Effects of Mineral Elements and Vitamins on Eggshell Color and Its Possible Mechanisms: Postprint

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Abstract

Eggshell color is not directly related to the nutritional content of the egg itself; however, it influences egg marketability and can partially reflect the health status of laying hens and egg quality. Eggshell pigments are primarily composed of three components: protoporphyrin IX, biliverdin, and their chelates. Although eggshell color exhibits high heritability and is regulated by multiple genes, feed nutrients can also influence eggshell color. This paper describes the formation process of eggshell pigments, reviews the effects of certain minerals, vitamins, and other nutrients on eggshell color, and analyzes their potential mechanisms, thereby providing insights for further research on the mechanisms underlying eggshell pigment formation and variation, as well as for regulating eggshell color through micronutrients.

Full Text

Review on the Effects and Possible Mechanisms of Mineral Elements and Vitamins on Eggshell Color

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Abstract: Although there is no direct relationship between eggshell color and the nutritional value of eggs, eggshell color can influence egg marketing and, to some extent, reflect the health status of laying hens and egg quality. Eggshell pigments are primarily composed of three components: protoporphyrin , biliverdin, and its chelate. While eggshell color exhibits high heritability and is regulated by multiple genes, feed nutrients can also affect eggshell color. This review introduces the formation process of eggshell pigments and summarizes the effects of certain mineral elements and vitamins on eggshell color, analyzing their possible mechanisms. This provides insights for further research on the formation and alteration mechanisms of eggshell pigments and for regulating eggshell color through micronutrient supplementation.

Keywords: eggshell color; mineral elements; vitamins; protoporphyrin ; heme; mechanism

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Eggshell color is not directly related to intrinsic egg quality or the nutritional value of the egg itself. However, changes in eggshell color and gloss can indirectly reflect the health status of laying hens and their egg quality, serving as the most intuitive indicator for consumers to evaluate egg quality and make purchasing decisions. Eggshell color has high heritability (0.58-0.76) [1], but in production, laying hens are susceptible to various stressors, such as excess or deficiency of certain nutrients and environmental changes, which can lead to decreased production performance, reduced egg quality, lighter eggshell color, and compromised hen health [2]. Lighter eggshell color directly affects egg appearance, reduces sales price and profit, and hinders the development of the egg production industry.

1 Formation of Eggshell Pigments

Eggshell pigments are mainly composed of three components: protoporphyrin (C H N O), biliverdin (C H O N), and biliverdin zinc chelate [3-4]. Protoporphyrin produces brown, yellow, or pink colors, while biliverdin and its zinc chelate produce green. These three components combine in different proportions to create various colors [5]. The pigment in brown eggshells is primarily protoporphyrin , whereas the pigment in green eggshells is mainly biliverdin.

Eggshell pigments are deposited on the eggshell. Except for the yolk, all other parts of the egg form in the oviduct: the magnum secretes albumen, the isthmus forms the shell membranes, and the shell forms in the uterus. During the final stage of shell formation, pigments secreted by the shell gland epithelial cells are primarily deposited on the eggshell surface. The eggshell structure consists of six layers, from inner to outer: inner shell membrane, outer shell membrane, mammillary layer, palisade layer, vertical crystal layer, and cuticle [6]. Eggshell pigments are mainly deposited in the cuticle layer.

Eggshell pigments are closely related to heme. Heme synthesis requires protoporphyrin , and biliverdin can be formed from heme degradation. Heme oxygenase (HO) is the key enzyme in heme catabolism, functioning to decompose heme into biliverdin, ferrous iron (Fe^{2}), and carbon monoxide (CO) [7]. The heme synthesis pathway is divided into four processes [8]: formation of the pyrrole ring, assembly of tetrapyrrole, modification of tetrapyrrole side chains, and oxidation of protoporphyrinogen to protoporphyrin and its chelation with Fe^{2} , as shown in Figure 1 [Figure 1: see original paper] [9].

In the first process of the heme synthesis pathway, -aminolevulinic acid (ALA) is synthesized from succinyl-CoA and glycine via condensation in mitochondria, catalyzed by -aminolevulinic acid synthase (ALAS). ALAS is a crucial enzyme that limits the overall efficiency of heme synthesis, and its activity is subject to inhibitory feedback regulation by heme levels in the environment [10]. The synthesized ALA is then transported to the cytosol. Once in the cytosol, two molecules of ALA are dehydrated and condensed into one molecule of porphobilinogen (PBG) under the catalysis of -aminolevulinic acid dehydratase (ALAD), forming the pyrrole ring structure. The second process occurs in the cytosol, where four molecules of PBG are condensed to form hydroxymethylbilane (HMB), creating an unstable tetrapyrrole polymer, under the action of porphobilinogen deaminase (PBGD). In the third process, HMB is converted to uroporphyrinogen (UROgen) by uroporphyrinogen synthase (UROS), which inverts the D ring and closes the tetrapyrrole macrocycle. UROgen is then decarboxylated by uroporphyrinogen decarboxylase, sequentially removing four carboxyl groups from the acetic acid side chains to form coproporphyrinogen (CPgen). The CPgen generated in the cytosol is transported into mitochondria and converted to protoporphyrinogen by coproporphyrinogen oxidase (CPOX). The fourth process involves oxidation catalyzed by protoporphyrinogen oxidase (PPOX) to generate protoporphyrin . Finally, ferrochelatase (FECH) catalyzes the binding of one Fe^{2} to protoporphyrin to synthesize heme.

However, the formation mechanism of protoporphyrin is not yet fully understood. Protoporphyrin is synthesized from ALA. In the heme synthesis pathway, protoporphyrin is generated from protoporphyrinogen through oxidation by PPOX, which removes six hydrogen atoms. Research indicates that protoporphyrin production is oxygen-dependent; protoporphyrinogen can only be oxidized to protoporphyrin in the presence of oxygen, and within a certain range, the synthesis rate of protoporphyrin increases with oxygen content [11].

In mammalian cells, the key enzyme for protoporphyrin formation is PPOX. However, PPOX has not been identified in avian species; instead, protoporphyrinogen is oxidized to protoporphyrin through interaction with oxygen [12]. Sparks et al. [13] speculated that colorless protoporphyrinogen synthesized by tubular gland cells in the uterine lamina propria is transferred to epithelial cells, where it undergoes auto-oxidation to form colored protoporphyrin that accumulates in the shell gland. Therefore, the presence of oxygen may be a critical factor for protoporphyrin formation in the shell gland. Li et al. [14] discovered through transcriptome sequencing and proteomics that differentially expressed transcripts and proteins between dark brown and light brown eggshell groups were both involved in oxidative phosphorylation, a process that consumes oxygen. In the shell glands of light brown eggshell layers, four differentially expressed transcripts and fourteen differentially expressed proteins were upregulated, indicating higher oxidative phosphorylation levels and thus greater oxygen consumption. This may reduce intracellular oxygen content, thereby inhibiting the conversion of protoporphyrinogen to protoporphyrin and resulting in lighter eggshell color. Consequently, intracellular oxygen content affects protoporphyrin generation, but excessive free radicals produced by redox reactions in the body can cause oxidative stress, which impacts eggshell color.

The mechanism of biliverdin formation is relatively well understood and is regulated by the major gene SLC01B3. Wang et al. [15] reported that in the shell glands of green eggshell layers, a complete insertion of an EAV-HP endogenous virus element into the 5' flanking region of the organic anion transporter family member SLC01B3 gene enables it to transport biliverdin, which is deposited on the eggshell surface to form green eggs.

2 Effects of Certain Micronutrients on Eggshell Color

The formation and deposition of eggshell pigments are determined by multiple genes and exhibit high heritability (0.58–0.76) [1], while also being influenced by many factors. Among these, dietary nutrients can affect eggshell color, and in practice, eggshell color often changes with dietary modifications. Iron participates in heme formation; vanadium induces oxidative stress and apoptosis; vitamins are essential for maintaining epithelial cell integrity and antioxidant function, playing important roles in protoporphyrin and heme generation. Moreover, mineral elements and vitamins can have synergistic or antagonistic interactions. Therefore, the levels of certain micronutrients in the diet can influence eggshell color changes.

2.1.1 Iron

Iron participates in heme formation, and supplementing with appropriate amounts of iron can improve eggshell color. Seo et al. [16] significantly increased eggshell color by adding 100 mg/kg of iron-soy proteinate to the diet, with a concurrent increase in plasma hemoglobin. Park et al. [17] found that adding 100 mg/kg of ferrous sulfate and iron-methionine chelate to the diet also significantly increased eggshell color. Paik et al. [18] similarly observed that adding 100 mg/kg of organic iron to the diet significantly improved pigment deposition on eggshells. However, Yuan et al. [19] found no significant difference in eggshell color when adding 80 mg/kg iron compared to 60 mg/kg iron, possibly due to insufficient dosage to alter eggshell color and the relatively short experimental period. Additionally, interactions among mineral elements can have an impact. For instance, iron utilization requires the presence of copper; iron or copper deficiency can cause nutritional anemia in layers, but high doses of copper can affect iron absorption, potentially lightening eggshell color. Iron also has an antagonistic relationship with phosphorus; excessive dietary iron can reduce phosphorus absorption in the gastrointestinal tract, causing calcium-phosphorus imbalance that may hinder shell formation and affect eggshell color [20].

The effect of iron on eggshell color may be achieved through two pathways: influencing protoporphyrin /heme synthesis and transport. Protoporphyrin and Fe^2 generate heme via PPOX in mitochondria, which is a critical step limiting the overall efficiency of heme synthesis. Protoporphyrin may originate from the blood; increased dietary iron promotes erythropoiesis and is important for heme generation. Furthermore, as Fe^2 is a raw material for heme synthesis, increased iron in mitochondria leads to more heme production, which requires more protoporphyrin . If intracellular Fe^2 concentration is insufficient, heme synthesis will be impaired, affecting protoporphyrin generation [21].

Heme synthesis occurs jointly in both the cytosol and mitochondria. Proteins on the mitochondrial membrane are crucial for the transport of iron, heme, and protoporphyrin . Li et al. [14] identified differentially expressed proteins that are primarily mitochondrial membrane proteins: translocator protein (TSPO), transferrin (TF), adenine nucleotide translocator (ANT2), and iron-sulfur protein assembly factor (Iba57). TSPO and ANT2 transport heme and protoporphyrin , respectively; TF primarily transports iron ions into mitochondria; and Iba57 forms iron-sulfur clusters from Fe^2 , which acts on succinyl-CoA, a raw material for heme synthesis. Therefore, iron is important for transporter proteins on the mitochondrial membrane, thereby affecting protoporphyrin and heme synthesis and transport. The concentration of iron ions in mitochondria will influence the efficiency of protoporphyrin /heme synthesis and its transport, consequently affecting protoporphyrin accumulation in the shell gland.

2.1.2 Vanadium

Vanadium is one of the essential trace elements in animals, participating in the metabolism of the three major nutrients and maintaining growth and development. Excessive vanadium intake affects pigment deposition, resulting in whiter eggshell color. Vanadium in layer diets mainly comes from dicalcium phosphate. Sullivan et al. [22] reported vanadium levels of 36–185 mg/kg in several feed-grade phosphates. Huang [23] also reported vanadium content of 10–100 mg/kg in feed-grade dicalcium phosphate. Henry et al. [24] indicated that the tolerance level of vanadium for layers is 10 mg/kg. When high-quality dicalcium phosphate is used, corn-soybean meal diets contain less than 5 mg/kg vanadium. However, typical dicalcium phosphate can easily cause excessive vanadium levels in layer diets (with phosphate supplementation at approximately 1.5%). Odabaşı et al. [25] found that adding 15 mg/kg or more vanadium to the diet lightened eggshell color. Yuan et al. [26] also observed that adding 5 mg/kg or more vanadium significantly decreased eggshell redness (a*) and yellowness (b*) values while increasing lightness (L*), resulting in noticeably lighter eggshell color. This demonstrates the bleaching effect of vanadium on eggshell color.

The bleaching effect of vanadium may occur through oxidative stress and apoptosis in uterine epithelial cells, impairing the structural and functional integrity of the shell gland, weakening protoporphyrin synthesis and secretion, and consequently causing whiter eggshells [26].

However, there are two hypotheses regarding the synthesis site of protoporphyrin : in the blood or in the shell gland. Li et al. [27] compared the expression levels of key genes for heme synthesis and transport in the shell glands and livers of dark brown and light brown eggshell layers. They found that expression levels of ALAS, CPOX, and ATP-binding cassette transporters (ABCB6, ABCB7, and ABCG2) in the shell gland were higher than those in the liver for both groups, while only ALAS expression in the liver was significantly higher in the dark brown group. ALAS gene expression in both liver and shell gland was significantly higher in dark brown layers compared to light brown layers. Furthermore, protoporphyrin content in eggshells and shell glands was significantly higher in dark brown layers than in light brown layers, but showed no significant differences in serum, bile, or feces, indicating that protoporphyrin is synthesized and accumulated in the shell gland. Zhou [28] also reported that protoporphyrin is synthesized by shell gland epithelial cells rather than derived from hemoglobin degradation. If protoporphyrin were synthesized in the blood, hypoxia would stimulate erythropoietin secretion in the kidneys, increasing red blood cells and thus heme synthesis, which would darken eggshell color. This contradicts the finding by Li et al. [14] that lighter brown eggshell color may result from oxygen deficiency. Therefore, protoporphyrin in eggshell pigments is most likely synthesized in the shell gland. Consequently, damage to shell gland structure and function would hinder protoporphyrin synthesis. Yuan et al. [26] indeed found that adding 10 mg/kg vanadium to the diet caused oxidative stress and apoptosis in uterine epithelial cells, destroying shell gland structure and function, weakening protoporphyrin synthesis and secretion, and resulting in lighter eggshell color.

Additionally, vanadium disrupts the redox balance, causing oxidative stress that reduces HO activity, decreasing biliverdin production from heme via HO. This may lead to excessive heme accumulation in mitochondria, impairing heme synthesis and consequently reducing protoporphyrin synthesis, thereby lightening eggshell color.

2.1.3 Magnesium

Eggshells contain certain amounts of magnesium. Seo et al. [16] reported that adding 3 g/kg magnesium oxide to the diet had no significant effect on eggshell L, *a*, or b* values. Kim et al. [29] reported that as dietary magnesium increased to 3 g/kg, eggshell a* and L* values decreased linearly, while b* values also showed a decreasing trend, though all L, *a*, and b* values remained within the normal eggshell color range. This suggests that higher dietary magnesium content can lighten eggshell color. However, there is currently no direct evidence that increased serum magnesium from higher intake affects red blood cell catabolism in the shell gland, thereby altering pigment deposition. This effect may be due to the deposition of magnesium itself, a white element, in the eggshell, causing slight lightening. This indicates that magnesium does not affect eggshell color by influencing pigment formation and deposition.

2.2 Effects of Vitamins on Eggshell Color

In addition to mineral elements, certain vitamins also affect eggshell color, such as vitamin A, which is essential for maintaining uterine epithelial cell structure and function integrity. Some reports indicate that adding 300 mg/kg of multivitamins to the diet tends to improve eggshell color, though long-term supplementation with 400 mg/kg shows no significant effect [30].

2.2.1 Vitamin A Vitamin A is necessary for maintaining the integrity of all epithelial tissues and is related to the development and integrity of reproductive tract mucosal epithelium. Since protoporphyrin can be secreted by the shell gland in uterine wall epithelial cells, vitamin A may affect protoporphyrin generation and deposition, thereby influencing eggshell color [31]. Morales et al. [32] found that dietary carotenoid supplementation increased biliverdin content in green eggs, but McDonald et al. [33] reported that adding 3,000 IU/kg vitamin A had almost no effect on biliverdin formation and deposition, possibly due to breed differences. When dietary vitamin A levels are low in layers, the structural and functional integrity of epithelial cells is compromised, causing epithelial tissue dryness and excessive keratinization, making them susceptible to bacterial infection and affecting the secretory function of reproductive organ epithelial cells [34]. Damage to shell gland structure and function subsequently affects the formation and deposition of protoporphyrin or biliverdin pigments, impacting eggshell color.

Furthermore, vitamins A and E have antagonistic effects in chickens; high dietary vitamin A can reduce plasma and body fat vitamin E levels. Vitamin E promotes the conversion of carotenoids to vitamin A. When dietary zinc content is low, carotenoids cannot be efficiently converted to vitamin A, hindering vitamin A absorption in layers [35]. Vitamin A maintains epithelial cell integrity and is involved in mucopolysaccharide synthesis in the body, which serves as the prosthetic group for glycoproteins or mucins secreted by mucus-secreting epithelial cells. Vitamin A deficiency affects uterine epithelial cell structure and mucus secretion, which may consequently influence secreted eggshell pigments.

2.2.2 Vitamin C Vitamin C is an antioxidant in the body that can also enhance thyroid activity and promote calcium metabolism, thereby improving eggshell quality and color, making shells smooth and enabling uniform pigment deposition. Additionally, vitamin C can improve iron utilization. Wang et al. [36] found an interactive effect between vitamin C and ALA; vitamin C can reduce Fe^{3} to Fe^{2}, improving iron nutrition absorption in layers and darkening eggshell color. Vitamin C also promotes iron absorption and transport in the intestinal tract of layers and reduces Fe^{3} to Fe^{2} in transfer proteins, allowing its release for binding with ferritin. Increased available Fe^{2} in the body facilitates protoporphyrin /heme formation and transport, promoting eggshell pigment formation and improving eggshell color [20]. Odabaşı et al. [25] found that adding 100 mg/kg vitamin C to the diet alleviated vanadium-induced eggshell lightening, but Wang et al. [37] observed no improvement from adding 100 mg/kg vitamin C on vanadium-induced eggshell color lightening, possibly due to a short feeding period. As an antioxidant, vitamin C primarily alleviates oxidative stress caused by vanadium, improving eggshell color under oxidative stress conditions but having little direct effect on eggshell color under normal rearing conditions. Moreover, vitamin E deficiency can affect endogenous vitamin C synthesis, while vitamin C can alleviate symptoms resulting from deficiencies in vitamins A, E, thiamine, riboflavin, B12, and pantothenic acid, thereby improving eggshell color to some extent.

2.2.3 Vitamin D Vitamin D regulates calcium and phosphorus absorption and is important for eggshell strength. However, under strong natural light, free-range layers with increased endogenous vitamin D synthesis may also produce lighter brown eggshells. Ryan [38] reported that layers fed diets containing 3,000 IU/kg vitamin D supplemented with ultraviolet light produced eggs with different colors compared to those receiving lower vitamin D levels with UV light; eggshell color became progressively lighter and paler over time, reaching a high reflectance coefficient of 46%. This may be because UV light enables layers to synthesize additional vitamin D , resulting in excessive vitamin D levels. Therefore, dietary vitamin D levels should be appropriately reduced for free-range layers under natural light.

However, Roberts [39] found that increasing vitamin D supplementation in free-range layer diets did not significantly improve eggshell color, possibly due to breed differences. There is no direct evidence that increased vitamin D content lightens eggshell color; its effects are more related to eggshell strength and thickness. Vitamin D and its hormonal metabolites act on small intestinal mucosal cells to form calcium-binding proteins, promoting calcium, magnesium, and phosphorus absorption [40]. Since eggshells primarily contain calcium carbonate and magnesium carbonate, this enables uniform deposition of eggshell pigments on the eggshell surface, making shells smooth and glossy.

2.2.4 Other Vitamins Other vitamins significantly affecting eggshell color include vitamin E and vitamin B6. Vitamin E influences eggshell color gloss through its antioxidant function, which maintains cell membrane integrity and function, and its protective effect against hemolysis caused by peroxides during heme synthesis, thereby protecting red blood cells [41]. Shi et al. [42] reported that adding 50 IU/kg vitamin E to the diet significantly increased eggshell L* values under stress conditions but had no significant effect on a* or b* values.

Vitamin B6, as a coenzyme for all amino acid transaminases in cellular metabolism, promotes heme synthesis, and heme is the raw material for porphyrin-iron synthesis. Vitamin B6 deficiency can easily lead to oviduct atrophy, significantly reducing hemoglobin content in red blood cells [43], consequently affecting eggshell color.

Eggshell pigments containing protoporphyrin and biliverdin are most likely synthesized in the uterine shell gland. Protoporphyrin formation is related to oxidative phosphorylation, though its mechanism requires further investigation. Eggshell color is primarily genetically determined with high heritability (0.58-0.76) [1], but nutrition influences pigment formation and deposition. In practice, eggshell color is mostly regulated through nutritional approaches, though excess or deficiency of certain nutrients will alter eggshell color. Although eggshell color is not directly related to the nutritional composition of eggs themselves, it affects egg marketing and may indicate that layers are experiencing significant stress from disease, environment, or diet, compromising their health. Current research on the effects of mineral elements and vitamins on eggshell color is limited, and studies elucidating their mechanisms are scarce. Therefore, investigating the effects of mineral elements and vitamins on eggshell color is of great significance and will promote development in the layer industry.

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