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Endocrine and molecular regulation of oviposition sequence and eggshell biomineralization in commercial poultry: mechanisms, genetic control, and nutritional interventions: A review

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Abstract

Sequential oviposition in commercial laying hens is the highly integrated reproductive process, wherein neuroendocrine signaling, follicular development, oviductal transport, and uterine biomineralization are precisely coordinated. In modern laying strains, the major challenge is not only to maintain oviposition every 24-27 h but also to sustain eggshell quality, skeletal integrity, and laying persistency during extended production cycles. Thus, understanding the endocrine and molecular mechanisms of oviposition is necessary to improve the reproductive efficiency and eggshell quality of commercial poultry. This review presents the current knowledge of endocrine, physiological, and molecular regulations of sequential oviposition and their practical implication to enhance eggshell formation in modern laying hens. Ovulatory ovipositional cycle is under the control of the hypothalamic-pituitary-gonadal axis wherein photoperiodic and circadian signals regulate GnRH secretion, pituitary FSH and LH release, preovulatory progesterone production, and the LH surge for ovulation to be initiated. Following ovulation, the egg is moved through the oviduct; however, the uterine (shell gland) phase is the longest and most physiologically demanding because it includes the entire process of eggshell calcification. Eggshell formation is a highly regulated biomineralization process that involves the coordination of calcium mobilization, bicarbonate production, ion transport, acid-base regulation, and secretion of specialized organic matrix proteins such as ovocleidin-116 and osteopontin, which control crystal nucleation and shell ultrastructure. Recent transcriptomic, proteomic, and genomic studies indicate that most of the strength of the eggshell is determined in the early stage of calcification, primarily through the regulation of mammillary layer formation, organization of extracellular matrix, and uterine bicarbonate transport. Recent breeding strategies have shifted from conventional phenotypic selection to more advanced genomic, transcriptomic, and multitopic approaches targeting genes and biological pathways related to shell quality persistence and reproductive efficiency. Recently, precision nutritional strategies have been proposed as feasible approaches to enhance calcium utilization and eggshell quality while egg production is not adversely affected; these include split-feeding systems and optimized calcium supplementation in the afternoon and evening, with vitamin D supply synchronized with the circadian rhythm of the hens. Overall, current evidence supports an integrated biological framework in which endocrine regulation, molecular signaling, uterine ion transport, biomineralization, genetic selection, and precision nutrition function collectively to regulate sequential oviposition and optimize eggshell quality in modern commercial laying hens.

Keywords: Laying hens, sequential oviposition, eggshell biomineralization, eggshell quality, shell gland, calcium metabolism, genomic selection, precision nutrition

1. Introduction

Commercial layers have been subjected to relentless genetic selection for increased egg production, prolonged persistency of lay, and efficient feed conversion over the past few decades. Modern commercial hybrids can now lay more than 300 eggs per year, and long-cycle hybrids targeted to lay approximately 500 eggs over 100 weeks have recently been developed [1-3]. The advancements in genetic potential have resulted in increased productivity and profitability of the poultry industry but have also magnified the physiological challenges posed to the reproductive and skeletal system of the laying hen [4] [5] [4, 5] [4] [5]. Thus, the challenge of maintaining

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eggshell quality in long laying cycles has become one of the most important issues in modern egg production [6-8].

Eggshell quality is a primary determinant of biological performance and economic return because defects in the shell increase egg breakage, downgrade rates, microbial contamination, and losses of hatchability in breeder flocks, as well as financial losses through the production chain [7-9]. Furthermore, to support uninterrupted eggshell formation, a close to 2.0-2.5 g quantity of calcium carbonate must be deposited per day, which places a significant demand on calcium absorption in the intestine, medullary bone reserves, endocrine regulation, and uterine mineralization mechanisms. With the aging of laying hens, these mechanisms progressively become less efficient; therefore, shell thinness, weakness, and variability in ultrastructure gradually become more pronounced. Sequential oviposition is not just a repetitive biological process but rather the result of a well-coordinated system integrating neuroendocrine function, follicular hierarchy, ovulation, oviductal transport, uterine ion exchange, and controlled biomineralization [10-13]. Each ovulation under normal physiological conditions should take place within an endocrine window of proper regulation after the oviposition of the previously laid egg to maintain the sequence of clutch and to ensure continuous egg production [14, 15]. Even minor perturbations in hormonal synchronization, uterine ion transport, calcium metabolism, or extracellular matrix formation can disrupt oviposition timing, shell mineralization, and hence eggshell quality [16-18].

Recent progress in reproductive endocrinology, molecular biology, and high-throughput omics technologies has brought a much better understanding of the mechanism behind oviposition and the formation of the eggshell [19-22]. Studies on transcription, protein, metabolism, epigenetics, and single-cell sequencing have identified many genes, signaling pathways, and regulatory networks for calcium transport, bicarbonate secretion, organization of the extracellular matrix, nucleation of calcite crystals, response to oxidative stress, regulation of the immune response, and differentiation of uterine epithelial cells [23-27]. These findings have changed the strategy of commercial breeding from the selection based on conventional phenotypes to selection based on genomics and pathways to improve shell quality, persistence of laying, and skeletal health [28-30].

Precision nutritional support for eggshell formation over longer production cycles has also come to be of greater importance. At present, split-feeding systems, optimized calcium particle size, evening calcium supplementation, vitamin D metabolites, and trace minerals including probiotics and phytochemical additives, as well as other functional feed ingredients, are being implemented to synchronize nutrient availability with the hen's circadian pattern of shell calcification. All these would improve calcium utilization and reduce skeletal depletion [31-35].

In fact, there have been major advances in single research sectors, but current knowledge, covering endocrinology, reproductive physiology, molecular genetics, biomineralization, and poultry nutrition, remains fragmented [36-38]. A comprehensive synthesis is still limited, particularly for the modern high-producing laying strains maintained under extended laying cycles [39].

Therefore, this review concentrates on the crosstalk between endocrine regulation of sequential oviposition and uterine molecular biology, ion transport, biomineralization, and precision nutritional management in the determination of eggshell competence in the modern commercial laying hen. Specific consideration is directed toward the means by which

the hormonal signal pathways are linked with molecular mechanisms, recent multi-omics technologies, and realizable measures for improving eggshell quality, laying persistency, and long-term reproductive efficiency.

2. Endocrine Regulation of Sequential Oviposition

2.1 The Hypothalamic–Pituitary–Gonadal Axis

The egg-laying cycle in commercial laying hens is mainly controlled by the hypothalamic–pituitary–gonadal (HPG) axis. This axis integrates photoperiodic information, circadian rhythms, and metabolic status, as well as ovarian feedback on follicular development and ovulation [1, 6-8]. Light stimulation of hypothalamic neurons via retinal and extra-retinal photoreceptors leads to the release of gonadotropin-releasing hormone (*GnRH*) which in turn stimulates the anterior pituitary to secrete follicle-stimulating hormone (*FSH*) and luteinizing hormone (*LH*) [1]. Follicular recruitment and establishment of the preovulatory hierarchy depend primarily on *FSH* responsiveness. Terminal maturation of the dominant follicle is increasingly governed by *LH*-induced steroidogenesis and progesterone secretion [1, 7, 8].

2.2 Hormonal Control of Ovulation

The hallmark endocrine event that precedes ovulation is the tightly coordinated preovulatory rise in circulating levels of progesterone and *LH*. Progesterone, which is synthesized by the granulosa cells of the largest preovulatory follicle, was later shown by Scanes [1] to act through positive feedback on the hypothalamus and pituitary to induce the characteristic *LH* surge in an estrogen-dependent endocrine system. This surge represents the immediate hormonal trigger for ovulation and occurs several hours before rupture of the follicle.

Ovulation usually takes place about 30 minutes after the laying of the last egg, and the interval between successive oviposition generally varies from 24 to 27 h in commercial chickens [1, 7]. Such precise endocrine coordination accounts for the formation of egg-laying sequences (clutches) [1, 7] where successive oviposition take place on consecutive days until the ovulatory rhythm is interrupted by a pause day [1].

2.3 Endocrine Regulation of Follicular Hierarchy

The follicular hierarchy has to be constantly reestablished for sequential oviposition to occur. Normally, the largest yellow follicle (F1) ovulates first. The smaller follicles will then sequentially advance through the hierarchy to replace the ovulated follicle. At the cellular level, *FSH* increases granulosa-cell differentiation by enhancing cyclic AMP (cAMP) signalling, steroidogenic acute regulatory protein (*STAR*) expression, cholesterol transport, and progesterone biosynthesis [1, 40].

Apart from gonadotropins, several growth factors that act locally regulate the selection of follicles before the formation of hierarchy. The members of the superfamily of transforming growth factor- β (*TGF- β*), such as *anti-Müllerian* hormone (*AMH*), bone morphogenetic proteins (*BMP2*, *BMP4*, and *BMP6*), growth differentiation factor-9 (*GDF9*), and inhibins, perform the functions of modulation on granulosa-cell proliferation, steroidogenic capacity, differentiation of follicles, and survival of follicles [1, 40-42].

2.4 Neuroendocrine and Circadian Regulation

Sequential oviposition is further modulated by the complex neuroendocrine pathways which integrate stimulatory and inhibitory hypothalamic signals. Norepinephrine, vasoactive

intestinal peptide (VIP), and neuropeptide Y (NPY) stimulate hypothalamic *GnRH* secretion. Secretion of dopamine and gonadotropin-inhibitory hormone (*GnIH*) suppresses gonadotropin release and delays ovulation. New evidence also suggests that indirect involvement in avian reproductive neuroendocrine function may be a property of kisspeptin signaling, although its physiological role is far less defined than in mammals.

Circadian regulation is another key factor in ovulatory timing. Clock genes such as *BMAL1*, *CLOCK*, *PER2*, and *CRY1* have been detected in ovarian follicles and tissues of the oviduct, suggesting that endocrine events are not directly initiated by mechanical events associated with oviposition but are rather synchronized with endogenous biological rhythms [1, 43, 10]. However, the exact mode of molecular communication between the ovary and the oviduct for synchronization of the next ovulation with completion of the previous laying cycle is not clear [1].

2.5 Hormonal Regulation of Oviposition

The final stage of the reproductive cycle oviposition is initiated by relaxation of the uterovaginal sphincter along with rhythmic contraction of the smooth uterine muscle, actions primarily mediated by arginine vasotonic (AVT), the principal avian neurohypophyseal hormone. AVT acts synergistically with locally synthesized prostaglandins, particularly prostaglandin F_{2a} (PGF_{2a}), to stimulate myometrial contractility and facilitate egg expulsion.

Oviposition, therefore, is not an isolated mechanical event but rather the terminal phase of an integrated endocrine cycle that simultaneously prepares the ovary for the next ovulation to ensure continuity of clutch formation in commercial laying hens. These facts were elegantly summarized in a review by Perry and co-workers [7] [1].

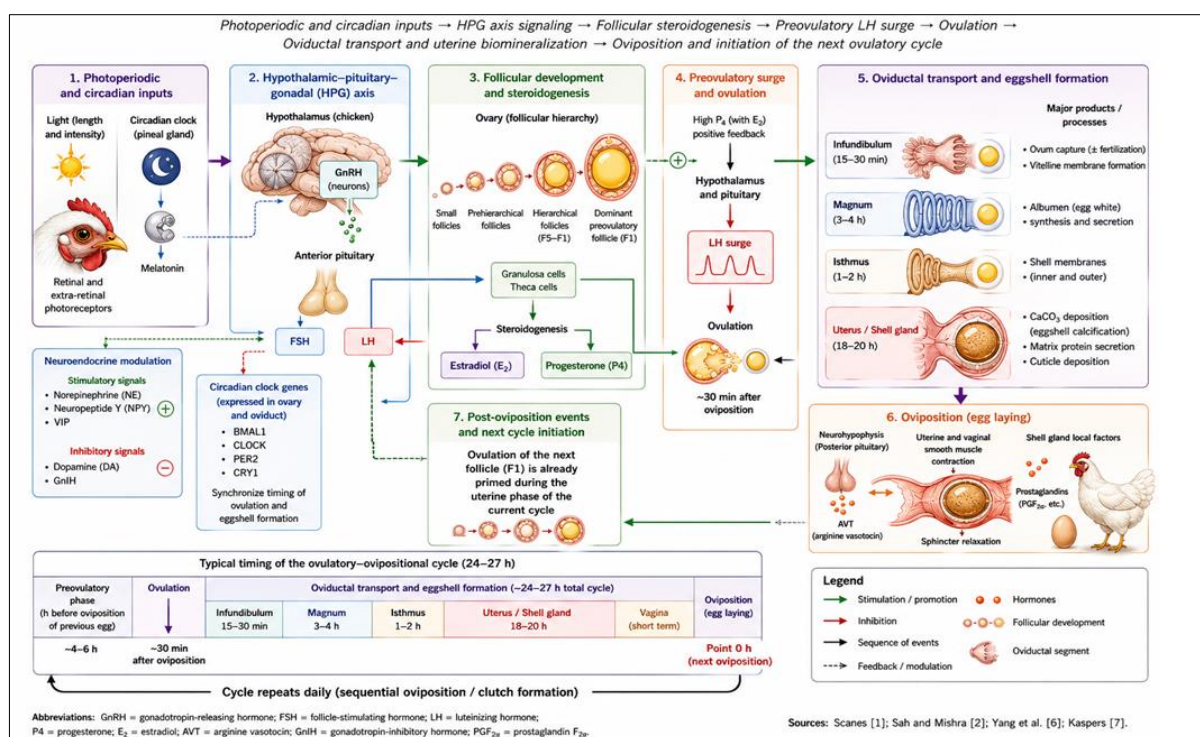


Fig 1: Schematic representation of the neuroendocrine and oviductal events underlying sequential oviposition in commercial laying hens. It depicts photoperiodic and circadian inputs, HPG axis signaling, ovarian follicular steroidogenesis, preovulatory LH surge, ovulation, oviductal transport, uterine biomineralization, and hormonally regulated oviposition, integrated with each other. The schematic draws its basis from the mechanistic framework as described by Scanes [1], Sah and Mishra [2], Yang et al. [6], and Kaspers [7]. Table 1. Principal endocrine regulators involved in sequential oviposition and their physiological functions in commercial laying hens.

Table 1: Principal endocrine regulators involved in sequential oviposition and their physiological functions in commercial laying hens.

Hormone / Regulatory Factor	Primary Source	Primary Target	Major Physiological Function	Representative References
Gonadotropin-releasing hormone (GnRH)	Hypothalamus	Anterior pituitary	Stimulates synthesis and secretion of FSH and LH, initiating the reproductive endocrine cascade	[1, 6]
Follicle-stimulating hormone (FSH)	Anterior pituitary	Small white, PR hierarchical, and granulosa follicles	Promotes follicular recruitment, granulosa-cell differentiation, steroidogenesis, and follicular hierarchy formation	[1, 7, 40]
Luteinizing hormone (LH)	Anterior pituitary	Dominant preovulatory follicle (F1)	Induces final follicular maturation, progesterone secretion, ovulation, and maintenance of clutch sequence	[1, 7]
Progesterone (P4)	Granulosa cells of the F1 follicle	Hypothalamus and pituitary	Triggers the preovulatory LH surge through positive feedback and initiates ovulation	[1, 9]

Estradiol (E2)	Ovarian follicles	Oviduct, liver, hypothalamus	Stimulates oviduct development, medullary bone formation, vitellogenesis, and progesterone responsiveness	[1, 6, 41]
Arginine vasotocin (AVT)	Neurohypophysis	Uterine smooth muscle and uterovaginal junction	Induces uterine contraction and facilitates oviposition	[7, 11]
Prostaglandin F _{2α} (PGF _{2α})	Shell gland (uterus)	Uterine smooth muscle	Acts synergistically with AVT to stimulate myometrial contraction during oviposition	[7, 12]
Neuropeptide Y (NPY)	Hypothalamus	GnRH neurons	Stimulates GnRH secretion and supports reproductive endocrine activity	[1, 44]
Norepinephrine	Central nervous system	Hypothalamic GnRH neurons	Enhances GnRH release and facilitates gonadotropin secretion	[1, 44]
Gonadotropin-inhibitory hormone (GnIH)	Hypothalamus	Pituitary and GnRH neurons	Suppresses LH and FSH secretion and delays ovulation	[1, 45]
Dopamine	Hypothalamus	Pituitary	Inhibits gonadotropin secretion and modulates reproductive timing	[1, 44]
Melatonin	Pineal gland	Hypothalamus and ovary	Synchronizes circadian rhythms with photoperiodic regulation of reproduction	[6, 43]

3. Oviductal Progression and the Dominant Role of the Shell Gland

Following ovulation, the ovum enters the oviduct where it undergoes a very well organized series of structural and biochemical changes that eventually result in the formation of a true egg^{[7] [2]}. Each anatomical region of the oviduct expresses specialized physiological functions closely coordinated with endocrine regulation and the progressive maturation of the egg. The infundibulum picks up the ovulated ovum; in breeder hens it helps to pick up sperm for fertilization and initiates formation of the vitelline membrane. It is in the magnum where most of the albumin proteins are synthesized and secreted. The isthmus is the site where the inner and outer shell membranes are deposited providing the structural scaffold on which the mineralized eggshell will later develop. The egg finally enters the uterus, which is commonly referred to as the shell gland; this is where extensive deposition of calcium carbonate occurs to form the hard eggshell^[13, 7, 2].

The temporal distribution of egg transit through the oviduct would obviously reflect the dominant physiological importance of the shell gland. Kaspers^[7] claims that ovum remains in the infundibulum for about 15 to 30 minutes, in the magnum for about 3 to 4 hours, and in the isthmus for 1 to 2 hours before going to the shell gland where it stays for about 18 to 20 hours. Thus, nearly three-quarters of the entire ovulatory-ovipositional cycle is devoted to uterine residence and eggshell formation^[7]. This length of a retention period obviously underscores the extremely complex nature of shell biomineralization continuous transport of calcium, secretion of bicarbonate, assembly of extracellular matrix, control over crystal nucleation, and progressive maturation of the ultrastructure of the shell. Instead of being a passive reservoir for calcium deposition, the shell gland is described as a highly specialized biomineralization organ that is endocrinologically regulated so that epithelial transport mechanisms, extracellular matrix proteins, acid-base balance, and immune defense are integrated for the production of a mechanically competent eggshell. Active transcellular calcium transport by uterine epithelial cells includes calcium channels, ATP-dependent calcium pumps, carbonic anhydrase for bicarbonate generation, and matrix protein secretion controlling calcite crystal orientation and shell microarchitecture. Therefore, the quality of the shell will depend not only on how much calcium is deposited but also on how well these molecular events take place at the right time. Thus, the time the egg stays in the uterus is one of the main factors that determine the quality of the eggshell. Changes in

the timing of hormones, environmental stress, aging, imbalance in food, or damage to the ways the uterus moves can all make the time an egg is in the shell gland shorter or longer. This will then affect shell thickness, strength, how the crystals are arranged, and how the cuticle is laid down^{[18] [16] [1]}. Even quite small differences in how well ovulation is matched with the time the shell forms can result in eggs with poor shell strength even though egg production seems normal.

From a commercial perspective, the shell gland is the primary physiological bottleneck in the quality of the eggshell for modern laying hens. Genetic selection has markedly increased egg number; thus, the capacity of the uterus to sustain efficient biomineralization has become even more critical for shell integrity during extended laying cycles^{[19] [17]}. Therefore, knowledge of the endocrine regulation and molecular physiology of the shell gland will provide the biological basis for the development of genetic, nutritional, and management strategies that will improve eggshell quality and increase the productive lifespan of current poultry strains.

4. Molecular Basis of Eggshell Biomineralization

The process of forming eggshells is not simply a passive one of carbonate calcium precipitating, but rather a highly coordinated biomineralization event. It is regulated via endocrine signals, uterine epithelial transport, extracellular matrix proteins, and the exact physicochemical conditions^[5, 6, 15]. Specialized organic macromolecules and mineral ions are secreted within the shell gland into the uterine fluid. This provides a cell-free mineralization environment where nucleation of calcite crystals can take place, as well as growth and assembly into the highly organized structure of the eggshell^[5]. Brionne *et al.*^[5] described the process of eggshell formation as a model system of biological biomineralization since microstructure, mechanical strength, and fracture resistance of the shell are determined by continuous interactions between inorganic mineral fluxes and the organic matrix.

The mineral phase of the eggshell is primarily composed of calcium carbonate (CaCO₃) in the calcite polymorph, which makes up about 95–97% of the weight of the shell; the rest is made up of organic matrix proteins, glycoproteins, proteoglycans, lipids, and water^[5, 15, 20]. Organics influence crystal nucleation and crystal orientation mamillary cone formation palisade layer organization and cuticle deposition, though they represent a small fraction of the total mass of the shell^[5, 16]. Therefore, shell quality does not only depend on the

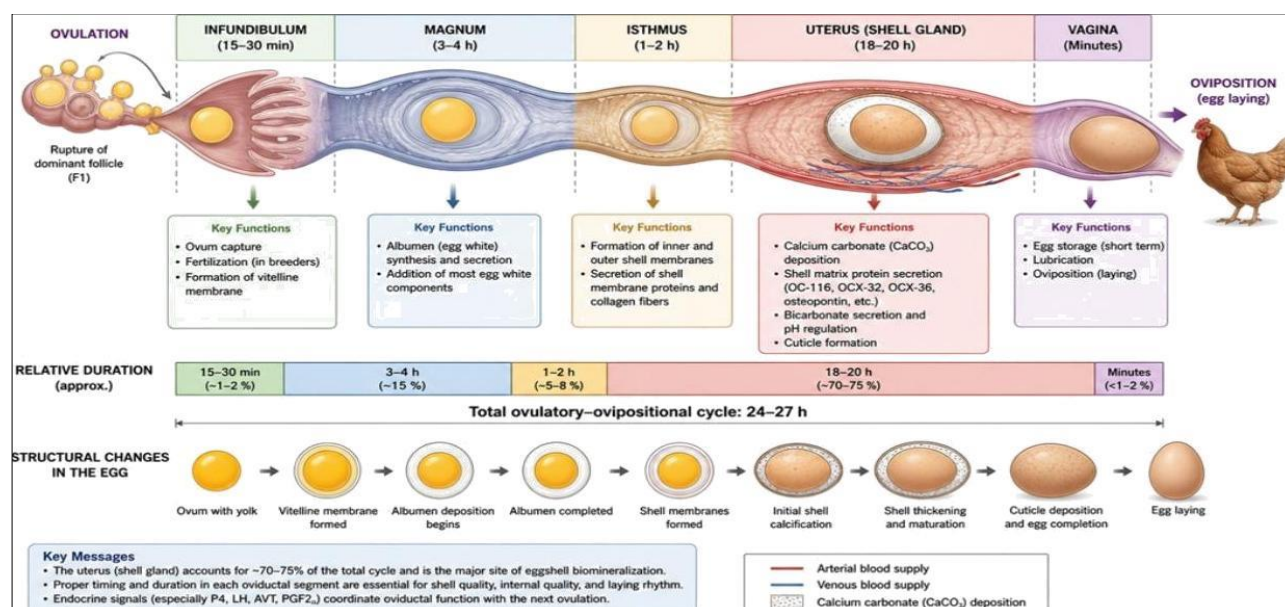


Fig 2: Transit of the egg through the avian oviduct and the relative duration of each developmental stage during the ovulatory ovipositional cycle. The schematic shows the egg's movement in order through the infundibulum, magnum, isthmus, uterus (shell gland), and vagina, underscoring the extended uterine stay during the formation of the eggshell and its importance in determining the final quality of the shell. Reference to Scanes ^[1], Yang *et al.* ^[6], and Kaspers ^[7].

Table 2: Functional characteristics of the oviductal segments during egg formation in commercial laying hens.

Oviductal Segment	Average Residence Time	Major Physiological Function	Principal Secretions / Products	Representative References
Infundibulum	15–30 min	Ovum capture, fertilization, initiation of vitelline membrane formation	Vitelline membrane proteins	[2, 7]
Magnum	3–4 h	Albumen synthesis and deposition	Ovalbumin, Ovo transferrin, ovomucin, lysozyme	[2, 7]
Isthmus	1–2 h	Formation of inner and outer shell membranes	Shell membrane proteins and collagen fibers	[2, 7]
Uterus (Shell gland)	18–20 h	Calcium carbonate deposition, biomineralization, shell ultrastructure formation, cuticle deposition	CaCO_3 , matrix proteins (OC-116, OCX-32, OCX-36, osteopontin), bicarbonate	[6, 7, 15–18]
Vagina	Minutes	Egg expulsion (oviposition) and temporary storage before laying	Mucus and lubricating secretions	[7, 11]

quantity of mineral matter deposited but also on molecular regulation of crystal architecture.

Uterine calcification requires an uninterrupted supply of calcium and bicarbonate. In the process of shell formation, calcium is supplied mainly from intestinal absorption; however, in cases where the dietary availability of calcium is not able to meet the very high mineral requirement, about 20–40% of the needed calcium may be mobilized from medullary bone ^[7]. Kaspers ^[7] estimated about 2.0–2.5 g of calcium to be needed for each eggshell, which depicts the remarkable mineral turnover that occurs daily in modern laying hens. Efficient formation of the eggshell is hence closely related to skeletal metabolism, endocrine regulation, and homeostasis of calcium. At the cellular level, uterine epithelial cells take an active part in the process of calcium transport from the blood into the uterine lumen by systems of transcellular transport coordinated ^[15, 17]. The major site of entry of calcium into epithelial cells is through apical calcium channels, one of which is transient receptor potential vanilloid 6 (TRPV6); it then binds to intracellular calcium-binding proteins like calbindin-D28k (CALB1), which facilitates cytoplasmic calcium diffusion while it checks any possibility of cytotoxic accumulation ^[15, 21]. Plasma membrane Ca^{2+} -ATPases (ATP2B1) and sodium–calcium exchangers provide the final step in the extrusion of

calcium into the uterine fluid for continuous supply as and when needed for calcite deposition ^[15, 21, 22].

At the same time, bicarbonate ions that are needed for the synthesis of calcium carbonate are prepared by hydration of carbon dioxide in uterine epithelial cells via carbonic anhydrase II (CA2) ^[17, 22]. Bicarbonate then gets into the uterine lumen by the action of anion exchangers and bicarbonate transporters, such as some members of the SLC26 family, where it joins with calcium ions to form calcite crystals ^[17, 22]. Proper crystal growth kinetics as well as the prevention of abnormal shell mineralization depend on the strict control of luminal pH and ionic composition.

The organic matrix is believed to provide a molecular template for the initiation of crystal growth and to control the ultrastructure of the shell. The most thoroughly characterized matrix proteins are ovocleidin-116 (OC-116), ovocalyxin-32 (OCX-32), ovocalyxin-36 (OCX-36), osteopontin (SPP1), and ovotransferrin, each contributing to different stages in the formation of calcite crystals and their subsequent maturation ^[5, 16, 23]. Instead of being structural proteins, these macromolecules determine the morphology of the crystals, the orientation of the crystals, the rate at which mineral is deposited, and the ability to resist crack propagation as an index of mechanical competence of the mature eggshell ^[5, 23, 24].

From recent studies at the transcriptomic and proteomic levels, it has been established that the very early stages of shell calcification play a more important role in determining final shell quality [15, 24-26]. A differential expression of genes related to calcium transport, synthesis of the extracellular matrix, oxidative phosphorylation, and ion homeostasis was found to be associated with marked differences in shell strength among commercial laying strains [24-26]. In fact, organization of the mamillary layer has appeared as one of the strongest structural predictors of shell mechanical properties; hence, quality of the eggshell is determined at the very early stages of uterine

mineralization rather than only during later thickening of the shell [24] [5].

These findings show that eggshell biomineralization is a closely coordinated biological process involving endocrine regulation, epithelial ion transport, assembly of the extracellular matrix, nucleation of crystals, and the metabolism of skeletal calcium. In other words, these molecular mechanisms are the basis for future genetic improvement as well as precision nutrition and management strategies for enhancing eggshell quality and sustaining reproductive performance of modern commercial laying hens.

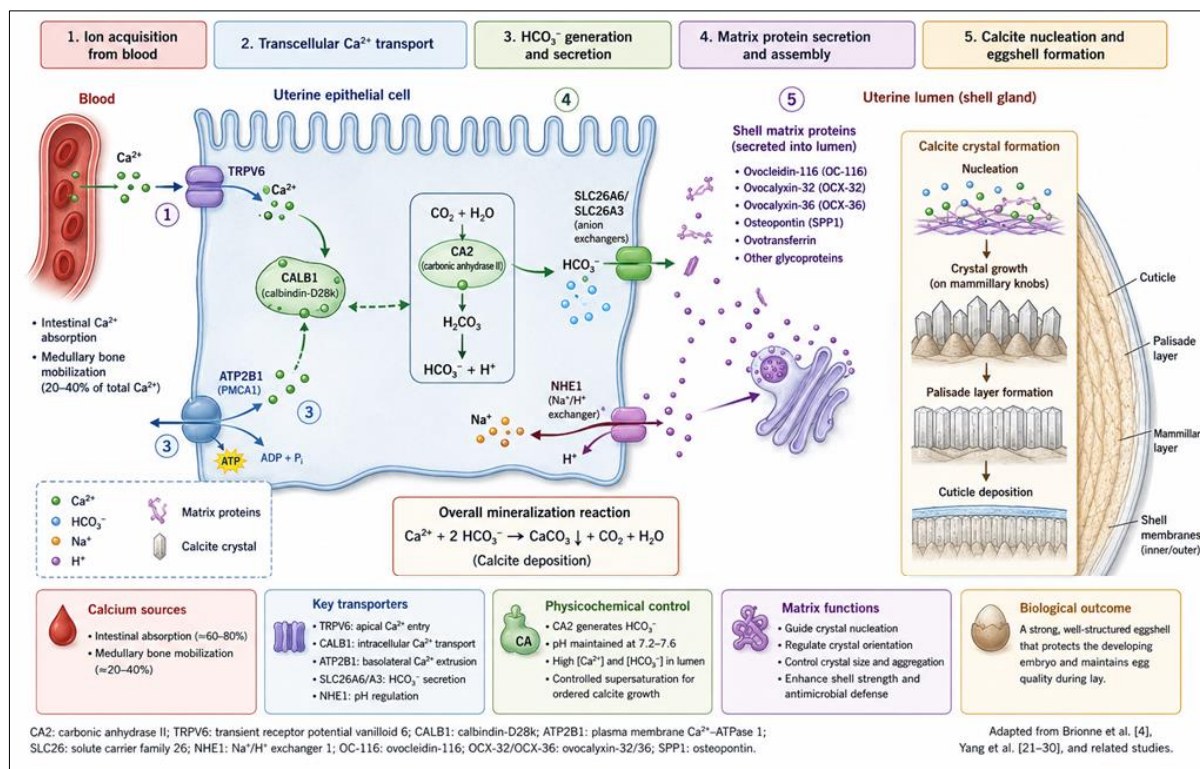


Fig 3: Molecular mechanisms of eggshell biomineralization in the uterine epithelium of commercial laying hens. The schematic illustrates transcellular calcium transport (TRPV6–CALB1–ATP2B1), CO₂ hydration by carbonic anhydrase II (CA2), SLC26 transporters for bicarbonate secretion, and extracellular matrix protein secretion (OC-116, OCX-32, OCX-36, osteopontin) along with nucleation of calcite crystals in the process of making the eggshell. The figure summarizes the coordinated interaction between ion transport, matrix assembly, and biomineralization based on current molecular evidence [4, 21–30]. Table 3. Principal molecular components involved in eggshell biomineralization and their physiological functions.

Table 3: Principal molecular components involved in eggshell biomineralization and their physiological functions.

Molecular component	Primary location	Principal function	Representative references
TRPV6	Uterine epithelium	Apical Ca^{2+} uptake	[15, 21]
CALB1 (Calbindin-D28k)	Uterine epithelial cytoplasm	Intracellular Ca^{2+} transport and buffering	[15, 21]
ATP2B1	Basolateral membrane	Ca^{2+} extrusion into uterine fluid	[15, 22]
Carbonic anhydrase II (CA2)	Uterine epithelial cells	HCO_3^- generation	[17, 22]
SLC26 transporters	Uterine epithelium	Bicarbonate secretion	[17, 22]
Ovocleidin-116 (OC-116)	Eggshell matrix	Crystal nucleation and shell organization	[5, 23]
Ovocalyxin-32 (OCX-32)	Eggshell matrix	Matrix stabilization and shell defense	[23, 24]
Ovocalyxin-36 (OCX-36)	Eggshell matrix	Shell maturation and antimicrobial activity	[23, 24]
Osteopontin (SPP1)	Eggshell matrix	Regulation of calcite crystal growth	[5, 23]

4.1 Calcium Transport and Intracellular Handling

High rates of calcium carbonate deposition during eggshell formation also point to the need for an effective, tightly regulated transcellular calcium transport system within the uterine epithelium. There is no passive diffusion in the movement of calcium from the maternal circulation to the uterine lumen, but rather, a network of membrane channels,

intracellular calcium-binding proteins, ATP-dependent pumps, and ion exchangers, all of which work in a coordinated fashion to achieve continuous calcium delivery while maintaining intracellular calcium homeostasis [3, 5, 15].

Jonchère *et al.* [3] provided a comprehensive uterine calcium ion-transport system in which TRPV6 was the primary apical calcium channel for uptake of calcium from the extracellular environment into uterine epithelial cells. Once in the cytoplasm,

calcium is immediately bound by calbindin-D28k (*CALB1*). This facilitates intracellular calcium diffusion while also preventing an excessive rise in free cytosolic Ca^{2+} concentration [5] [3] [5] [3]. This intracellular buffering system allows rapid trafficking of calcium towards the basolateral membrane without inducing cellular toxicity mediated by calcium.

Subsequently, calcium efflux to the uterine fluid is mediated by plasma membrane Ca^{2+} -ATPases, primarily *ATP2B1*, *ATP2B2*, and *ATP2B4* (PMCA isoforms), also known as the isoforms of PMCA, which actively transport calcium against its electrochemical gradient by ATP hydrolysis. In parallel, sodium/calcium exchangers (*SLC8A1* and *SLC8A3*) contribute to calcium efflux and help sustain the high extracellular calcium concentration, over which there is continuous growth of calcite crystals within the uterine lumen [3].

This underlines the importance of intracellular calcium buffering; this is further supported by the very high expression of *CALB1* in the uterus during the active phase of shell calcification. Brionne *et al.* [5] showed a large increase in *CALB* transcripts during eggshell formation, which points to its

major role in the control of calcium homeostasis in such high mineral flux conditions. In a previous study, Jonchère *et al.* [3] proposed that calbindin works together with other systems responsible for the storage and release of intracellular calcium such as sarco-endoplasmic reticulum Ca^{2+} ATPases (*ATP2A2* and *ATP2A3*) and inositol 1,4,5- triphosphate receptors (*ITPRs*) to control the cycling of intracellular calcium, refill endoplasmic reticulum calcium stores and protect uterine epithelial cells against calcium overload in fast biomineralization.

Together, these molecular transport systems make sure that large amounts of calcium are brought near the mineralization front with maximum efficiency while keeping the cells alive and their metabolism stable. Therefore, eggshell quality depends not only on the total amount of calcium available but also on the ability of uterine epithelial cells to quickly take up, buffer, recycle, and secrete calcium in a highly organized way. Any disruption to this network may lead to inadequate calcium availability at the mineralization site, thereby reducing shell thickness and strength as well as overall eggshell quality [3, 5, 15].

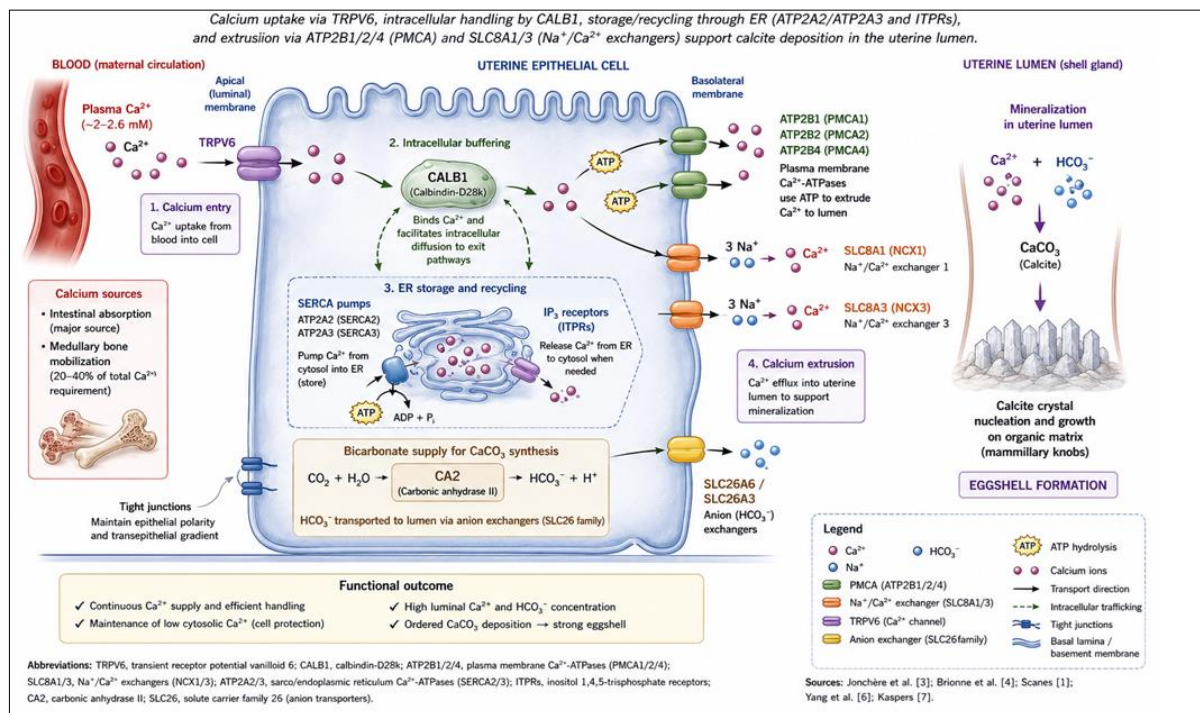


Fig 4: Schematic of transcellular pathway for calcium transport in uterine epithelial cells during eggshell biomineralization. The diagram describes the pathway of calcium uptake through TRPV6, intracellular buffering with CALB1, and storage plus recycling through ATP2A2/ATP2A3 and ITPRs in endoplasmic reticulum. Also included are pathways for extruding calcium into the uterine lumen through ATP2B1/ATP2B2/ATP2B4 (PMCA) and SLC8A1/SLC8A3 ($\text{Na}^+/\text{Ca}^{2+}$ exchangers) to support continuous Ca^{2+} calcite deposition during eggshell formation. Table 4. Major calcium transport proteins involved in uterine calcium homeostasis during eggshell biomineralization.

Table 4: Major calcium transport proteins involved in uterine calcium homeostasis during eggshell biomineralization

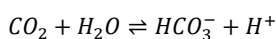
Protein / Gene	Protein family	Cellular localization	Principal physiological function	Representative references
TRPV6	Calcium channel	Apical membrane	Ca^{2+} entry into uterine epithelial cells	[3, 15]
CALB1 (Calbindin-D28k)	Calcium-binding protein	Cytoplasm	Intracellular Ca^{2+} buffering and transport	[3, 5]
ATP2B1 (PMCA1)	Ca^{2+} -ATPase	Basolateral membrane	ATP-dependent Ca^{2+} extrusion into uterine fluid	[3]
ATP2B2 (PMCA2)	Ca^{2+} -ATPase	Plasma membrane	Calcium export and maintenance of Ca^{2+} homeostasis	[3]
ATP2B4 (PMCA4)	Ca^{2+} -ATPase	Plasma membrane	Calcium secretion during shell mineralization	[3]
SLC8A1 (NCX1)	$\text{Na}^+/\text{Ca}^{2+}$ exchanger	Plasma membrane	Ca^{2+} extrusion coupled to Na^+ influx	[3]
SLC8A3 (NCX3)	$\text{Na}^+/\text{Ca}^{2+}$ exchanger	Plasma membrane	Supplemental Ca^{2+} efflux pathway	[3]

ATP2A2(SERCA2)	ER Ca ²⁺ -ATPase	Endoplasmic reticulum	Refilling intracellular Ca ²⁺ stores	[3]
ATP2A3(SERCA3)	ER Ca ²⁺ -ATPase	Endoplasmic reticulum	Intracellular calcium recycling	[3]
ITPRs	Calcium-release channels	Endoplasmic reticulum	Controlled release of stored Ca ²⁺ into cytoplasm	[3]

4.2 Bicarbonate Supply, pH Control, and Ionic Microenvironment

Efficient eggshell biomineralization requires not only an adequate supply of calcium ions but also a steady source of bicarbonate, which in its free state combines with calcium to form calcium carbonate, the principal mineral constituent of the eggshell. Therefore, uterine epithelial cells must synchronize bicarbonate production, bicarbonate secretion, proton removal, and luminal pH regulation in the overall scheme to maintain the physicochemical conditions under which calcite crystal growth can proceed.

Inside cells, bicarbonate is made mainly by carbonic anhydrase II (CA2), which quickly combines carbon dioxide and water to form bicarbonate and protons. This happens through the reversible reaction:



Jonchère *et al.* [3] identified CAAs as one of the most highly expressed enzymes in the uterine epithelium during active shell calcification, underlining its major function in sustaining the very high demand for bicarbonate associated with continuous deposition of calcium carbonate. Bicarbonate, once formed, is transported across the epithelial membrane by several anion transport systems, including SLC26A9 and members of the SLC4 bicarbonate transporter family, to enrich uterine fluid with bicarbonate ions which are required for the formation of calcite.

The uterine ionic microenvironment is more than just the availability of bicarbonate. It was argued earlier that since

CaCO₃ continuously generates H⁺ ions, in the absence of an effective pathway for removal of these protons, acidification would occur intracellularly and, in the lumen, [15] [3]. The authors went on to show later that systems involved in proton extrusion, like the Na⁺/H⁺ exchangers and the vacuolar H⁺ ATPases, are of critical importance for homeostasis at intracellular pH and for support to mineralization. Thus, production of bicarbonate and removal of protons are tightly coupled physiological processes, not independent mechanisms.

Transcriptomic data from recent studies highlight the fact that the regulation of acid–base parameters is important at the initial stage of eggshell formation. When Zhang *et al.* [4] compared the hens that laid eggs with strong shells to those that laid eggs with weak shells, they observed a significant upregulation of bicarbonate transport genes (SLCA1, SLCA2, and SLCA9) and proton pump subunits (ATP6V0D2, ATP6V1B, ATP6V1C2, and ATP6V1G3) during the initiation phase of calcification in the former group. They proposed that an ionic environment best suited for efficient nucleation of crystals and early formation of the mammillary layer would be one in which bicarbonate is secreted and protons are extruded.

Eggshell quality depends on calcium availability and the physicochemical properties of uterine fluid. Proper regulation of bicarbonate concentration, luminal pH, ionic gradients, and electrochemical balance determines kinetics of crystal growth, matrix–mineral interactions, and eventually ultrastructural organization of mature eggshell. Weak eggshells can be formed even with adequate calcium supply if the ionic microenvironment of the uterus does not support the efficient biomineralization process.

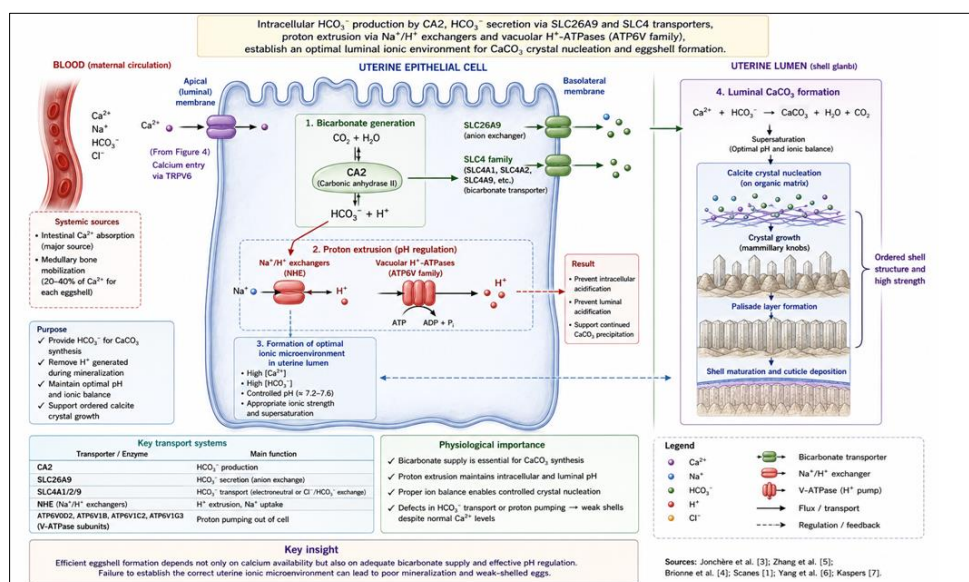


Fig 5: Bicarbonate generation, proton transport, and regulation of the uterine ionic microenvironment during eggshell biomineralization. This is a schematic which shows intracellular production of bicarbonate by carbonic anhydrase II (CA2), bicarbonate secretion via SLC26A9 and SLC4 transporters, and proton extrusion by Na⁺/H⁺ exchangers and members of the ATP6V family of vacuolar H⁺-ATPases. This leads to an optimal luminal ionic environment for nucleation of calcium carbonate crystals and eggshell formation.

Table 5: Major bicarbonate transporters and pH-regulatory proteins involved in uterine eggshell biomineralization.

Gene / Protein	Protein family	Primary physiological function	Functional significance during biomineralization	Representative references
CA2	Carbonic anhydrase	Catalyzes intracellular HCO_3^- production	Primary source of bicarbonate for CaCO_3 formation	[3]
SLC26A9	Anion transporter	HCO_3^- secretion into uterine lumen	Supplies bicarbonate for calcite crystal growth	[3]
SLC4A1	Bicarbonate transporter	HCO_3^- transport	Maintains luminal bicarbonate concentration	[4]
SLC4A2	Bicarbonate transporter	Acid-base regulation	Supports early shell calcification	[4]
SLC4A9	Bicarbonate transporter	HCO_3^- secretion	Associated with superior shell strength	[4]
ATP6V0D2	Vacuolar H^+ - ATPase	Proton extrusion	Prevents luminal acidification	[4]
ATP6V1B	Vacuolar H^+ - ATPase	Intracellular pH regulation	Maintains acid-base balance	[4]
ATP6V1C2	Vacuolar H^+ - ATPase	Proton transport	Supports crystal nucleation environment	[4]
ATP6V1G3	Vacuolar H^+ - ATPase	Proton pumping	Facilitates optimal mineralization pH	[4]

4.3 Matrix Proteins and Crystal Architecture

It is the organic eggshell matrix that provides the molecular framework through which nucleation, growth, orientation, and termination of calcite crystals are to be governed. In biomineralization, crystal formation is thus seen to be guided both spatially and temporally by the eggshell matrix. This matrix was considered merely as the scaffolding structure on which mineral deposition takes place passively; recent work has demonstrated its active participation in regulating crystal shape, kinetics of mineral deposition, ultrastructure of the shell, and mechanical properties of the mature eggshell [5, 4, 15]. Thus, eggshell quality results not only from the available minerals but also from an organized expression and secretion of matrix proteins over the entire process of calcification. Proteomic studies have shown that active shell formation leads to a very complex mixture of extracellular proteins present in the uterine fluid. Brionne *et al.* These authors identified 57 proteins secreted into the uterine fluid during the stage of calcification, most of them related to participation in the assembly of extracellular matrix, crystal nucleation, proteoglycan biosynthesis, molecular chaperoning, antimicrobial defense, and regulation of mineral deposition. These proteins are responsible for creating a dynamic molecular environment that controls both the initiation and subsequent maturation of calcite crystals.

The most frequently cited matrix protein in the literature as a major player in eggshell mineralization is ovocleidin-116 (OC-116). It is therefore assumed that ovocleidin-116 is first deposited in the mammillary layer as a structural template that will later facilitate appropriate nucleation of calcite crystals, their orientation, and uniformity of microarchitecture of the shell. High levels of expression of OC-116 have generally been

related to good shell organization and high mechanical strength. Osteopontin (SPP1) is mainly a negative regulator of calcium carbonate crystal growth. In fact, osteopontin was shown to specifically bind the surface of the growing calcite crystals in order to limit their overgrowth and reshape the morphology of the crystal by Brionne *et al.* This work was later confirmed by Zhang *et al.* [8] SPP1 was reported to be significantly higher in hens with weak-shelled eggs at the onset of calcification, a finding that strongly indicates excessive inhibitory controls during the formation of the mammillary layer, resulting in poorly organized crystals and weak shell [8].

Other matrix proteins include ovocalyxin-32 (OCX-32), ovocalyxin-36 (OCX-36), ovotransferrin, ovocleidin-17 (OC-17), clusterin, and several proteoglycans; they contribute to crystal stabilization, antimicrobial protection, extracellular matrix organization, and shell maturation [5, 23-25]. Even though they have different individual biological functions, these proteins maintain the balance between crystal nucleation, crystal growth, and matrix remodeling; thus, they ensure the formation of a mechanically competent eggshell. From the available evidence, one can therefore argue that the quality of the eggshell depends on a regulatory model wherein there is coordinated interaction between mineral transport and extracellular matrix proteins rather than calcium deposition alone. It is proposed that proper mechanical strength and integrity of the eggshell can only be attained when both crystalpromoting proteins (*e.g.*, OC-116 and OC-17) and crystal-modulating proteins (*e.g.*, SPP1) are precisely temporally expressed to allow for formation of an ordered mammillary layer and maintenance of appropriate crystal orientation [5, 4].

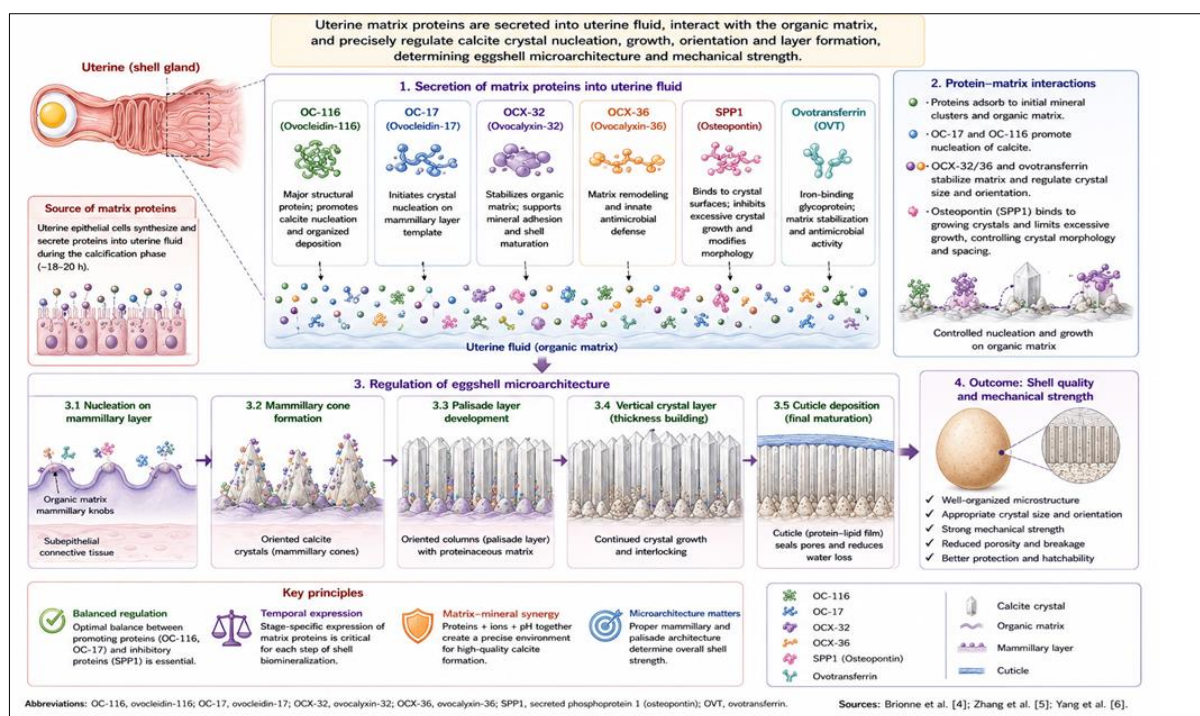


Fig 6: Schematic for matrix protein mediated regulation of calcite crystal nucleation and eggshell microarchitecture during uterine biomineralization. The schematic should represent the organic matrix protein secretion involving OC-116, OC-17, OCX-32, OCX-36, osteopontin, and Ovo transferrin, their interaction with the organic matrix, and regulation in the process of mammillary cone formation and crystal orientation with palisade layer development; leading to effects on the mechanical strength and ultrastructure of the eggshell.

Table 6: Principal eggshell matrix proteins involved in biomineralization and crystal architecture.

Matrix protein	Gene	Primary function	Effect on crystal architecture	Representative references
Ovocleidin-116	OC-116	Crystal nucleation and matrix organization	Promotes ordered calcite deposition	[5, 6]
Ovocleidin-17	OC-17	Initiation of crystal nucleation	Directs early calcite crystal formation	[5, 23]
Osteopontin	SPP1	Regulation of crystal growth	Inhibits excessive crystal growth; elevated in weak shells	[5, 4]
Ovocalsin-32	OCX-32	Matrix stabilization and antimicrobial activity	Supports shell maturation	[23, 24]
Ovocalsin-36	OCX-36	Matrix remodeling and innate immunity	Maintains shell integrity	[23, 24]
Ovo transferrin	TF	Iron-binding glycoprotein	Matrix stabilization and antimicrobial defense	[5, 24]
Proteoglycans	Multiple genes	Extracellular matrix assembly	Regulate crystal spacing and orientation	[5]
Molecular chaperones	Multiple genes	Protein folding and matrix assembly	Maintain proper matrix organization	[5]

5. Why Early Calcification Determines Final Shell Quality

Advances in transcriptomics and the study of eggshell ultrastructure have provided new insight into the biological determinants of shell quality. It is now believed that, rather than being formed progressively over the entire period of calcification, the earliest stages of uterine mineralization have the most significant effect on the structural integrity and mechanical properties of the mature eggshell [4, 15, 24]. In this context, it is proposed that the initiation phase of calcification should be considered a critical period during which the architecture of the shell is biologically programmed.

In a comparative uterine transcriptomic study, Zhang *et al.* [4] identified 1,777 differentially expressed genes (DEGs) during the initiation stage of calcification in hens that produce hard-shelled and weak-shelled eggs. The authors observed relatively

few differences in transcription between the subsequent rapid growth and termination phases of shell formation.

This suggests that most molecular mechanisms for quality determination are not primarily activated at the onset of biomineralization but much later during crystal thickening.

Ultrastructural analysis further supported these molecular findings. Hens that produce eggs with superior mechanical strength present significantly thinner mammillary layers, a smaller width of mammillary knobs, and a more organized transition between the mammillary and palisade layers. Since mammillary cones represent the primary nucleation sites for calcite crystals, the spatial organization of these structures will determine subsequent crystal orientation, as well as crystal fusion, and vertical column development throughout the palisade layer. Therefore, even minor irregularities established during the initial nucleation process shall be considered to

spread throughout the entire shell if not to reduce fracture resistance despite apparently normal shell thickness [4]. Transcriptomic evidence indicates that successful early calcification involves coactivation of multiple biological pathways, including calcium transport, secretion of bicarbonate, assembly of the extracellular matrix, regulation of acid-base, energy metabolism of mitochondria, organization of cytoskeletons, and differentiation of epithelial cells [3, 5, 4]. It suggests shell quality results from integrated regulation of mineral transport and crystal nucleation-not isolated changes in calcium availability. This was differentially expressed genes for bicarbonate transporters (SLCA1, SLCA2, and SLCA9), ATP6V family proton pumps, TRPV6 and CALB1 calcium transport proteins, and matrix proteins such as OC-116 and SPP1. These findings substantially change the classic concept of eggshell weakness in the second half of the laying period. Previously, low dietary calcium or an inability of the intestine to absorb calcium was mostly blamed for poor shell quality. However, molecular evidence at present indicates that shell

defects may occur due to disruption of the uterine microenvironment at the very early stage of calcification, including problems with crystal nucleation, extracellular matrix organization, bicarbonate transport, proton extrusion, or epithelial homeostasis. In such a situation, if the molecular machinery of biomineralization is already compromised, an increase in dietary calcium alone may not fully restore the quality of the shell. Thus, from a practical point of view, these results imply that the interventions aiming to sustain the physiological environment during the early stages of calcification, such as uterine ion transport optimization, and maintenance of acid-base balance, synthesis of matrix proteins, and nutritional strategies in precision, and genomic selection for biomineralization-related genes are likely to be more effective than those that focus primarily on calcium intake. Therefore, the initiation phase of eggshell calcification should be considered the main biological target for future genetic, nutritional, and management strategies aimed at improving shell quality and extending laying persistency in modern commercial hens [4, 15, 24].

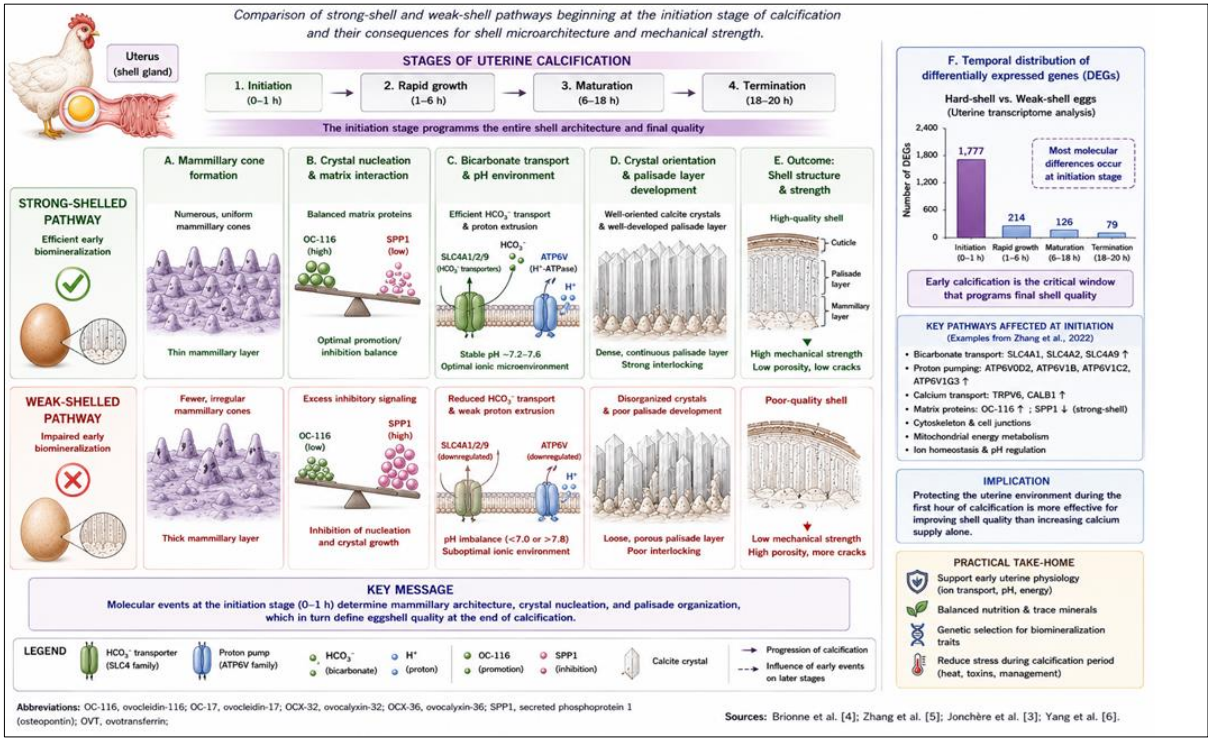


Fig 7: Early calcification as the critical determinant of eggshell quality in commercial laying hens. The figure should depict pathways for strong-shell and weak-shell comparison from the initiation stage of calcification. It should show differences in mammillary cone formation, crystal nucleation, bicarbonate transport, matrix protein expression (OC-116 vs. SPP1), crystal orientation, palisade layer development, and shell mechanical strength. A side panel may summarize the temporal distribution of differentially expressed genes (1,777 DEGs during initiation versus few during later calcification stages), framing the dominant influence of early biomineralization on final shell quality. Table 7. Biological characteristics distinguishing early calcification in strong- and weakshelled eggs.

Table 7: Biological characteristics distinguishing early calcification in strong- and weak-shelled eggs.

Characteristic	Strong-shelled eggs	Weak-shelled eggs	Biological consequence	Representative references
Differential gene expression	Extensive activation during initiation	Reduced or dysregulated expression	Altered biomineralization pathways	[4]
Mammillary layer thickness	Thinner, well organized	Thicker, irregular	Lower fracture resistance	[4]
Mammillary knob width	Smaller and uniform	Larger and variable	Poor crystal fusion	[4]
Crystal nucleation	Efficient and synchronized	Delayed or disorganized	Reduced shell strength	[5, 4]
Matrix protein regulation	Balanced OC-116 and SPP1 expression	Increased inhibitory signaling (SPP1)	Abnormal crystal architecture	[5, 4]

Bicarbonate transport	Efficient	Reduced	Suboptimal ionic microenvironment	[3, 4]
Final shell quality	High mechanical	Increased brittleness and	Economic losses	[4]

6. Genetics, Modern Layer Strains, and Long-Life Shell Efficiency

The remarkable increase in egg production achieved over the past few decades has been due to genetic selection for egg number, feed efficiency, sexual maturity, and laying persistency. Modern commercial layers can maintain high production rates for over 90-100 weeks, with some breeding programmers aiming at cumulative production approaching 500 eggs per hen housed [6, 9, 25]. This unprecedented reproductive performance has, however, put increased physiological pressure on calcium metabolism, skeletal integrity, uterine function, and eggshell biomineralization. Long-term shell quality has thus become one of the major constraints in prolonged laying cycles [6, 25].

Phenotypic selection for shell thickness, shell breaking strength, shell weight, and specific gravity was the primary focus of traditional breeding programmers. Success was achieved in the improvement of shell quality by these methods; however, very little information was attained regarding the intricate molecular mechanisms that control biomineralization. Genomics, transcriptomics, proteomics, metabolomics, and systems biology have redefined poultry breeding by showing that eggshell quality is determined by the coordinated action of biological pathways, rather than by individual genes acting independently [26].

Yang *et al.* [6] stressed that quality of shell is regulated by interconnected functions involving calcium transport, bicarbonate metabolism, assembly of extracellular matrix, endocrine regulation, immune function, responses to oxidative stress, metabolism of mitochondria, and differentiation of epithelium in the uterus. As a result, the selection of the pathway at the genomic level has become more important than the candidate genes.

Some other genes have been identified that play a key role in the regulation of eggshell mineralization. These genes are responsible for calcium transportation (*TRPV6*, *CALB1*, and *ATP2B1*), production of bicarbonates (*CA2*, *SLC26A9*, and *SLC*family), and formation of the extracellular matrix (*OC-116*, *OC-17*, *SPP1*, *OCX-32*, and *OCX-36*) [3, 5, 4, 6]. These same genes have also been linked with strength, thickness, organization of crystals, and efficiency in uterine calcification. More candidate genes that indirectly affect persistency of laying and quality of shell by regulating the follicular hierarchy, steroidogenesis, hypothalamic–pituitary–gonadal (HPG) [3] [5] [4] signaling, lipid metabolism, mitochondrial function, and innate immunity are also included [6, 27].

Genetic studies also reveal that eggshell quality has moderate-to-high heritability, though estimates of heritability vary among commercial populations based on breed, age, management system, and environmental condition [28]. The importance of genotype-by-environment interactions increases during late lay, since, at this stage, age-related deterioration in uterine function and calcium homeostasis may undermine shell quality even when egg production is sustained at high levels. Thus, breeding strategies for extended laying cycles must also consider the preservation of reproductive longevity and skeletal health, as well as the biomineralization capacity of the uterus.

Long-life commercial laying strains are one of the most interesting challenges in contemporary poultry breeding. The ability to support optimal shell quality over production periods approaching 100 weeks reflects sustained physiological coordination among calcium metabolism, bone remodeling, endocrine regulation, ion transport, extracellular matrix synthesis, and mitochondrial energy production [25]. Thus, failure of any one biological system may accelerate shell deterioration during late lay even when egg production remains relatively stable.

This has also recently been further shown by the latest advance in genomic prediction, that marker effects may differ much among chicken populations, that it is not possible to apply individual candidate genes universally [6]. Therefore, population-specific genomic evaluation, GWAS, whole-genome sequencing, GEBVs, and multitopic integration are growing in importance for commercial breeding programs to enhance the accuracy of predictions in the selection index [29, 28, 27, 26].

Altogether, the current evidence indicates that future breeding for long-life shell efficiency should give top priority to pathway resilience rather than single-gene effects. In this regard, selection programs should aim at ensuring the coordinated functions of the hypothalamic–pituitary–gonadal axis and uterine ion transport, bicarbonate metabolism, extracellular matrix assembly, bone turnover, mitochondrial metabolism, oxidative stress resistance, and clutch stability over extended laying cycles. System-based breeding strategies of this nature are expected to improve eggshell quality, reproductive longevity, and production efficiency in next-generation commercial laying hens.

7. Practical Strategies to Improve Eggshell Efficiency in Commercial Layers

7.1 Precision Calcium Supply and Circadian Nutrition

Since eggshell calcification follows a clearly defined circadian rhythm, nutritional interventions are most effective when synchronized with the physiological pattern of shell formation in the hen rather than being uniformly supplied throughout the day [9] [8] [9] [8]. Approximately two-thirds of shell calcification occurs during the dark period, when uterine calcium demand is at its maximum [7]. Therefore, the timing of calcium availability has become more and more important as a determinant of eggshell efficiency in modern commercial laying hens [8].

Akter *et al.* [8] reported that the afternoon drinking water supplemented calcium and vitamin D as an alternative to the high-calcium feeding program. In a 10-week trial, hens on a basal diet with 4.1% calcium and targeted afternoon supplementation attained laying performance similar to that of birds on a conventional 4.5% calcium diet [8]. This also raised serum vitamin D levels significantly, improved ileal energy digestibility, reduced water intake, and enhanced feather condition- all signs of better nutrient use and general physiological condition [8]. These results imply that it is possible to synchronize calcium delivery according to the period of maximum shell mineralization so that calcium utilization is optimized and undesirable morning calcium intake is avoided [8].

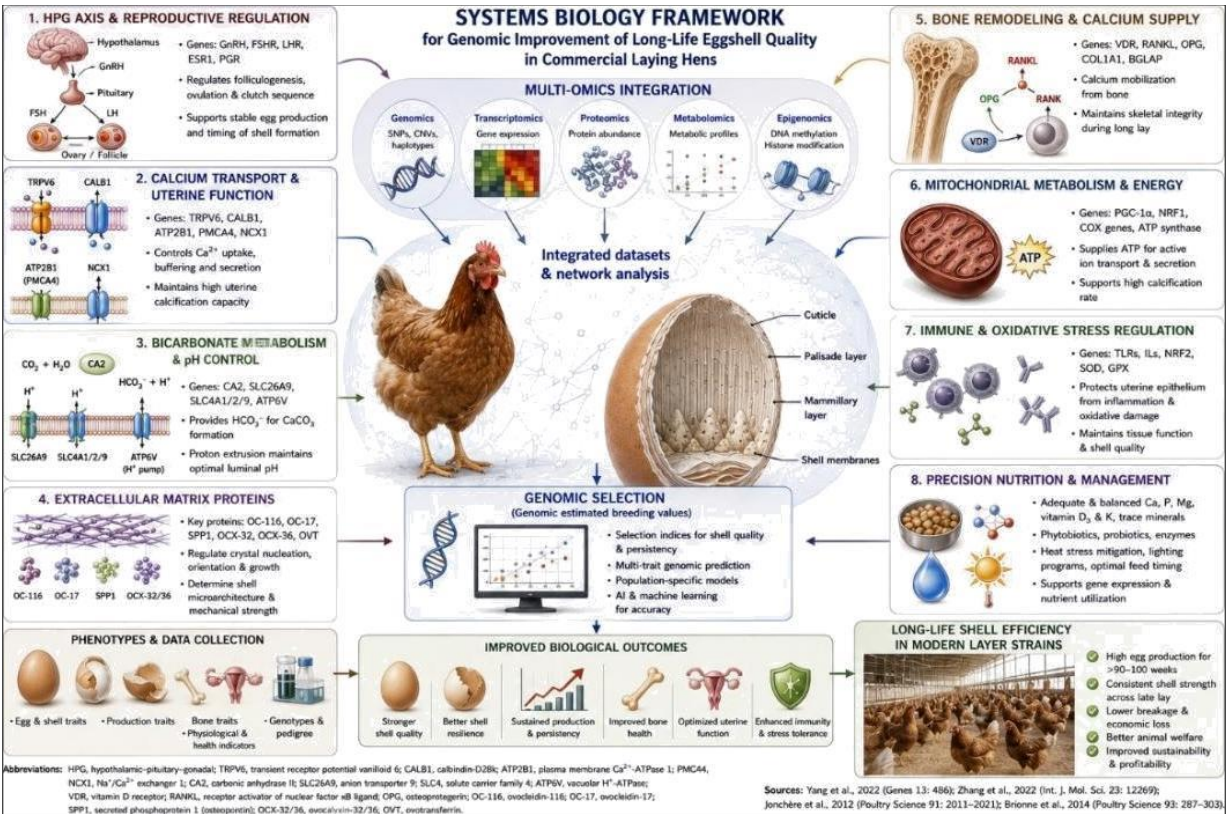


Fig 8: Systems biology framework for genomic improvement of long-life eggshell quality in commercial laying hens. This figure shows a schematic representation of how genomic selection improves eggshell quality and laying persistency in modern commercial strains by integrating many biological pathways, including the HPG axis, calcium transport, and bicarbonate metabolism, extracellular matrix proteins, bone remodeling, mitochondrial metabolism, and immune regulation, as well as precision nutrition. A large

panel in the center should portray multilayer omics (genomics, transcriptomics, proteomics, metabolomics, and epigenomics) converging to genomic selection and enhanced shell efficiency during long life.

Table 8: Major genetic pathways contributing to long-life eggshell quality in commercial laying hens.

Biological pathway	Representative genes	Primary physiological role	Contribution to shell quality	References
Calcium transport	TRPV6, CALB1, ATP2B1	Ca ²⁺ transport and secretion	Supports continuous mineralization	[3, 6]
Bicarbonate metabolism	CA2, SLC26A9, SLC4A1/2/9	HCO ₃ ⁻ production and transport	Maintains optimal calcification environment	[3, 4]
Matrix proteins	OC-116, OC-17, SPP1, OCX-32, OCX-36	Crystal nucleation and shell architecture	Determines shell ultrastructure	[5, 4, 6]
HPG-axis regulation	GnRH, FSHR, LHR, ESR1, PGR	Endocrine regulation of ovulation	Maintains clutch sequence	[1, 6]
Bone metabolism	VDR, RANKL, OPG	Calcium mobilization and skeletal homeostasis	Sustains calcium availability	[6, 25]
Mitochondrial metabolism	Multiple genes	ATP production	Supports active ion transport	[4, 6]
Oxidative stress defense	NRF2 pathway, antioxidant enzymes	Cellular protection	Preserves uterine epithelial function	[6]

7.2 Split-Feeding Strategies

The split-feeding systems exploit the fact that there are marked changes in nutrient requirements throughout the ovulatory-ovipositional cycle. In the morning, hens will have high protein and metabolizable energy requirements to support follicular growth, yolk deposition, and albumen synthesis; calcium requirement will be high in the afternoon and night when uterine shell calcification is most active. Feeding a high-protein, high-energy, lower-calcium diet in the morning, followed by a lower-protein, lower-energy, calcium-enriched diet in the afternoon, was also the practice that significantly improved calcium digestibility while keeping eggshell quality, as obtained by Akter *et al.* [9]. This proposal of feeding synchronizes nutrient supply with physiological demand, thereby enhancing nutrient-use efficiency without

raising total dietary calcium intake, as earlier reported by other workers [9] [8] [9] [8].

7.3 Vitamin D Optimization

Efficient eggshell formation is impossible without vitamin D because it controls calcium absorption in the intestines, medullary bone mobilization, renal conservation of calcium, and uterine transport of calcium [8]. Biologically active vitamin D increases the expression of calcium transport proteins including *TRPV6*, *CALB1*, and plasma membrane calcium ATPases bringing calcium to the bird's shell gland for shell biomineralization [3]. Therefore, a combination of calcium and vitamin D generally works better than increased dietary calcium alone. This is especially true in old laying hens, where calcium absorption efficiency gradually falls [6, 8].

7.4 Gut Health and Probiotic Supplementation

Another approach would be enhancing eggshell quality through improving intestinal health since calcium utilization is directly linked to gastrointestinal function and nutrient absorption. Probiotic supplementation has been reported to improve intestinal morphology as well as stabilizing gut microbiota and increasing mineral digestibility, indirectly supporting shell mineralization.

Onbaşilar *et al.* [9] stated that drinking water supplemented with probiotics increased the Haugh unit from 84.91 to 87.66, shell thickness from 378.38 to 386.48 μm , and shell breaking strength from 3.60 to 3.87 kg/cm^2 . Since the Haugh unit is mainly related to the quality of the contents of the egg rather than the shell, the simultaneous change of these three measurements indicates that

intestinal physiology can be optimized to positively affect more than one characteristic of egg quality [9].

7.5 Practical Implications for Commercial Egg Production

The above studies proved that the deposition of more efficient eggshells does not just involve increasing the concentration of dietary calcium [9] [8]. The most successful nutritional programs will, therefore, avail calcium when there is maximal uterine demand, optimize vitamin D status, improve intestinal nutrient utilization, and synchronize feeding time with the hen's circadian physiology [9, 8, 7]. Such integrated management strategies are particularly valuable for modern long-life laying strains because they have made the maintenance of shell quality during late lay a major breeding and production objective [6].

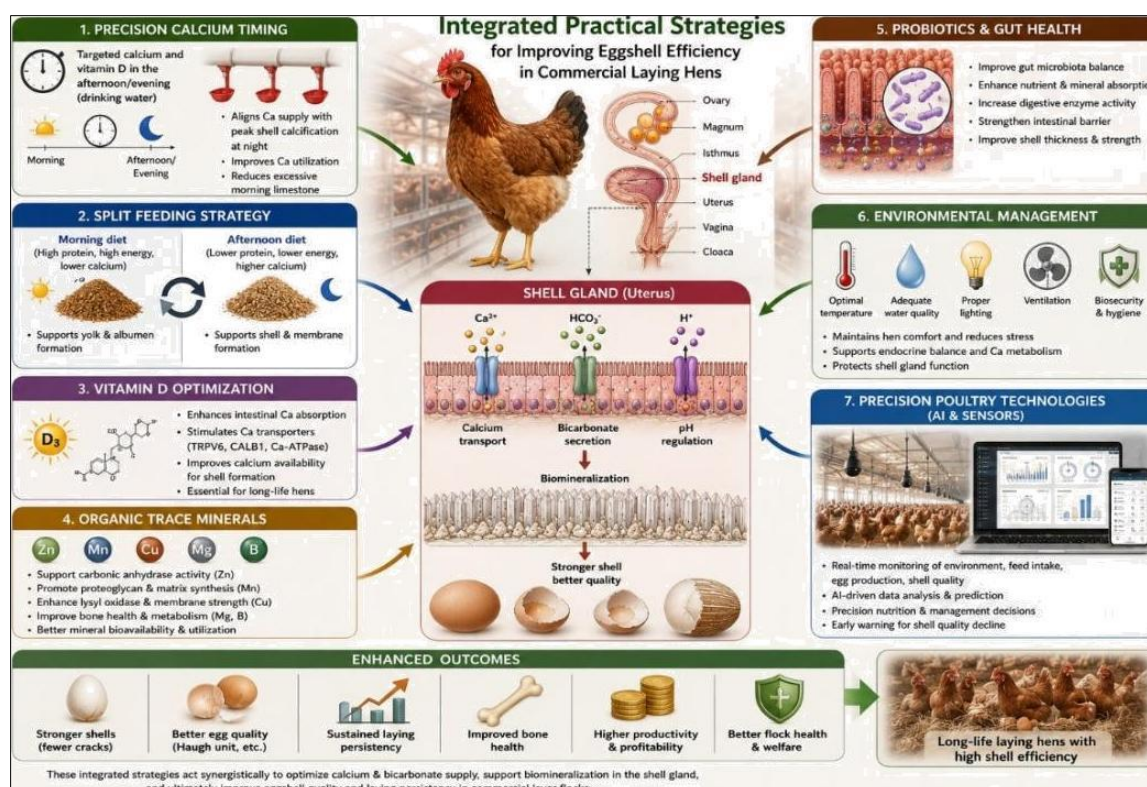


Fig 9: Integrated practical strategies for improving eggshell efficiency in commercial laying hens. Integrated practical strategies for improving eggshell efficiency in commercial laying hens.

Table 9: Practical nutritional strategies for improving eggshell efficiency in commercial laying hens. Practical nutritional strategies for improving eggshell efficiency in commercial laying hens.

Strategy	Primary mechanism	Major physiological effect	Expected outcome	References
Afternoon/evening calcium supplementation	Synchronizes calcium supply with uterine calcification	Improved calcium utilization	Increased shell thickness and shell strength	[8]
Split-feeding system	Matches nutrient supply with circadian physiological demand	Improved calcium digestibility	Maintained shell quality during extended lay	[9]
Vitamin D supplementation	Enhances calcium absorption and transport	Increased calcium bioavailability	Improved shell mineralization	[6, 8]
Probiotic supplementation	Improves gut health and mineral absorption	Enhanced nutrient utilization	Increased shell thickness and breaking strength	[40]

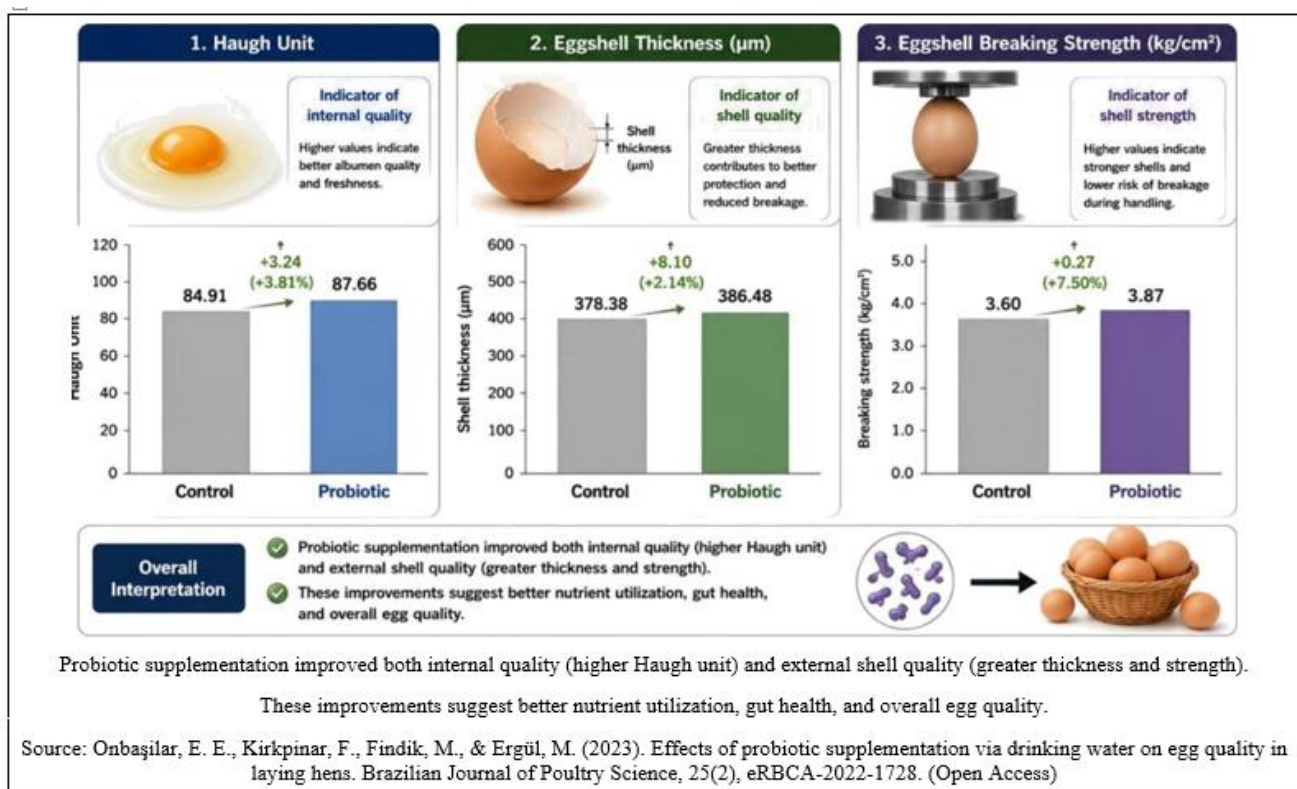


Fig 10: Comparative effects of probiotic supplementation on selected egg-quality traits in commercial laying hens. Comparative numerical illustration of selected egg-quality parameters in control and probiotic-supplemented laying hens using open-access data reported by Onbaşilar *et al.* [9]. Values include Haugh unit (84.91 vs. 87.66), eggshell thickness (378.38 vs. 386.48 μm), and eggshell breaking strength (3.60 vs. 3.87 kg/cm^2). It can be observed from the figure that both internal and external egg-quality characteristics improved together after probiotic supplementation. It serves as a quick visual summary of the experimental findings reported.

8. Future Perspectives

It is anticipated that the future of eggshell research will shift from single physiological mechanisms to integrate systems that encompass endocrinology, molecular biology, nutrition, genetics, and precision livestock management [1, 5, 6]. As commercial laying hens are continually selected for production cycles that exceed 100 weeks, maintaining shell quality throughout the extended laying period will become increasingly more challenging for both breeding programs and commercial egg production [6].

A major research priority will be to improve our understanding of the temporal coordination between ovulation, oviposition, and uterine biomineralization. Considerable progress has been made in the characterization of the hormonal regulation of the hypothalamic-pituitary-gonadal axis, but important questions remain regarding ovary-oviduct communication, circadian synchronization, and endocrine regulation of shell gland activity during prolonged laying cycles [1, 6]. Future investigations that integrate chronobiology with reproductive endocrinology may identify new physiological targets for improving shell formation efficiency.

Another area of rapid advancement is in the field of multi-omics technologies, which include genomics, transcriptomics, proteomics, metabolomics, epigenomics, and single-cell sequencing. These have been used to complement each other, and in doing so, they have proven that eggshell quality is not regulated by one or a few candidate genes but rather by very complex networks that include calcium transport, bicarbonate secretion, extracellular matrix assembly, mitochondrial metabolism, response to oxidative stress, regulation of immunity, and homeostasis of the epithelium. Systems biology and network analysis are thus projected to play ever-increasing

roles in the identification of biomarkers linked to not only superior shell quality but also laying persistency.

The use of artificial intelligence and precision technologies in poultry farming is expected to change commercial eggshell management as well [6]. Automated environmental sensors, computer vision systems, real-time production monitoring, and machine-learning algorithms can constantly assess egg production as well as shell quality and even compare feed intake by the hens to environmental conditions and flock health, so that producers can implement precision nutritional and management interventions before measurable shell deterioration sets in [6], and thus, significantly improve prediction accuracy for shell defects while enhancing production efficiency and animal welfare.

It is expected that future nutritional strategies would shift from fixed diet formulation to dynamic precision feeding systems wherein calcium, vitamin D, phosphorus, and other necessary nutrients would be provided at the physiological stage of shell formation. Automatic feeding technologies could be integrated with sensor-based monitoring and predictive algorithms to optimize nutrient utilization, reduce unnecessary mineral excretion, cut production costs, and improve environmental sustainability without compromising shell quality. The reference list is as mentioned in the original article [9] [8] [9] [8].

Genomic selection is also projected to make the transition from conventional selection based on phenotypic performance to genome-assisted breeding, genomic estimated breeding values (GEBVs), and pathway-based selection facilitated by artificial intelligence and systems biology [6]. Future breeding programs are expected to prioritize biological resilience, uterine functional capacity, skeletal integrity, and persistent shell

quality throughout extended laying cycles rather than early egg production as it was in the past ^[6].

Perhaps, the major advancements in eggshell research will be achieved when endocrinologists, chronicle uterine physiologists, and those undertaking multi-omics studies, precision nutritionists, digital phenotypes, and genomic selectors finally get their acts together as a big family of precision production. Such interdisciplinary approaches are anticipated to improve eggshell quality, as well as laying persistency, while reducing the economic losses linked to shell defects and promoting more sustainable commercial egg production in next-generation laying hens ^[1, 5, 6, 8, 9].

9. Conclusion

Sequential oviposition in modern laying hens demonstrates the operation of an integrated biological system in endocrine regulation, follicular development, oviductal transport, ion regulation in the uterus, and biomineralization of the eggshell. In this integrated reproductive framework, the shell gland is deemed the primary functional center for shell formation due to its activities in calcium transport and bicarbonate secretion with pH regulation and extracellular matrix assembly; together with calcite crystal growth, they define the structural integrity and mechanical strength of the eggshell.

Current evidence indicates that the quality of the shell is determined by gene networks and not by single genes. Good mineralization of the shell in quality shells implies integrated actions of calcium homeostasis, bicarbonate metabolism, matrix proteins, mitochondrial function, immune regulation, and integrity of the epithelium. Also, the initial phase of shell calcification can be considered as a critical period during which the mamillary layer is formed and the crystalline structure is organized to provide a foundation for the final quality of the shell.

Since the genetic selection programs for commercial laying hens have been directed toward increasing the length of productive life, eggshell quality in late lay has become a major problem. This would require an integrated approach in advancing our knowledge of reproductive physiology, molecular biology, genomics, and nutrition. In practice, such strategies as precision calcium supplementation, split-feeding systems, vitamin D optimization, improved gut health, and environmental management can be implemented to improve shell quality while persistency and sound skeletal condition can be maintained.

The next breakthroughs in eggshell quality will result from successful integration of all these measures into an overall production system on an interdisciplinary and holistic basis. Systems biology, multi-omics technologies, genomic selection, artificial intelligence, precision nutrition, and digital poultry management are the next breakthroughs in eggshell quality. This will lead to better laying persistence and production efficiency with enhanced animal welfare under conditions of sustainability in commercial egg production for the next generation of high-performing laying hens.

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