Green tea powder inclusion promoted hatchability through increased yolk antioxidant activity

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ABSTRACT Dietary supplementation of green tea powder (**GTP**) changes egg quality of hens, however, whether these changes affect incubation is still unknown. This study was to compare the proteomic difference of incubated eggs from hens with GTP supplemented or not. Huainan partridge chickens (1,080) at 35 wk of age were allocated into 2 groups, one group fed basal diet (**CG**) and one group fed basal diet plus 1% GTP (**EG**). After 4 wk feeding, artificially fertilized eggs were collected for yolk cholesterol determination and incubation. During incubation, 6 embryos from each group were randomly selected in each day for yolk protein extraction and quantification. Yolk cholesterol content was significantly lower, while the hatchability was significantly higher in EG than that of the CG group ($P < 0.05$). Yolk protein concentration at embryonic days (**ED**) of 0, 2, 6, and 13 showed significant changes and were selected for proteomic analysis by 2-dimensional gel electrophoresis combined with liquid chromatography-tandem mass spectrometry. Fifty-one differentially expressed (**DE**) protein spots were identified among different incubation stages between CG and EG group which were mainly classified into vitellogenin, immunoglobulin, and ovoinhibitor, and occupied 45.1, 23.5, and 15.7%, respectively, to the total DE proteins. Ovotransferrin, participated in extracellular sequestering of iron ion process, was significantly lower in EG group than that of the CG group ($P < 0.05$). Ig light chain precursor (Immunoglobulin) exhibited higher expression at ED6 in EG group as compared with that of the CG group, and was participated in immune response related processes. Ovoinhibitor, mainly involved in protease binding activity, showed lower abundance at ED13 in EG group as compared with that of the CG group. Vitellogenin-3, showed lower expression in EG group as compared with that of the CG group, was mainly participated in lipid transportation and localization according to GO enrichment. Chickens fed diet with GTP provided eggs more antioxidant ability that increased hatchability, indicated that GTP could be considered as additive in breeding layer.

Key words: Huainan partridge chicken, green tea powder, hatchability, proteomics

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INTRODUCTION

Green tea is one of the most popular beverages and produces nearly 6 million tons each year worldwide. Nearly 5 to 10 percent of green tea powder produced and been cast off during processing. Major components of polyphenols in green tea, such as catechins, alkaloids, and polysaccharide could enhance immunity of livestock and poultry, which make green tea a research hotspot as antibiotics replacement (Chandravanshi et al., 2020) Chen et al. (2019). demonstrated that green tea powder could be used as feed additive to improve meat color and *Lactobacillus* proliferation for broiler production Chen et al. (2020). found that green tea powder inclusion decreased plasma lipid concentration and increased the content of orexin A in Chinese indigenous chicken breed. Green tea powder could also decrease egg weight, egg specific gravity, eggshell strength, and thickness, and importantly, yolk cholesterol content (Chen et al., 2021). It is demonstrated that high cholesterol diet is positively associated with hyperlipidemia (Wang et al., 2019) and deteriorates bone health (Mandal, 2015). Therefore, farmers hope to reduce egg cholesterol content through genetic and nutritional methods (Elkin and Rogler, 1990; Carrillo et al., 2012). However, for layer hen breeders, it is not clear whether the

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decreased egg cholesterol could affect embryonic development.

Chicken egg yolk consists of approximately 50% water, 15% protein, and 33% fat, which will be internalized before hatch and provide immediate nourishment for hatchling (O'Sullivan et al., 1991). Therefore, reports suggested that yolk content influence chicken body composition and viability (Finkler et al., 1998). Embryos' successive development requires energy, moisture and DM, which exhibited distinctive association with yolk fatty acids (Peebles et al., 1999).

Although studies demonstrated that yolk fatty acid is the essential nutrient support embryonic development, little is known about the uptake of protein from egg yolk. What's more, all lipids of egg yolk are associated with proteins to form lipoproteins. Therefore, the importance of protein in egg yolk for embryonic development is self-evident, and has been supported by many publications stating that protein insufficiency could cause reduced hatchability and increased mortality (Mohanty et al., 2015).

As mentioned that green tea could reduce egg yolk cholesterol content, whether the reduced fat and lipoprotein could affect hatchability is still unknown. In this experiment, proteomic differences of total protein at different incubation stages were analyzed by 2-dimensional gel electrophoresis (**2-DE**) combined with liquid chromatography-tandem mass spectrometry (**LC-MS/MS**). The results would provide new insights on the regulatory mechanism of egg yolk protein for chicken embryo development, and whether green tea powder could affect egg hatchability and its mechanism.

MATERIALS AND METHODS

All the experimental protocols involving care, handling, and treatment of embryos were approved by the Institutional Animal Care and Use Committee of Anhui Agricultural University, Hefei, Anhui, China. The permission number is No. SYDW-P2018110702.

Materials

A total of 1,080 Huainan Partridge hens (provided by Huainan Partridge chicken breeding farm, Huainan, China) at 35 wk of age, with similar body weight (1.48 ± 0.13 kg), were allocated into 2 groups with 15 battery cages (as 15 replicates, one cage include 6 tiers and 6 cages per tier, 1 hen per cage) per group. Thirty eggs were collected from each group (1 egg per replicate) within 3 connective days for egg quality and yolk cholesterol determination. All chickens were treated under a light /dark cycle of 16L: 8D and free access to feed and water (Chen et al., 2021). Hens from the control group received a basal diet (without GTP, CG group), and hens from the experimental group received a basal diet plus 1% GTP instead of bran (EG group). After 1 wk adaption and 4 wk of feeding, artificially fertilized eggs were collected for 3 connective days for yolk cholesterol content determination and incubation. During incubation, 6 embryos from each group were randomly selected in each day for yolk sac separation and yolk protein extraction. After hatch, the hatchability was calculated in each group (the live embryos during sampling were considered to be able to hatch).

Cholesterol Content and Hatchability Determination

Yolk cholesterol content was measured according to Chen et al. (2021). Hatchability was calculated as number of hatchlings divided by incubated eggs. The mortality was calculated as number of dead embryos divided by incubated eggs.

Egg Yolk Protein Extraction and Quantification

Protein extraction from egg yolk was performed as previously described by Zhu et al. (2020). Bradford protein quantification kit was used for the quantification of protein samples (Yeasen, Shanghai, China). The inflection points of yolk protein concentration during incubation were selected for yolk proteomic analysis.

The 2-D Gel Electrophoresis and Proteins Identification

The 2-DE experiment was performed by using the PROTEAN i12 IEF cell (Bio-Rad, California, CA, USA). Protein sample of 100 μg was added in loading buffer II (loading buffer I with 65 Mm DTT, 0.2% Bio-Lyte) to a total volume of 125 μL, and then loaded on an IPG strip (7 cm, PH 3-10, Bio-Rad) with 2 mL mineral oil (Bio-Rad) coverage. The IPG was hydrated under 18°C for 14 h with the following procedures, 250 V for 0.5 h, 500 V for 0.5 h, 1,000 V for 0.5 h, 12,000 V for 1 h, 3,000 V for 1 h, 4,000 V for 2 h and finally 4,000 V for 20,000 Vh.

After isoelectric focusing, the IPG strip was then equilibrated for 15 min in equilibration buffer I (6 M urea, 2% SDS, 0.375 M Tris-HCl, pH 8.8, 20% glycerol and 0.1 M DTT) and then stand for 15 min in equilibration buffer II (6 M urea, 2% SDS, 0.375 M Tris-HCl, pH 8.8, 20% glycerol and 250 mM iodoacetamide). Second-dimensional electrophoresis was performed by using 12% SDS polyacrylamide gel (Zhu et al., 2020). Gel was stained by Coomassie brilliant blue (P0017F, Beyotime, Shanghai, China) for 30 min, and then washed by double distilled water for 3 times.

Gel Analysis and Protein Spots Identification by LC-MS/MS

All gels were scanned and calibrated using a GS-900 Calibrated Densitometer (Bio-Rad) according to Zhu et al. (2020). Protein spots were analyzed by PDQuest

Table 1. Yolk cholesterol content and hatchability from hens with green tea powder included or not.

Group	Cholesterol content (mg/egg)		Hatchability (%)	Mortality (%)
	35 wk of age	40 wk of age		
CG	216.3±18.9	189.6[a]±15.3	87.1[b]	3.6
EG	207.6±17.5	164.2[b]±9.8	89.3[a]	2.9

CG, control group fed basal diet; EG, experimental group with 1% green tea powder inclusion in the basal diet.
[a, b] Means in a column that possess different superscripts differ significantly ($P < 0.05$).

software (version 8.0.1, Bio-Rad). The differentially expressed protein spots were manually cut out for enzymatic hydrolysis. The digested protein samples were re-dissolved in Nano-LC mobile phase A (0.1% formic acid) for identification by LC-MS/MS. The Easy nLC 1200 (Thermo Fisher) consisted of C18 enrichment column (nanoViper, 3 μm, 100Å) and C18 analytical column (Acclaim PepMap RSLC, 75 μm×25 cm, 2 μm ,100 Å) was used for LC system. Mobile phase B (80% acetonitrile and 0.1% formic acid) was set as gradient increase from 5 to 38% within 10 min. The Thermo Fisher Q Exactive combined with Nano Flex Pump (Thermo Fisher, Massachusetts, MA, USA), was used for MS system. The positive ion mode was Vcap of 1,900 V, the drying gas temperature was set at 275°C, and the scanning mode was DDA (Data Dependent Analysis).

The identification of proteins was performed by searching the MASCOT program (http://www.matrixscience.com) in the nonredundant sequence database of NCBI, and those with identification score <30 were excluded.

Bioinformatic Analysis and Statistical Analysis

Cholesterol content was compared between the 2 groups by student t test using the SAS9.3 system. One-way ANOVA by software SAS9.3 was used for the expression analysis of different protein spots. Gene Ontology (**GO**) analysis was performed by the online website (https://david.ncifcrf.gov/home.jsp), and the GO term of differential protein was clustered into biological processes, cell components, and molecular functions. For GO analysis, the protein spots at 0 embryonic day (**ED**) was set as control when compared with 2 ED, and proteins at 2 ED was set as control when compared with 6 ED, which was also set as control when compared with 13 ED. Student's t test was used for comparing the expression level of proteins between the 2 groups. The online website (http://www.heatmapper.ca/) was used to make the heatmap of differentially expressed protein, and the data was normalized by using the z-value method. The P value less than 0.05 was considered as significant difference.

RESULTS

Yolk Cholesterol Content and Hatchability in the Two Groups

After 4 wk feeding, green tea powder inclusion resulted in a significantly lower yolk cholesterol content and higher hatchability as compared to the control group ($P < 0.05$). The mortality between the 2 groups showed no significant difference (Table 1).

Concentration of Yolk Protein During Incubation

The concentration of yolk protein was determined on each day of incubation (Figure 1). The protein concentration significantly increased at second embryonic day (**ED2**), and then decreased to a very low point of nearly 1 μg/mL at sixth embryonic day (**ED6**). The protein concentration maintained in low level during ED6 to 13th embryonic day (**ED13**) and then increased to a high level similar to that of the ED2. Therefore, embryos at the day of 0, 2, 6, and 13 were selected for proteomic analysis.

Differentially Expressed Yolk Protein During Incubation

A total of 51 differentially expressed (**DE**) protein spots were identified among different incubation stages between the 2 groups (Figures 2 and 3). The identified proteins were mainly classified to Vitellogenin, Immunoglobulin, and Ovoinhibitor, which occupied 45.1, 23.5, and 15.7%, respectively, to the total DE proteins (Table 2).

Differential Expression of Ovotransferrin During Incubation Between the Two Groups The relative molecular weight (**MW**) of protein spots (spot 1, 2, 3, 4, and 5), which represent Ovotransferrin, was near to its theoretical molecular value. The protein abundance of spots 1 and 2 at embryonic day 0 (**ED0**) was significantly higher, while the protein abundance of spots 4 and 5 at ED0 was significantly lower than that of ED13 in eggs from CG group. The protein abundance of spots 1 to 5 at ED0 and ED6 was significantly higher than that of ED2 and ED13. As compared with eggs from CG group, the protein spots 1 to 5 were significantly lower at ED2, ED6, and ED13 in EG group (Table 3).

Differential Expression of Immunoglobulin During Incubation Between the Two Groups Three kinds of Immunoglobulin were identified, Ig gamma chain, immunoglobulin lambda light precursor, and Ig light chain precursor V-J region. The relative MW of Ig gamma chain was higher than that of the theoretical molecular value. While the relative MW of immunoglobulin lambda light precursor and Ig light chain precursor V-J region was near the theoretical molecular value

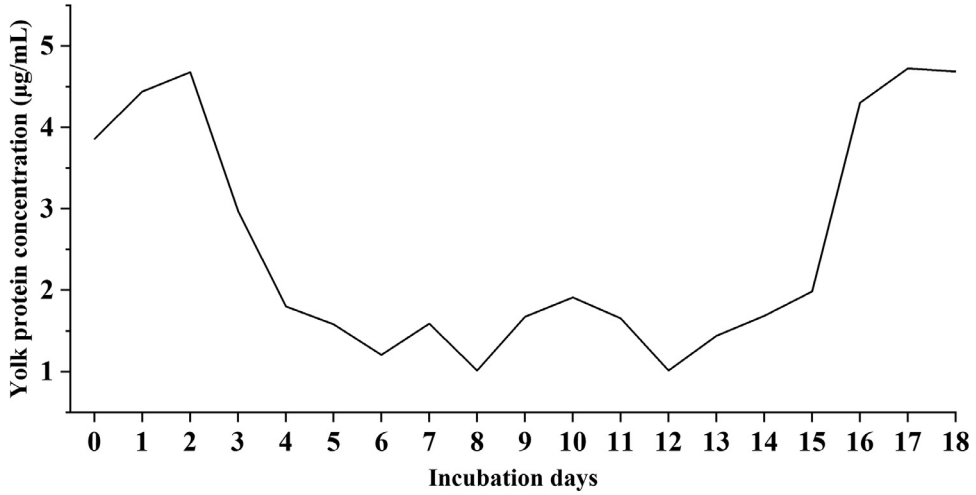

Figure 1. Concentration of yolk protein at each embryonic day.

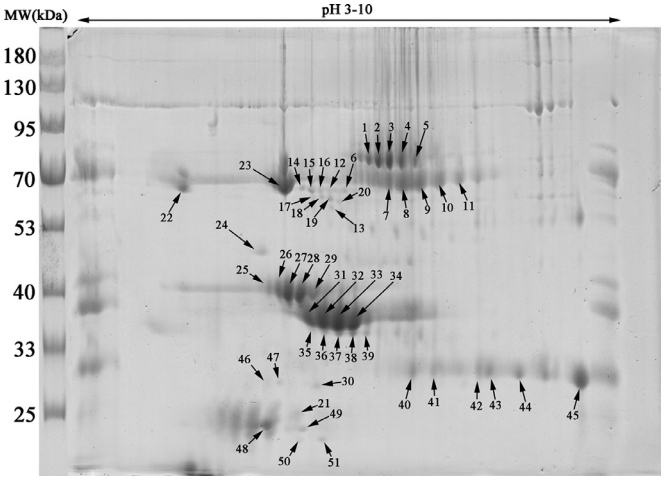

Figure 2. Representative 2-DE gel images of proteins from chicken egg yolk prepared by IEF/SDS-PAGE separation followed by staining with Coomassie Brilliant Blue G-250. Spots that significantly changed in abundance during incubation were indicated by numbers and arrows. The molecular weight (MW) and pI scales were indicated. 2-DE means 2-dimensional gel electrophoresis. IEF means isoelectric focusing.

(Table 2). The protein abundance of Ig gamma chain (spots 6−7 and 11−13) exhibited the lowest at ED6 in the CG group, while the protein abundance of spots 8 to 12 was significantly higher at ED6 than that of ED2 and ED13 in the EG group. As compared with CG group, the protein abundance was decreased at ED2 and ED13, while increased at ED6 in EG group. The abundance of immunoglobulin lambda light (spots 48−50) showed highest at ED2 in CG group, while spot 49 showed lower at ED2 than that of ED6 in EG group, and spots 49 and 50 were not detected at ED13. The abundance of immunoglobulin lambda light showed no significant difference between EG and CG group. The abundance of Ig light chain precursor V-J region (spot 51) at ED0 was significantly higher than that of ED6 and ED13. Ig light chain precursor V-J region was significantly expressed at ED6 in EG group, and showed lower expression at ED0 and ED2, and higher expression at ED6 as compared with that of the CG group (Table 3).

Differential Expression of Ovoinhibitor During Incubation Between the Two Groups The relative MW of protein spots 14 to 20 that represent Ovoinhibitor showed digitally higher theoretical molecular value, while spot 21

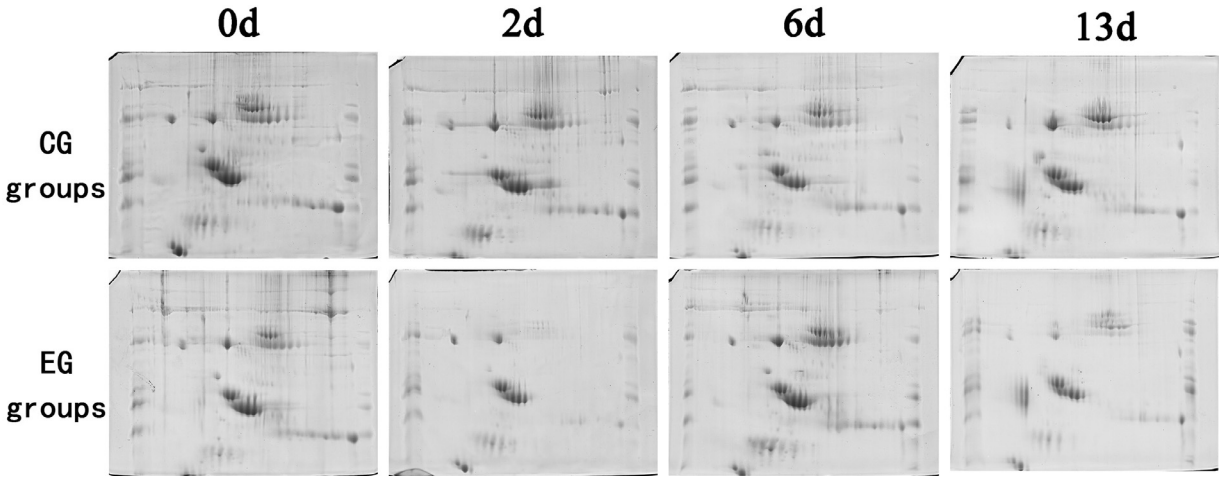

Figure 3. Representative 2-DE gel images of egg yolk proteins from chickens fed diet with green tea powder (EG) or not (CG) at each incubation stages. Each gel was representative of 3 independent replicates.

Table 2. List of significantly altered fertilized chicken egg yolk proteins during incubation.

Spot[1]	Accession[2]	Protein name	Score[3]	% Sequence coverage	MW(kDa/pI)	
					Thero.	Obs.
1	71274075	Ovotransferrin BB type [*Gallus gallus*]	962	22	79.6/7.08	78.9/6.81
2	71274075	Ovotransferrin BB type [*Gallus gallus*]	1,148	23	79.6/7.08	78.9/6.91
3	71274075	Ovotransferrin BB type [*Gallus gallus*]	1,222	23	79.6/7.08	78.9/7.06
4	71274075	Ovotransferrin BB type [*Gallus gallus*]	484	14	79.6/7.08	78.9/7.24
5	71274075	Ovotransferrin BB type [*Gallus gallus*]	857	20	79.6/7.08	78.9/7.42
6	86318	Ig gamma chain (clone 36) - chicken [*Gallus gallus*]	246	18	54.5/6.84	65.9/6.53
7	86318	Ig gamma chain (clone 36) - chicken [*Gallus gallus*]	255	24	54.5/6.84	68.8/7.06
8	86318	Ig gamma chain (clone 36) - chicken [*Gallus gallus*]	384	23	54.5/6.84	68.8/7.24
9	86318	Ig gamma chain (clone 36) - chicken [*Gallus gallus*]	376	18	54.5/6.84	68.8/7.46
10	86318	Ig gamma chain (clone 36) - chicken [*Gallus gallus*]	439	18	54.5/6.84	68.8/7.71
11	86318	Ig gamma chain (clone 36) - chicken [*Gallus gallus*]	473	24	54.5/6.84	68.8/7.97
12	86318	Ig gamma chain (clone 36) - chicken [*Gallus gallus*]	357	22	54.5/6.84	65.9/6.32
13	86318	Ig gamma chain (clone 36) - chicken [*Gallus gallus*]	156	22	54.5/6.84	59.1/6.32
14	212485	Ovoinhibitor [*Gallus gallus*]	275	25	54.4/6.16	65.9/5.98
15	212485	Ovoinhibitor [*Gallus gallus*]	100	21	54.4/6.16	65.9/6.11
16	212485	Ovoinhibitor [*Gallus gallus*]	80	17	54.4/6.16	65.9/6.20
17	212485	Ovoinhibitor [*Gallus gallus*]	102	24	54.4/6.16	63.2/6.11
18	212485	Ovoinhibitor [*Gallus gallus*]	62	18	54.4/6.16	63.2/6.20
19	212485	Ovoinhibitor [*Gallus gallus*]	61	22	54.4/6.16	63.2/6.32
20	212485	Ovoinhibitor [*Gallus gallus*]	65	21	54.4/6.16	63.1/6.42
21	212485	Ovoinhibitor [*Gallus gallus*]	73	17	54.4/6.16	24.9/5.86
22	13434994	PIT 54 [*Gallus gallus*]	327	36	52.7/4.61	67.2/4.35
23	63748	Serum albumin [*Gallus gallus*]	255	16	71.9/5.51	65.9/5.71
24	63748	Serum albumin [*Gallus gallus*]	279	16	71.9/5.51	47.8/5.42
25	1871444	Vitellogenin-1 precursor [*Gallus gallus*]	226	5	212.6/9.16	39.8/5.52
26	1871444	Vitellogenin-1 precursor [*Gallus gallus*]	601	4	212.6/9.16	39.8/5.63
27	1871444	Vitellogenin-1 precursor [*Gallus gallus*]	618	4	212.6/9.16	39.8/5.74
28	1871444	Vitellogenin-1 precursor [*Gallus gallus*]	273	3	212.6/9.16	39.8/5.92
29	1871444	Vitellogenin-1 precursor [*Gallus gallus*]	180	2	212.6/9.16	39.8/6.11
30	1871444	Vitellogenin-1 precursor [*Gallus gallus*]	83	3	212.6/9.16	28.2/6.11
31	63887	Vitellogenin-2 [*Gallus gallus*]	183	1	206.7/9.23	35.9/6.04
32	63887	Vitellogenin-2 [*Gallus gallus*]	667	5	206.7/9.23	35.9/6.20
33	63887	Vitellogenin-2 [*Gallus gallus*]	159	2	206.7/9.23	35.9/6.42
34	63887	Vitellogenin-2 [*Gallus gallus*]	293	2	206.7/9.23	35.9/6.60
35	63887	Vitellogenin-2 [*Gallus gallus*]	579	4	206.7/9.23	34.7/6.04
36	63887	Vitellogenin-2 [*Gallus gallus*]	356	3	206.7/9.23	34.5/6.20
37	63887	Vitellogenin-2 [*Gallus gallus*]	243	2	206.7/9.23	34.5/6.42
38	63887	Vitellogenin-2 [*Gallus gallus*]	361	3	206.7/9.23	34.5/6.60
39	63887	Vitellogenin-2 [*Gallus gallus*]	264	1	206.7/9.23	34.5/6.78
40	63887	Vitellogenin-2 [*Gallus gallus*]	391	5	206.7/9.23	29.9/7.40
41	63887	Vitellogenin-2 [*Gallus gallus*]	224	5	206.7/9.23	29.9/7.64
42	63887	Vitellogenin-2 [*Gallus gallus*]	266	5	206.7/9.23	29.9/8.25
43	63887	Vitellogenin-2 [*Gallus gallus*]	76	5	206.7/9.23	29.9/8.39
44	63887	Vitellogenin-2 [*Gallus gallus*]	261	5	206.7/9.23	29.9/8.75
45	63887	Vitellogenin-2 [*Gallus gallus*]	365	4	206.7/9.23	28.4/9.55
46	971408444	Vitellogenin-3 [*Gallus gallus*]	157	4	193.3/8.93	28.1/5.47
47	971408444	Vitellogenin-3 [*Gallus gallus*]	112	2	193.3/8.93	28.5/5.63
48	266634462	Immunoglobulin lambda light chain precursor, partial [*Gallus gallus*]	65	11	23.2/5.66	23.9/5.49
49	266634462	Immunoglobulin lambda light chain precursor, partial [*Gallus gallus*]	169	11	23.2/5.66	23.4/5.91
50	266634462	Immunoglobulin lambda light chain precursor, partial [*Gallus gallus*]	130	11	23.2/5.66	22.9/5.88
51	104728	Ig light chain precursor V-J region - chicken [*Gallus gallus*]	175	25	22.8/5.36	22.8/6.18

[1]Spot ID represents the protein spot number on the 2-DE gel image.
[2]Accession numbers of matched proteins according to the NCBInr database.
[3]MASCOT score. The Mascot threshold score for all of these identified proteins is 59.

showed a lower theoretical molecular value (Table 2). In the CG group, spots 15 to 18 showed no significant difference among incubation stages, while spots 14 and 19 showed higher expression, and spot 21 showed lower expression at ED2. In the EG group, spots representing ovoinhibitor all showed higher expression at ED6 and lower expression at ED13 except for spots 20 and 21. Protein spots showed lower abundance at ED13 in EG group as compared with that of the CG group (Table 3).

Differential Expression of Other Proteins During Incubation Between the Two Groups The relative MW of protein PIT54 (spot 22) was higher than that of the theoretical molecular value (Table 1). The abundance of PIT54 was significantly higher at ED0 than that of the other stages in CG group. The abundance of PIT54 was higher at ED2 and ED6 as compared with ED0 and ED13 in the EG group. As compared with CG group, the expression of protein PIT54 in EG group was lower at ED0 and higher at ED6 (Table 3).

The relative MW of serum albumin (spots 23 and 24) was lower than that of the theoretical molecular value (Table 2). In CG group, the abundance of protein spot 24 was higher at ED0 and ED6. In EG group, the serum albumin was in high expression at ED0, ED2, and ED6. The abundance of serum albumin decreased at ED13 in EG group as compared with that of the CG group (Table 3).

Three vitellogenins, Vitellogenin-1 precursor (spots 25−30), Vitellogenin-2 (spots 31−45), and Vitellogenin-3

Table 3. Comparison of protein abundance of egg yolk during incubation.

Spot	CG groups (day)				EG groups (day)				SEM
	0	2	6	13	0	2	6	13	
1	37.5[a]	15.9[b,*]	13.0[b]	11.2[b,*]	20.4[a]	2.6[c]	7.9[b]	1.6[c]	4.1
2	34.0[a]	26.3[a,*]	32.2[a,*]	7.8[b]	29.3[a]	4.3[b]	14.7[a]	6.6[b]	4.4
3	33.3	32.6*	38.1	38.6*	28.4[a]	3.4[b]	21.8[a]	2.6[b]	5.1
4	15.9[b]	22.0[ab,*]	24.9[ab,*]	38.2[a,*]	17.9[a]	1.5[c]	7.8[b]	1.9[c]	4.4
5	6.5[b,*]	9.8[b,*]	11.6[b,*]	34.4[a,*]	2.4[ab]	1.3[b]	2.8[a]	2.8[a]	3.9
6	1.1[bc]	1.6[ab,*]	0.6[c]	2.6[a,*]	1.3[a]	0.2[c]	1.2[a,*]	0.7[a]	0.3
7	3.0[b]	11.8[a,*]	1.1[c,*]	11.3[a,*]	2.5[a]	0.3[b]	0.3[b]	3.6[a]	1.7
8	3.2[d]	32.1[a,*]	15.9[b]	7.1[c,*]	11.1[a,*]	0.6[c]	9.2[a]	3.0[b]	3.6
9	5.7[b]	22.3[a,*]	31.4[a,*]	1.8[c]	3.5[b]	0.2[c]	13.3[a]	3.7[b,*]	4.0
10	25.3	13.5*	26.0	24.9*	18.4[b]	0.9[d]	65.2[a,*]	8.0[c]	6.8
11	17.7[a]	19.0[a,*]	3.8[c]	7.6[b]	38.2[a,*]	0.3[c]	24.0[a,*]	7.2[b]	4.4
12	2.2[a]	1.9[a,*]	0.4[b]	2.5[a,*]	2.6[a]	0.8[b]	2.8[a,*]	0.2[c]	0.4
13	3.2[ab]	2.2[ab]	2.1[b]	4.3[a,*]	2.0[a]	2.0[a]	2.8[a]	0.8[b]	0.4
14	3.3[ab,*]	4.9[a]	2.1[b]	4.0[ab,*]	1.4[b]	2.8[a]	4.7[a,*]	0.8[b]	0.5
15	4.2	5.2	3.1	4.1*	2.5[b]	2.7[b]	5.8[a]	0.7[c]	0.6
16	1.6	2.6	1.6	2.6*	4.2[ab,*]	2.6[b]	5.2[a,*]	1.0[c]	0.5
17	1.5	2.8*	1.8	2.4	1.2[b]	0.2[c]	2.7[a]	-	0.4
18	2.6	2.6*	2.4	2.4*	2.3[a]	0.5[b]	1.9[a]	0.4[b]	0.3
19	1.7[b]	4.7[a,*]	4.0[a]	2.6[ab,*]	2.2[ab]	1.4[b]	3.4[a]	0.7[c]	0.5
20	1.8[b,*]	2.9[ab,*]	1.6[b,*]	3.7[a,*]	0.9	0.9	0.5	0.5	0.4
21	26.1[a,*]	1.8[b]	42.1[a,*]	28.3[a]	4.9[b]	20.7[a,*]	4.2[b]	22.1[a]	5.0
22	93.7[a,*]	37.8[b]	18.2[c]	34.6[bc]	27.5[b]	70.8[a]	84.3[a,*]	19.6[b]	10.6
23	149.4	97.6	147.5	123.1*	140.7[a]	61.8[b]	141.0[a]	30.8[c]	15.7
24	30.5[a]	10.5[b]	29.8[a]	9.6[b,*]	16.3[a]	14.4[a]	19.0[a]	0.7[b]	3.6
25	17.7[a]	7.3[b]	6.6[b]	28.8[a]	24.0	14.2	23.8*	17.5	2.8
26	27.9	30.0	16.7	26.4	28.0[ab]	27.2[b]	29.1[ab]	55.9[a,*]	4.0
27	34.7	56.7	49.1	50.4	43.1[ab]	38.1[ab]	31.3[b]	72.7[a]	4.7
28	30.3	36.5	42.0*	24.7	44.1[a]	70.1[a]	15.4[b]	16.8[b]	6.3
29	0.2[c]	7.1[a]	1.1[b,*]	4.6[a]	5.7[b,*]	33.4[a,*]	0.3[c]	4.4[b]	3.9
30	0.9[b]	6.7[a,*]	3.9[a]	3.5[a,*]	2.3[a,*]	3.0[a]	2.9[a]	0.5[b]	0.7
31	45.3*	35.3	35.8	54.2*	12.6[b]	18.3[ab]	28.8[a]	16.7[ab]	5.2
32	106.8[a,*]	69.4[ab]	72.2[ab]	51.2[b]	44.0[b]	65.5[ab]	61.4[ab]	98.6[a]	7.7
33	65.4	77.2	97.3	67.4	53.8	65.1	49.1	67.1	5.2
34	37.2	52.7	53.9*	44.3*	60.8[a]	37.1[ab]	26.7[bc]	16.9[c]	5.2
35	-	0.9[c]	1.8[b]	19.8[a,*]	-	-	6.2*	5.0	3.4
36	9.0[a,*]	3.1[b]	2.2[b]	8.8[a]	1.7[c]	2.5[bc]	4.3[ab]	7.5[a]	1.1
37	13.3[a,*]	0.7[c]	1.3[c]	4.6[b]	2.3[b]	2.0[b,*]	10.9[a,*]	8.0[a]	1.7
38	3.3[a,*]	1.1[b]	3.7[a,*]	3.2[a]	1.2[b]	1.9[b]	1.5[b]	7.4[a,*]	0.7
39	-	7.9*	5.5	9.5*	18.2[a]	3.4[c]	9.1[b]	2.8[c]	2.0
40	6.0[b]	22.4[a,*]	2.3[c]	5.9[b,*]	4.0[a]	0.4[b]	3.9[a]	0.5[b]	2.5
41	2.9[b,*]	11.4[a,*]	0.9[c]	2.5[b]	1.0[b]	5.2[a]	1.1[b]	3.2[a]	1.2
42	10.4*	10.4	10.7*	11.9	0.2[c]	10.9[a]	2.9[b]	8.1[a]	1.5
43	17.7[b,*]	32.1[ab,*]	40.6[a,*]	49.7[a,*]	3.6[b]	3.3[b]	17.2[a]	6.4[b]	6.2
44	9.2[b]	46.3[a,*]	39.4[a]	9.0[b,*]	11.1[b]	4.5[c]	33.6[a]	4.3[c]	6.1
45	19.4[a]	6.6[b]	20.5[a]	8.8[b]	101.2[a,*]	40.1[b,*]	42.7[b,*]	117.3[a,*]	14.9
46	8.1[a]	1.1[b]	4.3[a,*]	8.4[a,*]	8.8[a]	-	1.4[c]	3.1[b]	1.3
47	1.9	3.0	2.1	-	-	9.0*	6.0*	-	1.4
48	53.6[ab,*]	71.8[a]	35.8[b]	60.7[ab,*]	24.9	38.2	34.4	22.8	6.2
49	1.2[b]	6.5[a,*]	3.8[a]	4.8[a]	2.5[ab,*]	1.4[b]	4.7[a]	-	0.7
50	0.6[c]	2.2[ab]	1.9[b]	4.0[a]	1.0	-	1.3	-	0.5
51	8.7[a,*]	5.1[ab,*]	2.8[b]	2.7[b]	2.8[b]	1.7[b]	6.3[a,*]	-	0.9

[abc]Indicate significant difference among incubation stages.
[*]Indicate significant difference between groups.

(spots 46−47), were identified in this experiment with a relative MW far lower than that of the theoretical value (Table 2). The abundance of spots 26 to 28 representing vitellogenin-1 precursor showed no significant difference among incubation stages, while spots 29 to 30 showed a higher expression at ED2 and ED13 than that of the ED0 in CG group. In EG group, spots 28 to 30 showed highest abundance at ED2. There's no significant difference of protein abundance between CG and EG group (Table 3). The abundance of vitellogenin-2 was lower at ED2 or ED6 in CG group, and was lower at ED0 as compared to ED13 in EG group. Vitellogenin-2 showed higher abundance at ED0 and ED13 in EG group as compared to CG group (Table 3). Protein spot 46 representing vitellogenin-3 showed lower expression at ED2 than that of the other incubation stages in the CG group, and showed lower expression at ED6 and ED13 than that of the ED0 in EG group. The abundance of vitellogenin-3 was lower in EG group than that of the CG group at ED13 (Table 3).

GO Enrichment Analysis for the DE Proteins

The identified DE proteins were classified into ten categories according to the GO enrichment analysis, which was performed on biological processes, cellular components and molecular functions (Figure 3, Supplementary Table S1).

GO Annotation of Differentially Expressed Proteins in Biological Process During incubation, the DE proteins of Vitellogenin-1 precursor, Vitellogenin-2, and Vitellogenin-3 were mainly participated in lipid transport (GO:0006869) and lipid localization (GO:0010876). Immunoglobulin lambda light chain precursor and Ig gamma chain were participated in immune response related processes, such as humoral immune response (GO:0006959), complement activation, classical pathway (GO:0006958), humoral immune response mediated by circulating immunoglobulin (GO:0002455), complement activation (GO:0006956), protein activation cascade (GO:0072376), immunoglobulin mediated immune response (GO:0016064), and B cell mediated immunity (GO:0019724). As compared with ED2, immunoglobulin lambda light chain precursor and Ig gamma chain also participated in lymphocyte mediated immunity process at ED6. Ovotransferrin BB type participated in the extracellular sequestering of iron ion process (GO:0006881) at ED2/ED0 and ED13/ED6 stages (Figure 4). There's similar enriched biological process between GW and GT group, except that Immunoglobulin lambda light chain precursor, Ig gamma chain and Ig light chain precursor V-J region participated in immune response related processes in GT group at ED6/ED2 and ED13/ED6 stages.

Compared with GW group, DE proteins of immunoglobulin lambda light chain precursor, Ig gamma chain and Ig light chain precursor V-J region participated in immune response related processes at ED0. Proteins Vitellogenin-1 precursor, Vitellogenin-2, and Vitellogenin-3 participated in lipid transport (GO:0006869) and lipid localization (GO:0010876), and Ovotransferrin BB type participated in extracellular sequestering of iron ion (GO:0006881) at ED6 (Figure 5).

GO Annotation of Differentially Expressed Proteins in Cellular Component Differentially expressed proteins between GW and GT group, and between connective incubation stages were all enriched in extracellular region (GO:0005576), yolk (GO:0060417), extracellular space (GO:0005615), and extracellular region part (GO:0044421), etc (Figures 4 and 5).

GO Annotation of Differentially Expressed Proteins in Molecular Function In GW group, differentially expressed proteins of Vitellogenin-1 precursor, Vitellogenin-2, and Vitellogenin-3 were participated in nutrient reservoir activity (GO:0045735) and lipid transporter activity (GO:0005319) at all connective stages. Immunoglobulin lambda light chain precursor and Ig gamma chain participated in antigen binding (GO:0003823). Serum albumin participated in toxic substance binding (GO:0015643) and fatty acid binding (GO:0005504). Ovoinhibitor participated in potassium channel regulator activity (GO:0015459) and potassium channel inhibitor activity (GO:0019870). At ED2/ED0 and ED6/ED2 stages, ovoinhibitor also involved in ion channel inhibitor activity (GO:0008200) and channel inhibitor activity (GO:0008201). While ovotransferrin BB type involved in ferric iron binding (GO:0008199) at ED2/ED0 and ED13/ED6 stages. As compared with the GW group, proteins Vitellogenin-1 precursor, Vitellogenin-2, and Vitellogenin-3 also participated in substrate-specific transporter activity (GO:0022892) and transporter activity (GO:0005215) at ED6/ED2 stage, and participated in substrate-specific transporter activity (GO:0022892) at ED2/ED0 in GT group. Ovoinhibitor was involved in protease binding (GO:0002020) at ED2/ED0 in GT group (Figure 5).

DISCUSSION

Fertilized eggs need to be stored for 2 to 5 d to form a larger gas exchange for the early embryo by moisture

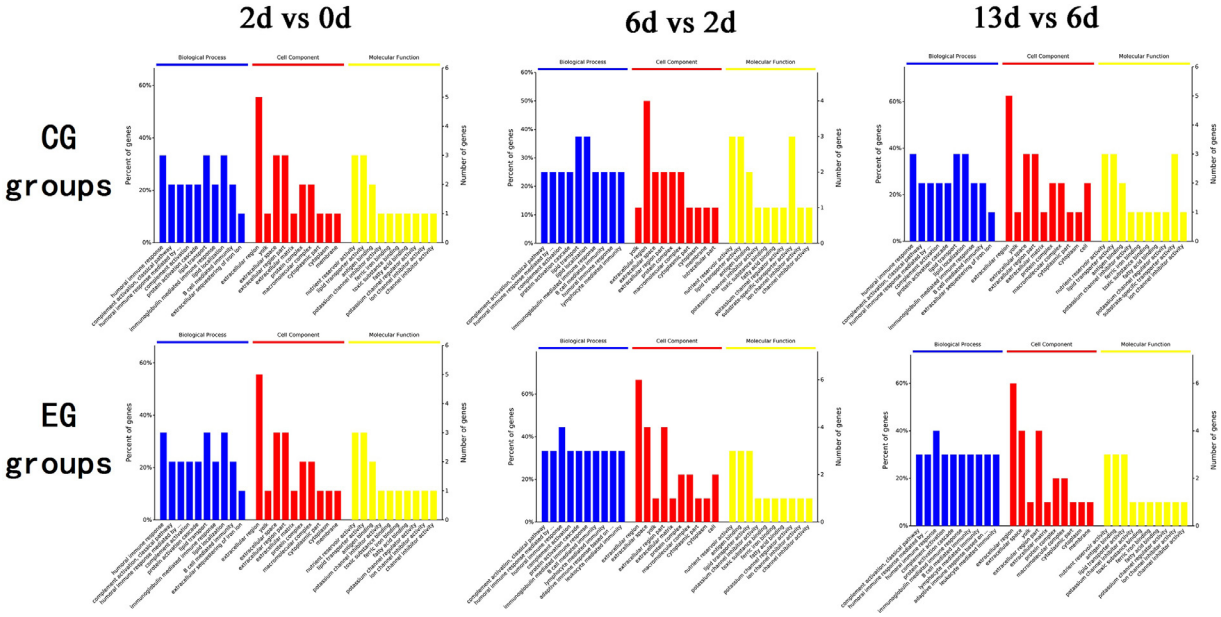

Figure 4. GO annotation of differentially expressed proteins in 3 categories including biological process, cellular component and molecular function during incubation. Only the 10 top annotations with $P < 0.05$ were listed.

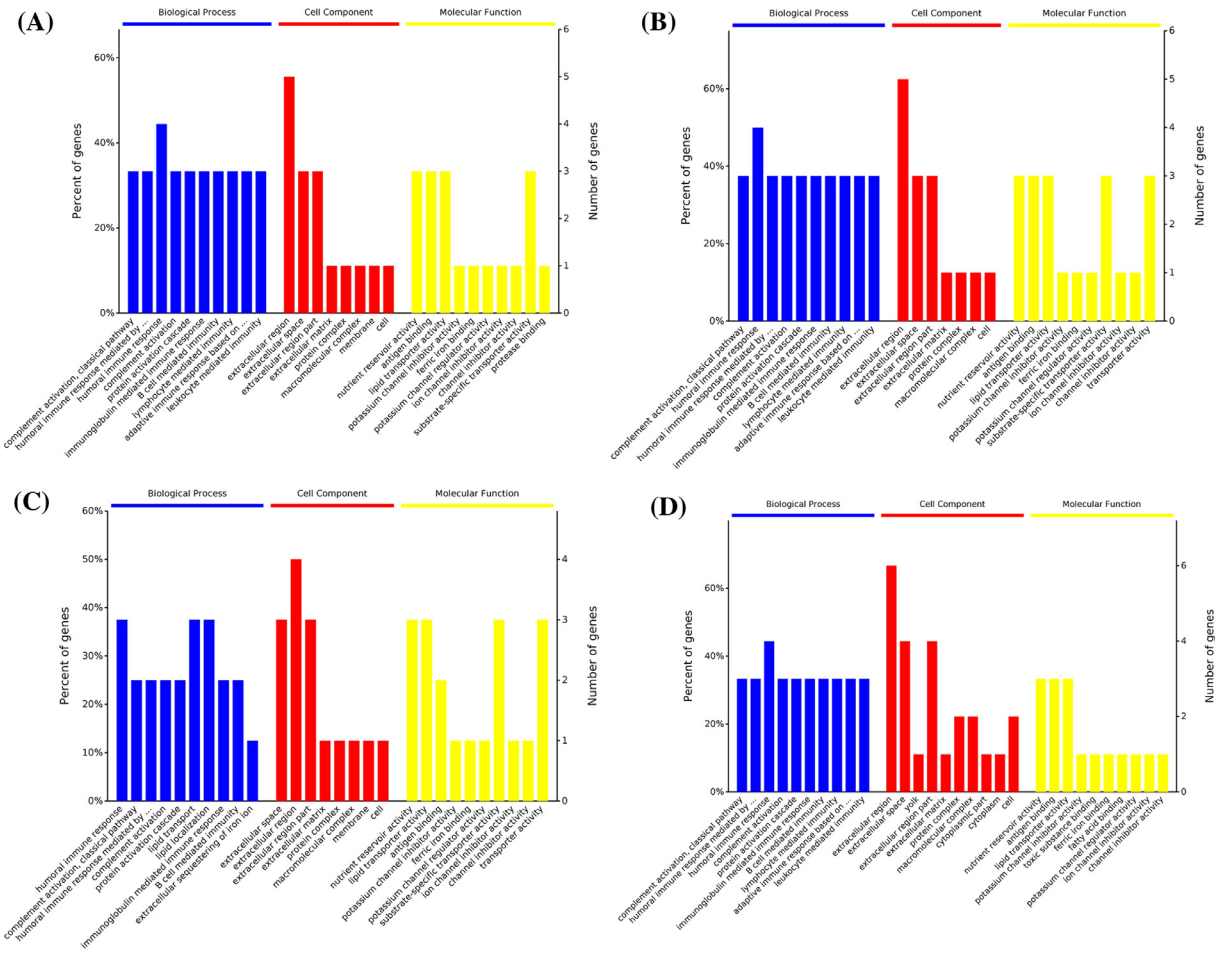

Figure 5. GO annotation of differentially expressed proteins in 3 categories including biological process, cellular component and molecular function in GT group compared with GW group. Only the 10 top annotations with $P < 0.05$ were listed. (A) EG vs. CG at 0 embryonic day; (B) EG vs. CG at 2 embryonic day; (C) EG vs. CG at 6 embryonic day; (D) EG vs. CG at 13 embryonic day.

loss before incubation (Şahan et al., 2003). Eggs from indigenous breed tend to have thicker eggshell and albumen (Franco et al., 2020) which is not helpful for incubation. While eggs from chickens fed diet with green tea powder has thinner eggshell as compared with that of the normal commercial diet (Chen et al., 2021), which could provide a higher moisture loss and vital gas exchange for embryo.

Yolk Protein Concentration Varied in Embryos by the Developmental Stages

Yolk content influences chicken body composition and viability (Finkler et al., 1998; Uni et al., 2012). The embryo's requires for particular protein may differ among successive developmental stages during incubation. The percentage of fraction higher than 30 KDa decreased gradually up to ED9 and then began to increase, indicated that chemical synthesis of new protein molecules occurred during incubation (Duan et al., 2013). Differential accumulation of moisture and dry matter in embryos during incubation exhibit distinctive associations with concentrations of various yolk proteins. However, the increased protein concentration at ED2 might due to the evaporative loss of water during egg storage. During incubation, water from albumen permeated into the yolk to make it larger and soluble for the embryo to absorb nutrients (Meuer and Egbers, 1990). In the late incubation stage, less yolk moisture would facilitate internalize of yolk sac into the body cavity of the embryo at the final incubation (Uni et al., 2012). Therefore, a decreased protein concentration at the early incubation stage and an increased protein concentration at the late incubation stage were observed in this experiment.

Protein Vitellogenin, Immunoglobulin, and Ovoinhibitor Were the Main DE Protein During Incubation

The main components of yolk proteins are lipovitellins (36%), livetins (38%), phosvitin (8%), and low-density lipoproteins (17%) (Benedé and Molina, 2020).

Egg yolk proteins are synthesized in the liver of the laying hens and then transported and deposited in the developing oocyte via blood fluent. Vitellogenin, known as the only phosphoprotein in the plasma, could be cleaved to form the egg yolk phosphoproteins, lipovitellin, and phosvitin (Deeley et al., 1975). During incubation, protein phosphorylation plays important role in yolk protein structure and function. Phosphorylation

also plays regulatory role in embryonic development during embryogenesis (Sun et al., 2019). Phosvitin, known as strong metal-chelating and antioxidant activity, could phosphorylate proteins and promote metal chelation (Benedé and Molina, 2020). Therefore, dephosphorylation occurred at the middle and late incubation stage to promote chicken embryonic bone growth. Some plant extracts contain specific functional peptides possessing a strong ability to chelate minerals (Walters et al., 2018). Green tea powder in the diet might cause mineral chelation. The lower expression of vitellogenin-2 and -3 in EG group after ED6 suggested that green tea inhibited transportation of vitellogenin to oocyte to compensate the chelating ability.

By use of isoelectric focusing, Ternes (1989) has reported at least 25 bands for livetins focusing between pH 4.3 and 5.5, which also known as immunoglobulin because of its immunological properties. The higher abundance of immunoglobulin detected before ED6 suggested a gradual permeation of immunoglobulin into embryo to provide passive immune protection for chicken embryos. It is widely proven that green tea incorporate essential components acting as immune modulator (Zhang et al., 2018). In this experiment, a higher abundance of Ig gamma at ED6 could endow the embryo higher level of protection at the later incubation.

Ovoinhibitor, as serine proteases occurred in albumen, inhibits the activity of trypsin, chymotrypsin, subtilisin, elastase, etc, and usually be detected in albumen in fertilized (Zhu et al., 2020) and unfertilized eggs (Gao et al., 2017). The higher expression of ovoinhibitor before ED6 in both CG and EG groups suggest its transfer from albumen to yolk at the early incubation stage. While with the increased protein concentration, proteases need to be activated to facilitate the embryo absorb yolk protein. Therefore, a decreased abundance of ovoinhibitor was observed at ED13.

Other DE Proteins Play Crucial Role for Egg Incubation

Ovotransferrin has been reported as the main component in egg white, which occupied 12% in total albumin protein (Superti et al., 2007). Ovotransferrin has the activity of antimicrobial and antioxidant. When binding with metals such as iron, magnesium and copper, and conjugated with small molecules such as catechin, or autoclaved treatment improved the antioxidant property of ovotransferrin (Benedé and Molina, 2020). Ovotransferrin also plays an important role in bacteriostatic activity for its high affinity for metal iron (Abdou et al., 2013). Movement of minerals from the yolk is facilitated via endocytosis by the yolk sac membrane. Hopcroft et al (2019) revealed that yolk transitional mineral like Fe, Zn, etc, could be decreased during incubation for embryo absorption. In this experiment, a decreased abundance of ovotransferrin was also observed at ED13. In addition, as mentioned above that components from green tea has strong ability to chelate minerals (Walters et al., 2018). Therefore, a decreased abundance of ovotransferrin in EG group was observed because of the chelating ability from green tea as compensation. As mentioned by Georgieva (2010), the PIT54 is the major hemoglobin-binding protein in chicken plasma. A higher expression of PIT54 in EG group at ED6 suggested that green tea powder enhanced the antioxidant activity to protect nitric oxide signaling pathway during embryonic development.

Serum albumin is the most important protein in egg white (Benedé and Molina, 2020). The occurrence of serum albumin in yolk protein during incubation might because that the thinner yolk and materials of egg white could flow into yolk to provide nutrient for embryo development. During incubation, proteins from egg white was decomposed and utilized which caused a decreased abundance of serum albumin at ED13 and could even not identified at ED18 as reported by Zhu et al. (2020).

CONCLUSIONS

Chickens fed diet with green tea powder provided a thinner eggshell would be helpful for egg incubation. Vitellogenin, immunoglobulin, and ovoinhibitor were the main differentially expressed proteins during incubation. Green tea powder provided eggs more antioxidant ability that made yolk protein lower expression of vitellogenin, ovotransferrin, and serum albumin. While as immune modulator, green tea powder provided eggs higher abundance of immunoglobulin. Therefore, a higher hatchability observed in the EG group indicated that a certain range of lower yolk cholesterol did not affect hatchability and green tea powder could be considered as additive in breeding layer.

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DISCLOSURES

The authors have no conflicts of interest to declare.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2022.101804.

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