Research Note: Integrated transcriptomic and metabolomic analysis reveals potential candidate genes and regulatory pathways associated with egg weight in ducks

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ABSTRACT Egg weight is an important indicator of egg phenotypic traits, which directly affects the economic benefits of the poultry industry. In the present research, laying ducks were classified into high egg weight (**HEW**) and light egg weight (**LEW**) groups. To reveal the underlying mechanism that may be responsible for the egg weight difference, the integrated analysis of transcriptomes and serum metabolomics was performed between the two groups. The results showed extremely significant differences ($P < 0.01$) in the total egg weight at 300 d, and average egg weight between the HEW and LEW groups. 733, 591, 82, and 74 differentially expressed genes (**DEGs**) were identified in the liver, magnum, F1, and F5 (hierarchical follicles) follicle membrane, respectively. The candidate genes were screened further from the perspective of forming an egg. In terms of egg yolk formation, the functional analysis revealed fatty acid metabolism-related pathways account for 36% of the liver's top pathways, including fatty acid biosynthesis, folate biosynthesis, fatty acid metabolism, and glycerol lipid metabolism pathways. *FASN* gene was identified as the key candidate gene by comprehensive analysis of gene expression and protein-protein interaction (**PPI**) network. In the follicle membrane, the DEGs were mainly enriched in protein processing in the endoplasmic reticulum, and MAPK signaling pathway, and *HSPA2, HSPA8, BAG3* genes were identified as crucial candidate genes. In terms of egg white formation, the functional analysis revealed protein metabolism-related pathways account for 40% of the magnum's top pathways, which includes protein processing in the endoplasmic reticulum pathway. *HSP90AA1* and *HSPA8* genes were identified as key candidate genes. In addition, the integrated transcriptomic and metabolomic analysis showed that arginine and proline metabolism pathways could contribute to differences in egg weight. Thus, we speculated that the potential candidate genes, regulatory pathways, and metabolic biomarkers mentioned above might be responsible for the egg weight difference. These findings might provide a theoretical basis for improving the egg weight of ducks.

Key words: ducks, egg weight, candidate genes, regulatory pathways, metabolic biomarkers

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INTRODUCTION

The demand for animal protein production continues to increase in many developing countries. The World Food and Agriculture Organization (**FAO**) has reported that the poultry industry is an efficient way to meet the demand for animal protein. In poultry breeding, increasing egg production and weight is an important breeding goal. Previous studies have focused on candidate genes of poultry egg production, and these results provide data for revealing the mechanism of poultry egg production and the development of molecular markers (Bello et al., 2021; Yan et al., 2022). Increasing egg weight while maintaining egg amount is also an effective way to increase animal protein supply. However, research on candidate genes related to poultry egg weight is still relatively rare.

Egg yolk and egg white are the two most important components that affect egg weight. The egg yolk consists

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of lipids mainly from egg yolk precursors, including very low-density lipoprotein (**VLDL**) and vitellogenin (**VTG**) synthesized in the liver. Egg yolk precursors are dissolved in the blood and then transported to the follicle through the blood circulation system (Ratna et al., 2016). The magnum is the longest part of the oviduct and the core part where egg white is mainly formed. Egg white protein is secreted within 3 to 5 h in the process of egg formation (Ren et al., 2017). Considering the egg formation process is so complicated, it is necessary to reveal the underlying mechanism responsible for egg weight from a new comprehensive view.

We performed an integrated analysis of transcriptomes of the liver, magnum, F1 follicle membranes, F5 follicle membranes, and serum metabolomics. These works were to reveal the potential candidate genes and regulatory pathways associated with egg weight difference through a perspective of formation in egg yolk and egg white in Nonghua Ma ducks, which is a strain with the advantages of strong adaptability, high reproduction performance, and a high feed conversion ratio. These results will provide a theoretical basis for improving duck egg weight in poultry breeding programs.

MATERIALS AND METHODS

Experimental Animals

Three hundred sixty Nonghua Ma ducks were obtained from the Waterfowl Breeding Experimental Farm of Sichuan Agricultural University, Ya'an, Sichuan province, China. The ducks were fed and managed under the same environmental conditions with free access to feed and water. Ducks were transferred to a single cage at 120 d, and we recorded daily the egg numbers and egg weight from the age of the first egg to 300 d. Ducks were selected and divided into HEW and LEW groups, with similar body weights and egg numbers at 300 d, but extremely significant differences in egg weight. Twenty-two ducks with the highest egg weight were defined as the HEW group, and 22 ducks with the lowest egg weight were defined as the LEW group.

Internal Egg Quality Evaluation and Sample Collection

Twenty-two ducks were evaluated for the yolk and albumen weight in each group for 3 consecutive days. After breaking the egg, separated the yolk and white, measured the weight respectively, then measured the eggshell's weight after natural drying for 24 h. Six ducks were selected for blood and liver, magnum, F1 follicle membrane, F5 follicle membrane tissue sample collection in each group. We recorded the number and weight of hierarchical follicles according to the color and diameter of the follicles. By the standard of follicle diameter of 6 to 10 mm, 2 to 6 mm, and <2 mm, prehierarchical follicles were grouped and weighed.

RNA Isolation and cDNA Library Preparation

Total RNA of the liver, magnum, F1 follicle membrane, and F5 follicle membrane was extracted using Trizol reagent (Invitrogen, CA) following the manufacturer's instructions. RNA that meets the criteria (A260/A280 ≥1.8, A260/A230 ≥2.0, and RNA integrity number >7.0) was selected for transcriptome sequencing. The cDNA library was prepared using the VAHTS mRNA-seq V3 Library Prep Kit for Illumina. Then, libraries were sequenced by the Illumina sequencing platform (Nova Seq 6000 Illumina), and 150 bp paired-end reads were generated. The quality of raw reads was analyzed by Fast QC analyzed, and high-quality reads (Q > 20) were obtained by NGS Toolkits (version: 2.3.3).

Transcriptome Sequencing, Differential Expression, and Functional Analysis

The HISAT2 (V2.1.0) software mapped all paired clean transcriptome reads to the duck genome. Samtools software converts the Sam files obtained by mapping them into bam files and sorting them. StringTie was used to assemble transcripts, quantitatively express genes, and estimate gene expression abundance. Gffcompare detects gene annotation and transcript assembly. Gene expression was calculated by the FPKM method and TPM method. The differential expression analysis in the liver, magnum, F1 follicle membrane, and F5 follicle membrane was performed using the DESeq2 R package (1.20.0). The genes that reached the standard of $|\log2FC| > 1$, $P < 0.05$ were screened as the DEGs. Gene ontology enrichment analysis software tools were used to analyze the Gene Ontology (**GO**) functions. KOBAS3.0 was used to analyze the Kyoto Encyclopedia of Genes and Genomes (**KEGG**) pathway enrichment analysis functions. It is generally considered that GO terms and KEGG pathways with $P < 0.05$ are significantly enriched.

Metabolome Analysis of Serum

Serum samples were prepared for LC-MS, and the Electrospray ionization mass spectrometry experiments were executed on the Thermo Q Exactive mass spectrometer. The high-energy collision dissociation scan was used for data-dependent acquisition in MS/MS experiments. The proteowizard software (v3.0.8789) was used to convert the raw data into mzXML format, and the XCMS package for R (v3.3.2) was used for a subsequent series of analyses, including peak identification, peak filtering, and peak alignment. To obtain accurate metabolite information, we matched and annotated fragment information obtained from MS/MS mode in the following databases, HMDB, Metlin, Massbank, LipidMaps, and MzCloundto obtain accurate metabolite information. The metabolites that reached the standard of $P < 0.05$ and VIP > 1 were screened as the differentially accumulated metabolites (**DAMs**). The PHEATMAP package in R (v3.3.2) obtained a hierarchical

clustering map of relative quantitative values of metabolites. Moreover, Metabo Analyst 5.0 was used to analyze the metabolic pathways of DAMs.

Statistical Analysis

Data were analyzed using the SPSS statistical software version 20.0 (IBM Corp., Armonk, NY). T-test and one-way ANOVA were used to analyze the difference between the two groups. The data were presented as Mean ± SEM, and $P < 0.05$ was used as a significant statistical difference.

RESULTS AND DISCUSSION

Comparison of Egg Weight between The HEW and LEW Groups

As shown in Figure 1A, the total egg weight at 300 d (11.45 ± 0.08, 9.71 ± 0.05) and average egg weight (89.71 ± 3.50, 77.28 ± 0.92) showed extremely significant differences ($P < 0.01$) between the HEW and LEW groups. There were no significant differences in total egg number at 300 d, the number and weight of hierarchical, and the weight of prehierarchical follicles ($P>0.05$). Egg weight varies significantly between the two groups, indicating it is possible to enrich candidate genes associated with egg weight in this study. Egg formation is a dynamic and complex process in poultry, and it is essential to understand the molecular mechanism in the liver, magnum, and follicle to improve egg weight in poultry.

Transcriptome analysis showed that a total of 170.54 Gb clean bases were obtained from 24 samples through mRNA sequencing, with 93.09% of bases scoring Q30 and 86.49%−92.55% of the sequencing reads aligned to the duck reference genome. Based on the transcriptomic studies, 733, 591, 82, and 74 DEGs were identified in the liver, magnum, F1 follicle membrane, and F5 follicle membrane between the two groups, respectively (Figure 1B). In the present research, the number of

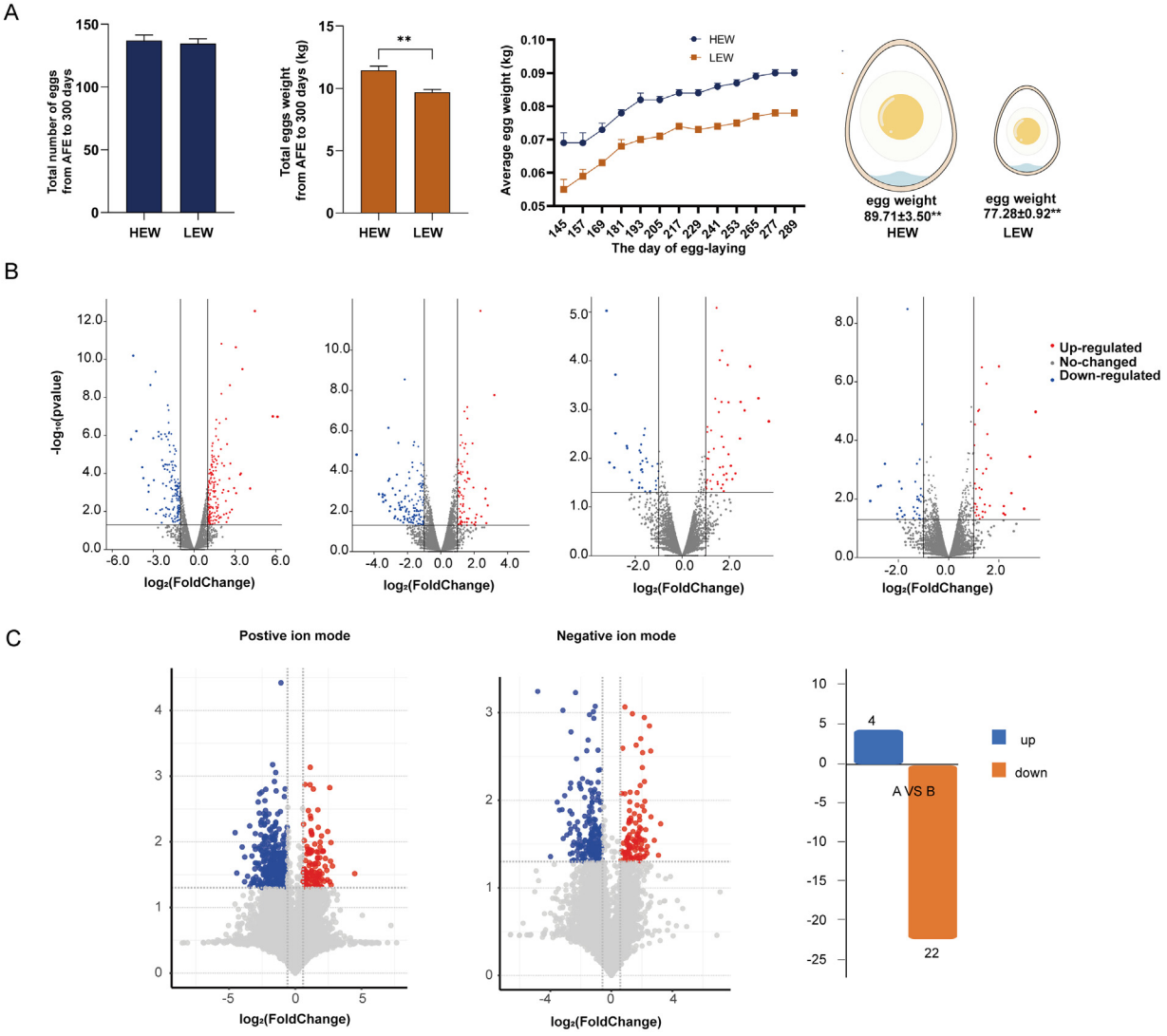

Figure 1. Overview of egg weight traits, DEGs, DAMs between the HEW and LEW groups. (A) Comparison of the total number of eggs at 300 d, the total egg weight at 300 d, the curve of egg weight, and the whole egg weight in 2 groups. (B) Volcano map of DEGs in the liver, magnum, F1 follicle membrane, and F5 follicle membrane, respectively. (C) Volcano map of DAMs in positive ion mode, volcano map of DAMs in negative ion mode, and histogram of the annotated DAMs, respectively.

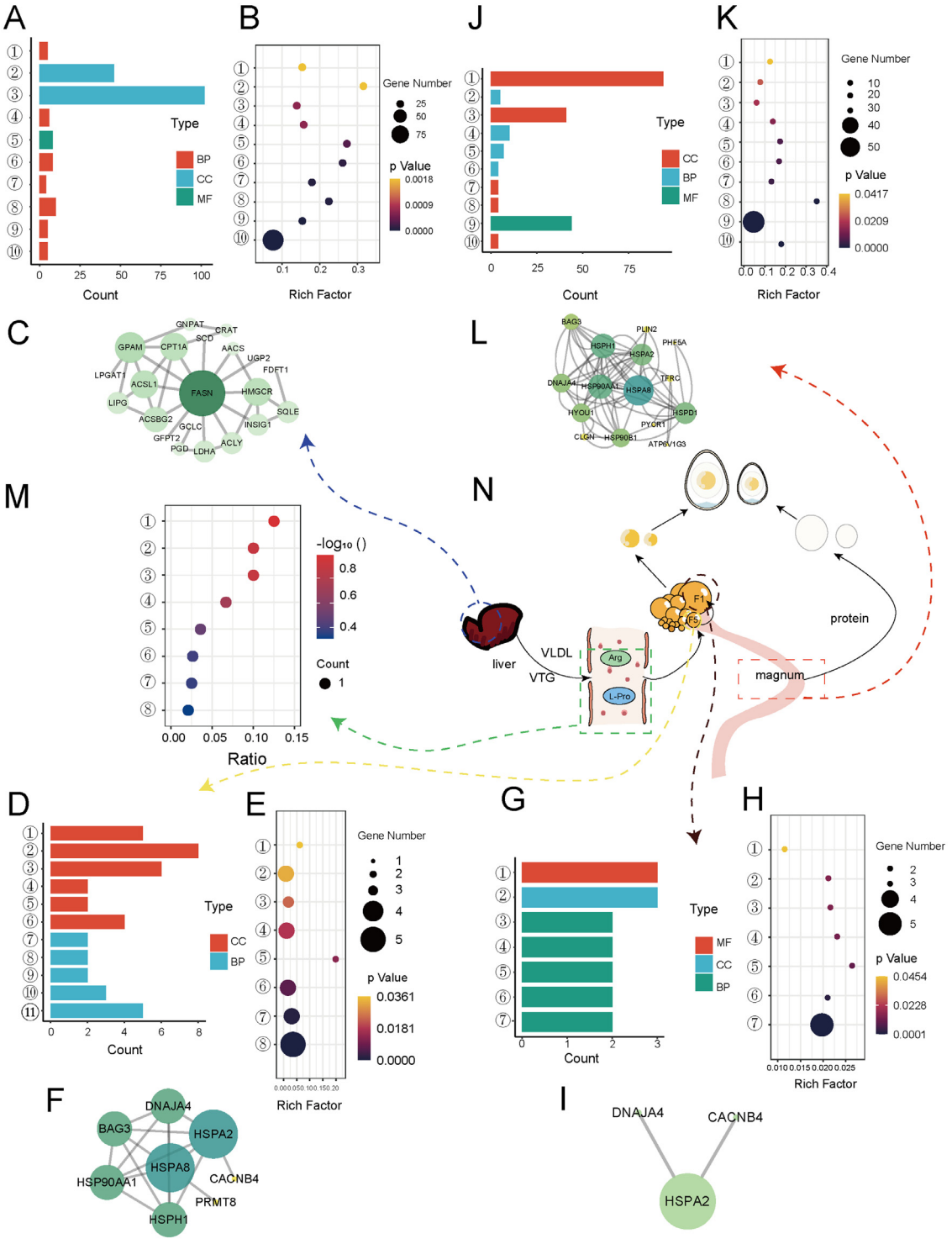

Figure 2. The potential candidate genes and regulatory pathways responsible for egg weight difference through a perspective view of egg yolk and egg white formation. (A) Top GO classification of DEGs identified in the liver between the HEW and LEW groups, ①–⑩ GO terms include phosphatidylethanolamine biosynthetic process; cytosol; extracellular exosome; negative regulation of endothelial cell apoptotic process; steroid hormone receptor activity; lipid metabolic process; protein refolding; glucose homeostasis; response to cold; negative regulation of extrinsic apoptotic signaling pathway via death domain receptors, respectively. (B) Top KEGG pathways enriched by the DEGs in the liver between the HEW and LEW groups, ①–⑩ represents fatty acid metabolism; steroid biosynthesis; peroxisome; adipocytokine signaling pathway; pentose phosphate pathway; pyruvate metabolism; PPAR signaling pathway; glycolysis/gluconeogenesis; carbon metabolism; metabolic pathways. (C) PPI interaction networks of the top expression level of DEGs in the liver (F) F1 follicle membrane (I) F5 follicle membrane (L)magnum. (D) Top GO classification of DEGs identified in the F1 follicle membrane between the HEW and LEW groups, ①–⑪ GO terms include blood microparticle; cytosol; extracellular space; fibrinogen complex; synaptonemal complex; myelin sheath; negative regulation of inclusion body assembly; chaperone-mediated protein folding requiring cofactor; regulation of protein ubiquitination; protein refolding; signal transduction. (E) Top KEGG pathways enriched by the DEGs in the F1 follicle membrane between the HEW and LEW groups, ①–⑧ KEGG pathways include Phenylalanine metabolism; neuroactive ligand-receptor interaction; cell adhesion molecules (CAMs); MAPK signaling pathway; phenylalanine, tyrosine and tryptophan biosynthesis; cytokine-cytokine receptor interaction; spliceosome; protein processing in endoplasmic reticulum. (G) Top GO classification of DEGs identified in the F5 follicle membrane between the HEW and LEW groups, ①–⑦ GO terms include voltage-gated potassium channel activity; voltage-gated potassium channel complex; negative regulation of inclusion body assembly; protein refolding; energy homeostasis; regulation of heart contraction; positive

DEGs in the liver was higher than in the magnum, indicating that egg yolk formation involves more gene regulation. Similarly, a previous study reported that egg weight increases by 11% in laying turkey, egg yolk increases weight by 21%, but egg white increases by 7%. These results indicate that egg yolk has more influence on the whole egg weight than egg white(Reidy et al., 1994).

Serum metabolome indicated that 509 and 325 annotated metabolites were detected in the positive and negative ion modes, respectively. A total of 26 annotated DAMs ($P < 0.05$) were obtained in the HEW and LEW groups. After being annotated, 26 metabolites were mainly concentrated in amino acid, which provided the basis for the subsequent study in revealing the biomarkers associated with a different egg weight of ducks (Figure 1C).

The Candidate Genes Responsible for Egg Weight Difference through a Perspective View of Yolk Formation

In the liver, GO analysis showed that DEGs were significantly enriched in 20 terms, of which 5 were involved in biological processes (Figure 2A). The KEGG found DEGs were enriched in 138 pathways, and 25 pathways were screened significantly different according to the criterion ($P < 0.05$), of which 9 pathways (36%) were related to fatty acid metabolism-related pathways. DEGs were mainly enriched in fatty acid biosynthesis, folate biosynthesis, fatty acid metabolism, and glycerol lipid metabolism pathways, indicating that these pathways are critical for egg yolk formation (Figure 2B). These results also consistent with a previous report that fatty acid synthesis is associated with the synthesis and accumulation of yolk precursors (Wu, et al., 2022). Furthermore, the gene expression top 30 DEGs from acid metabolism-related pathways with significant enrichment were merged to construct the PPI network, *FANS* gene had the largest number of protein interaction nodes (Figure 2C). Previous research reported that *FASN* gene synthesizes fatty acids in the liver and degrades triglycerides (**TG**) through β-oxidation, TG and apolipoproteins, phospholipids, cholesterol, etc., form VLDL, and these yolk precursors are transported to developing oocytes through blood circulation (Nikolay, et al., 2013). Therefore, it can be hypothesized that the egg weight difference in ducks may be related to the different capacities of the liver for egg yolk precursor synthesis. *FASN* gene and pathways involved in regulating lipogenesis were identified as crucial candidate genes and regulatory pathways, which may be responsible for the egg weight difference from the perspective of egg yolk formation.

Follicles accumulate large amounts of egg yolk precursors, and proliferate, the development and maturation of follicular were associated with yolk synthesis and accumulation. DEGs were enriched in the protein processing in the endoplasmic reticulum pathway, and MAPK signaling pathway in the F1 follicle membrane (Figure 2D, E) and F5 follicle membrane (Figure 2G, H). These pathways' function is to degrade misfolded proteins and maintain the normal function of the endoplasmic reticulum. Accumulation of misfolded proteins in the endoplasmic reticulum leads to endoplasmic reticulum stress, autophagy is an important protective mechanism triggered by endoplasmic reticulum stress, it has been reported that autophagy of ovarian granulosa cells can regulate follicle atresia and development in poultry (Yu et al., 2016). Furthermore, comprehensive gene expression analysis and PPI network showed that *HSPA2, HSPA8,* and *BAG3* genes were identified as the key candidate genes in the F1 follicle membrane (Figure F) and F5 follicle membrane (Figure I). Specifically, *BAG3* gene is considered an anti-apoptotic and autophagic factor that can bind to *HSP70*, which involves the degradation of misfolded or damaged proteins in autophagosomes (Xiao et al., 2014). Previous research reported that external stimuli such as limited activity and temperature changes might lead to a severe reduction in egg production of laying hens by affecting lipogenesis lipid transportation (Wu et al., 2022). Therefore, we speculated that heat stress-induced endoplasmic reticulum stress affects lipid accumulation in follicles, *HSPA2, HSPA8,* and *BAG3* might affect egg yolk formation by regulating the lipid accumulation, which may be responsible for the difference in egg weight.

The Candidate Genes Responsible for Egg Weight Difference through a Perspective View of Albumin Formation

In the magnum, GO analysis showed that DEGs were significantly enriched in 30 terms, of which 21 terms were classified into biological process categories

regulation of smooth muscle cell proliferation. (H) Top KEGG pathways enriched by the DEGs in the F5 follicle membrane between the HEW and LEW groups, ①−⑦ KEGG pathways include cytokine-cytokine receptor interaction; spliceosome; glycerophospholipid metabolism; GnRH signaling pathway; ErbB signaling pathway; protein processing in endoplasmic reticulum; MAPK signaling pathway. (J) Top GO classification of DEGs identified in the magnum between the HEW and LEW groups, ①−⑩ GO terms include extracellular exosome; chaperone-mediated protein folding; cytosol; protein folding; toxin transport; protein refolding; nuclear heterochromatin; endoplasmic reticulum chaperone complex; poly(A) RNA binding; chaperonin-containing T-complex. (K) Top KEGG pathways enriched by the DEGs in the magnum between the HEW and LEW groups, ①−⑩ KEGG pathways include glutathione metabolism; phagosome; endocytosis; N-Glycan biosynthesis; arginine and proline metabolism; ferroptosis; biosynthesis of amino acids; protein export; metabolic pathways; protein processing in endoplasmic reticulum. (M) KEGG pathways enriched by the DAMs in the serum metabolomics between the HEW and LEW groups, ①−⑧ KEGG pathways include valine, leucine and isoleucine biosynthesis; phenylalanine metabolism; biotin metabolism; butanoate metabolism; alanine, aspartate and glutamate metabolism; arginine and proline metabolism; valine, leucine and isoleucine degradation; aminoacyl-tRNA biosynthesis. (N) Schematic diagram of egg yolk and egg white formation in the liver, follicle, and magnum tissues.

(Figure 2J). KEGG analysis found that DEGs were enriched in 124 categories, and 45 pathways were screened significantly different according to the criterion ($P < 0.05$), of which 18 pathways (40%) related to protein metabolism-related pathways, including protein processing in the endoplasmic reticulum (Figure 2K). Furthermore, the gene expression top 30 DEGs from the above pathways were merged to construct the PPI network. The results showed that *HSP90AA1* and *HSPA8* genes are located in the core position in the regulatory network (Figure 2L). Previous research reported that *HSP90* interacted with steroid hormones regulated by the hypothalamic-pituitary-gonadal axis, ultimately affecting ovulation, *HSPs* can combine with unfolded or misfolded proteins and make these problematic proteins return to normal, which is crucial for ovulation (Yu et al., 2016). Thus, *HSP90AA1, HSPA8*, and protein processing in the endoplasmic reticulum pathway were identified as key candidates genes and regulatory pathways responsible for the egg weight difference from the perspective of egg white formation.

Integrated Analysis of Transcriptome and Metabolome Data

As shown in Figure 2M, N, it was worth noting that arginine and proline metabolism is the common pathway enriched by the integrated analysis of transcriptome and metabolome data. It is demonstrated that the key enzymes and products in the arginine metabolic pathway are involved in the growth of granulosa cells and regulate the development of follicles (Braw-Tal, 2002). Therefore, we speculated that arginine and proline might be the key biomarkers affecting the egg weight difference in ducks.

CONCLUSION

The results of the present study revealed the underlying mechanism of the egg weight difference in ducks from the perspective of yolk and albumin formation by comparison of transcriptome and metabolome data. *FASN* gene in fatty acid metabolism-related pathways, *HSPA2, HSPA8, BAG3* genes in pathways of protein processing in the endoplasmic reticulum pathway, and MAPK signaling pathway, which may be responsible for egg weight from the perspective of egg yolk formation. *HSP90AA1* and *HSPA8* genes in protein processing in the endoplasmic reticulum pathway were crucial for egg white formation. Furthermore, the integrated transcriptomic and metabolomic analysis showed that arginine and proline metabolism pathways could contribute to egg weight differences. This study provides theoretical references for improving egg weight in ducks.

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DISCLOSURES

The authors have no conflicts of interest to report.

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