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Review

Eggshell color in brown-egg laying hens — a review

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ABSTRACT The major pigment in eggshells of brown-egg laying hens is protoporphyrin IX, but traces of biliverdin and its zinc chelates are also present. The pigment appears to be synthesized in the shell gland. The protoporphyrin IX synthetic pathway is well defined, but precisely where and how it is synthesized in the shell gland of the brown-egg laying hen is still ambiguous. The pigment is deposited onto all shell layers including the shell membranes, but most of it is concentrated in the outermost layer of the calcareous shell and in the cuticle. Recently, the genes that are involved in pigment synthesis have been identified,

but the genetic control of synthesis and deposition of brown pigment in the commercial laying hen is not fully understood. The brown coloration of the shell is an important shell quality parameter and has a positive influence on consumer preference. The extent of pigment deposition is influenced by the housing system, hen age, hen strain, diet, stressors, and certain diseases such as infectious bronchitis. In this article, the physiological and biochemical characteristics of the brown pigment in commercial brown-egg layers are reviewed in relation to its various functions in the poultry industry.

Key words: brown eggs, laying hens, protoporphyrin IX, eggshell quality, stress factors

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INTRODUCTION

The eggshell, which is a primary packaging container and also a microbial barrier, is of great importance to poultry producers. The eggshell layers are deposited in a precise order as the egg descends through the highly differentiated parts of the oviduct (Gautron et al., 1997). The hen eggshell is composed of three main layers, each having its own complex morphology: the cysteine-abundant proteinaceous shell membrane, the mineralized hard complex layer, and the outer non-mineralized cuticle (Kodali et al., 2011). The shell membrane is synthesized over a period of 1.0 to 2.0 h, when the immature egg travels through the proximal isthmus (Nys et al., 2004). The mineralized multilayered complex layer is formed in the distal isthmus and shell gland over an approximately 19 to 20-h time period. The cuticle is deposited onto the eggshell in the uterus 1.5 to 2.0 h before oviposition (Nys and Guyot, 2011). The eggshell is composed mainly of calcite, but a thin layer of hydroxyapatite is also present in the inner cuticle (Dennis et al., 1996).

There is an extensive scientific literature regarding eggshell pigmentation of wild birds, but relatively little

in relation to eggshell pigment in commercial brown-egg laying hens. The study of eggshell pigments has a long history, and the major pigment extracted from brown eggshells was initially named oorhodeine (Sorby, 1875). The first published report in English describing brown eggshell pigment as protoporphyrin was that of Punnett (1933), although earlier studies had acknowledged its presence in avian eggshells (Fischer and Kogl, 1923, as cited in Kennedy and Vevers, 1973). Later, it was confirmed that the brown pigment is protoporphyrin IX (Kennedy and Vevers, 1973). Protoporphyrin IX belongs to a group of families of biologically active tetrapyrrole compounds. Structurally, protoporphyrin IX is a tetrapyrrole ring containing a highly conjugated planar and a rigid macrocycle consisting of four pyrrole rings connected by methane groups (Bhosale et al., 2013). The nomenclature of this tetrapyrrole compound often includes a numerical suffix such as IX, which refers to the position of the side chain (Sparks, 2011). In addition to protoporphyrin IX, biliverdin, coproporphyrin, and uroporphyrin were also identified from domestic hen eggshell and shell gland (With, 1974; Baird et al., 1975). However, the dominant eggshell pigment in brown-egg laying hens is protoporphyrin IX, with traces of other porphyrins (Lang and Wells, 1987). The complex nature of eggshell color in laying hens is still under investigation, and the fact that certain hens lay brown eggs, others lay white, and some lay even blue-shelled

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eggs has long indicated a genetic basis for shell color, possibly involving sex linked genes (Hall, 1944). More recent studies have confirmed the high heritability of shell color (Forster et al., 1996; Francesch et al., 1997; Zhang et al., 2005). Brown eggshell color has been positively correlated with some shell characteristics such as shell strength and hatchability (Sekeroglu and Duman, 2011). Apart from quality functions, in the presence of light, brown pigment has shown bactericidal activity against certain gram positive bacteria (Ishikawa et al., 2010).

In this review article, some important aspects of the brown pigment in the eggshells of the laying hen are discussed.

METHODS OF CALCULATING EGGSHELL COLOR

A traditional scientific method is to use a reflectivity meter for measuring shell color intensity. Shell reflectivity, expressed as a percentage, is the amount of light that is reflected from the surface of an egg. It is an indication of shell color lightness — the higher the value, the lighter the color of the eggshell and vice versa. Shell reflectivity is less time consuming; however, it is not as accurate as other methods for measuring shell color. The reason could be that the reflectivity meter takes only one reading by targeting a small circular surface area (1 cm) of the eggshell. The most commonly used method for eggshell color quantification is spectrophotometry. One of the recent versions of spectrophotometer is the Konica Minolta hand-held spectrophotometer (CM-2600d) that works on the $L^*a^*b^*$ color space system, where L^* has a maximum of 100 (white) and a minimum of 0 (black) and is the important component for simply measuring shell chromaticity. For a^* , green is towards the negative end of the scale and red towards the positive end. For b^* , blue is towards the negative end and yellow towards the positive end of the scale (Roberts et al., 2013). The average value appears on the screen and is read as SCI $L^*a^*b^*$ and SCE $L^*a^*b^*$. The SCI (specular component included) and SCE (specular component excluded) are very similar to each other, and usually SCI values are used for interpretation. The lower the L^* value on the scale the darker the color of the shell surface and vice versa. One of the problems with spectrophotometer (CM-2600d) is how to interpret the results of a^* and b^* individually when the reading is taken on an unstained eggshell surface. For the amount of cuticle estimation, $L^*a^*b^*$ values are usually interpreted as single score. The single score, as described by Leleu et al. (2011), measures the L^* , a^* , and b^* values, before and after staining, and calculates a single value, ΔE_{ab}^* :

$$\Delta E_{ab}^* = \sqrt{[(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]}$$

A higher ΔE_{ab}^* denotes a higher staining affinity and hence more cuticle coverage (Leleu et al., 2011; Roberts et al., 2013). A Hunter lab calorimeter (working on the $L^*a^*b^*$ system) has been used for measuring eggshell color, but the values are less standardized compared to Minolta calorimeter values (Wei and Bitgood, 1990). A Color Computer Vision machine is an alternative to the Konica Minolta Spectrophotometer ($L^*a^*b^*$). This machine measures the color of a larger surface area of eggshell and can also detect dirt (Mertens et al., 2005). The Color Computer Vision machine operates on the same $L^*a^*b^*$ color space system (Odabasi et al., 2006). Image analysis is another effective tool used for eggshell color estimation. In this method, a rectangular area, region of interest (ROI), of the shell is selected and the machine calculates the average values of intensity of the pixels in red, green, and blue bands (Sezer and Tekelioglu, 2009). The values are then divided by 255 in order to produce a scale ranging from 0 to 1. This method is good for eggshells having numerous color spots. A High Performance Liquid Chromatography (HPLC) has been previously used to quantify the pigment from eggshell, uterine fluid, and parts of the shell gland (Liu et al., 2010). In HPLC, the sample is usually mixed with diluent (e.g. 50% HCl and acetonitrile), centrifuged, and read its absorbance in the machine. A shell (0.4 g) without shell membrane is dissolved in 0.08 g of celite and the pigment is then extracted with HCl, acetone, and oxalic acid (1:1:1). The extract is glass filtered and its absorbance is read in spectrometry at 409 to 412 nm (Nys et al., 1991). This method seems to be quite tedious.

In certain cases, eggshell color is masked by extraneous calcium deposition and thus the intensity of color is not measured precisely. In addition, the shell color measurement does not indicate how much pigment (g) is present in different shell layers. In order to confirm the intensity of shell color, the brown pigment in the eggshell (with and without cuticle) is quantified. Pigment (protoporphyrin IX) quantification is a more accurate way of determining the intensity and amount of pigment present in the shell. A method developed by Poole (1965) and modified by others (Wang et al., 2007, 2009a; Samiullah and Roberts, 2013) may be used for pigment quantification.

PROTOPORPHYRIN IX IN THE BROWN EGGSHELL AND SHELL GLAND

Microscopic examination of cross sections of the eggshell has shown that protoporphyrin is deposited in layers at different depths between the crystals within the eggshell (Tamura and Fujii, 1967a). Samiullah and Roberts (2013) found more protoporphyrin IX in the calcareous shell than in the cuticle. Other reports that most pigment is contained in the cuticle may reflect the methodology used in those studies (Tamura and Fujii, 1967a; Schwartz et al., 1975; Lang and Wells,

1987; Miksik et al., 2007). Protoporphyrin IX is also present in the shell membrane (Tamura et al., 1965). Using a subjective assessment of the extent of hen eggshell cuticle cover, it has been found that there is great variation in cuticle deposition on eggs of different breeds and of individual hens (Bain et al., 2013). A lack of significant genetic correlation has been recorded between the amount of cuticle and shell color intensity in brown eggs (Bain et al., 2013).

Protoporphyrin IX is primarily present in the surface epithelial cells of the shell gland (Baird et al., 1975), but in Japanese quail the lower isthmus may contribute to pigment production (Baird et al., 1975). The eggshell pigment appears in quail uterine tissue as granular and diffuse coloring in the cytoplasm of apical cells in the epithelium, and its concentration varies inversely with the amount of the pigment deposited into eggshell (Poole, 1967). The hen eggshell gland is considered to be the primary site of pigment production, and it produces twice as much porphyrin from δ -aminolevulinic acid compared to liver cells *in vitro* (Polin, 1957). Various hormones, such as estrogen, progesterone, and certain prostaglandins, have been shown to be involved in pigment production (Soh and Koga, 1994, 1997, 1999). It has not been confirmed where the pigment is synthesized in the brown-egg laying hen; however, enzymatic activity of δ -aminolevulinic acid synthase was detectable in the uterine glandular tissue of Rhode Island hens, but not that of White Leghorn hens (Stevens et al., 1974). The δ -aminolevulinic acid dehydratase activity was higher in the shell gland followed by isthmus and magnum, and the activity was also higher in Rhode Island hens than White Leghorn hens, even in the absence of an egg (Stevens et al., 1974). Pigment granules of porphyrin were detected in apical ciliated cells of the uterine epithelium of Rhode Island Red hens, but were absent in the uterus of White Leghorn hens (Tamura and Fujii, 1967b).

The timing of pigment secretion by shell gland epithelial cells and its deposition into the eggshell varies among species and from bird to bird within a species (Liu and Cheng, 2010). Pigment deposition and its rate of secretion are different in brown, blue, and white eggshell hens (Liu et al., 2010). More research has been conducted using Japanese quail rather than the commercial hen, but the histological structure of the uterus of Japanese quail is very similar to the commercial hen (Poole, 1967). The pigment on the surface of quail eggshells is accumulated in the last 3.0 to 3.5 h prior to oviposition (Poole, 1965; Soh et al., 1993). Similarly, the eggshell pigmentation in Dongxiang blue-shelled chickens is thought to be secreted mainly 2.0 to 3.0 h before oviposition (Zhao et al., 2006). It is assumed that, in the hen, pigment is deposited onto brown eggs throughout the shell formation process, but 50 to 74 % of it is deposited in the last 5.0 h before oviposition (Warren and Conrad, 1942). From the presence of the pigment in all shell layers in brown-egg laying hens, it is assumed that pigment deposition follows a similar pattern to

that seen in Japanese quail. It is also assumed that the same factors (hormonal, calcium, and phosphorus concentrations in shell gland) that are responsible for cuticle completion and termination (Nys et al., 1991; Soh and Koga, 1999) are also responsible for pigment deposition and its ultimate termination in brown-egg laying hens. More work is needed to measure precisely the timing of pigment deposition in the brown-egg laying hen.

METABOLIC PATHWAY OF PROTOPORPHYRIN IX

Early reports suggest that protoporphyrin IX is derived from blood heme, although more recent findings indicate that pigment is synthesized in the tissues of the hen's shell gland.

Evidence for Synthesis of Protoporphyrin IX from RBCs

Early researchers suggested that eggshell pigments are derived from normal erythrocyte destruction in the mucous layer of the oviduct, transported by special cells that enter the uterine epithelium in the final stages of calcification of shell, and from there deposited into shells (Giersberg, 1921; as cited in Kennedy and Vevers, 1973). The protoporphyrin IX in the shell gland is reported to originate either from free erythrocytes during circulation (Kennedy and Vevers, 1973), or as a metabolite from the heme released from aging erythrocytes (Wang et al., 2009a). It was suggested that hemoglobin degrades to bile pigments, whereas protoporphyrin IX is synthesized from heme via the δ -aminolevulinic acid pathway (Polin, 1957). Support for the synthesis of protoporphyrin IX from red blood cells was based partly on the histological work of Giersberg (1921; as cited in (Kennedy and Vevers, 1973), who linked pigment derivation with the erythrocyte aging and destruction process.

Evidence for Synthesis of Protoporphyrin IX in the Shell Gland

Most authors are of the opinion that protoporphyrin IX is first synthesized in the shell gland, from where it is secreted and deposited into eggshell layers (Baird et al., 1975; Solomon, 2002). This view is supported by evidence that previously free heme, protoporphyrin, and biliverdin in sufficient quantity have been quantified from the shell gland of hens (Gorchein et al., 2012). The extraction of precursor enzymes and protoporphyrin IX from the shell gland (Schwartz et al., 1980) suggests that porphyrin synthesis occurs in the shell gland of the hen. A comparison of protoporphyrin IX in different tissues of light- and deep-brown-egg laying Rhode Island Red hens indicated that eggshell pigment is synthesized in the shell gland, and there was a significantly higher gene expression of δ -aminolevulinic

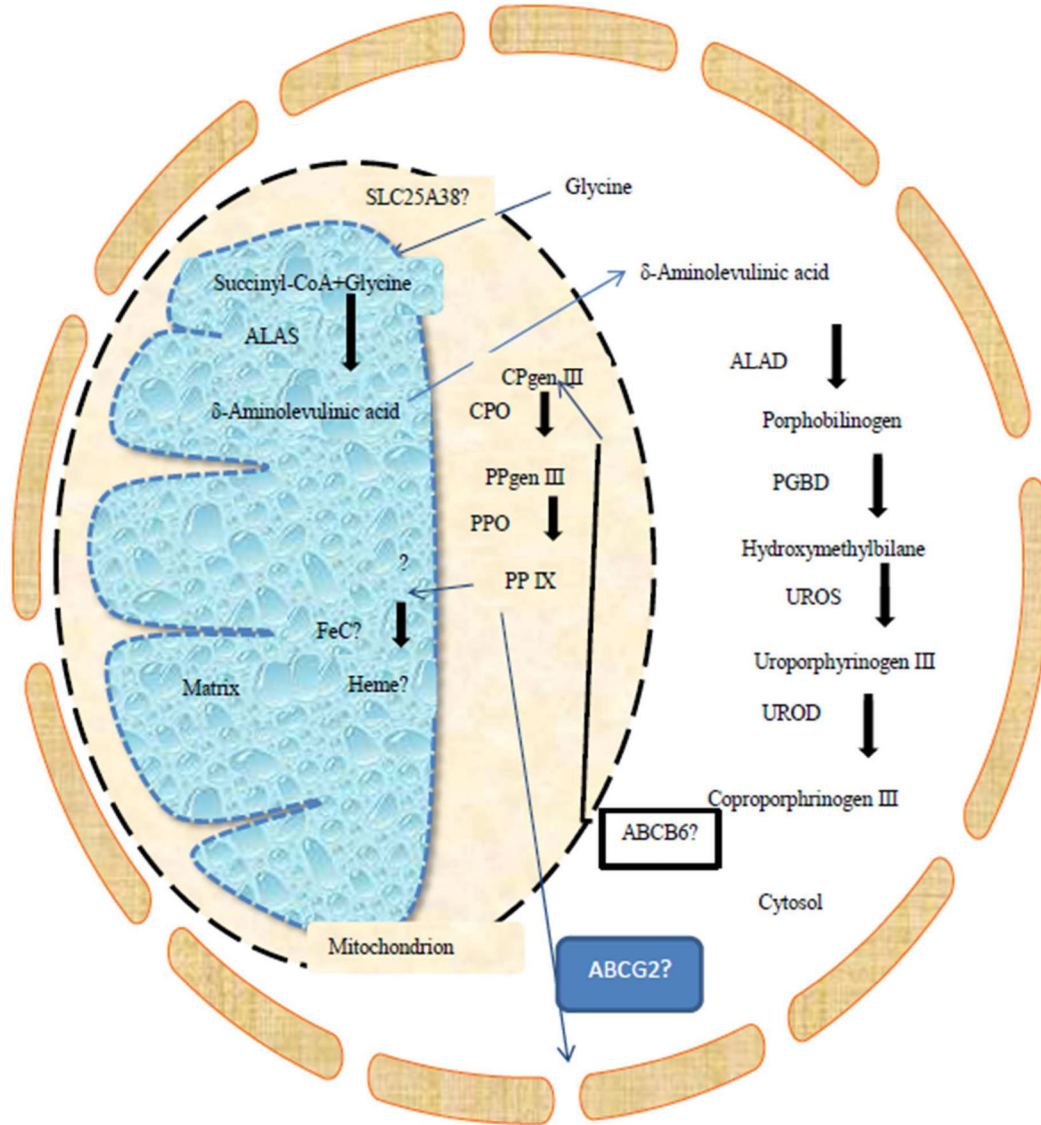


Figure 1. The protoporphyrin IX biosynthesis pathway presumed to be in the shell gland of brown-egg laying hen. Figure 1 has been partly adapted from Ajioka et al. (2006), Khan and Quigley (2011), and Miller and Kappas (1974). ALAS is Aminolevulinic acid synthase; ALAD is Aminolevulinic acid dehydratase; PGBD is Porphobilinogen deaminase; UROS is Uroporphyrinogen III synthase; UROD is Uroporphyrinogen decarboxylase; CPgen III is Coproporphyrinogen III; CPO is Coproporphyrinogen oxidase; PPgen III is Protoporphyrinogen III; PPO is Protoporphyrinogen oxidase; PP IX is protoporphyrin IX.

acid synthase recorded in the deep-brown-egg laying hens compared with hens laying lighter colored eggs (Li et al., 2013). The shell gland tissue of laying hens has the capability of transforming δ -aminolevulinic acid into porphyrin *in vitro* (Polin, 1957) at greater capacity than do tissues from other segments of the oviduct (Polin, 1959). The histological and radiographic study of the shell gland at various stages of shell formation showed red fluorescence and pigment granules, which increased until cuticle deposition (Tamura and Fujii, 1967b; Baird et al., 1975). A higher activity of several porphyrin biochemical enzymes in the shell gland was recorded in Rhode Island Red hens compared to mutant and White Leghorn hens (Schwartz et al., 1980). There is much evidence that protoporphyrin IX is synthesized in the shell gland (Zhao et al., 2006; Wang et al., 2007), but this type of synthesis does not ex-

clude the possibility that the precursor material is derived from blood (Wang et al., 2009a). The biosynthetic pathway of protoporphyrin IX pigment is well established, as shown in Figure 1, but the cells involved and precursor origins still need to be investigated. The first step in protoporphyrin IX synthesis is thought to be transportation of glycine from cytosol to mitochondria. Protoporphyrin IX appears to be an immediate precursor of heme, following the same metabolic pathway. Many regulators are presumed to be involved in this pathway in the chicken shell gland, and these include some with known functions such as SLC25A38 and ABCB. In normal heme biosynthesis, the last step is conversion of PP IX into heme catalyzed by ferrochelatase. In the chicken shell gland, it is not clear how PP IX is not transformed into heme by ferrochelatase. ABCG2 is thought to export PP IX out of

the cell; however, it is not clear whether it is located on the mitochondrial outer membrane or on the cell membrane.

The availability of δ -aminolevulinic acid in the shell gland might determine the amount of porphyrin deposited into the eggshell (Polin, 1957). In attempting to clarify whether or not the enzyme catalyze reaction for heme synthesis is the same in erythroid and non-erythroid cells, Riddle et al. (1989) found that at least two separate genes were expressed in erythroid and non-erythroid cells, and the enzymes regulated by the genes also differ widely in biochemical properties. Brown eggshell pigment primarily contains protoporphyrin IX, but other pigments including uroporphyrin, coproporphyrin, and biliverdin and its zinc chelates are also present, which led With (1974) to conclude that protoporphyrin is synthesized in the oviduct. The precursor molecule, protoporphyrinogen, is colorless, and the brown color appears when this compound is auto-oxidized into protoporphyrin (Sparks, 2011).

HOUSING SYSTEM, NUTRITION, AND EGG SHELL COLOR

With the increase in free-range commercial farming in different parts of the world and particularly in Australia, there is interest in investigation of the factors causing pale shell color and how to improve eggshell color in hens laying brown-shelled eggs. Consumer preference is shifting from quantity to quality with free-range eggs gaining popularity due to the perception that it is a natural production system (Wang et al., 2009b). Egg color is generally maintained well in cage flocks, but there is anecdotal evidence that maintenance of shell color can be more challenging in free-range flocks (Sekeroglu et al., 2010). It has been reported that eggs from free-range flocks may be lighter in color compared to those from cage systems (Samiullah et al., 2014).

Hen nutrition is an area that needs to be tested in relation to eggshell color. Hooge (2007) reported that certain probiotics improved shell color in hens laying brown-shelled eggs. Feeding *Bacillus subtilis* supplemented feed to 63 wk Lohman Brown hens improved the intensity of brown shell color in the following two weeks of production. The mode of action of *Bacillus subtilis* is not clear and needs further investigation; however, a possible mechanism of action could be due to amino acid residues such as His183 and Glu264 in *Bacillus subtilis* ferrochelatase facilitating the insertion of metal ion into protoporphyrin (Hansson et al., 2007). Some elements, such as Fe, Cu, Mn, and Zn, function as chelating carriers at the central position of porphyrin molecules (Solomon, 1987). Feed supplemented with Fe soy proteinate significantly improved eggshell color in brown-egg laying hens (Seo et al., 2010).

Vanadium is usually found in commercial poultry feed at very low inclusion levels (Miles and Henry, 2004). Vanadium in poultry feed has a detrimental effect on shell color (Sutly et al., 2001), which can be overcome by feeding vitamin C at various levels, depending on the level of vanadium in the feed (Odabasi et al., 2006). The exact mechanism of vanadium toxicosis is not clear. The suggestion that loss of shell color in free-range laying hens could be due to elevated levels of vitamin D was tested in a study that found that vitamin D had no significant effect on shell color (Roberts et al., 2014).

GENETICS OF THE EGG SHELL COLOR

The molecular basis of brown pigment synthesis and its possible metabolic pathway in brown-egg laying hens need further exploration in order to determine the genes involved (Wang et al., 2013). The color of the eggshell is assumed to be controlled by several genes that encode proteins and enzymes, thereby regulating the production and deposition of pigment into the shell (Van Brummelen and Bissbort, 1993; Liu and Cheng, 2010), but the responsible genes in brown-egg laying hens are yet to be identified. The higher δ -aminolevulinic acid synthase activity in brown-egg laying hens compared to white-egg laying hens suggests that the trait is purely controlled by genes (Schwartz et al., 1980). Brown eggshell color is due to a dominant gene that is epistatic to the recessive white shell color gene (Punnett and Bailey, 1920); however, the phenotypic heritability calculations for dams ($s^2 = 0.9135$) for eggshell color in brown-shelled eggs showed some dominance over sire ($s^2 = 0.3035$) (Blow et al., 1950). Similarly, in measuring the heritability estimates of sire and dams for shell color in a Light Sussex flock, the dam heritability was higher than the sire, indicating the existence of a dominance effect (Hunton, 1962). Genetically, the quantitative trait loci (QTL) region on chromosomes 2, 4, 5, 6, and 11 influences eggshell color (Wardecka et al., 2002; Sasaki et al., 2004; Schreiweis et al., 2006). The genes coding for aminolevulinic acid synthase and aminolevulinic acid ferrochelatase are thought to be located in chromosome 1 (Schwartz et al., 1980). Based on the phenotypic observations, when white- and brown-egg laying chickens are crossed, a co-dominance effect is created with an intermediate eggshell color (Hall, 1944; Blow et al., 1950); however, when comparing the sire effects, pullets sired by the White Leghorn male produced eggs with less pigment than the pullets sired by the Rhode Island Red male, suggesting the involvement of sex-linked genes (Hall, 1944). Based on the available information, it is still unclear which genes are responsible for brown pigment synthesis in the shell gland.

The heritability of eggshell color in a brown-egg laying breed was closer to 0.50 (Francesch et al., 1997). The analysis of brown-egg laying strains and their crosses has shown a higher variance value for dam than sire (Blow et al., 1950; Hunton, 1962). A protein haplotype

of ovocalyxin 32 has been shown to affect eggshell color (Fulton et al., 2012). A gene that can reduce protoporphyrin has some value in breeding for eliminating the tinted eggshell problems occurring sometimes in white-egg laying hens (Shoffner et al., 1982). Recent molecular work has established that the expression of *SLCO1A2* and *SLCO1C1* genes in the shell gland of a brown-egg laying hen was associated with brown eggshell color (Zheng et al., 2014). In the heritability measurements, a low genetic correlation of eggshell color with quality traits suggests that eggshell color affects other shell traits at a very low level (Zhang et al., 2005). Summarizing the genetic work conducted, it can be concluded that the genetics of the brown eggshell color in the laying hen has not been studied sufficiently to understand the process of shell pigment synthesis in the shell gland. Dunn (2011) offers the opinion that traditional methods of selection for shell color may prove more useful than molecular methods.

HEN STRAIN, AGE, AND EGG SHELL COLOR

Production of uniform dark-brown colored eggshells through selective breeding is the goal of poultry breeders of brown-egg laying hens. Significant differences in shell color have been recorded among breeds laying eggs with brown shells (Grover et al., 1980). The amount of brown and blue pigments is higher in the shell gland and eggshell of brown and blue eggs when compared to white-shelled eggs, which indicates that pigment is breed specific (Liu et al., 2010). Hens that lay lighter-colored eggs at the start of lay also lay lighter-colored eggs in the late laying period (Odabasi et al., 2007).

Generally, the eggshell gets paler as the hen ages (Odabasi et al., 2007). It is not clear how this happens, but increase in egg size with hen age is considered one of the main factors (Hunton, 1962; Grover et al., 1980; Odabasi et al., 2007). In a longitudinal study of the effect of hen age on brown eggshell color, there was no significant difference between the eggshell color at 35 to 75 wk, but the 25 wk eggshell color was significantly darker than all other age groups (Samiullah et al., 2014). Following the same flock at different ages (longitudinal study) or different ages of different brown-egg laying flocks (horizontal study), eggshell color gets lighter as the flocks get older (Samiullah, 2012). The amount of protoporphyrin IX in 1 g of whole eggshell in 33, 50, and 67 wk old HyLine Brown flocks was not significantly different (Samiullah and Roberts, 2013); however, the amount of protoporphyrin IX measured in the cuticle itself was significantly higher at 50 wk compared with 33 and 67 wk eggs, which suggests that shell color gets lighter with flock age, and the amount of cuticle present on eggshell surface has positive influence on pigment present in the shell. It can be concluded that eggshell color generally gets lighter as the hen ages.

INFECTIOUS BRONCHITIS VIRUS AND EGG SHELL COLOR

Infectious bronchitis virus (IBV) is a coronavirus of economic importance to the poultry industry around the world. IBV can infect chickens of all ages and has the capability to multiply in various epithelial tissues, including trachea, lungs, kidney, ovaries, and oviduct (Ignjatovic et al., 2002). The incubation period of IBV in fully susceptible hens is about 18 to 36 h (Sevoian and Levine, 1957). Many IBV strains affect egg production and cause paleness of eggshell color in brown-egg laying hens (Chousalkar and Roberts, 2007). Previous studies show that IBV strains differentially affect the epithelial tissues in the shell-forming regions of the oviduct in brown-egg laying hens (Chousalkar et al., 2007). The Australian IBV strains are mainly respiratory and nephropathogenic with variation in pathogenicity, but they are also capable of infecting the oviduct (Ignjatovic et al., 2002). There was no significant difference in the histopathology induced by two strains of IBV (T and N1/88) in the oviduct of vaccinated and unvaccinated birds (Chousalkar et al., 2007). Comparing the histopathological severity of the different strains in the oviduct, T strain was more virulent followed by N1/88 and then the vaccine strains, Vic S and A3. All strains caused paler coloration of brown-shelled eggs (Chousalkar and Roberts, 2009; Chousalkar et al., 2009). The mechanism of action of IBV strains on pigment synthesis is not clear, but pathology induced in the oviduct by IBV may disrupt the cellular mechanisms responsible for the secretion and subsequent deposition of pigment onto the eggshell. Further research is needed to explore the mechanism of action of IBV on pigment secretion and deposition. IBV strains have been shown to cause disorder of eggshell formation by disrupting isthmus gene expression of collagen type I and calcium binding protein 28kDa (CaBP-D28K) in the uterus (Nii et al., 2014). The viral antigen localizes only in the epithelial cells lining the oviduct (Crimion and Hofstad, 1972). Different strains differentially reduce the beating of epithelial cilia (Raj and Jones, 1996). Other diseases that cause deterioration of brown shell color are *Mycoplasma* spp. (Gole et al., 2012), Newcastle disease, and egg drop syndrome 76 (Higashihara et al., 1987), but their mechanism of action is also not known. Some parasitic diseases (e.g. *Leucocytozoon caulleryi*) that cause damage to the oviduct (Nakamura et al., 2001) may result in the production of paler-shelled eggs.

STRESS AND EGG SHELL COLOR

One of the major problems in the assessment of poultry welfare is measurement of stress. Handling and relocation stress for about 4.5 h prior to subsequent oviposition has been shown to delay hen oviposition time by about 3.0 h (Reynard and Savory, 1999). Hens become unable to lay if the level and duration of stress exceed a

certain level (Reynard and Savory, 1999). Molting stress has been shown to severely affect eggshell color once the hen starts laying again, with the extent of the response being different among individual hens (Aygün, 2013). Hens kept at high densities in cages are more stressed compared to hens in individual cages or those at low stocking densities (Mills et al., 1987). Stress factors, such as cage design, high cage density, fear, and frequent disturbance can cause brown-egg laying hens to lay lighter-colored eggshells (Walker and Hughes, 1998). Shell abnormalities such as pale coloration or extraneous calcium may be caused by exposure of hens to environmental disturbances (Hughes et al., 1986), larger group size (Mills et al., 1987), certain drug infusions (e.g. adrenaline, nicarbazin, sulphonamides), and management failure. If such disturbances occur shortly prior to oviposition, the egg is retained in the shell gland and extra calcium coating takes place, which masks the brown shell color (Hughes et al., 1986). Physical stress, such as experimental removal of feathers and high environmental temperature, has a detrimental effect on pigment deposition onto the shell (Tangkere et al., 2001). An intrauterine injection of prostaglandin F_{2α} can cause the secretion of pigment and induce quick oviposition with paler shells (Soh and Koga, 1999). A drug, indomethacin, administered by intrauterine injection, has been shown to completely inhibit the secretion of pigment in the shell gland (Soh and Koga, 1999).

NICARBAZIN AND EGGSHELL COLOR

The coccidiostat drug nicarbazin, when fed to poultry, resulted in depigmented eggs at various levels within days of its administration (Hughes et al., 1991). The severity and duration of the effect depend on the drug concentration and duration of treatment (Schwartz et al., 1975; Hughes et al., 1991). This drug did not affect porphyrin synthesis in oviduct tissue (Polin, 1959; Schwartz et al., 1975), but how it affects deposition of protoporphyrin into the eggshell is not clear. Similarly, it did not prevent the elevation of erythrocyte protoporphyrin levels in the regenerative phase, following hen bleeding nor did it affect the formation of porphyrin from δ -aminolevulinic acid incubated with homogenates of uterus (Polin, 1959). The effect of the drug is reversible and shell color is restored usually within 6 to 8 days, once the drug is withdrawn (Hughes et al., 1991).

In an *in vitro* study, nicarbazin did not prevent tissue porphyrin synthesis from aminolevulinic acid, as tissues from hens fed nicarbazin formed the same level of porphyrin as the control group (Polin, 1959). These findings suggest that there could be another pathway for porphyrin synthesis other than the glycine succinate cycle. The decrease in shell pigment deposition in nicarbazin fed laying hens varied with the amount of nicarbazin-fed (Polin, 1959).

SHELL COLOR AND EGG QUALITY

Eggshell and egg internal quality are influenced by various factors such as egg weight, shell weight, specific gravity, shell breaking strength, shell deformation, shell thickness, albumen height, and yolk color. A significant correlation between brown shell color and shell strength (Yang et al., 2009) may indicate that brown eggshell pigment affects shell quality. A dark brown eggshell color has been linked to higher eggshell specific gravity, which is a shell quality indicator (Joseph et al., 1999). Brown eggshell color has been positively correlated with some shell characteristics such as shell strength and hatchability (Sekeroglu and Duman, 2011), while egg internal quality has no correlation with shell color (Yang et al., 2009). Further, it has been suggested that some shell quality parameters such as shell strength, shell weight, shell thickness, and shell ultrastructure can be assessed via shell color because of significant correlations between the shell quality indicator and shell color (Schreiweis et al., 2006; Yang et al., 2009); however, others have provided conflicting evidence (Joseph et al., 1999; Richards and Deeming, 2001), and thus shell color cannot be applied reliably as a quality assessment tool.

CONCLUSIONS AND FUTURE RESEARCH

It can be concluded that protoporphyrin IX is synthesized in the shell gland, but the mechanism of synthesis is not fully understood. Eggshell color has some positive correlations with egg quality parameters. The intensity and evenness of the shell color deposition are affected by multiple factors. Further research is needed to elucidate the metabolic pathway of brown pigment in the shell gland and find out the genes for which over-expression can significantly increase the intensity of the brown shell pigment.

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