Mechanisms associated with the depigmentation of brown eggshells: a review

Ming-Yuan Lu,[*,†] Li Xu,[†] Guang-Hai Qi,[*] Hai-Jun Zhang,[*] Kai Qiu,[*] Jing Wang,[*,1] and Shu-Geng Wu[*]

[*]*Laboratory of Quality & Safety Risk Assessment for Animal Products on Feed Hazards (Beijing) of the Ministry of Agriculture & Rural Affairs, Feed Research Institute, Chinese Academy of Agricultural Sciences, Beijing 100081, China; and* [†]*College of Animal Science and Technology, Northeast Agricultural University, Harbin, Heilongjiang 150030, China*

ABSTRACT Eggshell color is an important shell quality trait that influences consumer preference. It is also of particular importance with respect to sexual signaling and the physiological and mechanical properties of shell pigment. Pigments include protoporphyrin IX, biliverdin, and traces of biliverdin zinc chelates, with brown eggs being notably rich in protoporphyrin IX, the synthesis of which has a marked effect on the intensity of brown eggshell color. This pigment is initially synthesized in the eggshell gland within the oviduct of laying hens and is subsequently deposited throughout the cuticular and calcareous layers of brown eggshell. In this review, we describe the factors affecting brown eggshell color and potential targets for the regulation of pigment synthesis. Protoporphyrin IX synthesis might be compromised by synthetase-mediated pigment synthesis, the redox status of the female birds, and regulation of the nuclear transcription factors associated with δ-aminolevulinic acid synthetase1. We believe that this review will provide a valuable reference for those engaged in studying eggshell depigmentation.

Key words: brown eggshell depigmentation, protoporphyrin IX, δ-aminolevulinic acid synthase, redox status, nuclear transcription factor

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INTRODUCTION

Eggshell color is an important parameter for determining egg quality. Over recent decades, numerous hypotheses have been proposed regarding the function of shell color, particularly with respect to avian eggs and female birds. Evolutionarily, it has been established that the earliest avian eggs lacked pigmentation (Sparks, 2011), and that during the course of avian evolution, birds gradually developed processes for the synthesis pigments that were deposited in eggshells, which has been suggested to function in the protection of eggs from predators (the "camouflage hypothesis") (Stoddard et al., 2011). A further hypothesis, "sexual signaling function," maintains that the intensity of shell color serves as an attractant for male birds, by indicating the heritable phenotypic qualities of a potential female partner (Soler et al., 2005).

Pigments incorporated into eggshells, include protoporphyrin IX, biliverdin, and traces of biliverdin zinc chelate (Liu and Cheng, 2010). On the basis eggshell pigment properties, such as photoactive antimicrobial defense (Ishikawa et al., 2010) and high affinity to eggshell protein (Ostertag et al., 2019), it is believed protoporphyrin IX may provide a microbial barrier and enhance eggshell thickness, whereas eggshell biliverdin may facilitate the development of embryos owing to its antioxidative potential (Stocker et al., 1987; McDonagh et al., 2001; Kaur et al., 2003), protection against UV radiation (Maurer et al., 2011), and high permeability (Morales, 2020). In addition, it has been reported that the biliverdin content of eggshells can reflect the physiological status of females during laying (Hargitai et al., 2016). Collectively, these observations would thus tend to indicate that sexual signaling and the physiological and mechanical properties of shell pigments could, to varying extents, explain the function of eggshell color in avian eggs.

The eggshell color of eggs produced for human consumption has attracted extensive attention owing to the importance in influencing consumer preference (Johnston et al., 2011), with colorful eggs potentially being considered more favorable by consumers (Ayim-Akonor and Akonor, 2014). Brown eggs, for example, are rich in protoporphyrin IX, the concentration of which is reflected in eggshell color, as has been reported in numerous studies (Miksik et al., 1994;

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[1]Corresponding author: wangjing@caas.cn

Samiullah and Roberts, 2013). Interestingly in this regard, Li et al. (2013) found that the concentration of protoporphyrin IX in the eggshell and eggshell gland of hens laying darker colored eggs was higher than that in hens laying eggs of a lighter color. These results are consistent with a study conducted by our research group, indicating that the protoporphyrin IX content of eggshell has a predominant effect on brown eggshell color, whereas the content biliverdin has little effect (Lu et al., 2021). It is now well established that protoporphyrin IX is synthesized de novo in the epithelial cells of the eggshell gland region of the oviduct (Li et al., 2013; Samiullah et al., 2015; Hargitai et al., 2017), the metabolic pathway of which is shown in Figure 1. Schwartz et al. (1980) extracted the precursors of protoporphyrin IX synthesis from the eggshell gland of hen, whereas Polin (1957, 1959), found that eggshell glands of hens laying brown egg have a greater capacity to convert δ-aminolevulinic acid to porphyrin than other tissues. In a further study, in which Li et al. (2013) compared differences in the protoporphyrin IX content of the eggshell gland, serum, bile, and excreta of hens laying darker and lighter brown eggs, the authors found that these differed only with respect to the concentration of protoporphyrin IX in the eggshell gland. Samiullah et al. (2017) examined the expression patterns of eggshell gland protoporphyrin IX synthetase and transporter genes at 2, 5, 15, and 22.5 h postovulation, and found that some of these genes were gradually upregulated during eggshell formation, thereby providing compelling evidence to indicate that protoporphyrin IX is initially synthesized in the eggshell gland. It has, however, also been proposed that protoporphyrin IX is derived from free or aging erythrocytes, in a destruction process during which the heme of erythrocytes is degraded (Kennedy and Vevers, 1973; Wang et al., 2009a). However, given the lack of evidence for an associated metabolic pathway, this hypothesis needs to be further assessed.

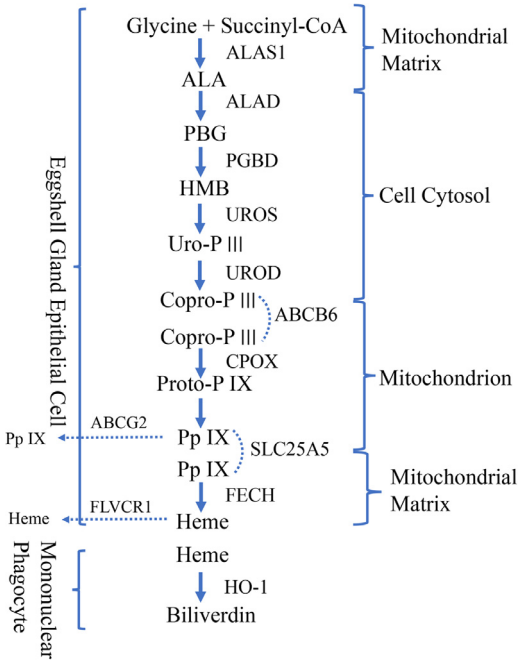

Figure 1. Pigment synthesis pathway in the eggshell gland of brown-egg hen. Abbreviations: ABCB6 and ABCG2, ATP-binding cassette trans- porter B6 and G2; ALA, δ-aminolevulinic acid; ALAD, δ-aminolevulinic acid dehydratase; ALAS1, δ-aminolevulinic acid synthase; Copor-p III, coproporphyrinogen III; CPOX, coproporphyrinogen oxidase; FECH, ferrochelatase; FLVCR1, feline leukemia virus subgroup C cellular receptor1; HMB, hydroxymethybilane; HO-1, heme oxygenase-1; PBG, porphobilinogen; PGBD, porphobilinogen deaminase; Pp IX, protoporphyrin IX; SLC25A5, solute carrier family 25 member 5; UROD, uroporphyrinogen decarboxylase; Uro-p III, uroporphyrinogen III; UROS, uroporphyrinogen synthase.

Protoporphyrin IX has been shown to be distributed in the cuticular and calcareous layers of brown eggshells. In this regard, Samiullah and Roberts (2013) determined the concentration of protoporphyrin IX in the calcareous layer based on mechanical scraping to remove the cuticular layer. They accordingly found that 80 to 87% of protoporphyrin IX is located in calcareous layer (0.12 nM/g shell), with the remaining 13 to 20% being distributed throughout the cuticular layer (0.02 nM/g shell), a finding that was subsequently confirmed by Samiullah et al. (2017). Paradoxically, however, the color of the calcareous layer is almost white. On the basis of analyses of reflectance, transmittance, and fluorescence spectra, it has been established that there are three structural types of protoporphyrin IX in eggshells, namely, highly fluorescent monomers, nonfluorescent dimers, and nonfluorescent poly-aggregates (Ostertag et al., 2019). Highly fluorescent monomers of protoporphyrin IX (color pigment) are embedded in the cuticular layer of the brown eggshell in a protein phase and represent only a small percentage of the total content (Ostertag et al., 2019). Contrastingly, non-fluorescent poly-aggregates, which are distributed in the calcareous layer, have been identified as the main structural of protoporphyrin IX in brown eggshells (Ostertag et al., 2019). In this paper, we provide an overview the major factors influencing the color intensity of brown egg and potential targets in the regulation of pigment synthesis, which we believe it will provide a valuable theoretical basis for future studies on eggshell depigmentation.

MEASUREMENT OF BROWN EGGSHELL COLOR

Eggshell color is typically estimated based on the L*a*b* system, with estimates being obtained using either a reflectometer or colorimeter (Samiullah et al., 2016a; Wang et al., 2018). Values of L*, representing eggshell lightness, range from 0 to 100 (black to white). This single score is typically used to rate the intensity of brown eggshell color (Lu et al., 2021), based on the correlation between L* values and protoporphyrin IX concentration, which has been reported to reach -0.75 (Li et al., 2013), with higher L* values being indicative of poorer eggshell coloration. For example, it has been found that an increase in eggshell L* values from 55.48 to 69.45 corresponds to a reduction in protoporphyrin IX concentration from 245.10 to 147.80 nmol/g (Li et al., 2013). Values of a* are based on a spectrum ranging from red toward the positive end of the scale and green toward the negative end, whereas b*

values reflect the range of colors from yellow toward the positive end of the scale and blue toward the negative end. Generally, brown eggs with lower L* values and higher positive a* and b* values are preferred by consumers, and in this regard, Cavero et al. (2012) defined the optimal brown colored egg as that with reference values: L* = 60, a* = 20, and b* = 30. Numerous studies have shown that brown eggshell depigmentation is characterized by an increase in the L* value and a reduction in the values of a* and b* (Li et al., 2013; Wang et al., 2018). A shell color index based on these 3 color parameters is typically used to measure the intensity of eggshell color (Lukanov et al., 2015), calculated using the formula SHELL COLOR INDEX = $L^* - a^* - b^*$. The higher mean coefficients of variation of values obtained using the shell color index compared with L* values indicate that this index might cover a larger range of chroma for the description of eggshell color.

A further method used to estimate eggshell color has been described by Hargitai et al. (2016), who applied a portable spectrometer with a bifurcated fiber-optic probe and a deuterium-halogen light source to measure the reflectance spectra of the egg surface, with reference calibration being obtained using a white WS-1-SS diffuse reflectance standard. Eggshell brightness was recorded over the range $R_{320\text{-}700\ nm}$, and brown eggshell color was calculated based on the proportion of absorbance $R_{600\text{-}700\ nm}/R_{320\text{-}700\ nm}$ ($R_{600\text{-}700\ nm}$, brown region of the spectrum), which is suggested to be correlated with protoporphyrin IX content (Hargitai et al., 2016).

In addition, numerous studies have used 2 further methods for the calculation of blue-green chroma, one of which is based on the calculation $R_{400\text{-}580\ nm}/R_{320\text{-}700\ nm}$ ($R_{400\text{-}5800\ nm}$, blue-green region of the spectrum), and has been reported to be related to biliverdin concentration (Siefferman et al., 2006; Honza et al., 2011; Butler and Waite et al., 2016). The other is based on values obtained using the calculation $(R_{480\ nm} - R_{370\ nm})/R_{480\ nm}$, where $R_{480\ nm}$ corresponds to the peak reflectance of the blue-green eggshell and $R_{370\ nm}$ indicates lowest reflectance (Hargitai et al., 2016). Moreover, determinations based on the formula $(R_{max\ nm} - R_{min\ nm})/R_{max\ nm}$ ($R_{max\ nm}$ corresponds to the peak reflectance of a certain chroma and $R_{min\ nm}$ indicates lowest reflectance), which is used to measure the carotenoid-based yellow chroma of plumage (Johnsen et al., 2003; Peters et al., 2008), could also be applied for the calculation of brown eggshell color. Although high-performance liquid chromatography has been reported to be more accurate in determining pigment content (Liu et al., 2010; Gorchein et al., 2012), the aforementioned methods are more readily applied in layer production and less expensive.

FACTORS AFFECTING BROWN EGGSHELL COLOR

Aging

It has been widely established that eggshell color gradually declines as hens age (Odabasi et al., 2007; Samiullah et al., 2016a; Bi et al., 2018; Sirri et al., 2018). To determine the causal factors associated with reductions in pigmentation, Odabasi et al. (2007) measured the eggshell color of Hy-line brown laying hens over a successive 10-mo period (hen age from 25 to 65 wk) and accordingly found that eggshell L* values increased and a* values decreased as the hens aged. However, when corrected for egg weight, the rate at which the values of L* and a* changed was found to show a declining tendency. The authors considered that older hens laid lighter colored eggs due to an increase in egg size related to no proportionate change in pigment content deposited over the shell surface, which is inconsistent with the findings of Bi et al. (2018), who found that egg size had a comparatively smaller effect on eggshell color as hens aged (average value of R = 0.07, $P > 0.05$). In this latter trial, the authors recorded shell color of eggs laid by 120 Rhode Island Red hens reared in individual cages at 26, 34, 42, 50, 60, and 70 wk of age, and established that the observed reduction in protoporphyrin IX content of eggshells was highly correlated with color (average value of R = 0.66, $P < 0.01$). Furthermore, in their analysis of differences in the eggshell pigment content of eggs laid by Hy-line brown laying hens at 33, 50, and 67 wk of age, Samiullah and Roberts (2013) found that the protoporphyrin IX content of the cuticular layer decreased with age, although they detected no significant differences with regards to total protoporphyrin IX per gram of shell. These observations accordingly revealed that the depigmentation of eggshells is conceivably attributable to changes in pigment concentration. Moreover, Park et al. (2017) suggested that fibrosis and atrophy of the endometrium, as a consequence of successive oviposition, is linked to the hypofunction of the eggshell gland, and thus the observed age-related decline in protoporphyrin IX synthesis may underlie the depigmentation of brown eggs.

Trace Minerals and Nutrients

Strong oxidants have been demonstrated to exert detrimental effects on eggshell pigmentation. For example, vanadium, a strong oxidant that has potentially adverse effects on egg production, is occasionally detected at low levels in commercial poultry feed, and numerous studies have reported that laying hens fed diets supplemented with 5 to 10 mg/kg vanadium produced eggs with a significantly reduced brown coloration (Miles and Henry, 2004; Odabasi et al., 2006). However, this depigmented effect can be reversed by supplementation with 100 mg/kg vitamin C or 130 mg/kg tea polyphenol epigallocatechin-3-gallate (Odabasi et al., 2006; Wang et al., 2017a; Wang et al., 2018). In this regard, it has been reported that vanadium results in paler eggshells via its effects in mediating the nuclear respiratory factor 2/heme oxygenase-1 signaling pathway (Wang et al., 2018). Similarly, the administration of high doses of zinc oxide, which is routinely used to induce molting in laying hens, has been found to be

associated with a gradual paling of egg color (Berry and Brake, 1985; Aygun, 2013).

In contrast, certain trace minerals have been reported to have positive effects with respect to brown eggshell color. For example, it has been demonstrated that dietary supplementation with 100 mg/kg iron (121 mg/kg iron-methionine chelate and 150 mg/kg iron-soy proteinate) can enhance the brown pigmentation of eggs during the peak laying period (Inkee et al., 2009; Seo et al., 2010). Consistently, Park et al. (2004), who examined the effects of iron-methionine chelate and iron sulfate on eggshell quality, found that hens fed diets supplemented with 100 to 300 mg/kg organic or inorganic iron produced egg with a more pronounced shell pigmentation. In porphyrin molecules, iron serves as a cofactor that acts as a chelating agent with beneficial effects on hemoglobin content (Theil, 2010). Dietary magnesium supplementation (3,000 mg/kg magnesium oxide) has similarly been shown to enhance the eggshell color of eggs laid by aged hens (Kim et al., 2013), although Seo et al. (2010) have reported that magnesium has no effect on eggshell color and hemoglobin concentrations during the peak production period. Consequently, the beneficial effects of trace minerals on eggshell color need to be further evaluated.

In addition to trace elements, diverse nutritional additives may also contribute to enhancing the intensity of eggshell color. Dietary supplementation with *Bacillus subtilis* spores, for example, has been reported to enhance eggshell pigmentation in 63 -wk-old Lohman Brown hens (Hooge, 2007), the mechanism of action of which has been suggested to be associated with an enhancement metal ion insertion into protoporphyrin compounds, promoted by certain amino acid residues produced by *Bacillus subtilis* (Hansson et al., 2007). Furthermore, diets supplemented with 0.2% organic acid and 0.4% available phosphorus has been found to facilitate brown pigment deposition (Inkee et al., 2009). It has been suggested that these beneficial effects of organic acids are associated with the maintenance of pigment synthesis in the eggshell gland by contributing to the retention of mucous membrane integrity. In future studies, it would accordingly be worthwhile examining a more diverse range of nutrients to determine their potential utility in enhancing eggshell color.

Stress and Environment

Commercial egg production is associated with a range of stresses that contribute to reducing the productive performance of layers, with most sources of stress being linked to the induction of oxidative stress at the cellular level, which is attributable to free radical production or inadequate antioxidant protection that cause an imbalance in redox potential. The synthesis of pigments in the eggshell gland during eggshell formation is known to be susceptible to disruption by stress (Ebeid et al., 2012), with stress-related factors such as high stocking density, uncomfortable cage design, and fear having been found to contribute to poor eggshell color (Walker and Hughes, 1998). Two types of stress-related egg abnormalities characterized by eggshell depigmentation have been identified, namely early and delayed oviposition. Stress-induced early oviposition can perturb pigment deposition, and may also result in the production of depigmented eggshells lacking a cuticular layer (Mertens et al., 2010). Delayed oviposition results in other abnormalities. For example, it has been reported that if hens suffer from an adverse stimulation prior to oviposition, they can delay laying for up to 3.0 h (Reynard and Savory, 1999). Such delayed oviposition results in the retention of eggs within the eggshell gland, thereby disrupting laying rhythmicity, during which time, additional calcium may be deposited on the surface of eggshells (Hughes, et al., 1986). The potential mechanisms whereby oxidation stability affects pigment synthesis are described below.

Compared with hens maintained in free-range systems, brown egg-laying hens reared in cages have been found to lay eggs with a darker color (Roberts and Chousalkar, 2014). In their study of the effects of cage, free-range, and barn production systems on brown eggshell color, Samiullah et al. (2016b) observed that more colorful eggs were obtained from those hens maintained in the cage system, and that these contained higher concentration of protoporphyrin IX in the calcareous layer. In this regard, it is plausible that cage systems may provide hens with a more congenial environment, including an appropriate temperature and humidity, which might be beneficial with respect to pigment synthesis. However, despite the fact that cage systems have been found to contribute to maintaining the chroma of eggshell color, from the perspective of meeting the demands of animal welfare and consumer preference, the adoption of free-range systems is encouraged for layer production (Wang et al., 2009b). Accordingly, if hens are to be raised free range, consideration should be given to the conditions necessary to resolve the problem of poor eggshell color obtained in these systems.

Protoporphyrin IX has photoactive properties that have been shown to be associated with the prevention of disease (Roberts et al., 2011). However, the effects of light on the pigmentation of brown eggs have yet to be sufficiently determined. In this regard, it has been reported that lighting-related changes occur in the shell color of egg specimens maintained in museums (Cassey et al., 2010). Using a broad-spectrum light source, Navarro and Lahti (2014) measured the reflectance of blue-green eggs and a range of eggshell colors from medium blue to white with dark red speckles, and observed a gradual decline in the reflectance of eggshells across a wavelength spectrum between 250 and 800 nm, with the reflectance of darker eggs being slightly lower than that of lighter eggs. By accelerating the degradation of eggshell pigments, it would appear that lighting has negative effects with respect to eggshell color, and it

can thus be concluded that the intensity of eggshell color is compromised to a certain extent by the external environment.

Medicinal Compounds and Diseases

Disease, not surprisingly, is the most significant problem facing laying production, and in this regard, it has been found that the administration of certain drugs to laying hens results in the production of eggs with a paler eggshell color (Mueller and Leach, 1974). For example, Hughes et al. (1991) have reported that coccidiostat nicarbazin reduces eggshell color and that the degree of depigmentation is dose dependent. Similarly, diets supplemented with 100 mg/kg nicarbazins have been found to be associated with an increase in brown eggshell L* values from 58 to 70 and a reduction in protoporphyrin IX content in eggshells and the eggshell gland (Samiullah et al., 2017), with the authors suggesting that nicarbazin reduced protoporphyrin IX synthesis in the eggshell gland by downregulating the expression of δ-aminolevulinic acid synthetase1. Nevertheless, it has been established that pigment synthesis is restored within 6 to 8 d after drug withdrawal (Hughes et al., 1991). Sulfonamides are known to adversely influence eggshell formation by inhibiting carbonic anhydrase activity, which leads to the production of soft-shelled eggs, and in some cases, even shell-less eggs (Ozensoy, et al., 2011). These eggshell defects are typically accompanied by the depigmentation of eggshells. For example, it has been reported that hens administered 3 to 5 mg/kg unsubstituted sulfonamides lay thin-shelled eggs with a paler color (Mueller and Leach, 1974). Furthermore, administration of prostaglandin F2α and indomethacin can induce rapid oviposition, which is associated with disrupted pigment deposition (Soh, 1999).

Numerous viral diseases, such as Newcastle disease, have been established to have detrimental effects on eggshell quality, which is attributed to virus-specific affinity for mucus membranes, resulting in damage to the reproductive tract (Butcher and Miles, 1995). Infectious bronchitis is a common disease in laying hens that causes substantial economic losses, one of the symptoms of which is the production of eggs with poor eggshell color (Chousalkar et al., 2009). On the basis of immunofluorescent staining, Crinion and Hofstad (1972) found that antigens of the causal virus are localized in cells lining the epithelium of the oviduct, and it had also been reported that eggshell gland mitochondrial counts were significantly lower compared with those in other segments of the oviduct in hens exposed to infectious bronchitis virus (Khan et al., 2019). It would thus appear that the infectious bronchitis virus induces certain pathological changes in the eggshell gland, which may have the effect of interrupting the synthesis and secretion of pigments.

The findings of these studies thus highlight the need to take into consideration the side effects of the drugs administered to hens and the quality of the eggs they produce, and accordingly take measures to reduce these detrimental effects.

POTENTIAL TARGETS FOR THE REGULATION OF PIGMENT SYNTHESIS

Pigment Synthetases and Transporters

The protoporphyrin IX synthesis pathway (shown in Figure 1) involves the generation of δ-aminolevulinic acid from glycine and succinyl-CoA, catalyzed by δ-aminolevulinic acid synthetase1 (**ALAS1**), as the initial step in a series of reactions in the synthesis of protoporphyrin IX, in which multiple synthetases and transporters are assumed to participate. Numerous studies have reported the functions of these synthetases and transporters (Zheng et al., 2014; Samiullah et al., 2017). For example, ALAS1, the first rate-limiting enzyme, is detected in the mitochondrial matrix, the activity of which determines the levels of δ-aminolevulinic acid (Li et al., 2013). The ATP-binding cassette family member ABCG2, located in the plasma and mitochondrial membranes has been shown to be associated with the export of synthesized protoporphyrin IX out of cells (Kobuchi, et al., 2012), whereas a translocator protein has been reported to export protoporphyrin IX from the mitochondria (Li et al., 2016). To investigate the effects of the various synthetases and transporters on pigment synthesis in laying hens, Zheng et al. (2014) compared gene expression levels in hens laying eggs with brown, white, and pink color, and accordingly detected high-level expression of coproporphyrinogen oxidase in a breed laying brown eggs, whereas high levels of ferrochelatase (conversion of protoporphyrin to heme) expression were identified in hens laying eggs with white and pink eggshell color. The authors concluded that higher levels of synthetase activity were associated with higher eggshell concentration of protoporphyrin IX, and that certain transporters play important roles in pigment synthesis (Zheng et al., 2014). These findings are consistent with those reported by Stevens et al. (1974), who demonstrated that coproporphyrinogen oxidase expression was higher in hens laying brown eggs than in those laying white eggs. In a further study, Li et al. (2013), who compared differences in then gene expression of enzymes and transporters of protoporphyrin synthesis in Rhode Island Red pure lines laying darker brown eggs and hens laying eggs of lighter colored eggshell, reported that *ALAS1*, coproporphyrinogen oxidase, feline leukemia virus subgroup C cellular receptor 1, and ATP-binding cassette family members *ABCB7* and *ABCG2* were all more highly expressed in the group laying darker eggs. Consistently, Samiullah et al. (2017) observed that the downregulation of *ALAS1* in hens administered nicarbazin was

associated with reduced pigment synthesis. Collectively, the findings of these studies highlight the prominent roles of synthetases and transporters in determining levels of protoporphyrin IX synthesis in the eggshell gland. It would thus be of interest to investigate the functions of other currently uncharacterized synthetases and transporters in future studies.

Redox Status Imbalance

Blue-green eggshell color was initially suggested to serve as a means of sexual signaling designed to attract male parents, given that eggshell biliverdin has certain antioxidant properties that contribute to promoting embryonic development (Soler et al., 2005), and that female birds laying eggs containing higher concentration of biliverdin have enhanced antioxidant properties (Hanley et al., 2008). Similarly, Navarro et al. (2011) have reported that the intensity of blue-green shell color is positively correlated with the antioxidant content of egg yolk, including that of carotenoids, vitamin A, and vitamin E, whereas Hargital et al. (2016) have proposed that the chroma of blue-green eggs reflects female body condition during laying and demonstrated that the average chroma can be increased by antioxidant supplementation. Notably, these authors detected no significant correlation between plasma antioxidant capacity and eggshell biliverdin content. However, given that they identified differences in the female plasma antioxidant capacities of control and treatment groups prior to antioxidant supplementation, they were unable to confirm whether plasma antioxidant capacity is associated with the intensity of blue-green eggshell color. Consequently, on the basis of the evidence obtained to date, it appears that female birds with higher antioxidant potential may lay more colorful blue-green eggs, although further confirmation is required.

The intensity of brown shell color is similarly assumed to be associated with the redox status of birds (Mertens et al., 2010), a view supported by evidence indicating that the conversion of a higher proportion of protoporphyrin IX to biliverdin, a strong antioxidant, leads to a reduction in brown pigmentation. In this regard, the findings of an interesting study conducted on Dongxiang chicken laying eggs with either brown or green eggshell color, indicated that the total amount of eggshell pigment was a relatively fixed value, and that the level of biliverdin and protoporphyrin IX were negatively correlated (Wang et al., 2009b). Given that protoporphyrin IX is a precursor of biliverdin (Samiullah et al., 2015), pigment conversion may result in a reduction in the concentration of protoporphyrin IX, a hypothesis subsequently confirmed by Lu et al., 2021. These authors, who examined hens laying eggs with darker brown color and hens laying eggs with a lighter color, found that the concentration of biliverdin and protoporphyrin IX in the eggshell gland of hens laying the lighter colored eggs were lower than those in the group laying darker eggs. In an iTRAQ-based quantitative proteomic analysis, Li et al. (2016) examined differences in potential regulatory proteins relative to protoporphyrin IX synthesis in the eggshell gland of hens laying darker and lighter brown eggs, and accordingly found that proteins contributing to the extracellular transport of protoporphyrin IX were downregulated in hens laying lighter brown eggs, whereas the expression of those mediating the import of protoporphyrin IX into mitochondria was increased. In a further proteomics-based analysis, it was demonstrated that protoporphyrin IX may serve as a precursor in biliverdin synthesis rather than ultimately undergoing deposition. This metabolic switch is assumed to be associated with changes in oxidation stability that increase the demand of the eggshell gland for biliverdin. A diet supplemented with vanadium was found to significantly reduce eggshell protoporphyrin IX concentration from 42.35 to 13.02 μg/g eggshell and antioxidative enzyme activity in the eggshell gland, including that of glutathione-*S*-transferase, glutathione peroxidase, and superoxide dismutase (Wang et al., 2018). Moreover, the findings of this study revealed that vanadium-induced reduction in the antioxidation potential of hens is mediated by the nuclear respiratory factor 1/heme oxygenase 1 signaling pathway, thereby indicating that larger amount of biliverdin, derived from the heme oxygenase 1 degradation of heme, may be required to counter an increased generation of reactive oxygen species (**ROS**). Accordingly, the higher concentration of biliverdin in the eggshell gland required to maintain oxidation stability may have the effect of reducing brown eggshell color.

Approximately 90% of the ROS generated in cells, which are produced in the mitochondria, can result in an imbalance in redox status (Balaban et al., 2005). When generated at excessive levels, ROS readily overwhelm the antioxidant capacities of cells, which is manifested in significant reductions in mitochondrial counts and function (Chan, 2006). In this regard, it has been reported that the synthesis of protoporphyrin IX is associated with the generation of ROS (Ryter and Tyrrell, 2000), which represent a potential source of redox imbalance. Given that pigment synthesis occurs partially in the mitochondria, it is conceivable that mitochondrial homeostasis has a certain influence on pigment synthesis. As mentioned previously, supplementation of the diets of brown egg-laying hens with nicarbazin has the effects of reducing eggshell color, as well as the expression of *ALAS1* mRNA and mitochondrial counts per cell in the eggshell gland (Samiullah et al., 2017). The findings of a transcriptomic study conducted by Li et al. (2017), in which the authors compared the differences in gene expression in the eggshell gland of hens laying darker and lighter brown egg, revealed that differentially expressed genes were enriched in the oxidative phosphorylation pathway, among which, that encoding heat-shock protein 70 was found to be significantly downregulated. The results indicated that those hens laying eggs of lighter color might be at a comparatively higher risk of redox imbalance, which is plausibly associated with electron leakage from the mitochondrial

respiratory system. This view is consistent with the findings of Li et al. (2016), who have suggested that an efficiently functioning mitochondrial respiratory chain might promote protoporphyrin IX synthesis in the eggshell gland. Indeed, given that impaired mitochondrial homeostasis results in ROS production and a reduction in pigment synthesis, and that the depigmentation of eggshells in brown egg-laying hens has been linked to an imbalance in oxidation stability, it might be assumed that mitochondrial oxidative phosphorylation plays important roles in pigment synthesis in the eggshell gland.

There is further evidence that the intensity of brown eggshell color may reflect the redox status of birds. For example, Javurkova et al. (2019), who evaluated the effects of the pigmentation of tinted, brown, dark brown, white, and blue eggs (among 23 traditional chicken breeds) on variability in the albumen concentrations of 2 major antimicrobial proteins (lysozyme and ovotransferrin), found that the concentrations of these 2 proteins were positively correlated with protoporphyrin content of the cuticular layer in tinted and dark brown eggs, although not in white, blue, or brown eggs, thereby indicating that the pigment content in eggshells may affect the synthesis of antimicrobial proteins by female birds. On the basis of a proteomic analysis of albumen content in response to vanadium treatment, with or without co-administration of a tea polyphenol, Wang et al. (2017b) demonstrated that the detoxification mechanisms of tea polyphenols are associated with the immune function-related proteins that contribute to alleviating cell apoptosis in the magnum region of the oviduct. The results indicated that vanadium-induced brown eggshell depigmentation might be associated with a reduction in the levels of immune function-related proteins. Consequently, monitoring the association between eggshell color and hen redox status may play an important role in the management of laying production. However, developing appropriate color detection methods that are accurate, rapid, and easy to use will represent a major challenge in future studies.

Nuclear Transcription Factors

The findings of numerous studies have provided consistent evidence that pigment concentration in the eggshell gland can be regulated by targeting ALAS1 and ALAS1-mediated protoporphyrin IX synthesis (Zhang et al., 2019; Lu et al., 2021). Regulation of ALAS1 activity is assumed to be associated with the intensity of brown eggshell color. The enzyme is initially encoded by nuclear genes, and its functional form is found in the mitochondrial matrix (Riddle, et al., 1989). Having isolated a rat ALAS1 gene from a genomic clone, Braidotti et al. (1993) analyzed ALAS1 gene promoter sequences, and accordingly identified two binding sites for nuclear respiratory factor 1 (**NRF1**). Independent mutagenesis of each of these NRF-1-binding motifs was found to result in a substantially downregulated expression of ALAS1 gene promoter sequences, whereas mutagenesis of both motifs led to a complete loss of expression (Braidotti et al., 1993). In further studies on this enzyme, Handschin et al. (2005) reported that elevation of the expression of peroxisome proliferator-activated receptor γ coactivator 1α (**PGC1α**), via transformation using an adenoviral vector, could enhance the synthesis of protoporphyrin IX precursors in rats, whereas the overexpression of ALAS1 in response to fasting was found to be suppressed in PGC1α knockout rats. Accordingly, these findings would tend to indicate that PGC1α may play a role in the regulation of ALAS1 activity, enhancing protoporphyrin IX synthesis directly, and thereby influencing brown eggshell color. These findings were confirmed by Shao et al. (2010), who suggested that overexpression of PGC1β may elevate ALAS1 expression in the presence of NRF1. In addition, a number of studies have reported that mitochondrial biogenesis is mediated by PGC1α/NRF1 activity (Lee and Wei, 2005), which is consistent with the aforementioned assumption that a reduction in pigment synthesis may be associated with reduced mitochondrial biogenesis. However, further studies will be necessary to verify whether the PGC1α/NRF1 system plays a prominent role in pigment synthesis by controlling mitochondrial levels of ALAS1.

CONCLUSIONS AND FUTURE RESEARCH

Substantial evidence obtained from searches of the scientific literature supports the importance of eggshell color with respect to both eggs and female birds, including roles in signaling and physiological and mechanical functions of shell pigment. Numerous studies have shown that protoporphyrin IX, which has prominent effects on egg color, is synthesized in the eggshell gland within the oviduct of laying hens and is primarily deposited in the calcareous layer of brown eggshell as nonfluorescent poly-aggregates. Numerous factors have been shown to affect eggshell color, including aging, nutrients, stress, and disease. In this review, we have described three factors that potentially influence the production of protoporphyrin IX, namely synthetase-mediated pigment synthesis, the redox status of female birds, and PGC-1α/NRF1-mediated ALAS1 activity.

Colorful eggs are generally perceived to be more attractive by consumers, and hence identifying appropriate nutritive approaches that aim to reduce the incidence of egg depigmentation would be particularly beneficial with respect laying production. In this regard, it will be desirable to develop suitable color detection methods that are accurate, rapid, and easy to use for the efficient monitoring of eggshell depigmentation, which would also contribute to assessments of the health status of laying hens.

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DISCLOSURES

The authors declared that there were no conflicts of interest to this work.

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