

Mitochondrial transcription factor A induces the declined mitochondrial biogenesis correlative with depigmentation of brown eggshell in aged laying hens

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ABSTRACT Eggshell color is an important characteristic for poultry eggs. Eggs from aged hens usually have poor shell color that is unacceptable for the table egg market. The objective of this study was to examine effects of pigment synthesis and mitochondrial biogenesis on brown eggshell color of aged laying hens. In this trial, 8 hens laying eggs with darker shell color and 8 hens laying eggs with lighter shell color were selected from 300 62-week-old Hy-Line brown-egg laying hens. Results showed that egg weight ($P < 0.05$), eggshell weight ($P < 0.01$), protoporphyrin IX (**Pp IX**) content of the eggshell and the shell gland ($P < 0.001$), and biliverdin content of the shell gland ($P < 0.001$) were significantly declined in the light-shell group compared with the dark-shell group. Relative mRNA expression of δ -aminolevulinic acid synthase1 (**ALAS1**) ($P < 0.05$), coproporphyrinogen

oxidase ($P < 0.01$), ATP-binding cassette transporter **ABCG2** ($P < 0.01$), and mitochondrial transcription factor A ($P < 0.05$) was reduced in hens laying lighter brown eggshell. Moreover relative mRNA expression of mitochondrial DNA copy number ($P < 0.01$), mitochondrial NADH dehydrogenase subunit 4 ($P < 0.05$), mitochondrial ATP synthase F0 subunit 8 ($P < 0.05$), and mitochondrial cytochrome c oxidase 1 ($P < 0.01$) was significantly decreased in the shell gland of the light-shell group. In addition, NAD^+ contents of the shell gland were increased in the dark-shell group ($P < 0.01$). Brown eggshell depigmentation is a result of decreased Pp IX content in the eggshell and the shell gland. Decreased mitochondrial biogenesis may contribute to the depigmentation of brown eggshell by targeting ALAS1 and ALAS1-mediated Pp IX biosynthesis.

Key words: brown eggshell depigmentation, protoporphyrin IX, mitochondrial biogenesis, aged laying hen

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INTRODUCTION

Eggshell color is an important shell quality parameter and has positive effects on consumer preference (Johnston et al., 2011). Darker brown is the favorable color for table eggs. Brown eggshell color gets paler as hens aged (Bi et al., 2018; Sirri et al., 2018), exposed to vanadium and nicarbazin supplementation (Samiullah et al., 2017; Wang et al., 2018), or challenged by diseases and

stresses (Ebeid et al., 2012; Wilson, 2017). Aging is an irreversible factor for paler brown eggs in practice. In addition, the heritability of eggshell color is closer to 0.50 in brown-egg laying breed (Franceschi et al., 1997). Individual differences might make poor eggshell color. It would be appropriate animal models that lay eggs with darker or lighter eggshell color for studying shell depigmentation.

Brown, blue, white, and tinted eggshells contain 2 major pigments, protoporphyrin IX (**Pp IX**) and biliverdin (**Bv**) (Ostertag et al., 2019). Blue eggshells contain a large amount of Bv, which is formed from the decomposed heme (Zhang et al., 2019) and have antioxidative activities toward free radicals and reactive oxygen species (Hargitai et al., 2016). Brown, white, and tinted eggshells are rich in Pp IX (Ostertag et al., 2019), which exhibits antibacterial activity as a protector on the egg surface

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(Ishikawa et al., 2010). The concentration of Pp IX has been reported to be positively correlated with antibacterial protein contents of the albumen (Javůrková et al., 2019) and cuticle (Mikšík et al., 2014). Moreover, a large amount of Pp IX is deposited in the cuticle region and the palisades region of brown eggshell (Samiullah and Roberts, 2013). The decreased Pp IX content results in the depigmentation of brown egg (Li et al., 2013; Bi et al., 2018). The concentration of Pp IX is responsible for the intensity of brown eggshell color.

It has been noted that Pp IX is synthesized in the epithelial cell of the shell gland (Samiullah et al., 2015; Li et al., 2016). Synthetases, transporter proteins, and other regulators are suggested to be associated with the synthesis of the pigment. The δ -aminolevulinic acid synthase1 (**ALAS1**), the first rate-limiting enzyme, plays a key role in Pp IX synthesis (Riddle et al., 1989). It has been reported that the downregulation of *ALAS1* mRNA by nicarbazin treatment results in a decline in Pp IX content in the shell gland (Samiullah et al., 2017).

In the epithelial cells of the shell gland, mitochondria provide ATP through electron transport and oxidative phosphorylation and play an important role in Pp IX biosynthesis (Li et al., 2016). Nicarbazin-induced depigmentation of brown eggshell characterizes with a decline in mitochondrial contents of the shell gland (Samiullah et al., 2017), indicating that the content of Pp IX might be positively related to the amount of mitochondrion. In addition, mitochondrial transcription factor A (**TFAM**) acts as the biogenesis and the maintenance of mitochondrial DNA (**mtDNA**) (Gustafsson et al., 2016). It has been reported that TFAM and ALAS1 are mediated by peroxisome proliferator-activated receptor- γ coactivators β (**PGC-1 β**) and nuclear respiratory factor 1 (**NRF1**) (Shao et al., 2010). These nuclear transcription factors may simultaneously regulate mitochondrial biogenesis and Pp IX synthesis. The link between pigment synthesis and mitochondrial biogenesis needs further study.

The objective of this study was to examine effects of pigment synthesis and mitochondrial biogenesis on brown eggshell color in aged laying hens. By selecting hens with the darker eggshell ($48 \leq L^*$ [lightness] ≤ 52) or the lighter eggshell ($58 \leq L^* \leq 68$), our study examined the changes of gene expression of pigment synthesis and mitochondrial biogenesis. Our findings may serve as a reference to enhance brown egg color of being more uniformity.

MATERIAL AND METHODS

Animal and Experimental Design

All experimental protocols were approved by the Animal Care and Use Committee of the Feed Research Institute of the Chinese Academy of Agricultural Sciences. A total of 300 62-week-old Hy-Line brown laying hens were reared in individual cages and were fed a corn-soybean meal diet (Table 1). They were allowed to access to food and water ad libitum. Room environment was controlled at 22°C with daily lighting schedule of

Table 1. Composition of the basal diet (air-dry basis).

Ingredients	Contents (g/kg of diet)
Corn	655
Soybean meal	235
Limestone	89.3
Sodium chloride	3.0
Dicalcium phosphate	13.5
Choline chloride (50%)	1.0
DL-Methionine (98%)	1.0
Multimineral ¹	2.0
Multivitamin ²	0.2
Nutrient levels	
ME (MJ/kg)	11.16
CP	165.0
Total phosphorus	5.4
Available phosphorus	3.3
Calcium	33.1
Lysine	8.6
Methionine	3.7

¹Supplied per kilogram of diet: Cu, 8 mg; Zn, 66 mg; Fe, 60 mg; Mn, 65 mg; Se, 0.3 mg; I, 1 mg.

²Supplied per kilogram of diet: vitamin A, 12,500 IU; vitamin D₃, 4,125 IU; vitamin E, 15 IU; menadione, 2 mg; thiamin, 1 mg; riboflavin, 8.5 mg; pyridoxine, 8 mg; cobalamin, 5 mg; pantothenic acid, 50 mg; niacin, 32.5 mg; biotin, 2 mg; folic acid, 5 mg; choline, 500 mg.

16L:8D. Eggs from all hens were collected for 14 successive days. Eggshell color was measured using a colorimeter (NH310; 3nh Cor., Shenzhen, China) with an 8-mm measuring aperture. The L*a*b* color system was used to assess eggshell color. Each egg was measured one time at the equator region of its surface. Owing to the correlation coefficient of L* value with Pp IX concentration being -0.75 (Li et al., 2013), L* value was used as the standard to select hens. Darker eggshells were those whose L* value was at least 2 times of standard deviation lower than the average of L* ($n = 300$). Lighter eggshells were those whose L* value was at least 2 times standard deviation higher than the average L* ($n = 300$). Finally, 24 hens with darker eggshell ($L^* = 50.00 \pm 1.87$) and 24 hens with lighter eggshell ($L^* = 62.35 \pm 2.92$) were selected. All selected hens laid eggs with stable L* within 14 d. Seven successive eggs from 48 hens were collected. The oviposition time was monitored for 5 successive days using Automatic-Monitoring Control System (FRI; CAAS, Beijing, China). Stable oviposition time within 5 d was a standard to choose hens. For reducing the stress, hens whose oviposition time was at the same period of the time were selected. Eight birds per group were slaughtered at 15 h after ovulation. Shell gland region of the oviduct was divided into 2 parts and stored at -80°C .

Eggshell Quality Determination

The collected eggs were measured for L*a*b* value, egg weight, shell strength, shell thickness. L*a*b* value was determined using a colorimeter (NH310; 3nh Co., Shenzhen, China) at the equator of the shell. The shell strength was measured using an egg force reader (Orka Technology Ltd., Ramat Hasharon, Israel). All eggs were measured at end of egg. Shell thickness was expressed as the mean values obtained at 3 locations

on the surface of the egg (i.e., the air cell, equator, and sharp end) using an Eggshell Thickness Gauge (ESTG1; Orka Technology Ltd., Ramat Hasharon, Israel). Shell proportion was defined as the percentage of shell weight to egg weight.

Measurement of Pigment Concentration in Tissue and Eggshell

After shell membrane removal by mechanical scraping, eggshells were rinsed. Air-dried eggshells were ground into powder. Shell glands were freeze-dried and were cut into pieces. Approximately 0.25 g eggshell powders and 0.1 g shell gland pieces were independently dissolved into 4 mL of the solvent ($V_{\text{methanol}}: V_{\text{concentrated HCl}} = 2:1$). Extraction of eggshell pigment was in the darkness at 4°C for 24 h. All extractions were centrifuged at 5,000 rpm for 10 min at 4°C. The supernatant (0.2 mL) was transferred for analysis with a microplate reader (Versa Max; Molecular Devices Co., Shanghai, China) at Pp IX-specific absorbance wavelength of 412 nm and Bv-specific absorbance wavelength of

670 nm. The measurement was undertaken in duplicate. Standards of Pp IX (TargetMol Co., Shanghai, China) and Bv (Toronto Research Chemicals Inc., Toronto, Canada) were separately weighed (0.003 g and 0.0035 g, respectively) and added into 6 mL solvent. Next, $2^{-7} \sim 2^{-13} \times$ Pp IX standard solutions and $2^{-4} \sim 2^{-11} \times$ Bv standard solutions were analyzed to make the standard curves that were used to calculate Pp IX and Bv content in the eggshell and shell gland (Wang et al., 2009).

RNA Isolation and Real-Time Quantitative PCR

Approximately 80 mg frozen shell glands was weighted and was added into an RNase-free tube containing 1 mL precooled TRIzol Reagent (TIANGEN Biotech. Co., Ltd., Beijing, China). All protocols were performed at 4°C or on the ice in accordance with the manufacturer's instructions. Each sample was conducted at 1 time. Extracted RNA was dissolved in RNase-free water. Quantity and purity of total RNA were

Table 2. Sequences for RT-PCR primers.

Genes ¹	Primer sequence ² (5'-3')	Accession no.
β -actin ³	F: ATGATATTGCTGCGCTCGTT R: TCTTCTGGCCCATACCAACC	L08165
ATP8 ³	F: ATCCTCACTACTGTCATCTTAAC R: AGTATGATGGAGAATCATGG	NC_040970.1
ND4 ³	F: CGCAGGCTCCATACTACTCG R: TTAGGGCACCTCATAGGGCT	NC_040970.1
COX1 ³	F: CCATACTACTTACCGACCGCAACC R: GTGTCTACGTCCATTCCGACTGTG	NC_040970.1
GADPH ⁴	F: GGTCACCAAGAAGGTGGAGA R: GACAGTGCCCTTGAAGTGTC	NC_006088.3
ALAS1 ⁴	F: GGTGGACAGGAAAGGTAAAGA R: ACTGGTCATACTGGAAGGTG	NM_001018012.1
CPOX ⁴	F: GAGAGGACGGTATGTGGAGT R: TTTGGGATGCGGAGAAC	XM_416596.6
ABCB6 ⁴	F: CTCAACTGGTTCCGGCACCTA R: TTTACTGCATCCTTACCTCC	XM_015290086.1
ABCG2 ⁴	F: CCTCCTTGTAACCTCCCTT R: GTAATCTTACCAGAGCACCTT	XM_421638.4
FLVCR1 ⁴	F: AGTGACATCGAGTGTGCTGCT R: GGTCAAAGTCTGGGAGATCTGGG	XM_419425.5
TSPO ⁴	F: GCGGTAGTTGAGCACAGAAGCC R: TTGGCAAGAAGCATCCAGA	NM_001278057.1
Drp1 ⁴	F: TGTGACCCGAAGACCCCTTA R: AGCATCTATCTCATTTTCATCTCCA	NM_001079722.1
MFN1 ⁴	F: GTTGGCAGTGATCGGTGAGGTG R: GACGGTCTTGACGCTTCTTCTC	XM_015291424.2
NRF1 ⁴	F: ACAGCAACAGACCACAACCACAC R: CTGCTTCTGCTAACGATGCTACCG	NM_001030646.1
PGC-1 β ⁴	F: AGAGGAGGAGGAGGAGGAGGAAG R: CACCGCTCACCGTTCTCACAC	XM_025155027.1
TFAM ⁴	F: CGAGGAAGCAAGGAAGACAGACTG R: CCTGAGCAAGCTGAAGGTATGGC	XM_015287950.2

¹Abbreviations: *ABCB6* and *ABCG2*, ATP-binding cassette transporter B6 and G2; *ALAS1*, δ -aminolevulinic acid synthase; *ATP8*, ATP Synthase F0 subunit 8; *COX1*, mitochondrial cytochrome c oxidase1; *CPOX*, coproporphyrinogen oxidase; *Drp1*, dynamin 1-like protein; *FLVCR1*, feline leukemia virus subgroup C cellular receptor1; *MFN1*, mitofusin1; *ND4*, NADH dehydrogenase subunit 4; *NRF-1*, nuclear respiratory factor 1; *PGC-1 β* , peroxisome proliferator-activated receptor- γ coactivators β ; RT-PCR, real-time quantitative polymerase chain reaction; *SLC25A5*, solute carrier family 25 member 5; *TFAM* mitochondrial transcription factor A; *TSPO*, translocator protein.

²Abbreviations: F, forward and R, reverse.

³Genes were used to amplify fragment of mitochondrial DNA.

⁴Genes were used to amplify fragment of cDNA.

examined using an UV/Visible spectrophotometer (Amersham Bioscience, Sweden) at an absorbance of 260 nm and were determined by the 18S and 28S bands using electrophoresis in 1% agarose gels stained with ethidium bromide. As per the TIANGEN QuantScript RT kit (TIANGEN Biotech. Co., Ltd., Beijing, China) instructions, cDNA samples were acquired by reverse transcription of total RNA. Gene expression was carried out by using a RealMasterMix-SYBR Green kit (TIANGEN Biotech. Co., Ltd., Beijing, China) in an iCycler iQ5 multicolor real-time quantitative polymerase chain reaction (**RT-PCR**) detection system (Bio-Rad Laboratories, Hercules, CA). The primer sequences for the target and reference genes are shown in Table 2. The protocol for gene determination was as follows: 95°C for 5 min, 40 cycles of 95°C for 10 s, and 60°C for 30 s. Each sample was analyzed in triplicate. Amplification efficiency of each gene was validated by constructing a standard curve through serial dilutions of cDNA. As per the melting curves, the specificity of the amplified products was confirmed. The results of relative mRNA expression of genes were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Determination of mtDNA Relative Expression and Copy Number

Mitochondrial genomic DNA was extracted from approximately 30 mg frozen shell glands by using a TIAN-Namp Genomic DNA Kit (TIANGEN Biotech. Co., Ltd., Beijing, China). All operations were completed at room temperature. Each sample was conducted at 1 time. Relative expression of mtDNA was measured by RT-PCR. The methods and calculation were followed by the cDNA quantitation and relative mRNA expression. The primer sequences for the target and reference genes are shown in Table 2. The mtDNA copy number was determined using the equation $\text{copies} = 2^{(-Ct_{mt})/(-Ct_{reference})}$. The copy number of mtDNA per cell was estimated by the average of 3 mtDNA copy numbers in a cell (Ballester et al., 2004).

Measurement of NAD^+ and NADP^+ Contents in Shell Gland

As per colorimetric kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) instructions, approximately 0.1 g frozen shell glands were used to analyze NAD^+ and NADP^+ contents. All procedures were carried out at room temperature.

Transmission Electron Microscopy

The mucosa layer of fresh shell gland was cut into 1 mm thickness soaking in 2.5% (vol/vol) glutaraldehyde. Samples were prepared as previously described (Anand et al., 2014) and were observed under a transmission electron microscope (HT7700; Hitachi, Ltd., Tokyo, Japan) at an acceleration voltage of 80 kV.

Statistical Analysis

The eggshell quality, the pigment concentration of the eggshell and the shell gland, gene expression of the shell gland, mtDNA copy number and relative expression of the shell gland, as well as NAD^+ and NADP^+ content of the shell gland between 2 groups were analyzed by Student *t* test with SAS 9.1 (SAS Institute Inc., Cary, NC). Differences were considered statistically significant at $P < 0.05$.

RESULTS

Eggshell Quality Parameters

Compared with the dark-shell group, L^* value was increased and a^* and b^* values were decreased in the light-shell group ($P < 0.001$, Table 3). There were no differences in shell thickness, shell strength, and shell proportion between the groups ($P > 0.05$, Table 3), but the egg weight was higher in the dark-shell group than that in the light-shell group ($P < 0.05$, Table 3).

Pigment Content in Eggshell and Shell Gland

Compared with the light-shell group, the content of Pp IX was higher in the eggshell and the shell gland of the dark-shell group ($P < 0.001$, Table 4). No difference in Bv content was observed in eggshell between the 2 groups ($P > 0.05$, Table 4), but Bv content of the shell gland was higher in the dark-shell group ($P < 0.001$, Table 4).

Pp IX-Synthesized Gene mRNA Expression

The expression levels of 7 genes in the shell gland of 2 groups are shown in Figure 1A – 1B. RT-PCR revealed that the *ALAS1* and coproporphyrinogen oxidase expression was significantly higher in the dark-shell group than in the light-shell group ($P < 0.05$ and $P < 0.01$, Figure 1A). As per Pp IX-related transporter proteins mRNA expression, *ABCG2* was upregulated in the dark-shell group ($P < 0.01$, Figure 1B), and there were no differences in ATP-binding cassette transporter

Table 3. Eggshell quality of dark- and light-shell groups.

Items ¹	Treatment		<i>P</i> -value
	Dark-shell group	Light-shell group	
L^* value	50.00 ± 1.87	62.35 ± 2.92	<0.001
a^* value	26.46 ± 0.87	20.13 ± 1.92	<0.001
b^* value	37.64 ± 1.70	30.21 ± 2.47	<0.001
Egg weight (g)	68.09 ± 3.55	65.38 ± 4.26	0.021
Shell thickness (mm)	0.44 ± 0.02	0.45 ± 0.03	0.16
Shell strength (N)	41.18 ± 5.37	41.68 ± 5.04	0.74
Shell proportion (%)	9.61 ± 0.65	9.53 ± 0.73	0.76
shell weight (g)	6.78 ± 0.51	6.35 ± 0.51	0.0044

¹ L^* value, a^* value, b^* value, egg weight, shell thickness, and shell strength were the mean of 7 replicates with each hen; shell proportion and shell weight were the mean of 3 replicates with each hen. Data were expressed as mean ± SD.

Table 4. Protoporphyrin IX and biliverdin content in the eggshell and shell gland.

Samples ¹	Treatment		P-value
	Dark-shell group	Light-shell group	
Pp IX in eggshell (µg/250 mg)	24.92 ± 3.35	13.96 ± 2.13	<0.001
Pp IX in shell gland (µg/100 mg)	20.09 ± 2.69	11.46 ± 1.57	<0.001
Bv in eggshell (µg/250 mg)	4.15 ± 1.33	4.10 ± 1.24	0.92
Bv in Shell gland (µg/100 mg)	14.49 ± 1.92	9.16 ± 2.33	<0.001

Abbreviations: Bv, biliverdin; Pp IX, Protoporphyrin IX.

¹Ten eggshells were analyzed; 8 eggshell glands were analyzed. Data were expressed as mean ± SD.

ABCB6, solute carrier family 25 member 5, translocator protein, and feline leukemia virus, subgroup C between the 2 groups ($P > 0.05$, Figure 1B).

Mitochondrial Copy Number and Relative Expression of mtDNA

Mitochondrial copy number was significantly higher in the dark-shell group than that of the light-shell group ($P < 0.01$, Figure 2A). Compared with the dark-shell group, the expression of mitochondrial ATP Synthase F0 subunit 8 ($P < 0.05$, Figure 2B), NADH dehydrogenase subunit 4 ($P < 0.05$, Figure 2B) and mitochondrial cytochrome c oxidase 1 ($P < 0.01$, Figure 2B) was decreased in light-shell group.

Mitochondrial Biogenesis-Related mRNA Expression

RT-PCR analysis of dynamin 1-like protein, mitofusin1, *NRF1*, *PGC-1β* and *TFAM* mRNA expression in the shell gland of 2 groups is shown in Figure 3. There were no differences in dynamin 1-like protein, mitofusin1, *NRF1* and *PCG-1β* mRNA level between the 2 groups ($P > 0.05$, Figures 3A, 3B). However, *TFAM*

was downregulated in the light-shell group in contrast with the dark-shell group ($P < 0.01$, Figure 3B).

Observation of Mitochondrial Morphology

Mitochondrial morphology is showed in the Figure 4A – 4D. No swelling and fragmental mitochondrion was observed between 2 groups (Figures 4A, 4C). There were no broken mitochondrial cristae and no existence of mitochondrial vacuoles between 2 groups (Figures 4B, 4D).

NAD⁺ and NADP⁺ Contents

The analysis of NAD⁺ and NADP⁺ contents in the shell gland is shown in Figure 5. NAD⁺ contents were significantly reduced in the light-shell group compared with the dark-shell group ($P < 0.01$, Figure 5A). There was no difference in NADP⁺ contents between 2 groups ($P > 0.05$, Figure 5B).

DISCUSSION

Whether poor eggshell color affect eggshell quality is opposing reports (ŞEkeroglu and Duman, 2009; Li et al., 2016). In the present study, there were no differences in shell thickness, shell strength, and shell

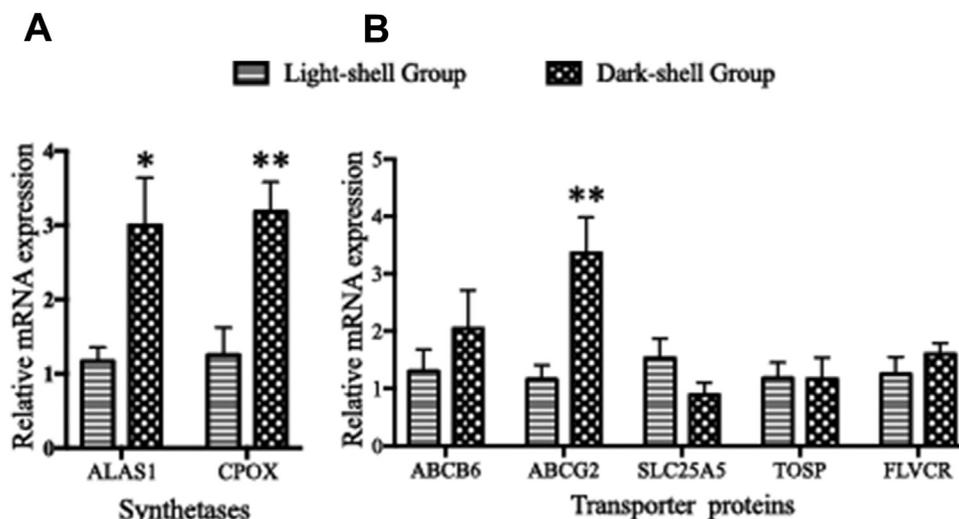


Figure 1. Protoporphyrin IX synthetase and transporter protein relative mRNA expression. Abbreviations: *ABCB6* and *ABCG2*, ATP-binding cassette transporter B6 and G2; *ALAS1*, δ-aminolevulinic acid synthase; *CPOX*, coproporphyrinogen oxidase; *FLVCR1*, feline leukemia virus subgroup C cellular receptor1; *SLC25A5*, solute carrier family 25 member 5; *TSP0*, translocator protein. Data were expressed as mean ± SE. * $P < 0.05$, ** $P < 0.01$.

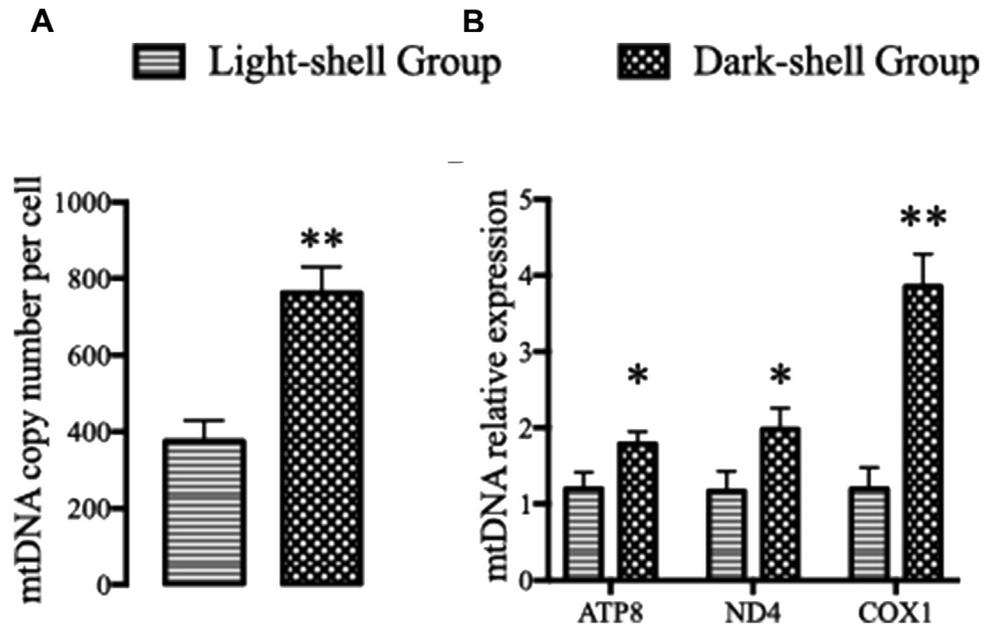


Figure 2. Mitochondrial DNA copy number and relative expression. (A) mitochondrial DNA copy number per epithelial cell of shell gland. Abbreviations: *ATP8*, ATP Synthase F0 subunit 8; *COX1*, mitochondrial cytochrome c oxidase1; mtDNA, mitochondrial DNA; *ND4*, NADH dehydrogenase subunit 4. Data were expressed as mean \pm SE. * $P < 0.05$, ** $P < 0.01$.

proportion between 2 groups. It was similar to the previous results (Li et al., 2016). Eggshell color may not be a direct indicator for eggshell quality (Samiullah et al., 2015). But, lower egg weight was observed in the light-shell group. It may be related to contents of antimicrobial and immune protein. Protoporphyrin IX content of eggshell has a positive correlation with antimicrobial protein contents of the albumen (Javůrková et al., 2019). In the model of eggshell depigmentation, deteriorated albumen quality by vanadium treatment results in the loss of egg weight (Yuan et al., 2016; Wang et al., 2018) and characterizes the decreased proteins related

to immune responses (Wang et al., 2017). But, it needs to be investigated in further study. Moreover, under no changes in shell proportion, the difference in eggshell weight might be association with the loss of egg weight. Eggshell color has no impacts on eggshell quality.

It has been found that decreased Pp IX is responsible for the depigmentation of brown eggshell (Li et al., 2013; Samiullah et al., 2017). In our study, reduced a^* and b^* values in the light-shell group indicated the decreased saturation of the color. In pigment content analysis, higher Pp IX and Bv content were observed in the shell gland of dark-shell hens, but there was no difference in

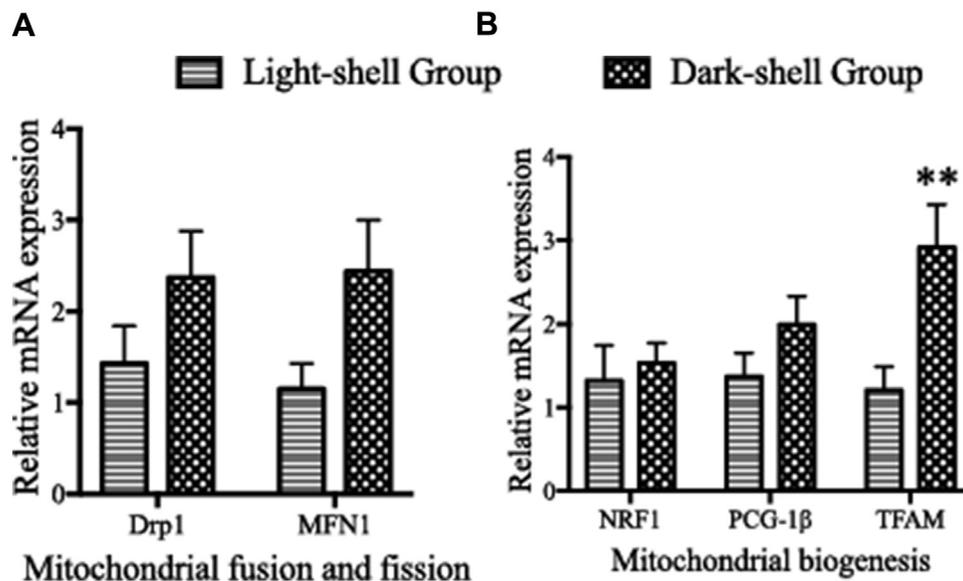


Figure 3. Mitochondria dynamic gene relative mRNA expression. Abbreviations: Drp1, dynamin 1-like protein; MFN1, mitofusin1; NRF-1, nuclear respiratory factor 1; PGC-1 β , peroxisome proliferator-activated receptor- γ coactivators β ; TFAM mitochondrial transcription factor A. Data were expressed as mean \pm SE. ** $P < 0.01$.

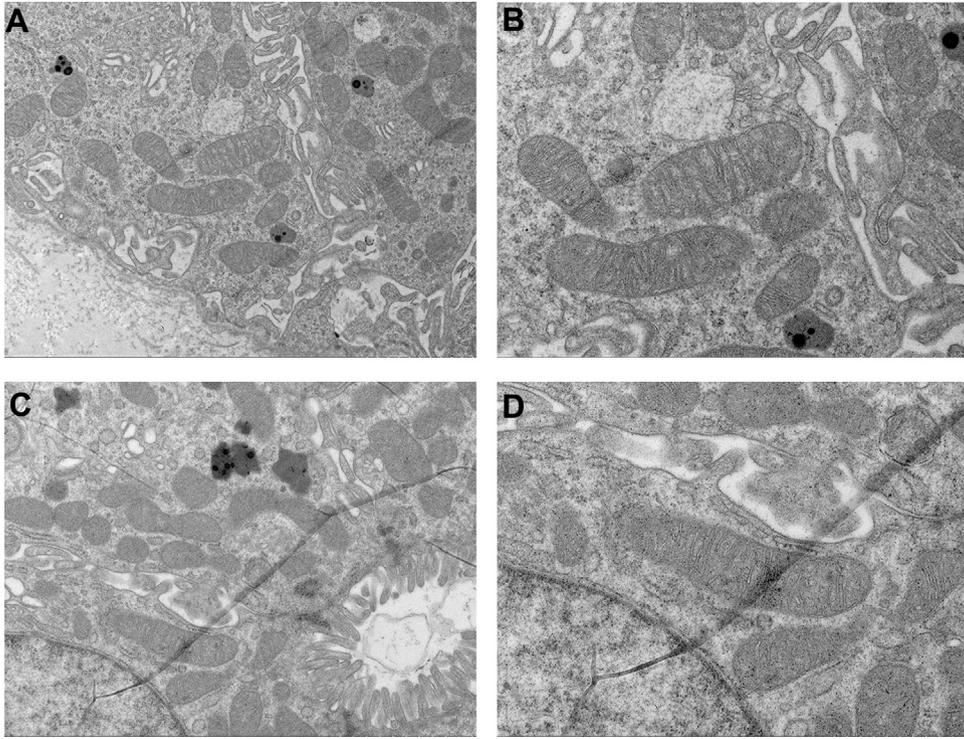


Figure 4. Mitochondrial morphology. (A) Mitochondria of the dark-shell group under 4,000 \times micrographs. (B) Mitochondrial cristae and vacuoles in the dark-shell group under 8,000 \times micrographs. (C) Mitochondria of light-shell group under 5,000 \times micrographs. (D) Mitochondrial cristae and vacuoles in light-shell group under 10,000 \times micrographs.

Bv content of the eggshell between 2 group. It is clear that Pp IX content of eggshell gland has a dominating effect on eggshell color (Li et al., 2013). But, what roles Bv acted in brown eggshell color were unknown. Our results suggested that Bv may play a limited role in brown eggshell color and the Bv transport and deposition in eggshell gland may be not as efficient as Pp IX in Hy-Line brown laying hens, which will need further study.

What was interesting in the study was the higher Bv detected by the shell gland of the dark-shell group. Biliverdin acts as an antioxidant (Hargitai et al., 2016), and its content reflects maternal antioxidant state of fowl (Moreno and Osorno, 2003; Hargitai et al., 2016). Higher level of Bv might indicate that shell glands of dark-shell hens showed stronger antioxidative properties compared with light-shell hens. Producing higher Pp IX content in

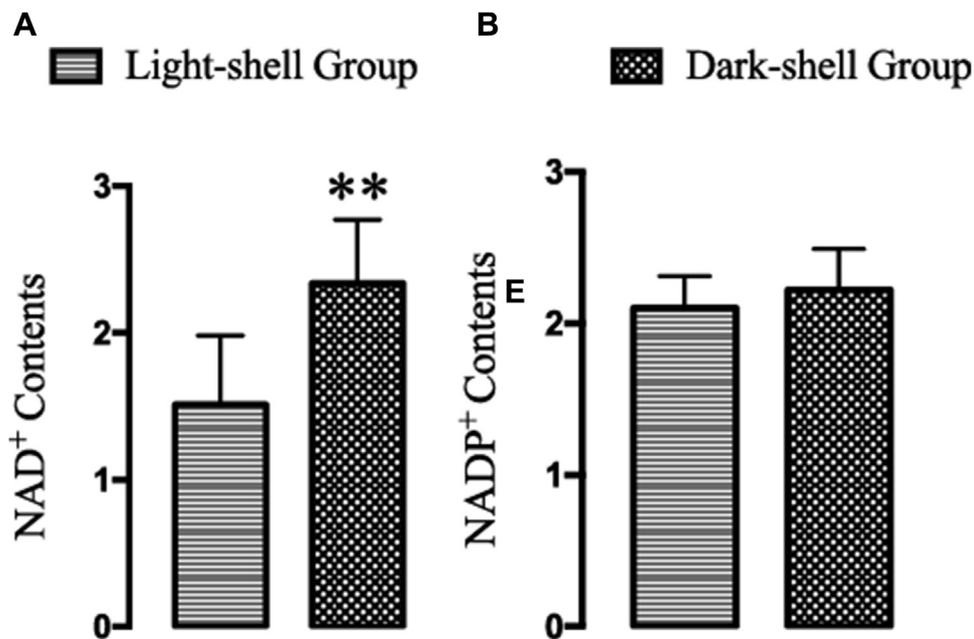


Figure 5. NAD⁺ and NADP⁺ Contents in the shell gland. (A) NAD⁺ contents (nmol/mg prot). (B) NADP⁺ contents (nmol/mg prot). Data were expressed as mean \pm SD. ** $P < 0.01$.

the eggshell gland contributes to darker brown eggshell color.

As the biosynthetic pathway of Pp IX showed (Figure 6), synthetases and transport proteins have effects on eggshell color (Zheng et al., 2014). For example, ALAS1 and δ -aminolevulinic acid dehydratase are detected in shell gland of Rhode Island Red but not in White Leghorn (Stevens et al., 1974). In our study, relative mRNA expression of *ALAS1* and coproporphyrinogen oxidase was reduced in the light-shell group. The results were in concordance with the previous study (Li et al., 2013). However, only *ALAS1* expression decreases in nicarbazin-induced shell depigmentation (Samiullah et al., 2017), indicating that *ALAS1* plays an important role in Pp IX synthesis in the eggshell gland. On the other hand, Pp IX content is controlled by the pigment transporter proteins in the shell gland (Li et al., 2013). Currently, *ABCG2* mRNA expression was increased in the dark-shell group. The *ABCG2* transporters are distributed not only in the plasma membrane but also in the mitochondrial membrane and export the Pp IX from mitochondrion to cytosol then out of the cell (Kobuchi et al., 2012). The higher expression of *ABCG2* might be the necessity of transporting higher Pp IX in dark-shell hens. δ -Aminolevulinic acid synthase1 regulates pigment synthesis in the shell gland.

A part of pigment synthesis occurs in the mitochondrion (Li et al., 2016). The functional form of *ALAS1* is found in intracellular compartment of the mitochondrial matrix (Riddle et al., 1989). Whether were mitochondrial counts associated with Pp IX synthesis? In the present study, mtDNA copy numbers and relative

expression of ATP Synthase F0 subunit 8, NADH dehydrogenase subunit 4, and mitochondrial cytochrome c oxidase 1 were reduced in the light-shell group. The results revealed that mtDNA depletion had impacts on Pp IX synthesis, which may be carried out through lessening activated *ALAS1*. In eggshell depigmentation by nicarbazin treatment, compared with 5 and 23.5 h after ovulation, mtDNA copies of shell gland are decreased at 15 h, the time in consistent with a decrease in Pp IX synthesis (Samiullah et al., 2017). On the other hand, the images of mitochondrial morphology showed no broken cristae and no vacuolations in the mitochondria of light- and dark-shell hens and no differences in the mRNA level of mitochondrial fusion and fission between 2 groups. In our trial, other changes of the mitochondrion were not detected between 2 groups except mtDNA depletion. As it has been acknowledged, mitochondrion is one of the important organelle as the energy metabolism and a small amount of the calcium store in the cell (Dedkova and Blatter, 2013), so it is important to maintain production performance for laying hens (Khan et al., 2019). Mitochondrial dysfunction has impacts on eggshell quality (Mashaly et al., 2004; Akbarian et al., 2016). The decreased mtDNA copies are one of the factors to limit pigments synthesis in the shell gland.

It has been noted that the replication, transcription, and translation of mtDNA are controlled by TFAM (Gustafsson et al., 2016). In our study, mitochondrial biogenesis was compromised by the low expression level of *TFAM* gene. In addition, *TFAM* and *ALAS1* translation are simultaneously regulated by nucleus transcription factors, such as PGC-1 α/β and NRF1 (Wu et al.,

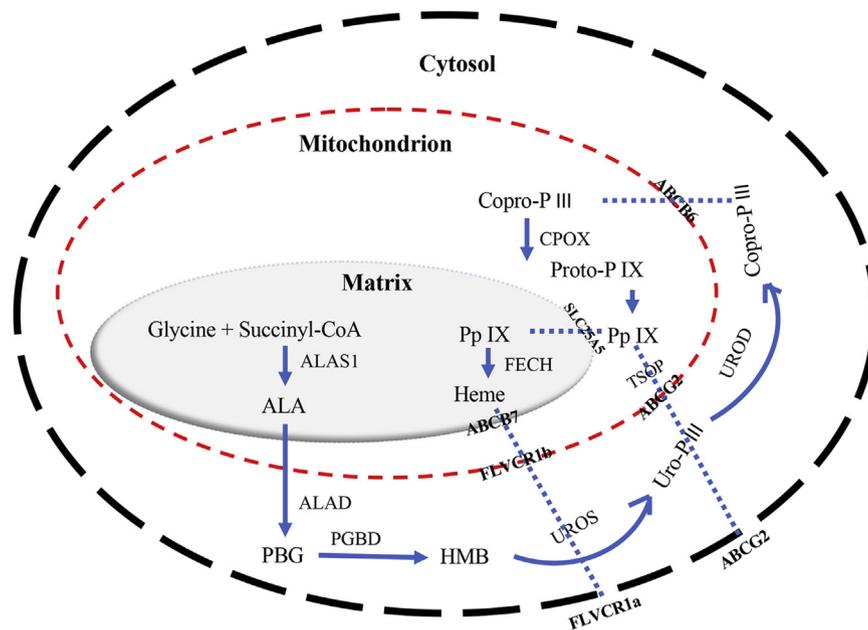


Figure 6. The heme and protoporphyrin IX biosynthetic pathway. Abbreviations: *ABCB6*, *ABCB7* and *ABCG2*, ATP-binding cassette transporter B6, B7 and G2; ALA, δ -aminolevulinic acid; ALAD, δ -aminolevulinic acid dehydratase; *ALAS1*, δ -aminolevulinic acid synthase; Copro-p III, coproporphyrinogen III; *CPOX*, coproporphyrinogen oxidase; *FECH*, ferrochelatase; *FLVCR1a* and *FLVCR1b*, feline leukemia virus subgroup C cellular receptor1 a and b; HMB, hydroxymethylbilane; PBG, porphobilinogen; *PGBD*, porphobilinogen deaminase; Pp IX, protoporphyrin IX; *SLC25A5*, solute carrier family 25 member 5; *TSPO*, translocator protein; *UROD*, uroporphyrinogen decarboxylase; Uro-p III, uroporphyrinogen III; *UROS*, uroporphyrinogen synthase.

1999; Handschin et al., 2005). They may have effects on mitochondrial biogenesis and pigment biosynthesis. Our results showed that there were no differences in the relative expression of *PGC-1 β* and *NRF1* genes between 2 groups. It has been reported that except for *PGC-1 α / β* -*NRF1* mediation, *PGC-1 α / β* -independent manner also affects mitochondrial counts during a brief period (Gomes et al., 2013), but it will need further study. Depigmentation-associated mtDNA depletion is mediated by *TFAM* beyond *PGC-1 β* -dependent manner.

Oxidative stresses alters the abundance of mitochondria, as well as the copy numbers and integrity of mtDNA (Lee and Wei, 2005). In our study, there was no difference in the system NADP^+ contents between 2 groups. But NAD^+ contents of the shell gland were reduced in the light-shell hens. It has been reported that in addition to maintain mtDNA by electron transport, NAD^+ directly governs the contents of sirtuin1 content, NAD^+ -dependent deacetylase that involves in mitochondrial biogenesis by regulating *TFAM* (Gomes et al., 2013). Increased NAD^+ level can reverse mtDNA depletion (Gomes et al., 2013). The increase in NAD^+ contents of the eggshell gland might facilitate eggshell color with the high uniformity, and it needs to be investigated in further study.

CONCLUSIONS

The remarkable traits of brown eggshell depigmentation resulted from decreased Pp IX content in the eggshell and shell gland. Decreased mitochondrial biogenesis may contribute to the depigmentation of brown eggshell by targeting *ALAS1* and *ALAS1*-mediated Pp IX biosynthesis.

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DISCLOSURES

The authors declare no conflicts of interest.

REFERENCES

- Akbarian, A., J. Michiels, J. Degroote, M. Majdeddin, A. Golian, and S. De Smet. 2016. Association between heat stress and oxidative stress in poultry; mitochondrial dysfunction and dietary interventions with phytochemicals. *J. Anim. Sci. Biotechnol.* 7:37.
- Anand, R., T. Wai, M. J. Baker, N. Kladt, A. C. Schauss, E. Rugarli, and T. Langer. 2014. The i-AAA protease YME1L and OMA1 cleave OPA1 to balance mitochondrial fusion and fission. *J. Cell Biol.* 204:919–929.
- Ballester, M., A. Castelló, E. Ibáñez, A. Sánchez, and J. M. Folch. 2004. Real-time quantitative PCR-based system for determining transgene copy number in transgenic animals. *Biotechniques* 37:610–613.
- Bi, H., Z. Liu, C. Sun, G. Li, G. Wu, F. Shi, A. Liu, and N. Yang. 2018. Brown eggshell fading with layer ageing: dynamic change in the content of protoporphyrin IX. *Poult. Sci.* 97:1948–1953.
- Dedkova, E. N., and L. A. Blatter. 2013. Calcium signaling in cardiac mitochondria. *J. Mol. Cell Cardiol.* 58:125–133.
- Ebeid, T., T. Suzuki, and T. Sugiyama. 2012. High ambient temperature influences eggshell quality and calbindin-D28k localization of eggshell gland and all intestinal segments of laying hens. *Poult. Sci.* 91:2282–2287.
- Francesch, A., J. Estany, L. Alfonso, and M. Iglesias. 1997. Genetic parameters for egg number, egg weight, and eggshell color in three Catalan poultry breeds. *Poult. Sci.* 76:1627–1631.
- Gomes, A. P., N. L. Price, A. J. Ling, J. J. Moslehi, M. K. Montgomery, L. Rajman, J. P. White, J. S. Teodoro, C. D. Wrann, and B. P. Hubbard. 2013. Declining NAD^+ induces a pseudohypoxic state disrupting nuclear-mitochondrial communication during aging. *Cell* 155:1624–1638.
- Gustafsson, C. M., M. Falkenberg, and N. G. Larsson. 2016. Maintenance and expression of mammalian mitochondrial DNA. *Annu. Rev. Biochem.* 85:133–160.
- Handschin, C., J. Lin, J. Rhee, A.-K. Peyer, S. Chin, P.-H. Wu, U. A. Meyer, and B. M. Spiegelman. 2005. Nutritional regulation of hepatic heme biosynthesis and porphyria through *PGC-1 α* . *Cell* 122:505–515.
- Hargitai, R., N. Boross, Z. Nyiri, and Z. Eke. 2016. Biliverdin- and protoporphyrin-based eggshell pigmentation in relation to antioxidant supplementation, female characteristics and egg traits in the canary (*Serinus canaria*). *Behav. Ecol. Sociobiol.* 70:2093–2110.
- Ishikawa, S. I., K. Suzuki, E. Fukuda, K. Arihara, Y. Yamamoto, T. Mukai, and M. Itoh. 2010. Photodynamic antimicrobial activity of avian eggshell pigments. *FEBS Lett.* 584:770–774.
- Javůrková, V. G., M. Pokorná, I. Mikšík, and E. Tůmová. 2019. Concentration of egg white antimicrobial and immunomodulatory proteins is related to eggshell pigmentation across traditional chicken breeds. *Poult. Sci.* 98:6931–6941.
- Johnston, N. P., L. K. Jefferies, B. Rodriguez, and D. E. Johnston. 2011. Acceptance of brown-shelled eggs in a white-shelled egg market. *Poult. Sci.* 90:1074–1079.
- Khan, S., J. Roberts, and S. B. Wu. 2019. Genes involved in mitochondrial biogenesis and function may not show synchronised responses to mitochondria in shell gland of laying chickens under infectious bronchitis virus challenge. *BMC Mol. Cell Biol.* 20:3.
- Kobuchi, H., K. Moriya, T. Ogino, H. Fujita, K. Inoue, T. Shuin, T. Yasuda, K. Utsumi, and T. Utsumi. 2012. Mitochondrial localization of ABC transporter ABCG2 and its function in 5-aminolevulinic acid-mediated protoporphyrin IX accumulation. *PLoS One* 7:e50082.
- Lee, H. C., and Y. H. Wei. 2005. Mitochondrial biogenesis and mitochondrial DNA maintenance of mammalian cells under oxidative stress. *Int. J. Biochem. Cell Biol.* 37:822–834.
- Li, G., S. Chen, Z. Duan, L. Qu, G. Xu, and N. Yang. 2013. Comparison of protoporphyrin IX content and related gene expression in the tissues of chickens laying brown-shelled eggs. *Poult. Sci.* 92:3120–3124.
- Li, G., C. Sun, G. Wu, F. Shi, A. Liu, and N. Yang. 2016. iTRAQ-based quantitative proteomics identifies potential regulatory proteins involved in chicken eggshell brownness. *PLoS One* 11:e0168750.
- Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. *Methods* 25:402–408.
- Mashaly, M., G. Hendricks, 3rd, M. Kalama, A. Gehad, A. Abbas, and P. H. Patterson. 2004. Effect of heat stress on production parameters and immune responses of commercial laying hens. *Poult. Sci.* 83:889–894.
- Mikšík, I., P. Ergang, and J. Pácha. 2014. Proteomic analysis of chicken eggshell cuticle membrane layer. *Anal. Bioanal. Chem.* 406:7633–7640.
- Moreno, J., and J. L. Osorno. 2003. Avian egg colour and sexual selection: does eggshell pigmentation reflect female condition and genetic quality? *Ecol. Lett.* 6:803–806.

- Ostertag, E., M. Scholz, J. Klein, K. Rebner, and D. Oelkrug. 2019. Pigmentation of white, brown, and green chicken eggshells analyzed by reflectance, transmittance, and fluorescence spectroscopy. *Chem. Open* 8:1084–1093.
- Riddle, R. D., M. Yamamoto, and J. D. Engel. 1989. Expression of delta-aminolevulinic synthase in avian cells: separate genes encode erythroid-specific and nonspecific isozymes. *Pro. Nati. Acad. Sci. U. S. A.* 86:792–796.
- Samiullah, S., and J. R. Roberts. 2013. The location of protoporphyrin in the eggshell of brown-shelled eggs. *Poult. Sci.* 92:2783–2788.
- Samiullah, S., J. R. Roberts, and K. Chousalkar. 2015. Eggshell color in brown-egg laying hens — a review. *Poult. Sci.* 94:2566–2575.
- Samiullah, S., J. Roberts, and S. B. Wu. 2017. Downregulation of ALAS1 by nicarbazin treatment underlies the reduced synthesis of protoporphyrin IX in shell gland of laying hens. *Sci. Rep.* 7:6235.
- Şekeroglu, A., and M. Duman. 2009. Effect of egg shell colour of broiler parent stocks on hatching results, chickens performance, carcass characteristics, internal organ weights and some stress indicators. *Kafkas Üniv. Vet. Fak.* 17:837–842.
- Shao, D., Y. Liu, X. Liu, L. Zhu, Y. Cui, A. Cui, A. Qiao, X. Kong, Y. Liu, and Q. Chen. 2010. PGC-1 β -regulated mitochondrial biogenesis and function in myotubes is mediated by NRF-1 and ERR α . *Mitochondrion* 10:516–527.
- Sirri, F., M. Zampiga, A. Berardinelli, and A. Meluzzi. 2018. Variability and interaction of some egg physical and eggshell quality attributes during the entire laying hen cycle. *Poult. Sci.* 97:1818–1823.
- Stevens, E. V., L. K. Miller, S. Weinstein, and A. Kappas. 1974. Biosynthesis of δ -aminolevulinic acid and porphobilinogen in the domestic fowl (*Gallus domesticus*). *Comp. Biochem. Physiol. B* 47:779–786.
- Wang, J., X. Bai, X. Ding, S. Bai, Q. Zeng, X. Mao, and K. Zhang. 2017. Quantitative proteomic analysis reveals the role of tea polyphenol EGCG in egg whites in response to vanadium stress. *Nutrition* 39:20–29.
- Wang, J., Z. Yuan, K. Zhang, X. Ding, S. Bai, Q. Zeng, H. Peng, and P. Celi. 2018. Epigallocatechin-3-gallate protected vanadium-induced eggshell depigmentation via P38MAPK-Nrf2/HO-1 signaling pathway in laying hens. *Poult. Sci.* 97:3109–3118.
- Wang, X. T., C. J. Zhao, J. Y. Li, G. Y. Xu, L. S. Lian, C. X. Wu, and X. M. Deng. 2009. Comparison of the total amount of eggshell pigments in Dongxiang brown-shelled eggs and Dongxiang blue-shelled eggs. *Poult. Sci.* 88:1735–1739.
- Wilson, P. B. 2017. Recent advances in avian egg science: a review. *Poult. Sci.* 96:3747–3754.
- Wu, Z., P. Puigserver, U. Andersson, C. Zhang, G. Adelmant, V. Mootha, A. Troy, S. Cinti, B. Lowell, and R. C. Scarpulla. 1999. Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. *Cell* 98:115–124.
- Yuan, Z. H., K. Y. Zhang, X. M. Ding, Y. H. Luo, S. P. Bai, Q. F. Zeng, and J. P. Wang. 2016. Effect of tea polyphenols on production performance, egg quality, and hepatic antioxidant status of laying hens in vanadium-containing diets. *Poult. Sci.* 95:1709–1717.
- Zhang, T., H. Liu, J. Wang, L. Li, C. Han, A. Mustafa, and X. Xiong. 2019. Evidences in duck (*Anas platyrhynchos*) by transcriptome data for supporting the biliverdin was mainly synthesized by shell gland. *Poult. Sci.* 98:2260–2271.
- Zheng, C., Z. Li, N. Yang, and Z. Ning. 2014. Quantitative expression of candidate genes affecting eggshell color. *Anim. Sci. J.* 85:506–510.