially acquires all of its components as it passes through specialized regions of the oviduct. The egg is composed of a central yolk surrounded by the albumen, eggshell membranes, calcified eggshell and cuticle (Roberts, 2004).

Following ovulation, the yolk is captured by the infundibulum where the developing egg remains for about 15 minutes while the perivitelline membrane is formed. The yolk is the main source of energy and fat soluble vitamins for the developing embryo because it contains all lipidic components of the egg (Burley and Vadehra, 1989). During the following 3–4 hour period, in which the yolk/ovum complex travels down the largest portion of the oviduct, the magnum, it progressively acquires the albumen (Nys *et al.,* 1999, 2004). The albumen is composed of water, salts and proteins that nourish the developing embryo and prevent the growth of micro-organisms. The alkaline pH of the albumen and the presence of proteins such as ovotransferrin and lysozyme significantly reduce the growth of micro-organisms

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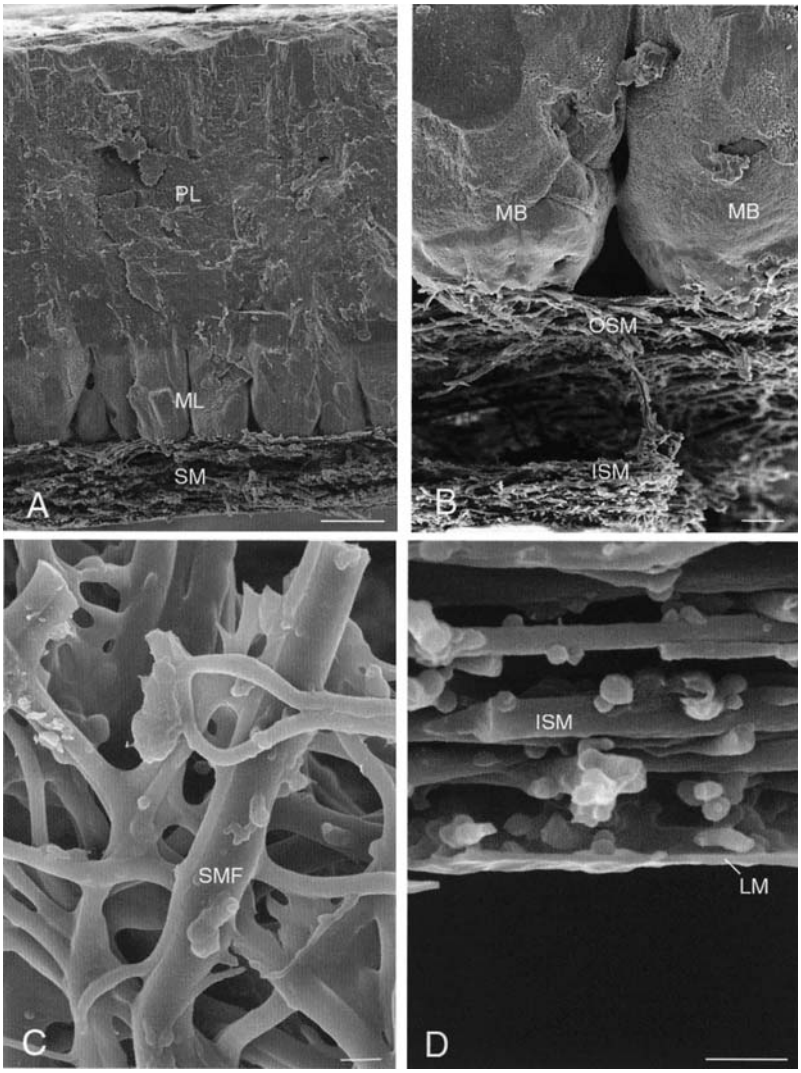

Fig. 1. **Scanning electron micrographs illustrating the morphology of the eggshell and eggshell membranes.**
A. Eggshell cross-fractured to reveal the shell membrane (SM), mammillary layer (ML) and palisade layer (PL); B. Higher magnification of the membrane - mammillary body interface. Outer shell membrane fibres (OSM) insert into the tips of the mammillary bodies (MB). Inner shell membranes (ISM); C. Enlargement of the shell membrane fibres (SMF) to reveal their interwoven and coalescing nature; D. Inner aspect of the inner shell membrane (ISM), demonstrating the limiting membrane (LM) that surrounds the egg white (here removed during sample preparation).
Scale bars: A, 50 mm; B, 20 mm; C and D, 2 mm.
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(Deeming, 2002). The albumen also stabilizes the developing embryo within the fertilized egg. The combination of albumen and yolk provide a perfect balance of the nutrients needed for embryonic growth; the egg possesses very high nutritional value as a human foodstuff because of its diverse and readily available components (Suess-Baum, 2007).

As the yolk and albumen complex next travel through the proximal (white) isthmus, the membranes are acquired in a 1 to 2 hour period. This meshwork of in-

terwoven fibres is considered to be the innermost component of the eggshell, and is organized into morphologically distinct inner and outer layers. The inner membranes remain uncalcified, while the fibers of the outer shell membrane penetrate the mammillary cones of the calcified shell (Arias *et al.,* 1993; Nys *et al.,* 2004) (Fig. 1A, B). The membranes are composed of 10% collagens (types I, V and X) and 70–75% of other proteins and glycoproteins containing lysine derived cross-links (Harris *et al.,* 1980; Wong *et al.,* 1984; Arias *et al.,* 1991; Fernandez *et al.,* 1997). Eggshell mineralization is subsequently initiated in the distal (red) isthmus (tubular shell gland) by calcification at distinct nucleation sites on the surface of the outer eggshell membrane; organic aggregates are deposited on the surface of the outer eggshell membranes in a quasi-periodic array, where calcium carbonate begins to aggregate, and are the origin of the mammillary knobs (Nys *et al.,* 2004). The mechanisms that prevent calcification towards the inner membranes and albumen are not well understood; one proposal is that collagen type X prevents a generalized calcification of the shell membrane (Arias *et al.,* 1997). Any modification of the eggshell membranes due to inhibition of fiber formation or crosslinking alters eggshell formation and its mechanical properties. For example, inhibition of the lysine-derived cross-linking of eggshell membrane by aminopropionitrile or by a copper deficiency (Chowdhury, 1990) affects the pattern of eggshell structure and degrades its mechanical properties.

The incomplete egg then enters the uterus (shell gland pouch), where fluid is pumped into the albumen, causing it to swell to its final size at oviposition. During the 16–17 hour period of calcification, rapid calcium carbonate deposition continues outward to give rise to the inner mammillary body (cone) layer and outer palisade (calcitic) layers (Nys *et al.,* 1999, 2004). Mineralization occurs in the uterine fluid, an acellular milieu containing ionized calcium and bicarbonate greatly in excess of the solubility product for calcite (Nys *et al.,* 1991). Thus, eggshell formation occurs in an acellular milieu, in contrast to other mineralized tissues, and its particular mineral structure results from self organization of mineral and organic precursors that are secreted into the milieu bathing the eggshell during its deposition. The ionic and organic constituents of the uterine fluid change progressively during eggshell formation and can be subdivided into the successive stages of initiation (5 hr), growth (12 hr) and termination (1.5 hr) of eggshell mineralization. The calcified eggshell consists primarily of calcite, the most stable polymorph of calcium carbonate and is progressively composed of the inner mammillary cone layer, central palisades and the outer vertical crystal layers (Nys *et al.,* 1999, 2004). The mammillary layer is a regular array of cones or knobs, with highly organic cores, into which are embedded the individual fibres of the outer eggshell membrane. Within the mammillary cone layer, microcrystals of calcite are arranged with spherulitic texture which facilitates the propagation of cracks during piping as well as the mobilization of calcium to nourish the embryo by dissolution of highly reactive calcite microcrystals (Nys *et al.,* 2004). The palisade layer is made up of groups of columns that are perpendicular to the eggshell surface and extend outwards from the mammillary cones (Fig. 1). This layer ends at the vertical single crystal layer which has a crystalline structure of higher density than that of the palisade region. The outer region of the palisade layer is a tough structure made of large crystals where the external impacts are absorbed by thin inter-crystalline organic layers that make intracrystalline crack propagation difficult (Nys *et al.,* 2004). Pores that traverse the eggshell permit the diffusion of metabolic gases and water vapor.

The outermost layer is the eggshell cuticle, a relatively thin, noncalcified layer which is deposited on the mineral surface. It is of variable thickness and may even be missing, and is composed of glycoprotein, polysaccharides, lipids and inorganic phosphorus including hydroxyapatite crystals (Dennis *et al.,* 1996; Whittow, 2000; Fernandez *et al.,* 2001). This layer, as well as the outer portion of the calcified shell, contains the eggshell pigments responsible for shell color.

3. Eggshell Mineralization

The avian eggshell is one of the fastest calcifying processes known in biology; 6 g of mineral is deposited in less than 20 hr during the daily production cycle of the chicken egg. Calcium transport mechanisms that underlie this process have been studied extensively (Nys, 1993; Bar, 2009). The mineralized shell is about 96% calcium carbonate; the remaining components include the organic matrix (2%), of which approximately half can be readily solubilized after decalcification. The native and soluble precursors of the eggshell matrix are present in the uterine fluid, from which they become incorporated into the calcifying shell. 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 nucleation site occurs initially in all direction but, due to 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. This anisotropy results from inhibition of crystal growth on the faces parallel to the C axis resulting in an elongation of the calcite crystal. This inhibition is likely to result from some organic components that are present in the uterine fluid, and then integrated into the eggshell. These are termed "matrix proteins", and are released by demineralization of the shell. These components are suspected to influence the texture of the eggshell by controlling the size, shape and orientation of this polycrystalline structure and therefore the mechanical properties of this material.

One of the first observations in favor of a role of the

matrix proteins in control of egg calcification was that the protein composition of uterine fluid varies during the initial, calcification and terminal phases of eggshell deposition (Gautron *et al.,* 1997). A large number of eggshell proteins have been identified by recent proteomic approaches (>500) (Mann *et al.,* 2006; Miksik *et al.,* 2007). The most abundant of these had already been identified by classic approaches: *Eggshell-specific proteins* such as the Ovocleidins and Ovocalyxins (Hincke *et al.,* 1995, 1999; Gautron *et al.,* 2001a, 2007); *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 (Hincke, 1995; Hincke *et al.,* 2000; Gautron *et al.,* 2001b). Lastly, *osteopontin,* which seems to be an invariant feature of biological calcification in birds and mammals, is also an eggshell matrix protein (Pines *et al.,* 1994; Lavelin *et al.,* 1998; Hincke and St. Maurice, 2000; Fernandez *et al.,* 2003; Chien *et al.,* 2008). Sequential incorporation of matrix proteins into the calcifying eggshell results in their differential localization between the inner (mammillary) and outer (palisade) layers of the mineralized shell (Hincke *et al.,* 1992). We hypothesize that their specific localization pattern provides functional insight into the role of such proteins during eggshell formation.

In general, the soluble matrix proteins of calcitic biomaterials modify crystal growth, and therefore regulate the macroscopic properties of the resulting bioceramic. For example, in the mollusk shell, specific proteins control phase switching between the calcite and aragonite forms of calcium carbonate (Falini *et al.,* 1996; Belcher *et al.,* 1996). A number of experimental observations support the role of the eggshell matrix proteins in determining the fabric of the eggshell and therefore influencing its resulting 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 a distinct protein electrophoretic profile at each phase of shell mineralization, suggesting specific roles for the organic contents during the calcification process (Gautron *et al.,* 1997). The nature of the interactions between the mineral phase and the eggshell matrix proteins has been intensely investigated. The presence of calcium binding proteins has been reported in eggshell extracts and in uterine fluid (Abatangelo *et al.,* 1978; Cortivo *et al.,* 1982; Hincke *et al.,* 1992; Gautron *et al.,* 1997). Whole uterine fluid modifies calcium carbonate precipitation kinetics, and alters the size and the morphology of calcite crystals grown *in vitro* (Dominguez-Vera *et al.,* 2000; Hernandez-Hernandez *et al.,* 2008). 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 (Gautron *et al.,* 1996). In the presence of uterine fluid, all calcium carbonate crystals formed *in vitro* were found to be calcite, demonstrating that this milieu promotes the calcitic polymorph found in the shell (Gautron *et al.,* 2005).

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 rhombohedric calcite crystals grown *in vitro* (reviewed in Nys *et al.,* 1999; 2004; Hernandez-Hernandez *et al.,* 2008). Low concentrations of goose eggshell ansocalcin (up to $10\mu g/ml$) induce calcite crystals with screw dislocations, while at higher concentrations ($>50\mu g/ml$), polycrystalline calcite aggregates are nucleated. These results are different from those obtained with calcite crystal growth in the presence of purified chicken Ovocleidin-17, where at low levels ($10–100\mu g/ml$) calcite crystals were twinned and less-aggregated (Lakshminarayanan *et al.,* 2005). On the other hand, other researchers found a different concentration-dependent aggregation of calcite crystals grown in the presence of Ovocleidin-17 ($50–200\mu g/ml$) (Reyes-Grajeda *et al.,* 2004). Differing results for Ovocleidin-17 may reflect differences in the experimental conditions of crystal growth. Ovotransferrin ($0.5\,mg/ml$) leads to smaller crystals and promotes the development of elongated crystals (Gautron *et al.,* 2001b). Lysozyme at high concentration ($>10\,mg/ml$) mainly affects the calcite faces parallel to the c axis, by inhibition of growth on {110} faces (Hincke *et al.,* 2000; Jimenez-Lopez *et al.,* 2003).

Finally, pure glycoaminoglycans also affect calcite morphology leading to crystal elongation (Arias *et al.,* 2002). Therefore, highly sulfated proteoglycans are likely to influence mineralization by electrostatic interactions. Protein phosphorylation is another post-translational modification that may be crucial, since 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 (Hincke and St. Maurice, 2000). Mann *et al.* (2007) demonstrated that among the major phosphorylated eggshell matrix proteins are osteopontin, Ovocleidin-17, Ovocleidin-116 and Ovocalyxin-32.

The results of *in vitro* experiments are supported by *in vivo* observations. Eggshell levels of certain matrix proteins are observed to correlate with variations in eggshell mechanical properties under specific conditions. The well-known improvement in shell quality (breaking strength) after moulting is correlated with increased levels of OC-17 and OC-116, and a relative decrease in ovalbumin, ovotransferrin and lysozyme in the eggshell matrix. This was correlated with a decrease in calcite grain size, which could be responsible for the improved mechanical properties (Rodriquez-Navarro *et al.,* 2002; Ahmed *et al.,* 2005).

In summary, matrix components play an active role in the control of calcite growth kinetics and crystal morphology during eggshell mineralization, and therefore regulate the textural properties and resulting biomechanical strength of the eggshell.

4. Eggshell Matrix Proteins

4.1 Proteomics

The eggshell mineral is associated with an organic matrix composed of proteins, glycoproteins and proteoglycans, termed "eggshell matrix proteins", which are progressively incorporated from the precursor milieu (uterine fluid) during calcification. Their function is thought to influence the fabric of this biomaterial and/or to participate in its antimicrobial defenses. These non-mineral constituents represent about 2% by weight of the calcified eggshell, and can be released for study by demineralization of the eggshell by calcium chelation (EDTA or EGTA) or acid demineralization (acetic acid or HCl), yielding soluble and insoluble constituents. A complex array of distinct protein bands was demonstrated in the soluble intra- and extra-mineral compartments by 1 D-electrophoresis (SDS-PAGE) (Hincke *et al.,* 1992; Gautron *et al.,* 1996), and in the precursor uterine fluid, showing different patterns between the 3 stages of the eggshell calcification process (initial, growth and terminal) (Gautron *et al.,* 1997). N-terminal sequencing of the electrophoretic bands allowed the egg white proteins ovalbumin, lysozyme and ovotransferrin to be identified (Hincke, 1995; Hincke *et al.,* 2000; Gautron *et al.,* 2001 b). N-terminal and internal amino acid sequencing of other protein bands revealed that they did not correspond to previously identified proteins and these have been subjected to more intensive investigation. Purification schemes using ion exchange (diethylaminoethyl (DEAE) - Sepharose and carboxymethyl (CM) - Sepharose) and hydroxyapatite were developed to isolate Ovocleidin-17 (Hincke *et al.,* 1995) and Ovocalyxin-32 (Hincke *et al.,* 2003) from eggshell extracts.

Ovocleidin-116 (OC-116) and Ovocalyxin-36 (OCX-36) were characterized by a combination of molecular cloning, immunochemistry and bioinformatics (Hincke *et al.,* 1999; Gautron *et al.,* 2007). Another associated approach was to compare the available Expression Sequence Tag (EST) sequences to partial protein or nucleotide sequences from egg components. This method was successfully used to characterize a 32 kDa band abundant in uterine fluid at the terminal phase of shell calcification (Ovocalyxin-32) (Gautron *et al.,* 2001a). Such studies led to the concept that eggshell matrix protein components form three characteristic groups:

i) "Egg white" proteins which are also present in the eggshell - these include ovalbumin, the most abundant egg white protein (Hincke, 1995), lysozyme, an antimicrobial protein with hydrolytic activity against peptidoglycans on cell walls of Gram-positive bacteria (Hincke *et al.,* 2000) and ovotransferrin, which sequesters iron necessary for bacterial growth (Gautron *et al.,* 2001b);

ii) Ubiquitous proteins that are found in many tissues - examples are osteopontin, a phosphorylated glycoprotein present in bone and other hard tissues of birds and mammals (Pines *et al.,* 1994; Hincke and St. Maurice, 2000; Fernandez *et al.,* 2003; Hincke *et al.,* 2008 b; Chien *et al.,* 2008, 2009), and clusterin, a widely distributed secretory glycoprotein that is also found in chicken egg white (Mann *et al.,* 2003); and

iii) Eggshell-specific matrix proteins unique to the shell calcification process that are secreted by cells in specific regions of the oviduct where eggshell mineralization is initiated (red isthmus) and continues to completion (uterus). These matrix components are 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), with distinction based on apparent molecular weight by SDS-PAGE when initially characterized.

Recently, a high-throughput tandem-mass spectrometry approach (MS/MS) identified more than 500 eggshell matrix proteins (Mann *et al.,* 2006), including the most abundant proteins that were already known (above). It is highly unlikely that all 520 proteins perform eggshell-specific functions or are involved in eggshell assembly. The majority of them are proposed to be remnants of previous stages of egg formation occurring in proximal segments of the oviduct, or intracellular proteins released by breakdown of the cells lining the oviduct during normal turnover (Mann *et al.,* 2006). According to this hypothesis, all proteins that are present in the uterine fluid during the calcification process become assimilated into the eggshell, many of them in a non-specific manner.

Two possible roles for eggshell-specific matrix proteins have been proposed; both reflect the protective function of the eggshell in avian reproduction: regulation of eggshell mineralization and antimicrobial defence. Egg calcification occurs in three distinct phases (initiation, active calcification, and termination of shell calcification); each phase of shell mineralization is associated with a specific protein electrophoretic profile for the uterine fluid, suggesting that these molecules play specific roles during the calcification process (Gautron *et al.,* 1997). The matrix proteins described in the next section are abundant components of the eggshell matrix and exhibit characteristics that are relevant to eggshell mineralization.

Ovocleidin-17 (OC-17) was the first eggshell-specific matrix protein to be isolated and characterized following its chromatographic purification after eggshell decalcification (Hincke *et al.,* 1995). OC-17 is an abundant eggshell-matrix specific protein (40 μg/g shell) (Mann *et al.,* 2002). It is secreted by the tubular gland cells in the shell gland; within the shell it is distributed throughout the shell matrix, but concentrated in the mammillary bodies (Hincke *et al.,* 1995). OC-17 is phosphorylated on two possible sites, Ser-61 and -67 (Mann and Siedler, 1999), and also can be glycosylated at Asn-59 to yield a 23 kDa

form: (Ovocleidin-23) (Mann, 1999). Glycosylation occurs at the N-glycosylation site consensus sequence, Asn-Ala-Ser which contains Ser-61. In the glycosylated form of the protein, Ser-61 is not phosphorylated, indicating that these modifications are mutually exclusive. In addition, peptides without modification at Asn-59 or Ser-61, with phosphorylation of Ser-67 only, or with no phosphorylation at all, have been detected (Mann *et al.,* 2007). 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.

Detailed studies have identified homologous eggshell matrix proteins in shell from other avian species. Comparison of their primary sequences revealed that ansocalcin (goose), struthiocalcin-1 & -2 (SCA-1 & -2, ostrich), dromaiocalcin-1 & -2 (DCA-1 & -2, emu) and rheacalcin-1 &-2 (RCA-1 & -2, rhea) form two groups based on sequence identity, serine phosphorylation and conservation of cysteine residues (Mann and Siedler, 2004; Lakshminarayanan *et al.,* 2003; Mann and Siedler, 2006). Goose ansocalcin aligns reasonably well with proteins of group 1 (63–70% identity with SCA-1, DCA-1, RCA-1), but OC-17 has much less sequence identity with group 2 where it is placed (37–39% with SCA-2, DCA-2, RCA-2). It can be proposed that homologous proteins are found in the shells of all other bird species. Database searches with these eggshell protein sequences reveal that they belong to a heterogeneous group of proteins consisting of a single C-type lectin domain (CTL) and display sequence homology to members of this family such as mammalian Reg (Regenerating islet-derived) proteins, pancreatic stone protein (lithostathine), fish Type II antifreeze proteins and anticoagulant proteins from snake venom (Zelensky and Gready, 2005). The x-ray structure of OC-17 has been determined; it reveals a mixed alpha helix/beta sheet structure and verifies the C-type lectin-like domain (Reyes-Grajeda *et al.,* 2002, 2004). Preliminary x-ray crystallographic studies of struthiocalcin-1 have been reported (Reyes-Grajeda *et al.,* 2007).

The properties of purified OC-17 and its goose homolog (ansocalcin), and their influence upon calcite crystallization patterns have been investigated and compared (Lakshminarayanan *et al.,* 2002, 2003, 2005; Reyes-Grajeda *et al.,* 2004). Functionally, OC-17 and ansocalcin do not appear to be completely equivalent in their effect on calcite crystal growth *in vitro* (Lakshminarayanan *et al.,* 2002; Reyes-Grajeda *et al.,* 2004). Ansocalcin showed reversible concentration-dependent aggregation in solution, and was reported to induce pits on growing calcite rhombohedral faces at lower concentrations ($<50\mu g/ml$) and to nucleate polycrystalline aggregates of calcite crystals at higher concentrations (Lakshminarayanan *et al.,* 2003). Aggregated ansocalcin may act as a template for the nucleation of calcite crystal aggregates (Lakshminarayanan *et al.,* 2002). However, under the same conditions, OC-17 was not observed to aggregate in solution nor induce the nucleation of calcite aggregates. Nevertheless, under different experimental conditions, Reyes-Grajeda and coworkers reported that OC-17 could modify the crystalline habit of calcium carbonate and the pattern of crystal growth at concentrations of $5–200\mu g/ml$ (Reyes-Grajeda *et al.,* 2004). Ovocleidin-17 and SCA-1, but not SCA-2, are reported to interact directly with carbonate anion, as a potential mechanism accounting for different effects upon calcite nucleation and crystal growth (Marin-Garcia *et al.,* 2008).

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 (Hincke *et al.,* 1999). OC-116 is the most abundant eggshell matrix protein, estimated at $80\mu g/g$ eggshell powder (Mann *et al.,* 2002). It is relatively eggshell specific; however, it is also present in young chick cortical bone, laying hen medullary bone and growth plate hypertrophic chondrocytes suggesting an additional role in bone mineralization (Horvat-Gordon *et al.,* 2008). 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 (Carrino *et al.,* 1997). Therefore, OC-116 is the core protein (predicted 75 kDa) corresponding to the doublet bands of an eggshell dermatan sulfate proteoglycan (116 kDa and 180 kDa). It is hypothesized that the 180 kDa form of OC-116 corresponds to the N-glycosylated core protein with attached glycoaminoglycans, while the 116 kDa form corresponds to the protein without glycoaminoglycans (Hincke *et al.,* 1999). 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 (Mann *et al.,* 2002). Detailed analysis of the carbohydrate structures attached to Asn-62 revealed 17 different oligosaccharide structures (Nimtz *et al.,* 2004); on the other hand, glycoaminoglycans associated with OC-116 have not yet been characterized. 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 (Mann *et al.,* 2007).

Ultrastructural immunocytochemistry indicates that OC-116 is synthesized and secreted from the granular cells of the uterine epithelium, and is incorporated into, and widely distributed throughout, the palisade region of the calcified eggshell (Hincke *et al.,* 1999). 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 corre-

spond to the walls of microvesicular holes (voids) in the calcitic eggshell (Hincke *et al.,* 1999). Such localization studies do not distinguish between the differentially phosphorylated, N-glycosylated or glycanated forms of OC-116, nor would possible differences in eggshell distribution between the 116 and 180 kDa forms be detected by this technique. Crystal growth studies have shown that pure glycoaminoglycans affect calcite morphology, leading to crystal elongation (Arias *et al.,* 2002), suggesting that the sulfated form of OC-116 could influence eggshell mineralization via electrostatic interactions. Single nucleotide polymorphisms (SNPs) in the OC-116 gene are significantly associated with eggshell elastic modulus and thickness and egg shape (Dunn *et al.,* 2008).

Ovocalyxin-32 (OCX-32) was originally identified as a 32 kDa uterine fluid protein that is abundant in the terminal phase of shell formation (Gautron *et al.,* 2001a; Hincke *et al.,* 2003). Sequencing of peptides derived from the purified protein allowed Expressed Sequence Tag sequences (EST's) to be identified that were assembled to yield a full-length composite sequence whose conceptual translation product contained the complete amino acid sequence of Ovocalyxin-32. Ovocalyxin-32 is expressed at high levels in the uterine and isthmus regions of the oviduct and is secreted by the surface epithelial cells that line the lumen (Gautron *et al.,* 2001a). 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 (Gautron *et al.,* 2001a; Hincke *et al.,* 2003; Miksik *et al.,* 2007). A study of eggshell phosphoproteins identified phosphorylation of Ovocalyxin-32 at serines and threonines between position 257 and 268, but exact sites were not determined (Mann *et al.,* 2007).

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 (Gautron *et al.,* 1997). This hypothesis originated from the observations of morphological changes in calcite crystals by uterine fluid collected during the terminal phase of calcification and the location of OCX-32 in the mineral pellet after its precipitation with calcium carbonate *in vitro* from fresh uterine fluid (Dominquez-Vera *et al.,* 2000; Hernandez-Hernandez *et al.,* 2008).

Ovocalyxin-36 (OCX-36) is a prominent 36 kDa protein present in the uterine fluid collected during the active calcification stage of shell mineralization. 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 (Gautron *et al.,* 2007). OCX-36 localizes to the calcified eggshell (predominantly in the inner part of the shell), and is abundant in the shell membranes. OCX-36 protein sequence displays significant identity with mammalian proteins that are associated with the innate immune response, such as lipopolysaccharide-binding proteins (LBP), bactericidal permeability-increasing proteins (BPI) and palate, lung and nasal epithelium clone (Plunc) family proteins. These belong to a superfamily of proteins that are key components of the innate immune system and act as the first line of host defense (Bingle and Craven, 2004). LBP proteins initiate the inflammatory host response upon the detection of a pathogen (Shumann *et al.,* 1990). OCX-36 may therefore participate in natural defense mechanisms that keep the egg and oviduct free of pathogens.

Osteopontin (OPN) is a phosphoglycoprotein associated with normal and pathological calcium mineralization (McKee and Nanci, 1996). In the chicken, osteopontin is found in both bone and eggshell (Pines *et al.,* 1994; Hincke *et al.,* 2008b). The oviduct expression of osteopontin is entirely uterine-specific and is temporally associated with eggshell calcification through coupling of physical distension of the uterus to osteopontin gene expression (Lavelin *et al.,* 1998).

Localization studies show that OPN is concentrated in the palisades region of the eggshell (Fernandez *et al.,* 2003, Hincke *et al.,* 2008b; Chien *et al.,* 2009). Osteopontin exists as two to three predominant forms in both eggshell and bone, with an apparent size by SDS-PAGE ranging between 46 and 54 kDa (Hincke *et al.,* 2008b), indicating that bone and eggshell OPN differ in their posttranslational modifications. A number of phosphorylated residues in chicken eggshell and osteoblast OPN have been identified, which partially overlap between the two tissues (Salih *et al.,* 1997; Mann *et al.,* 2007). Dephosphorylation of eggshell OPN greatly diminishes its ability to inhibit precipitation of calcium carbonate from a supersaturated solution (Hincke and St. Maurice, 2000). Osteopontin is synthesized and secreted by the granular epithelial cells of the shell gland (Pines *et al.,* 1994; Fernandez *et al.,* 2003; Hincke *et al.,* 2008b). The granular cells also synthesize and secrete ovocleidin-116 (Hincke *et al.,* 1999), suggesting that this epithelial cell type plays a major role in secretion of the eggshell matrix.

After decalcification and processing of the eggshell for TEM and Scanning electron microscopy (SEM), 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 fibers. OPN is associated with protein sheets in the highly mineralized palisades region, but not with the vesicular structures (Hincke *et al.,* 2008b; Chien *et al.,* 2008; 2009). The association of OPN with parallel sheets of matrix, and more diffusely with the {104} crystallographic faces of eggshell calcite, may function in regulating palisade growth by orienting calcite crystals and by regulating the speed of mineralization. The elongated calcite crystals in the palisades region 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 (Silyn-Roberts and Sharp, 1986; Rodriguez-Navarro *et al.,* 2002). The {104} calcite face is the natural cleavage plane, and specific osteopontin 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 (Chien *et al.,* 2008).

Unusual patterns of uterine OPN expression may underlie certain defects in eggshell mineralization. In birds laying eggs with normal eggshells, OPN is expressed uniformly by all the epithelial cells facing the uterine lumen (Arazi *et al.,* 2009). Decreased OPN gene expression is correlated with reduction in eggshell thickness that is observed after xenoestrogen treatment (Kamata *et al.,* 2009). Moreover, xenoestrogen treatment resulted in thinning of the mammillary layer at the same site where OPN is localized (Hincke *et al.,* 2008b; Kamata *et al.,* 2009). Reduced or absent OPN expression in specific regions of the uterine luminal epithelium is correlated with eggshell defects such as corrugations, pimples and cracks (Arazi *et al.,* 2009). A candidate gene association analysis with eggshell matrix genes recently revealed that OPN SNP's were associated with eggshell fracture toughness (Dunn *et al.,* 2008).

4. 2 Transcriptomics

There are few reports of global gene expression in chickens. Oviduct gene expression has been compared in mature and juvenile birds, in which 266 over-expressed genes were related to dramatic changes due to sexual maturity and the onset of egg production (Dunn *et al.,* 2009). Included in this list of differentially expressed genes are a number with known involvement in shell gland function, such as ion transporters and shell matrix proteins (OC-116, OCX-36, OCX-32 and OCX-21). Another recent study has focused on the uterus (shell gland) during deposition of the eggshell to identify genes that are differentially expressed in the uterus during eggshell calcification (Jonchere *et al.,* 2010). In this study gene expression in the uterus where the eggshell is formed was compared with two other segments of the oviduct (magnum, white isthmus) to detect genes encoding proteins involved in supplying mineral and organic precursors that participate in eggshell formation. Using this unique approach, differential expression of genes reveals specific functions of each specialized region that secrete egg components. More than 600 differentially expressed uterine transcripts were detected, corresponding to over 460 different genes. Gene Ontology (GO) analysis was used for interpretation of protein function. The most highly represented GO terms were related to genes encoding ion transport proteins, which provide eggshell mineral precursors (Nys *et al.,* 1999; Bar, 2009).

Uterine expression of 52 previously characterized eggshell proteins was confirmed, as well as transcripts for several new proteins not yet characterized in the eggshell. This limited number is partly due to the fact that some eggshell proteins are also expressed in other tissues along the oviduct. Consequently, these proteins are present in the eggshell, but not revealed by analysis of transcriptional data. Proteomic analysis allows minute amounts of biologically active proteins in tissue or fluid to be identified; the eggshell proteome contains a complex mixture of uterine-derived proteins including proteins derived from degraded cells or basement membranes and those derived from the proximal oviduct (i.e., egg white, egg yolk and vitelline membrane proteins) (Mann *et al.,* 2006). The number of eggshell proteins identified by mass spectrometry (528 proteins) is 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) (D'Ambrosio *et al.,* 2008; Farinazzo *et al.,* 2009; Mann, 2007; Mann, 2008; Mann and Mann, 2008). Thus, it is likely that the eggshell also passively incorporates proteins from the proximal oviduct. Protein sequences derived from upregulated genes were examined for the presence of a signal peptide, in order to identify those that could be potentially secreted by uterine cells for deposition in the shell. Amongst the upregulated uterine transcripts, more than 50 corresponded to proteins with signal peptide sequences; these proteins were classified according to their proposed biological function in the eggshell. Proteins believed to be involved in the biomineralization of the shell, such as osteopontin and ovocleidin-116 (OC-116) were identified with this approach. The restricted uterine expression of other shell-specific proteins, ovocalyxin-36 (OCX-36) and ovocalyxin-21 (OCX-21), was also noted. OCX-21 contains a brichos domain and consequently, could play a role as molecular chaperone (Gautron and Nys, 2007). Upregulation was noted for transcripts encoding up to 10 calcium binding proteins, which could be deposited in the shell to interact with mineral during shell formation. In addition, a number of proteins involved in protein folding, which could play an important role in proper folding of eggshell matrix components, were also upregulated. Finally, this study also revealed upregulation of transcripts encoding proteins with antimicrobial properties that could play a role in the protection of the egg.

In summary, global gene expression profiling of the hen's oviduct during sexual maturation and eggshell formation have revealed a large number of differentially expressed genes (Dunn *et al.,* 2009; Jonchere *et al.,* 2010). Upregulation of genes coding for eggshell-specific matrix proteins (OC-116 and ovocalyxins) occurs during both of these physiologically distinct processes, while OPN expression is only upregulated during eggshell calcification. This approach complements earlier focused proteomic analysis of the eggshell (Mann *et al.,* 2006; 2007) 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 putative 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 a marker assisted selection to improve egg and eggshell quality. For example, the OCX-32 gene is expressed at higher levels in a low egg production strain (compared to a high production strain) of Taiwanese country chickens (Yang *et al.,* 2007).

4.3 Genomics

The release of the chicken genome sequence in 2004 was a tremendous advance permitting molecular biology - based approaches to avian biology, physiology, breeding etc. (International Chicken Genome Sequencing Consortium, 2004). Inspection revealed that the chromosomal localization of OC-116 is adjacent to that of osteopontin on chromosome 4. These 2 genes are contiguous with other mineralization-specific genes (Bone Sialoprotein, Dentin Matrix Protein 1), that form the SIBLING (small integrin-binding ligand, N-linked glycoprotein) mineralization gene locus first reported in mammalian genomes. This correspondence reflects synteny between avian and mammalian genomes (Hincke *et al.,* 2008a). Moreover, investigations into 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 (Kawasaki and Weiss, 2003; 2006). Based on its position within this gene locus, OC-116 is predicted to be the avian ortholog of mammalian MEPE (matrix extracellular phosphoglycoprotein), and its N-terminus displays about 30% identity with the *Homo sapiens* MEPE protein sequence (Hincke *et al.,* 2008a). As expected, there is synteny between the two avian genomes that have been sequenced to date: *Gallus gallus* and *Taeniopygia guttata* (Zebra Finch) (Stapley *et al.,* 2008). Further study of common features of different avian OC-116's should help to define its features that are important for mineralization.

Proteins originating from the SCPP genes have a common characteristic; they bind calcium ions via acidic amino acids such as Glu, Asp and phospho-Ser (Kawasaki and Weiss, 2003). One member, OPN, regulates calcification in vertebrate biominerals such as bone and teeth (McKee and Nanci, 1996). Eggshell OPN inhibits calcium carbonate precipitation (Hincke and St. Maurice, 2000), and interacts specifically with the {104} calcite crystal face that may regulate shell calcification (Chien *et al.,* 2008; Hincke *et al.,* 2008b). Another SIBLING member, Dentin Matrix Protein 1 (DMP1), is also an eggshell matrix protein (Mann *et al.,* 2006; Horvat-Gordon *et al.,* 2008) but its influence upon calcite crystallization is not yet known. Of these proteins, OC-116 is uniquely specialized as an avian member of this mineralization-specific family, supporting the hypothesis that it has a unique role in calcitic mineralization of the avian shell.

A complementary approach to establish the role of matrix proteins in the variability of the eggshells 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 and Ovocalyxins, OPN, ovalbumin) and measurements of eggshell biomechanical properties (Dunn *et al.,* 2008). Single nucleotide polymorphisms (SNPs) in the OC-116 gene are significantly associated with the 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. Conclusion/Perspectives

This article reviews the results of recent proteomic, transcriptomic and genomic analyses of the eggshell constituents and draws attention to the impact of this data on current understanding of eggshell mineralization. The majority of constituents of the chicken eggshell have been identified. An exciting new approach is to determine which genes are upregulated during the onset of mineralization. Future effort to compare and contrast the chicken eggshell matrix proteins with those of other avian eggshells will pay dividends to better determine the function of the eggshell matrix proteins in the eggshell. Two functional roles have been proposed: (i) regulation of eggshell mineralization and (ii) antimicrobial protection of the egg and its contents. New information from studies with purified native or recombinant eggshell proteins are necessary for *in vitro* tests to gain insight into the role of each isolated matrix component, and eventually to learn how they may function synergistically. One important goal will be to determine the impact and importance of posttranslational modification of matrix components (glycosylation, glycanation, phosphorylation, etc.), which could greatly alter their properties and interactions. These investigations will continue to provide new insights into function of integrated defense strategies that operate at biomineralized barriers.

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References

Abatangelo G, Daga-Gordini D, Castellani I and Cortivo R. Some observations on the calcium ion binding to the eggshell matrix. Calcified Tissue Research, 26: 247–252. 1978.

Ahmed AM, Rodriguez-Navarro AB, Vidal ML, Gautron J, Garcia-Ruiz JM and Nys Y. Changes in eggshell mechanical properties, crystallographic texture and in matrix proteins induced by moult in hens. British Poultry Science, 46: 268–279. 2005.

Arazi H, Yoselewitz I, Malka Y, Kelner Y, Genin O and Pines M. Osteopontin and calbindin gene expression in the eggshell gland as related to eggshell abnormalities. Poultry Science, 88: 647–53. 2009.

Arias JL, Fernandez MS, Dennis JE and Caplan AI. Collagens of the chicken eggshell membranes. Connective Tissue Research, 26: 37–45. 1991.

Arias JL, Fink DJ, Xiao SQ, Heuer AH and Caplan AI. Biomineralization and eggshells: cell-mediated acellular compartments of mineralized extracellular matrix. International Review of Cytology, 45: 217–250. 1993.

Arias JL, Jure C, Wiff JP, Fernandez MS, Fuenzalida V and Arias JL. Effect of sulfate content of biomacromolecules on the crystallization of calcium carbonate. Materials Research Society Symposium Proceedings, 711: 243–248. 2002.

Arias JL, Nakamura O, Fernández MS, Wu JJ, Knigge P, Eyre DR, Caplan AI. Role of type X collagen on experimental mineralization of eggshell membranes. Connective Tissue Research, 36: 21–33. 1997.

Bar, A. Differential regulation of calbindin in the calcium-transporting organs of birds with high calcium requirements. Japanese Poultry Science, 46: 267–285. 2009.

Belcher AM, Wu XH, Christensen RJ, Hansma PK, Stucky GD and Morse DE. Control of crystal phase-switching and orientation by soluble shell proteins. Nature, 381: 56–58. 1996.

Bingle CD and Craven CJ. Meet the relatives: a family of BPI- and LBP-related proteins. Trends in Immunology, 25: 53–55. 2004.

Burley RW and Vadehra DV. The Avian Egg. Chemistry and Biology. Wiley Interscience. New York. 1989.

Carrino DA, Rodriquez JP and Caplan AI. Dermatan sulfate proteoglycans from the mineralized matrix of the avian eggshell. Connective Tissue Research, 36: 175–193. 1997.

Chien YC, Hincke MT and McKee MD. Avian eggshell structure and osteopontin. Cells, Tissue, Organs, 189: 38–43. 2009.

Chien YC, Hincke MT, Vali H and McKee MD. Ultrastructural matrix-mineral relationships in avian eggshell, and effects of osteopontin on calcite growth *in vitro.* Journal of Structural Biology, 163: 84–99. 2008.

Chowdhury SD. Shell membrane system in relation to latyrogen and copper deficiency. World's Poultry Science Journal, 46: 153–169. 1990.

Cortivo R, Castellani I, Martelli M, Michelotto G and Abatangelo G. Chemical characterization of the hen eggshell matrix: isolation of an alkali-resistant peptide. Journal of Chromatography, 237: 127–135. 1982.

D'Ambrosio C, Arena S, Scaloni A, Guerrier L, Boschetti E, Mendieta ME, Citterio A and Righetti PG. Exploring the Chicken Egg White Proteome with Combinatorial Peptide Ligand Libraries. Journal of Proteome Research, 7: 3461–3474. 2008.

Deeming DC. Avian Incubation: Behavior, Environment, and Evolution. Oxford University Press. New York. 2002.

Dennis JE, Xiao S-Q, Agarwal M, Fink DJ, Heuer AH and Caplan AI. Microstructure of matrix and mineral components of eggshells from white leghorn chickens (*Gallus gallus*). Journal of Morphology, 228: 287–306. 1996.

Dominguez-Vera JM, Gautron J, Garcia-Ruiz J and Nys Y. The effect of avian uterine fluid on the growth behavior of calcite crystals. Poultry Science, 79: 901–907. 2000.

Dunn IC, Joseph NT, Bain M, Edmond A, Wilson PW, Milona P, Nys Y, Gautron J, Schmutz M, Preisinger R and Waddington D. Polymorphisms in eggshell organic matrix genes are associated with eggshell quality measurements in pedigree Rhode Island Red hens. Animal Genetics, 40: 110–114. 2008.

Dunn IC, Wilson PW, Lu Z, Bain MM, Crossan CL, Talbot RT and Waddington D. New hypotheses on the function of the avian shell gland derived from microarray analysis comparing tissue from juvenile and sexually mature hens. General and Comparative Endocrinology, 163: 225–232. 2009.

Falini G, Albeck S, Weiner S and Addadi L. Control of aragonite or calcite polymorphism by mollusk shell macromolecules. Science, 271: 67–69. 1996.

Farinazzo A, Restuccia U, Bachi A, Guerrier L, Fortis F, Boschetti E, Rasoli E, Citterio A and Righetti PG. Chicken egg yolk cytoplasmic proteome, mined via combinatorial peptide ligand libraries. Journal of Chromatography A, 1216: 1241–1252. 2009.

Fernandez MS, Araya M and Arias JL. Eggshells are shaped by a precise spatio-temporal arrangement of sequentially deposited macromolecules. Matrix Biology, 16: 13–20. 1997.

Fernandez MS, Escobar C, Lavelin I, Pines M and Arias JL. Localization of osteopontin in oviduct tissue and eggshell during different stages of the avian egg laying cycle. Journal of Structural Biology, 143: 171–180. 2003.

Fernandez MS, Moya A, Lopez L and Arias JL. Secretion pattern, ultrastructural localization and function of extracellular matrix molecules involved in eggshell formation. Matrix Biology, 19: 793–803. 2001.

Gautron J, Bain M, Solomon S and Nys Y. Soluble matrix of hen's eggshell extracts changes *in vitro* the rate of calcium carbonate precipitation and crystal morphology. British Poultry Science, 37: 853–866. 1996.

Gautron J, Hincke MT and Nys Y. Precursor matrix proteins in the uterine fluid change with stages of eggshell formation in hens. Connective Tissue Research, 36: 195–210. 1997.

Gautron J, Hincke MT, Panheleux M, Bain M, McKee MD, Solomon SE and Nys Y. Ovocalyxin-32, a novel chicken eggshell matrix protein. Isolation, amino acid sequencing, cloning, and immunocytochemical localization. Journal of Biological Chemistry, 276: 39243–39252. 2001a.

Gautron J, Hincke MT, Panheleux M, Garcia-Ruiz JM, Boldicke T and Nys Y. Ovotransferrin is a matrix protein of the hen eggshell membranes and basal calcified layer. Connective Tissue Research, 42: 255–267. 2001b.

Gautron J, Murayama E, Vignal A, Morisson M, McKee MD, Rehault S, Labas V, Belghazi M, Vidal ML, Nys Y and Hincke MT. Cloning of Ovocalyxin-36, a novel chicken eggshell protein related to lipopolysaccharide-binding proteins (LPB) bactericidal permeability-increasing proteins (BPI), and Plunc family proteins. Journal of Biological

Chemistry, 282: 5273–5286. 2007.
Gautron J and Nys Y. Eggshell matrix proteins. In: Bioactive egg compounds. (Huopalahti R, Lopez-Fandino R, Anton M and Schade R eds.) pp. 103–108. Springer-Verlag. 2007.
Gautron J, Rodriguez-Navarro AB, Gomez-Morales J, Hernandez-Hernandez MA, Dunn IC, Bain M, Garcia-Ruiz JM and Nys Y. Evidence for the implication of chicken eggshell matrix proteins in the process of shell mineralization. In: Proceeding of the 9th international symposium on biomineralization (Biom 09). Pucon. Chile. 2005.
Harris ED, Blount JE and Leach RM Jr. Localization of lysyl oxidase in hen oviduct: implications in egg shell membrane formation and composition. Science, 208: 55–56. 1980.
Hernandez-Hernandez A, Gomez-Morales J, Rodriguez-Navarro AB, Gautron J, Nys Y and Garcia-Ruiz JM. Identification of some active proteins in the process of hen eggshell formation. Crystal Growth & Design, 8: 4330–4339. 2008.
Hincke MT. Ovalbumin is a component of the chicken eggshell matrix. Connective Tissue Research, 31: 227–233. 1995.
Hincke MT, Bernard A-M, Lee ER, Tsang CPW and Narbaitz R. Soluble protein constituents of the domestic fowl's eggshell. British Poultry Science, 33: 505–516. 1992.
Hincke MT, Chien YC, Gerstenfeld LC and McKee MD. Colloidal-gold immunocytochemical localization of osteopontin in avian eggshell gland and eggshell. Journal of Histochemistry and Cytochemistry, 56: 467–476. 2008b.
Hincke MT, Gautron J, Mann K, Panhéleux M, McKee MD, Bain M, Solomon SE and Nys Y. Purification of Ovocalyxin-32, a novel chicken eggshell matrix protein. Connective Tissue Research, 44: 16–19. 2003.
Hincke MT, Gautron J, Panhéleux M, Garcia-Ruiz J, McKee MD and Nys Y. Identification and localization of lysozyme as a component of eggshell membranes and eggshell matrix. Matrix Biology, 19: 443–453. 2000.
Hincke MT, Gautron J, Tsang CPW, McKee MD and Nys Y. Molecular cloning and ultrastructural localization of the core protein of an eggshell matrix proteoglycan, Ovocleidin-116. Journal of Biological Chemistry, 274: 32915–32923. 1999.
Hincke MT and St Maurice M. Phosphorylation-dependent modulation of calcium carbonate precipitation by chicken eggshell matrix proteins. In: Chemistry and Biology of Mineralized Tissues (Goldberg M, Boskey A, Robinson C eds) pp. 13–17. American Academy of Orthopaedic Surgeons. Rosemont, IL. 2000.
Hincke MT, Tsang CPW, Courtney M, Hill V and Narbaitz R. Purification and Immunochemistry of a soluble matrix protein of the chicken eggshell (Ovocleidin 17). Calcified Tissue International, 56: 578–583. 1995.
Hincke MT, Wellman-Labadie O, McKee MD, Gautron J, Nys Y and Mann K. Biosynthesis and structural assembly of eggshell components. In: Egg Bioscience and Biotechnology, (Mine Y. ed.). pp. 97–128. John Wiley and Sons. Hoboken. 2008a.
Horvat-Gordon M, Yu F, Burns D and Leach Jr R M Ovocleidin (OC 116) is present in avian skeletal tissues. Poultry Science, 87: 1618–1623. 2008.
International Chicken Genome Sequencing Consortium. Sequence and comparative analysis of the chicken genome provide unique perspectives on vertebrate evolution. Nature, 432: 695–716. 2004.
Jimenez-Lopez C, Rodríguez-Navarro A, Domingez-Vera JM and Garcia-Ruiz JM. Influence of lysozyme on the precipitation of calcium carbonate. Kinetic and morphological study. Geochimica Cosmochimica Acta, 67: 1667–1676. 2003.
Jonchere V, Rehault-Godbert S, Hennequet-Antier C, Cabau C, Sibut V, Cogburn LA, Nys Y and Gautron J. Gene expression profiling to identify eggshell proteins involved in physical defense of the chicken egg. BMC Genomics, 11: 57. 2010.
Kamata R, Shiraishi F, Izumi T, Takahashi S, Shimizu A, Shiraishi H. Mechanisms of estrogen-induced effects in avian reproduction caused by transovarian application of a xenoestrogen, diethylstilbestrol. Archives of Toxicology, 83: 161–171. 2009.
Kawasaki K and Weiss K. Mineralized tissue and vertebrate evolution: the secretory calcium-binding phosphoprotein gene cluster. Proceedings of the National Academy of Science of the United States of America, 100: 4060–4065. 2003.
Kawasaki K and Weiss K. Evolutionary genetics of vertebrate tissue mineralization: the origin and evolution of the secretory calcium-binding phosphoprotein family. Journal of Experimental Zoology B, Molecular Development and Evolution, 306: 295–316. 2006.
Lakshminarayanan R, Joseph JS, Kini RM and Valiyaveettil S. Structure-function relationship of avian eggshell matrix proteins: a comparative study of two major eggshell matrix proteins, ansocalcin and OC-17. Biomacromolecules, 6: 741–51. 2005.
Lakshminarayanan R, Kini RM and Valiyaveettil S. Investigation of the role of ansocalcin in the biomineralization in goose eggshell matrix. Proceedings of the National Academy of Science of the United States of America, 99: 5155–5159. 2002.
Lakshminarayanan R, Valiyaveettil S, Roa VS and Kini RM. Purification, characterization, and *in vitro* mineralization studies of a novel goose eggshell matrix protein, ansocalcin. Journal of Biological Chemistry, 278: 2928–2936. 2003.
Lavelin I, Yarden N, Ben-Bassat S, Bar A and Pines M. Regulation of osteopontin gene expression during egg shell formation in the laying hen by mechanical strain. Matrix Biology, 17: 615–623. 1998.
Mann K. Isolation of a glycosylated form of the eggshell protein Ovocleidin and determination of the glycosylation site. Alternative glycosylation/phosphorylation at an N-glycosylation sequon. FEBS Letters, 463: 12–14. 1999.
Mann K. The chicken egg white proteome. Proteomics, 7: 3558–3568. 2007.
Mann K. Proteomic analysis of the chicken egg vitelline membrane. Proteomics, 8: 2322–2332. 2008.
Mann K, Gautron J, Nys Y, McKee MD, Bajari T, Schneider WJ and Hincke MT. Disulfide-linked heterodimeric clusterin is a component of the chicken eggshell matrix and egg white. Matrix Biology, 22: 397–407. 2003.
Mann K, Hincke MT and Nys Y Isolation of Ovocleidin-116 from chicken eggshells, correction of its amino acid sequence and identification of disulfide bonds and glycosylated Asn. Matrix Biology, 21: 383–387. 2002.
Mann K, Maček B and Olsen JV. Proteomic analysis of the acid-soluble organic matrix of the chicken calcified eggshell layer. Proteomics, 6: 3801–3810. 2006.
Mann K and Mann M. The chicken egg yolk plasma and granule proteomes. Proteomics, 8: 178–191. 2008.

Mann K, Olsen JV, Macek B, Gnad F and Mann M. Phosphoproteins of the chicken eggshell calcified layer. Proteomics, 7: 106–115. 2007.

Mann K and Siedler F. The amino acid sequence of Ovocleidin-17, a major protein of the avian eggshell calcified layer. Biochemistry and Molecular Biology Journal, 47: 997–1007. 1999.

Mann K and Siedler F. Ostrich (Struthio camelus) eggshell matrix contains two different C-type lectin-like proteins. Isolation, amino acid sequence and posttranslational modifications. Biochimica Biophysica Acta, 1696: 41–50. 2004.

Mann K and Siedler F. Amino acid sequences and phosphorylation sites of emu and rhea eggshell C-type lectin-like proteins. Comparative Biochemistry and Physiology B, 143: 160–170. 2006.

Marin-Garcia L, Fraontana-Uribe BA, Reyes-Grajeda JP, Stojanoff V, Serrano-Posada HJ and Moreno A. Chemical Recognition of Carbonate Anions by Proteins Involved in Biomineralization Processes and Their Influence on Calcite Crystal Growth. Crystal Growth and Design, 8: 1340–1345. 2008.

McKee MD and Nanci A. Osteopontin: an interfacial extracellular matrix protein in mineralized tissues. Connective Tissue Research, 35: 197–205. 1996.

Miksik I, Eckhardt A, Sedlakova P and Mikulikova K. Proteins of insoluble matrix of avian (*Gallus gallus*) eggshell. Connective Tissue Research, 48: 1–8. 2007.

Nimtz M, Conradt H and Mann K. LacdiNAc (GalNAcβ1 – 4 GlcNAc) is a major motif in N-glycan structures of the chicken eggshell protein Ovocleidin-116. Biochimica Biophysica Acta, 1675: 71–80. 2004.

Nys Y. Regulation of plasma 1,25(OH)2D3, of osteocalcin and of intestinal and uterine calbindin in hens. In: Avian Endocrinology (Sharp PJ ed) pp. 345–357. Journal of Endocrinology Ltd. Bristol. Great Britain. 1993.

Nys Y, Gautron J, Garcia-Ruiz JM and Hincke MT. Avian eggshell mineralization: biochemical and functional characterization of matrix proteins. Comptes Rendus Paleovol, 3: 549–562. 2004.

Nys Y, Hincke MT, Arias JL, Garcia-Ruiz JM and Solomon S. Avian Eggshell Mineralization. Poultry Avian Biology Reviews, 10: 143–166. 1999.

Nys Y, Zawadzki J, Gautron J and Mills AD. Whitening of brown-shelled eggs: mineral composition of uterine fluid and rate of protoporphyrin deposition. Poultry Science, 70: 1236–1245. 1991.

Pines M, Knopov V and Bar A. Involvement of osteopontin in egg shell formation in the laying chicken. Matrix Biology, 14: 765–771. 1994.

Reyes-Grajeda JP, Jauregui-Zuniga D, Rodriguez-Romero A, Hernandez-Santoyo A, Bolanos-Garcia VM and Moreno A. Crystallization and preliminary x-ray analysis of Ovocleidin-17 a major protein of the Gallus gallus eggshell calcified layer. Protein Peptide Letters, 9: 253–257. 2002.

Reyes-Grajeda JP, Marin-Garcia L, Stojanoff V and Moreno A. Purification, crystallization and preliminary X-ray analysis of struthiocalcin 1 from ostrich (*Struthio camelus*) eggshell. Acta Crystallographica F, 63: 987–989. 2007.

Reyes-Grajeda JP, Moreno A and Romero A. Crystal structure of Ovocleidin-17, a major protein of the calcified Gallus gallus eggshell: implications in the calcite mineral growth pattern. Journal of Biological Chemistry, 279: 40876–40881. 2004.

Roberts JR. Factors affecting egg internal quality and egg shell quality in laying hens. Journal of Poultry Science, 41: 161–177. 2004.

Robey PG. Vertebrate mineralized matrix proteins: structure and function. Connective Tissue Research, 35: 131–136. 1996.

Rodriguez-Navarro A, Kalin O, Nys Y and Garcia-Ruiz JM. Influence of the microstructure on the shell strength of eggs laid by hens of different ages. British Poultry Science, 43: 395–403. 2002.

Salih E, Ashkar S, Gerstenfeld LC and Glimcher MJ. Identification of the phosphorylated sites of metabolically 32P-labeled osteopontin from cultured chicken osteoblasts. Journal of Biological Chemistry 272: 13966–13973. 1997.

Schumann RR, Leong SR, Flaggs GW, Gray PW, Wright SD, Mathison JC, Tobias PS and Ulevitch RJ Structure and function of lipopolysaccharide binding protein. Science, 249: 1429–1431. 1990.

Seuss-Baum I. Nutritional Evaluation of Egg Compounds. In: Bioactive egg compounds. (Huopalahti R, Lopez-Fandino R, Anton M, Schade R eds) pp. 117–140. Springer-Verlag. Berlin. 2007.

Silyn-Roberts H and SharpRM. Crystal growth and the role of the organic network in eggshell biomineralization. Proceedings of the Royal Society, London B Biological Sciences, 227: 303–324. 1986.

Stapley J, Birkhead TR, Burke T and Slate J A linkage map of the zebra finch Taeniopygia guttata provides new insights into avian genome evolution. Genetics, 179: 651–667. 2008.

Whittow GC. Sturkie's Avian Physiology. 5th ed. Academic Press. San Diego. 2000.

Wong M, Hendrix MJC, Von der Mark K, Little C and Stern R. Collagen in the egg shell membranes of the hen. Developmental Biology, 104: 28–36. 1984.

Yang KT, Lin CY, Liou JS, Fan YH, Chiou SH, Huand CW, Wu CP, Lin EC, Chen CF, Lee YP, Lee WC, Ding ST, Cheng WT and Huang MC. Differentially expressed transcripts in shell glands from low and high egg production strains of chickens using cDNA microarrays. Animal Reproduction Science, 110: 113–124. 2007.

Zelensky AN and Gready JE. The C-type lectin-like domain superfamily. FEBS Journal, 272: 6179–217. 2005.