Transcriptomic analysis of the liver in aged laying hens with different eggshell strength

G. P. Han, J. H. Kim, J.-M. Kim, and D. Y. Kil

Department of Animal Science and Technology, Chung-Ang University, Anseong-si, Gyeonggi-do 17546, Republic of Korea

ABSTRACT Eggshell is composed of a very ordered and mineralized structure and is important for egg quality. Eggshell strength is particularly important because of its direct association with economic outcomes and egg safety. Various factors related to laying hens and their environment affects eggshell strength. However, the molecular mechanisms of liver functions related to decreased eggshell strength of aged laying hens are largely unknown. Therefore, this study aimed to identify potential factors affecting eggshell strength in aged laying hens at the hepatic transcriptomic level. A total of five hundred 92-wk-old Hy-line Brown laying hens were screened to select those exhibiting the greatest variation in eggshell strength. Based on the final eggshell strength, 12 hens producing eggs with strong eggshell strength (SES) and weak eggshell strength (WES) were finally selected (n = 6) for liver tissue sampling. The RNA-sequencing was performed to identify differentially expressed genes (DEGs) between the 2 groups. We identified a total of 2,084 DEGs, of which 1,358 genes were upregulated and 726 genes were downregulated in the WES group compared with SES group. According to the Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis, the DEGs indicated the mammalian target of rapamycin signaling pathway, the Janus kinase-signal transducer and activator of transcription pathway, the mitogen-activated protein kinase signaling pathway, and the insulin resistance pathways. Genes related to fatty liver disease were upregulated in WES group compared with SES group. In addition, expression of several genes associated with oxidative stress and bone resorption activity was altered in aged laying hens with different eggshell strength. Overall, these findings contribute to the identification of genes involved in different intensity of eggshell strength, enabling more understanding of the hepatic molecular mechanism underlying in decreased eggshell strength of aged laying hens.

Key words: aged laying hen, eggshell strength, liver, transcriptome

INTRODUCTION Egg quality is determined based on internal and external characteristics (Jacob et al., 2000). Eggshell strength is considered the most important quality among external qualities because of its direct association with economic outcomes and egg safety (Gole et al., 2014). Thus, many poultry scientists have a large attention on an improvement in eggshell strength of laying hens by environmental and nutritional managements.

Eggshells have a highly ordered and mineralized structure composed of an organic component that includes the matrix, eggshell membrane, mammillary knob, and cuticle layer as well as an inorganic component, comprised largely of calcium (Ca) carbonate, which accounts for approximately 95% of the weight of the eggshell (Qiu et al., 2020). Several animals (e.g., age, genetics, and diseases) and environmental factors (e.g., stress, diets, and raising facility) are known to affect eggshell formation and, subsequently, eggshell strength (Roberts, 2004; Solomon, 2010). Among these factors, age is the one of the most influential factors. It is well-recognized that eggshell formation cannot fully support the increase in egg size associated with the aging of laying hens (Washburn, 1982; Park and Sohn, 2018). Moreover, it is also likely that the ability of eggshell formation is decreased by increasing ages of laying hens (Gu et al., 2021). This decreased eggshell strength with age of laying hens is often deteriorated by other additional factors impairing eggshell strength such as environmental stress and disease outbreaks (Solomon, 2010). A lot of efforts have been made to ameliorate decreased eggshell strength of aged laying hens, especially by dietary manipulations. Increasing supply or modified supply ratios of Ca, phosphorus (P), and other potential minerals such as zinc and magnesium (Mg) in diets have been widely...
practiced to improve eggshell strength of aged laying hens (Frost and Roland, 1991; Cufadar et al., 2011; An et al., 2016; Yu et al., 2020; Belkameh et al., 2021). However, the results have been variable, which may be attributed to the limited knowledge of altered nutrient metabolism with age of laying hens.

The application of transcriptomic analysis in poultry science is recently increasing because this analysis can provide a useful information for the molecular changes based on the identification of vital genes and their expression levels, which are related metabolic pathways for poultry raised under different environment and managements (Coble et al., 2014; Li et al., 2015a; Li et al., 2017; Wan et al., 2017). Several previous studies have performed transcriptomic analysis to reveal the molecular mechanisms related to eggshell strength of laying hens with an emphasis of uterine tissues where eggs are actually formed (Sah et al., 2018; Feng et al., 2020). According to the uterine transcriptomic results from laying hens with different intensity of eggshells, Zhang et al. (2019) reported that differences in gonadal hormones in the initial calcification stage of eggshells were related to eggshell strength of laying hens (Zhang et al., 2019). However, there is still a lack of molecular information for the other pivotal tissues in the body of laying hens.

The liver plays a role in various key metabolic functions (carbohydrate, protein, lipid metabolism, bile secretion, etc.), immune response, and detoxification processes (Zaeffarian et al., 2019). Thus, maintaining liver health and functions is imperative to support the proper egg production rate and high egg quality of laying hens (Lera, 2018). However, the liver health is generally decreased with age of laying hens. For instance, increasing de novo fat synthesis in the liver of aged laying hens frequently induces an increasing occurrence of fatty liver diseases, which is highly associated with decreased egg production and egg quality (Galea, 2011). However, other molecular mechanisms for liver functions related to decreased eggshell strength of aged laying hens are still largely unknown. In addition, to our knowledge, no hepatic transcriptomic analysis has been performed to investigate novel genes and metabolic pathways of aged laying hens with the different intensity of eggshell strength.

Accordingly, the present study aimed to compare hepatic transcriptomes between aged laying hens with different intensity of eggshell strength based on RNA-seq analysis and to identify the differentially expressed genes (DEGs) regarding hepatic molecular functions related to eggshell strength of aged laying hens.

**MATERIALS AND METHODS**

**Animals and Tissue Collection**

All experimental procedures were reviewed and approved by the Animal Care and the Use Committee at Chung-Ang University (IACUC: 2018-00002). A total of five hundred 92-wk-old Hy-Line Brown laying hens were used to investigate the eggshell strength at the start of the current experiment. All hens were placed in individual cages (37 × 30 × 40 cm) and raised according to the recommendation of environment and feeds in Hy-Line Brown laying hens (Hy-Line, 2016). The eggshell strength of all eggs produced from 500 hens was measured using the Texture analyzer (model TAHDi 500, Stable Micro System, Godalming, UK) for 10 d. After assessment of initial eggshell strength, 200 hens (100 laying hens with strong eggshell strength and 100 laying hens with weak eggshell strength) were separately selected. The eggshell strength of all eggs produced from the 200 selected laying hens was continuously re-examined for 7 d. A total of 40 hens (20 laying hens with strong eggshell strength and 20 laying hens with weak eggshell strength) were selected again. Within each group of eggshell strength, 6 hens were finally selected based on an additional 6 d of eggshell strength measurements and were assigned to strong eggshell strength (SES) and weak eggshell strength (WES) group. In addition to eggshell strength, eggshell thickness, and eggshell color of eggs from the SES and WES groups were also measured using a dial pipe gauge (model 7360, Mitutoyo Co., Ltd., Kawasaki, Japan) and the eggshell color fan (Samyangsa, Kangwon, Korea), respectively. All 12 hens were euthanized by CO2 asphyxiation at 6 h after their oviposition, which is considered the initiation period of calcification in the uterus (Zhang et al., 2019). The liver was rapidly harvested and immediately frozen in liquid nitrogen for RNA-seq analysis. All liver tissue samples were stored at −80°C until further RNA extraction analysis. The detailed procedure of hen selection and liver sampling is presented in Figure 1.

**RNA Extraction, Library Construction, and Sequencing**

Total RNA was extracted from the liver tissue using TRIzol reagent (Invitrogen, Carlsbad, CA) after grinding the frozen liver sample under liquid nitrogen condition. A total of 12 RNA integrities were checked using an Agilent Technologies 2100 Bioanalyzer and RiboGreen dye (Invitrogen) and quantified using a Trinean DropSense96 spectrophotometer (Trinean, Gentbrugge, Belgium). Quality control criteria were set as the ratio of 28S:18S > 1 and RNA integrity number >7. Only high quality RNA extracts were used for library preparation. As a result, 8 of 12 RNAs were passed and subjected to RNA-seq (SES = 4 samples and WES = 4 samples). The RNA-seq libraries were synthesized from total RNA using the TruSeq Stranded mRNA Sample Preparation Kit (Illumina, San Diego, CA). PolyA+ RNA was purified from total RNA using AMPure XP beads and fragmented to 200 to 700 bp (average 350 bp). A total of 8 cDNA was synthesized using reverse transcriptase (SuperScript II, Invitrogen) and random primers. Strand-specific RNA-seq libraries were prepared by following a modified deoxy-UTP strand-
marking protocol. The mRNA was used to synthesize double-stranded cDNA. This double-stranded cDNA was subsequently ligated to the barcoded Truseq adapters. Library construction was performed by PCR on the size selected fragments. The resultant library was sequenced on an Illumina Nextseq 500 platform (Illumina) using a paired-end run (2 £ 75 bases).

Transcriptomic Analysis

Quality of the raw reads was assessed using FastQC (Andrews, 2010). The raw reads containing adapters or low-quality reads were trimmed using Trimmomatic ver 0.38 (Bolger et al., 2014). The remaining high-quality reads were aligned to the chicken reference genome (GRCg6a, GCA_000002315.5) of the Ensembl genome browser (http://www.ensembl.org/Gallus_gallus/) using HISAT2 ver 2.1.0 (Kim et al., 2015). The mapped reads were quantified based on the exons in the Gallus gallus genome GTF (Ensembl ver. 100) using the featureCounts ver. 1.6.3 of the subread package (Liao et al., 2014). Total DEGs analyses for the acquired raw counts were counted using R package edgeR ver. 3.26.5 and normalization of the raw counts was carried out using the trimmed mean of M-value method (Robinson et al., 2010; Robinson and Oshlack, 2010). Genes with a false discovery rate (FDR) < 0.05 and an absolute log2 fold-change (FC) ≥1 were identified as DEGs. The DEGs obtained from WES group were compared with those obtained from SES group. The sequencing data were submitted to the NCBI Sequence Read Archive database under the accession number PRJNA728451.

Multidimensional scaling (MDS) was conducted to prove the similarities among samples. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were conducted using the Database for Annotation, Visualization and Integrated Discovery (DAVID ver 6.8; Dennis et al., 2003). The GO enrichment analysis was performed with biological process (BP), cellular component (CC), and molecular function (MF).

Protein—Protein Interaction Network Analysis and Modules Selection

The Search Tool for the Retrieval of Interacting Genes (STRING) database was performed to acquire protein-protein interaction (PPI) data. The cutoff DEGs based on an absolute value of log2FC of ≥1.5 were mapped to STRING measuring the interactive relationship. The PPI network of the cutoff DEGs was visualized by Cytoscape (ver 3.8.2; http://www.cytoscape.org/). A high confidence score (more than 0.9) was considered significant and then disconnected nodes were finally removed. The CytoHubba app in Cytoscape was performed to analyze the hub genes using 5 centrality calculation methods, including Degree, EPC, Eccentricity, MCC, and MNC (Liu et al., 2018). The Molecular Complex Detection (MCODE) app in Cytoscape was utilized to investigate the modules of the PPI network. The criteria settings of MCODE were as follows: degree cutoff = 2, node score cutoff = 0.2, k-core = 2, maximum depth = 100 (Yang et al., 2020). Furthermore, the interesting genes derived from these 4 calculation methods, and the function and pathway enrichment analyses were conducted for interesting genes in the modules.

Validation of Differentially Expressed Genes Identification by Reverse Transcription Polymerase Chain Reaction

Nine mRNAs identified as differentially expressed between SES and WES groups in the RNA-seq were selected for verification based on the relative quantification by reverse transcription polymerase chain reaction (RT-PCR). The total RNA was used for cDNA synthesis using the RevertAid First Strand cDNA Synthesis Kit (Thermo scientific, Waltham, MA) according to the manufacturer’s guidelines.

All primer sequences for RT-PCR were designed using Primer-BLAST in the NCBI and are listed in (Supplementary Table 1). To validate the specificity of the primer to a target gene, PCR amplification was performed by modifying the protocol described by Aznar and Alarcon (2002). Briefly, PCR amplification was conducted using cDNA samples and Dream Taq Green PCR Master Mix (Thermo
scientific). The PCR cycling conditions were: an initial cycle at 95°C for 5 min followed by 35 cycles at 95°C for 30 s, annealing temperature for 30 s, 72°C for 1 min, and a final 5 min extension at 72°C. Reactions were performed in a MyCyclerTM Thermal Cycler System (Bio-rad, Hercules, CA). The RT-PCR amplifications were carried out in 20 µL PCR reaction mixture containing diluted 1 µL of cDNA (100 ng), 10 µL AMPIGENe qPCR Green Mix Lo-ROX (Enzo life science, Farmingdale, NY), 1 µL of each forward and reverse primer (10 pmole) 7 µL of nuclease-free water. The RT-PCR was performed using a CFX connect real-time PCR Detection system (Bio-rad). The cycling conditions were as follows: 95°C for 2 min, followed by 40 cycles of amplification (95°C for 5 s and annealing temperature for 25 s). The relative quantification of gene expression was calculated after normalization to chicken glyceraldehyde-3-phosphate dehydrogenase and by using $2^{-\Delta\Delta Ct}$ method.

**Statistical Analysis**

The GLM procedure of SAS was used to compare with egg quality in laying hens between SES and WES (SAS Institute, Cary, NC). The statistical significance was set at $P < 0.05$.

**RESULTS**

**Comparison of Eggshell Quality Between Hens With SES or WES**

The result for eggshell quality between SES and WES groups is presented in Table 1. Consistent with the screening model, SES group had a greater ($P < 0.01$) eggshell strength and thickness by 54.0% and 16.1% than WES group, respectively; however, no difference in eggshell colors was observed between groups.

**Transcriptome Alignment and Mapping Statistics**

After removing low-quality and adaptor sequences, a total of 136.48 million reads with an average of 17.06 million reads per sample (range: 14.54–19.09 million) were obtained. The GC contents of the libraries ranged from 47 to 50%, which were very close to 50%. The Q20 and Q30 quality values were 99.95% and 94.74%, respectively (Supplementary Table 2). The reads in each sample were mapped to the *Gallus gallus* genome at average alignment rates of 96.29% and 96.70% for SES and WES groups, respectively.

**DEGs Profiling**

The DEG profiles were investigated between the SES and WES groups. The entire DEGs were identified 2,084 genes. Of the 2,084 genes, 1,358 genes were upregulated and 726 genes were downregulated in WES group compared with SES group (Figure 2A). The details of those DEGs are presented in Supplementary Table 3. The MDS plots of the DEGs were visualized for their distinct transcriptional profiles between SES and WES groups (Figure 2B).

**GO Enrichment and KEGG Pathways Analysis for DEG**

All DEGs were analyzed by GO enrichment and KEGG pathway enrichment analyses using the DAVID ver. 6.8 online tool to identify the differences in the hepatic physiological processes between SES and WES groups. A total of 107 GO terms were enriched ($P$-value < 0.1; Supplementary Table 4) and 45 GO terms were significant ($P$-value < 0.05, fold enrichment >2.0) as shown in Figure 3. The most significantly enriched GO terms included protein phosphorylation, canonical Wnt signaling pathway, activation of GTPase activity, regulation of vesicle fusion, epidermal growth factor receptor signaling pathway, positive regulation of osteoblast differentiation, protein processing, insulin receptor signaling pathway, and response to calcium ion in the BP category. In the CC category, external side of plasma membrane was enriched and was associated with processes such as membrane raft, exocyst, contractile fiber, and membrane attack complex. Protein kinase activity, which is related to 16 genes, was the most enriched term within MF category.

Moreover, the DEGs were enriched in 141 KEGG pathways (Supplementary Table 5) and the most significant pathways ($P < 0.05$; Figure 4) included mammalian target of rapamycin (mTOR) signaling pathway, Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway, mitogen-activated protein kinase (MAPK) signaling pathway, insulin resistance (IR), and insulin signaling pathways. Additionally, the DEGs were predicted to play important roles in energy metabolism, particularly for fatty acid metabolism, and ascorbate and aldarate metabolism based on the KEGG pathway enrichment analysis.

**Identification of Hub Genes and Pathways Through PPI Network Analysis of DEG**

The PPI network was constructed including 418 nodes and 674 edges (Figure 5A). Based on the STRING database, the top 10 DEGs were evaluated in the PPI network using 5 centrality methods (Table 2). Furthermore, we identified the intersections of these 5 centrality
methods and created a Venn plot (Figure 5B) to recognize significant hub genes using an online website (http://jvenn.toulouse.inra.fr/app/example.html). Ultimately, the 3 hub genes, including growth factor receptor bound protein 2 (GRB2), transferrin/ovotransferrin (TF), and Cbl proto-oncogene (CBL) were confirmed. The hub gene extracted from 5 centrality methods may correspond to key candidate genes with important regulatory functions to make a different intensity of eggshell strength of aged laying hens.

Furthermore, 3 significant modules, including module 1 (MCODE score = 10.6), module 2 (MCODE score = 8), and module 3 (MCODE score = 7) were created from the PPI network through the cut-off DEGs by MCODE (Figure 6). Genes of each module were investigated by biological functional enrichment analysis using DAVID database. Module 1 (Figure 6A), including 11 nodes and 53 edges, were remarkably enriched in ‘endocytosis’, ‘ErbB signaling pathway’, ‘Jak-STAT signaling pathway’, and ‘insulin signaling pathway’. Moreover, module 2 (Figure 6B), including 8 nodes and 28 edges, were involved in ‘ubiquitin mediated proteolysis’, ‘endocytosis’, and ‘calcium signaling pathway’. Furthermore, module 3 (Figure 6C) contains 15 nodes and 49 edges that are primarily related to ‘calcium signaling pathway’, ‘regulation of actin cytoskeleton’, and ‘neuroactive ligand-receptor interaction’ (Table 3).

**Validation of DEG in the RNA-Sequencing Results**

To validate RNA-seq expression results, 9 DEGs (Top 9 DEGs; 5 upregulated and 4 downregulated genes) were selected for qRT-PCR, which possessed the characteristics of abundant expression and higher FC. These genes were acid phosphatase 3 (ACP3), regenerating family member 1 alpha (REG1A), rhodopsin (RHO), adenylate cyclase 2 (ADCY2), transmembrane protein 86A (TMEM86A), regulator of G-protein signaling 16 (RGS16), angiotensin I converting enzyme (ACE), transient receptor potential cation channel subfamily M member 8 (TRPM8), and solute carrier family 7 member 11 (SLC7A11). The samples of qRT-PCR were same as those used for RNA-seq. The results confirmed
that the expression patterns of selected genes were consistent with those of RNA-seq (Figure 7).

**DISCUSSION**

Eggshell strength is one of the most important egg quality parameters because high incidence of soft or easily-cracking eggshells leads to a considerable economic loss in the layer industry (Stefanello et al., 2014). The main factors affecting eggshell strength have been associated with both animal (e.g., age, genetics, and health) and environmental factors (e.g., nutrition, temperature, management system; Roberts, 2004; Guo et al., 2015; Ketta and Tunová, 2016). Therefore, many efforts have been made to improve eggshell strength by controlling those factors involved in eggshell strength of laying hens (Zhang et al., 2017; Qiu et al., 2020). Mineral nutrition has received the most attention, especially for Ca and P because nutritional management is convenient and practical in the layer industry and those specific minerals are the main components of eggshells (Ademosun and Kalango, 1973; Lim et al., 2003; Jiang et al., 2013b). It has been reported that increasing Ca concentrations with maintaining proper available P concentrations in diets improved eggshell strength of aged laying hens (Lim et al., 2003; An et al., 2016). In addition, Mg concentrations in diets was also reported to improve eggshell strength in laying hens (Seo et al., 2010; Kim et al., 2013a,b). However, the results from previous experiments have been inconsistent (Lim et al., 2003; Pelicia et al., 2009; An et al., 2016), such that no promising regimens have been identified. One possible reason for variable results is a lack of information for molecular mechanisms such as nutrient metabolism associated with eggshell quality in laying hens. Therefore, in the current study, we applied hepatic transcriptomic analysis to identify the role and regulation of the liver for eggshell strength using 2 distinct groups of laying hens with either strong eggshells or weak eggshells.

For the molecular analysis regarding eggshell strength in laying hens, Zhang et al. (2019) conducted a uterine transcriptomic analysis in laying hens with high or low eggshell strength during 3 different uterine calcification periods, and showed differences in extracellular proteins and ion transporters associated with eggshell...
calcification. However, most of molecular studies related to eggshell strength have emphasized specifically the uterine function because eggshell formation occurs exclusively in the uterus (Brionne et al., 2014; Sah et al., 2018). Thus, few studies have analyzed other pivotal tissues that can potentially affect egg production and eggshell strength in laying hens.

The liver is the central metabolic organ associated with the metabolism of various nutrients, detoxification of exogenous substances, and host defense against invading pathogens (Crispe, 2009; Chiang, 2014). In addition, the liver plays a role in egg formation by participating in egg yolk synthesis and Ca transport (Galea, 2011). Therefore, the knowledge of the hepatic transcriptome might provide novel molecular functions of the liver, which may influence the eggshell strength in laying hens. However, a research pertaining to hepatic molecular functions on eggsheen strength is largely lacking in laying hens.

As we intended, eggshell strength was significantly different between SES and WES groups (eggshell strength = 4.17 vs. 1.92 for SES and WES, respectively). Likewise, eggshell thickness for SES group was greater than for WES group. This result confirmed the high positive correlation between eggshell strength and thickness as reported previously (Zhang et al., 2005), which confirmed that the selected layer groups in the current study provided the reliable data for hepatic transcriptomic analysis of laying hens with different eggshell strength.

Based on the hepatic transcriptomic profile in the current experiment, liver homeostasis capacity (e.g., liver regeneration or fatty liver disease) is likely an important factor affecting eggshell strength of laying hens. In the current experiment, mTOR signaling pathway was enriched in DEGs between SES and WES group. The mTOR is a serine/threonine protein kinase that integrates various nutrient and hormonal signals to regulate cell growth and metabolism (Dann and Thomas, 2006). Bottje et al. (2014) reported that the mTOR pathway genes were upregulated in the liver of broiler chickens with high feed efficiency as compared to those with low feed efficiency, which indicates that mTOR pathway is highly involved in muscle development and protein metabolism. On the contrary, laying hens with low egg production had upregulated mTOR pathway-related genes in the pituitary gland than those with high egg production (Mishra et al. 2020). Moreover, previous poultry studies have reported that apoptosis caused by external stimulus (e.g., Salmonella infection or thermal stress) upregulated mTOR signaling (Arsenault et al., 2013; Napper et al., 2015).
Similar upregulation of genes related to mTOR signaling pathway in the liver was observed for WES group than SES group in the current experiment. The phosphatidylinositol-3 kinases (PI3Ks) family is involved in stimulating differentiation, proliferation, and survival of cells and participates in the signaling pathways related to the liver injury caused by fluorosis in the human body (Okkenhaug and Vanhaesebroeck, 2003; Fan et al., 2015). According to Zhu et al. (2017), phosphoinositide-3-kinase regulatory subunit 1 (PIK3R1) is a candidate gene for non-alcoholic fatty liver disease (NAFLD) in rats, indicating that the upregulation of this gene may represent the development of NAFLD. In the current experiment, PIK3R1 was upregulated by a log₂ FC of 1.62 in WES group than in SES group, indicating that decrease in eggshell strength is likely associated with the development of fatty liver disease in laying hens. Likewise, in another mTOR pathway-related gene, we also observed upregulation of ribosomal protein s6 kinase a3 and ribosomal protein s6 kinase a6 by a log₂ FC of 1.33 and 1.16, respectively, in WES group. These genes encode a member of the ribosomal S6 kinase family of serine/threonine kinases that regulate diverse cellular processes such as cellular growth, motility, survival, and proliferation (Carriere et al., 2008). In addition, the expression of these genes in the liver was also reported to be increased in mice with fatty liver diseases (Wang et al., 2014; Simoes et al., 2020).

The present study also showed other evidence of alterations in gene expressions related to liver homeostasis.
Figure 6. The three protein-protein interaction (PPI) hub network modules. The three significant modules, including (A) module 1 [molecular complex detection (MCODE) score = 10.6], (B) module 2 (MCODE score = 8), and (C) module 3 (MCODE score = 7), were constructed from PPI network of differentially expressed genes using MCODE. The red node represents the upregulated gene, while the blue node represents the downregulated gene. The seed node of each module was shaped as diamond and highlight the green gene symbol. Node size indicated the fold change of each gene.

Table 3. The biological function enrichment analysis of the three protein-protein interaction (PPI) hub network modules.

| Module   | Database | GO term/pathway                                      | P-value | Genes          |
|----------|----------|------------------------------------------------------|---------|----------------|
| module 1 | KEGG     | Endocytosis                                          | 0.0074  | ARPC2, STAM, CBL |
|          |          | ErbB signaling pathway                               | 0.0525  | GRB2, CBL      |
|          |          | Jak-STAT signaling pathway                           | 0.0777  | GRB2, STAM      |
|          |          | Insulin signaling pathway                            | 0.0777  | GRB2, CBL      |
|          | GO_MF    | Protein binding                                      | 0.0351  | TF, GRB2       |
| module 2 | KEGG     | Ubiquitin mediated proteolysis                        | 0.0284  | ITCH, SMURF1   |
|          | GO_BP    | Protein ubiquitination involved in ubiquitin-dependent | 0.0000  | ITCH, SMURF1   |
|          | GO_CC    | Protein catabolic process                            | 0.0351  | SMURF1, KLHL11 |
|          |          | Proteasome-mediated ubiquitin-dependent protein      |         |                |
|          |          | catabolic process                                    |         |                |
|          | GO_MF    | Calcium signaling pathway                             | 0.0123  | KLHL11, FBXL15 |
| module 3 | KEGG     | Ubiquitin-protein transferase activity                | 0.0011  | ITCH, SMURF1, FBXL15 |
|          | GO_BP    | Ligase activity                                      | 0.0223  | ITCH, SMURF1   |
|          | GO_CC    | Ubiquitin-protein ligase activity                     | 0.0483  | SMURF1, FBXO30 |
|          |          | Calcium signaling pathway                             | 0.0256  | BDKRB1, PLCB1, NTSR1 |
|          |          | Regulation of actin cytoskeleton                     | 0.0306  | BDKRB1, PIK3R1, FGF23 |
|          |          | Neuroactive ligand-receptor interaction               | 0.0627  | BDKRB1, LPAR3, NTSR1 |
|          | GO_BK    | Negative regulation of apoptotic process             | 0.0187  | GHRL, PIK3R1, NTSR1 |
|          |          | Negative regulation of osteoblast differentiation     | 0.0355  | IGFBP5, FGF23  |
|          |          | Activation of MAPK activity                           | 0.0483  | LPAR3, GHRL   |
|          | GO_CC    | Extracellular space                                  | 0.0039  | IGFBP5, GOLM1, GHRL, FGF23, FBN1 |
|          |          | Axon                                                 | 0.0058  | GRNRH1, LPAR3, GHRL |

1Abbreviations: BP, biological process; CC, cellular component; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes pathway; MF, molecular function.
2Upregulated genes in WES groups are highlighted in bold and downregulated genes in normal typeface.
capacity or fatty liver disease: Jak-STAT signaling pathway, MAPK signaling pathway, and IR pathway. The Jak-STAT signaling pathway plays a crucial role in signaling of cytokine receptors, various cell functions such as differentiation, proliferation, and apoptosis (Kisseleva et al., 2002; Truong et al., 2017). A previous study reported that Jak-STAT signaling pathway was significantly enriched in the liver of broiler breeder hens with low egg production induced by overfeeding as compared to restricted feeding (Wei et al., 2019). Moreover, it was reported that Jak-STAT signaling pathway was downregulated in the blood sample of laying hens fed with a dried olive pomace, indicating that Jak-STAT signaling pathway is also correlated with amelioration of immune responses (Iannaccone et al., 2019). This pathway was also highlighted in the adipose tissue of an experiment comparing with fatty liver hemorrhagic syndrome (FLHS) chickens and normal chickens (Zhu et al., 2021). In the current study, similar upregulation of genes related to Jak-STAT signaling pathway in the liver were observed for WES group compared with SES group. Interleukin (IL)-22 is a member of the IL-10 family of cytokines that act as mediators in inflammatory response and immune system in several tissues including the liver and pancreas (Khawar et al., 2016). The IL-22 is mostly produced by dendritic cells, activated T cells, innate lymphoid cells (Khawar et al., 2016). The IL-22 receptor subunit alpha 2 (IL-22RA2), which is known as IL-22BP, is an inhibitor of IL-22 and is positively correlated with the severity of liver damage and fibrosis (Khawar et al., 2016; Weiskirchen and Tacke, 2016). In the present study, the expression of IL-22RA2 was upregulated by a log2 FC of 2.25 in the WES group compared with SES group. Therefore, it is likely that WES group may suffer from liver damages compared with SES group. Moreover, in another Jak-STAT signaling pathway-enriched gene, AKT serine/threonine kinase 1 (AKT1) is a member of AKT responsible for the regulation of glucose uptake and plays a similar role to the PI3Ks family (Turinsky and Damrau-Abney, 1998; Fan et al., 2015). The expression of AKT1 and PIK3 has been related to increase protection against liver injury, suggesting that a link between hepatocyte damage and impaired eggshell strength (Fan et al., 2015). Furthermore, PI3K and AKT signaling were upregulated in lipopolysaccharides-induced mice, demonstrating that these 2 genes may control oxidative stress-inducing inflammation (Zhong et al., 2016). In other words, upregulation of IL-22 and AKT1 observed in WES group compared with SES group may indicate that decreased eggshell strength may be linked to the liver damage or oxidative stress in laying hens. A recent study reported that laying hens induced oxidative stress by dietary arsenic supplementation had significantly lower eggshell strength, indicating that oxidative stress may be related to the deterioration of eggshell strength (Ma et al., 2021).

The MAPK signaling pathway is a regulator of hepatic metabolism (e.g., glucose metabolism and fatty acid metabolism) and participates in the modulation of inflammation response (Lawan et al., 2015; Shen et al., 2019). Luan et al. (2014) reported that MAPK signaling pathway was differentially expressed in the goose ovarian tissue at 2 stages of the egg-laying cycle (ceased period vs. laying period). Most of previous studies investigating MAPK signaling pathway in the liver have proved that NAFLD is alleviated via inactivation of the MAPK signaling pathway (Zhang et al., 2016; Shen et al., 2019). In the MAPK signaling pathway, fibroblast growth factor 23 (FGF-23) plays a role in maintaining normal phosphate homeostasis (Perwad et al., 2005). According to He et al. (2018), FGF-23...
regulates fat content and distribution and is associated with IR in the liver. Furthermore, the patients with NAFLD had significantly higher serum FGF-23 levels as compared to those without NAFLD (He et al., 2018). In the eggshell quality-related study, FGF-23 signaling was involved in vitamin D metabolism and correlated with eggshell calcification, indicating that blocking the FGF-23 signaling pathway increased eggshell strength of laying hens (Ren et al., 2018). In our results, FGF-23 gene was also upregulated by a log2 FC of 2.73 in WES group compared with SES group. In another MAPK signaling pathway-enriched gene, serine/threonine-protein kinase 4 (STK4) is a stress-induced kinase and is correlated with apoptosis (Ready et al., 2017). It was reported that STK4 was highly expressed in hematopoietic cells to promote the naive T lymphocyte proliferation and survival (Li et al., 2015b). In the present study, STK4 expression in the liver was upregulated by a log2 FC of 2.31 in laying hens with WES than those with SES. Therefore, it can be hypothesized that hepatic injury stimulating the immune response in the liver of WES group may occur, leading to an increase in STK4 expression.

The IR refers to a physiological condition in which the body’s response to insulin is reduced below the normal standard response (Bugianesi et al., 2005). It is intimately related to NAFLD (Bugianesi et al., 2005; Watt et al., 2019). Pathological observations have shown a high level of IR in the FLHS model, which is known to be similar to NAFLD (Zhuang et al., 2019). Among the upregulated DEGs in IR pathway, Insulin receptor substrate proteins 1 (IRSI) is an important target of the insulin receptor tyrosine kinase (Copps and White, 2012). The IRS1 signaling is required for glucose homeostasis and systemic growth in the liver (Dong et al., 2006). It appears that IRS1 is a crucial regulator for the synthesis and oxidation of fatty acids in the liver of non-alcoholic steatohepatitis (NASH), which was significantly increased in the NASH rat model (Matsumani et al., 2011). Therefore, based on previous IRS1 results, we speculate that high IRS1 expression in WES group is closely related to FLHS. In another IR pathway-enriched gene, MAX-like protein X interacting protein (MLXIP), also known as MondoA, is responsible for glucose/fructose metabolism in human skeletal muscles and directs the transcription of genes in cellular metabolic pathways involved in diverse nutrient metabolism including fatty acid desaturation and elongation, triacylglyceride biosynthesis, glycogen storage (Ahn et al., 2019). Previous experiment indicated that mice-deficient MondoA have partially protected from IR and muscle fat accumulation (Ahn et al., 2019). In the current study, MLXIP expression was upregulated by a log2 FC of 2.64 in WES group compared with SES group. Therefore, we suggest that different intensity of eggshell strength in laying hens may be related to IR, and furthermore, laying hens with WES may show abnormal lipid metabolism such as fatty liver diseases.

Another functional pathway identified by KEGG analysis showed genes involved in nutrient metabolism, including the glycerolipid metabolism, and the glycine, serine, and threonine metabolism. Interestingly, all DEGs in both KEGG pathways were upregulated in WES group compared with SES group in this study. A previous uterine transcriptomic study regarding eggshell strength in laying hens showed similar results to our observation, indicating the glycerolipid metabolism in the liver may also influence on eggshell strength (Zhuang et al., 2019). Furthermore, both KEGG pathways were enriched in the hepatic transcriptome of broiler breeder hens fed diets supplemented with genistein, an isoflavone phytoestrogen known to have antioxidant effects and alleviate fibrosis of the liver (Lv et al., 2018).

In the glycerolipid metabolism pathway, cystathionine-beta-synthase like (CBSL) is known to be involved in catalyzing the conversion of serine and homocysteine to cystathionine and water (Jhee and Kruger, 2005). Homocysteine is proposed to modulate bone remodeling and strength (Zhou et al., 2019). A previous transcriptome study comparing the tibia from laying hens with good or poor bone quality showed that CBSL was highly expressed by 9-fold increase in laying hens with poor bone quality (De Koning et al., 2020). The bone-related disorders were reported to affect egg production and egg quality in laying hens because skeletal damage in laying hens leads to various problems with Ca metabolism associated with eggshell formation (Whitehead, 2004). In the present study, CBSL expression in the liver was upregulated by a log2 FC of 2.35 in laying hens with WES than those with SES. However, it remains unclear how the upregulation of CBSL in the liver affects poor bone quality. Further studies on CBSL functions in the liver of laying hens are necessary for elucidating its role in eggshell formation and strength.

The alignment of PPI networks allows a further investigation into the fundamental relationships among DEGs by associated genes and protein complexes with network pathways (Athanasios et al., 2017). The PPI network in the current study was created with DEGs, and then the top centrality hub genes were obtained using 5 centrality methods. Ultimately, we confirmed 3 hub genes, including GRB2, TF, and CBL, which were upregulated in WES group compared with SES group. GRB2 contributes to the regulation of cellular functions, including proliferation and differentiation, as an important adaptor protein (Ge et al., 2017). A previous study suggested that downregulation of GRB2 improved hepatic steatosis, glycogen metabolism, oxidative stress, and apoptosis by modulating the insulin signaling pathway in HepG2 cells (Shan et al., 2013). According to Amanatidou and Dedoussis (2021), GRB2 is one of the candidate NAFLD-related proteins. TF is one of the major matrix protein of hen eggshell membranes that consists of 12 to 13% of total egg white protein (Ko and Ahn, 2008). TF was reported to play a role as an acute phase protein in chickens and the level of TF in the blood was increased in response to inflammation (Rath et al., 2009). A comparison of eggshell matrix proteins between young and aged laying hens revealed that
aged laying hens had less eggshell strength than young laying hens and high concentrations of TP protein was observed in aged laying hens than in young laying hens (Panheleux et al., 2000). The expression of TP observed in this study is also coincident with the result from the previous study investigating the gene expression using integrated transcriptome and whole genome re-sequencing in the uterus of laying hens with different eggshell strength (Zhang et al., 2015). However, to our knowledge, no previous studies have revealed the mechanism involving TP expression and eggshell strength in poultry. The CBL is a protein coding gene, which functions as ubiquitin ligases and multifunctional adaptor molecules and plays role in the control of cell proliferation, differentiation and morphology, as well as in pathologies such as autoimmune diseases (Dikic et al., 2003). Overexpression of CBL is reported to reduce in vitro bone resorption (Miyazaki et al., 2004). Our results also showed that the expression of CBL was upregulated in WES groups compared with SES groups. These findings lead us to hypothesize that differential expression of CBL impacts eggshell strength, which means that WES group may exhibit a lower ability of bone resorption than SES group. However, this hypothesis should be validated in further researches.

We composed 3 significant modules to further analyze the PPI network. In the present study, the genes of module 1 were upregulated in WES group and enriched in ‘endocytosis’, ‘Jak-STAT signaling pathway’, and ‘insulin signaling pathway’. The seed node of module 1 is GRB2, which is one of the hub genes as mentioned above. Genes of module 2 enriched in ‘ubiquitin mediated proteolysis’ and ‘calcium signaling pathway’. The seed node of module 2 is FBXO30, which acts as an ubiquitin ligase. A recent study indicated that expression of protein ubiquitination in goose fatty liver was higher than in the normal liver (Yan et al., 2020). This result appears to support other results regarding fatty liver development. Genes of module 3 were associated with ‘calcium signaling pathway’ and ‘negative regulation of osteoblast differentiation’. The seed node of module 3 is SERPINC1, one of the biomarkers for the prediction of NAFLD, showing upregulated in WES group (Pirola and Sookoian, 2018). Therefore, it is speculated that the pathways and genes associated with fatty liver and liver homeostasis in laying hens influence eggshell strength deterioration. However, there were no studies on the closed relationship between fatty liver diseases and the deterioration of eggshell strength. However, several previous studies in laying hen with fatty liver disorder found decreased egg production rate (Walzem et al., 1993; Jiang et al., 2013a) and increased eggs with a bad eggshell quality as compared to the control group (Galea, 2011).

In conclusion, we conducted a comparative transcriptomic analysis in the liver of aged laying hens displaying different eggshell strength. KEGG pathway analysis reveals that the relevant genes are enriched in the pathways related to fatty liver and liver homeostasis capacity. Genes related to fatty liver development are upregulated in laying hens with WES as compared to those with SES. Several genes associated with age-related alterations in oxidative stress and bone resorption are also identified in aged laying hens with WES. These findings of the current study provide some potential clues for understanding the molecular mechanism in the liver of aged laying hens with different intensity of eggshell strength, and therefore, serve as valuable reference data for future researches regarding improvements in eggshell strength of aged laying hens.

DISCLOSURES

The authors declare no conflict of interest for the data presented in this experiment.

ACKNOWLEDGMENTS

This research was carried out with the support of the Cooperative Research Program for Agriculture Science and Technology Development (Project No. PJ01256701), Rural Development Administration, Republic of Korea.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2022.102217.

REFERENCES

Ademosun, A. A., and I. O. Kalango. 1973. Effect of calcium and phosphorus levels on the performances of layers in Nigeria: 1. Egg production, egg shell quality, feed intake and body weight. Poult. Sci. 52:1383–1392.

Ahn, B., S. Wan, N. Jaiswal, R. B. Vega, D. E. Ayer, P. M. Tichenell, X. Han, K. J. Won, and D. P. Kelly. 2019. MondoA drives muscle lipid accumulation and insulin resistance. JCI Insight 4:e129119.

Amanatidou, A. I., and G. V. Dedoussis. 2021. Construction and analysis of protein-protein interaction network of non-alcoholic fatty liver disease. Comput. Biol. Med. 131:104243.

An, S. H., D. W. Kim, and B. K. An. 2016. Effects of dietary calcium levels on productive performance, eggshell quality and overall calcium status in aged laying hens. Asian-Australas. J. Anim. Sci. 29:1477–1482.

Andrews, S. 2010. FastQC: a quality control tool for high throughput sequence data. Accessed Sep. 2020. http://www.bioinformatics.babraham.ac.uk/projects/fastqc.

Arsenault, R. J., S. Napper, and M. H. Kogut. 2013. Salmonella enterica Typhimurium infection causes metabolic changes in chicken muscle involving AMPK, fatty acid and insulin/mTOR signaling. Vet. Res. 44:35.

Athanasios, A., V. Charalampos, T. Vasileios, and G. M. Ashraf. 2017. Protein-protein interaction (PPI) network: recent advances in drug discovery. Curr. Drug Metab. 18:5–10.

Aznar, R., and B. Alarcón. 2002. On the specificity of PCR detection of Listeria monocytogenes in food: a comparison of published primers. Syst. Appl. Microbiol. 25:109–119.

Belkameh, M. M., M. Sedghi, and A. Azarfar. 2021. The effect of different levels of dietary magnesium on eggshell quality and laying hen’s performance. Biol. Trace Elem. Res. 199:1566–1573.

Bolger, A. M., M. Lahaye, and B. Usadel. 2014. Trimomatic: a flexible trimmer for Illumina sequence data. Bioinform. 30:2114–2120.

Bottje, W. G., B. -W. Kong, J. Lee, T. Washington, J. I. Baum, S. Dridi, T. Wing, and J. Hardiman. 2014. Potential roles of
nTOR and protein degradation pathways in the phenotypic expression of feed efficiency in broilers. Biochem. Physiol. 3:1.

Brionne, A., Y. Nys, C. Hennequet-Antier, and J. Gautron. 2014. Hen uterine gene expression profiling during eggshell formation reveals putative proteins involved in the supply of minerals or in the shell mineralization process. BMC Genomics 15:220.

Bugianesi, E., A. J. McCullough, and G. Marchesini. 2005. Insulin resistance: a metabolic pathway to chronic liver disease. Hepatol. 42:987–1000.

Carriere, A., H. Ray, J. Blenis, and P. P. Roux. 2008. The RSK factors of activating the Ras/MAPK signaling cascade. Front. Biosci. 13:4258–4275.

Chiang, J. 2014. Liver physiology: metabolism and detoxification. Pages 1770–1782 in Pathobiology of Human Disease. A Dynamic Encyclopedia of Disease Mechanisms. L. M. McManus and R. N. Mitchell, ed. Elsevier, Amsterdam, the Netherlands.

Coble, D. J., D. Fleming, M. E. Persia, C. M. Ashwell, M. F. Rothschild, C. J. Schmidt, and S. J. Lamont. 2014. RNA-seq analysis of broiler liver transcriptome reveals novel responses to high ambient temperature. BMC Genomics 15:1084.

Copps, K. D., and M. F. White. 2012. Regulation of insulin sensitivity by serine/threonine phosphorylation of insulin receptor substrate proteins IRS1 and IRS2. Diabetologia 55:2565–2582.

Crise, I. N. 2009. The liver as a lymphoid organ. Annu. Rev. Immunol. 27:147–163.

Cufadar, Y., O. Olgun, and A. Ö. Yi ldiz. 2011. The effect of dietary calcium concentration and particle size on performance, eggshell quality, bone mechanical properties and tibia mineral contents in moulted laying hens. Br. Poult. Sci. 52:761–768.

Danna, S. G., and G. Thomas. 2006. The amino acid sensitive TOR pathway from yeast to mammals. FEBS Lett. 580:2821–2826.

De Koning, D.-J., N. Dominguez-Gasca, R. H. Fleming, A. Gill, D. Kurian, A. Law, H. A. McCormack, D. Morrice, E. Sanchez-Rodriguez, A. B. Rodriguez-Navarro, R. Reisinger, M. Schmutz, V. Smidlová, F. Turner, P. W. Wilson, R. Zhou, and I. C. Dunn. 2020. An eQTL in the eQTL grid database. Nat. Genet. 52:13.

Frost, T. J., and D. A. Roland. 1991. The influence of various calcium and phosphorus levels on tibia strength and eggshell quality of pullets during peak production. Poult. Sci. 70:963–969.

Gole, F. 2011. Nutrition and feed management and their influence on egg quality. XLVIII Scientific Symposium of Poultry. Ge, S., Y. Xiong, W. Xu, J. Xie, F. Liu, J. He, T. Xiang, N. Cheng, L. Lai, and Y. Zhong. 2017. Role of growth factor receptor-bond-2 in CCI4-induced hepatic fibrosis. Biomed. Pharmacother. 92:942–951.

Guo, J., M. Ma, L. Qu, M. Shen, T. Dou, and K. Wang. 2015. Estimation of genetic parameters related to eggshell strength using random regression models. Br. Poult. Sci. 56:645–650.

Hy-Line. 2016. Hy-Line Brown Commercial Layer Management Guide. Hy-Line Int, West Des Monies, IA.

Iannaccone, M., A. Ianni, S. Ramazzotti, L. Grotta, E. Marchioni, A. Cichelli, and G. Martin. 2019. Whole blood transcriptome analysis reveals positive effects of dry diets on inflammation and cholesterol in laying hens. Animals 9:127.

Jacob, J. P., R. D. Miles, and F. B. Mather. 2000. Egg quality. Institute of Food and Agricultural Science-University of florida, FL, PS24.

Jhee, K.-H., and W. D. Kruger. 2005. The role of cystathionine β-synthase in homocysteine metabolism. Antioxid. Redox Signal. 7:813–822.

Jiang, S., H. W. Cheng, L. Y. Cui, Z. L. Zhou, and J. F. Hou. 2013a. Changes of blood parameters associated with bone remodeling following experimentally induced fatty liver disorder in laying hens. Poult. Sci. 92:1443–1453.

Jiang, S., L. Cui, C. Shi, X. Ke, J. Luo, and J. Hou. 2013b. Effects of dietary energy and calcium levels on performance, egg shell quality and bone metabolism in hens. Vet. J. 198:252–258.

Ketta, M., and E. Ťimův. 2016. Eggshell structure, measurements, and quality-affecting factors in laying hens: a review. Czech J. Anim. Sci. 61:299–309.

Khawar, M. B., F. Azam, N. Sheikh, and K. A. Mugeeb. 2016. How does Interleukin-22 mediate liver regeneration and prevent injury and fibrosis? J. Immunol. Res. 2016:2148129.

Kim, C. H., I. K. Paik, and D. Y. Kil. 2013a. Effects of increasing supplementation of magnesium in diets on productive performance and eggshell quality of aged laying hens. Biol. Trace Elem. Res. 151:38–42.

Kim, C. H., I. K. Paik, D. Y. Kil, and M. B. Chang. 2013b. Effects of dietary magnesium concentrations on performance and eggshell quality of laying hens. J. Anim. Vet. Adv. 12:104–107.

Kim, D., B. Langmead, and S. L. Salzberg. 2015. HISAT: a fast spliced aligner with low memory requirements. Nat. Methods 12:357–360.

Kisseleva, T., S. Bhattacharya, J. Braunstein, and C. W. Schindler. 2002. Sequencing through the JAK/STAT pathway: recent advances and future challenge. Gene 285:1–24.

Ko, K. Y., and D. U. Ahn. 2008. An economic and simple purification procedure for the large-scale production of ovotransferrin from egg white. Poult. Sci. 87:1441–1450.

Lawan, A., L. Zhang, F. Gatze, K. Min, M. J. Jurczak, M. A. Mutairi, P. Richter, J. P. G. M. Campoen, A. Couvillon, D. Pesce, R. J. R. Flach, G. I. Shulman, and A. M. Bennett. 2015. Hepatic mitogen-activated protein kinase phosphatase 1 selectively regulates glucose metabolism and energy homeostasis. Cell. Mol. Biol. 35:26–40.

Lena, R. 2018. Nutrition and metabolism: preventing fatty liver. Accessed May 2021. https://www.isa-poultry.com/en/news/nutritio-and-metabolism-preventing-fatty-liver/.

Li, H., T. Wang, C. Wu, D. Wang, J. Ren, Y. Li, Y. Tian, Y. Wang, Y. Xiao, X. Kang, and X. Liu. 2015a. Transcriptome profile of liver at different physiological stages reveals potential mode for lipid metabolism in laying hens. BMC Genomics 16:763.

Li, J., X. Liu, L. Xing, H. Liu, and J. Bao. 2017. Gene expression profiling of broiler liver under cold stress by high-throughput sequencing technology. J. Poult. Sci. 54:185–196.

Li, W., J. Xiao, X. Zhou, M. Xu, C. Hu, X. Xu, Y. Lu, C. Liu, S. Xue, L. Nie, H. Zhang, Z. Li, Y. Zhug, F. Ji, L. Hui, W. Tao, B. Wei, and H. Wang. 2015b. STK4 regulates TLR pathways and protects against chronic inflammation–related hepatocellular carcinoma. J. Clin. Invest. 125:4239–4254.

Liao, Y., G. K. Smyth, and W. Shi. 2014. featureCounts: an efficient and memory- and core-limited implementation of read mapping for RNA sequencing. Genome Biol. 15:520.

Lim, H. S., H. Namkung, and I. K. Paik. 2003. Effects of phytase supplementation on the performance, egg quality, and phosphorus excretion of laying hens fed different levels of dietary calcium and nonphytate phosphorus. Poult. Sci. 82:92–99.
Liu, Z., J. Meng, X. Li, F. Zhu, T. Liu, G. Wu, and L. Zhang. 2018. Identification of hub genes and key pathways associated with two subtypes of diffuse large B-Cell lymphoma based on gene expression profiling via integrated bioinformatics. Biomed. Res. Int. 2018:3574534.

Luan, X., D. Liu, Z. Cao, L. Luo, M. Liu, M. Gao, and X. Zhang. 2014. Transcriptome profiling identifies differentially expressed genes in young goose ovaries between the laying period and ceased period. PLoS One 9:e113211.

Lv, Z., H. Fan, B. Zhang, C. Ning, K. Xing, and Y. Guo. 2018. Dietary genistein supplementation in laying broiler breeder hens alters the development and metabolism of offspring embryos as revealed by hepatic transcriptome analysis. FASEB J. 32:4214–4228.

Ma, Y., Y. Shi, Q. Wu, and W. Ma. 2021. Dietary arsenic supplementation induces oxidative stress by suppressing nuclear factor erythroid 2-related factor 2 in the livers and kidneys of laying hens. Poult. Sci. 100:982–992.

Matsunami, T., Y. Sato, S. Ariga, T. Sato, T. Shimomura, H. Kashimura, Y. Hasegawa, and M. Yukawa. 2011. Regulation of synthesis and oxidation of fatty acids by adiponectin receptors (AdipoR1/R2) and insulin receptor substrate isoforms (IRS-1/2–) of the liver in a nonalcoholic steatohepatitis animal model. Metabolism 60:805–814.

Mishra, S. K., B. Chen, Q. Zhu, Z. Xu, C. Ning, H. Yin, Y. Wang, X. Zhao, X. Fan, M. Yang, D. Yang, Q. Ni, Y. Li, M. Zhang, and D. Li. 2020. Transcriptome analysis reveals differentially expressed genes associated with high rates of egg production in chicken hypothalamic-pituitary-ovarian axis. Sci. Rep. 10:5976.

Miyaizaki, T., A. Sanjay, L. Neff, S. Tanaka, W. C. Horne, and R. Baron. 2004. Src kinase activity is essential for osteoclast function. J. Biol. Chem. 279:17660–17666.

Napper, S., S. Dadgar, R. J. ArsenaULT, B. Trost, E. Scruton, A. Kusulik, and P. Shank. 2015. Induction of tissue- and stressor-specific kinomic responses in chickens exposed to hot and cold stressors. Poult. Sci. 94:1333–1345.

Okkenhaug, K., and B. Vanhaesebroeck. 2003. PI3K in lymphocyte development, differentiation and activation. Nat. Rev. Immunol. 3:317–330.

Panhelez, M., Y. Nys, J. Williams, J. Gautron, T. Boldicke, and M. T. Hincke. 2000. Extraction and quantification by ELISA of eggshell organic matrix proteins (ovocleidin-17, ovalbumin, ovotransferrin) in shell from young and old hens. Poult. Sci. 79:580–588.

Park, J.-A., and S. -H. Sohn. 2018. The influence of hen aging on eggshell ultrastructure and shell mineral components. Korean J. Food Sci. 38:1080–1091.

Pelica, K., E. A. Garcia, A. B. G. Faitarone, A. P. Silva, D. A. Berto, Roberts, J. R. 2004. Factors affecting egg internal quality and egg shell quality in laying hens. Poult. Sci. 41:161–177.

Robinson, M. D., D. J. McCarthy, and G. K. Smyth. 2010. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinform. 26:139–140.

Robinson, M. D., and A. Oshlack. 2010. A scaling normalization method for differential expression analysis of RNA-seq data. Genome Biol. 11:R25.

Sah, N., D. L. Kuehn, V. S. Khadka, Y. Deng, K. Peplowska, R. Jha, and B. Mishra. 2018. RNA sequencing-based analysis of the hen uterus revealed the novel genes and biological pathways involved in the eggshell biominalization. Sci. Rep. 8:16853.

Shi, Y. M., K. S. Shin, A. R. Rhee, Y. S. Chi, J. Han, and I. K. Paik. 2010. Effects of dietary Fe- and MgO on egg production and quality of eggshell in laying hens. Asian-Australas. J. Anim. Sci. 23:1043–1048.

Shan, X., Y. Miao, R. Fan, C. Song, G. Wu, Z. Wan, J. Zhu, G. Sun, W. Zha, X. Mu, G. Zhou, and Y. Chen. 2013. Suppression of Grb2 expression improved hepatic steatosis, oxidative stress, and apoptosis induced by palmitic acid in vitro partly through insulin signaling alteration. In Vitro Cell. Dev. Biol. Anim. 49:576–582.

Shen, X., H. Guo, J. Xu, and J. Wang. 2019. Inhibition of IncRNA HULC improves hepatic fibrosis and hepatocyte apoptosis by inhibiting the MAPK signaling pathway in rats with nonalcoholic fatty liver disease. J. Cell. Physiol. 234:18169–18179.

Simoes, I. C. M., A. Karkicina-Wieckowska, J. Jaukiewicz, S. Szymanska, M. Pronicki, P. Dobrzan, M. Dabrowski, A. Dobrzan, P. J. Oliveira, H. Zischka, Y. Potes, and M. R. Wieckowski. 2020. Western diet causes obesity-induced nonalcoholic fatty liver disease development by differentially compromising the autophagic response. Antioxidants 9:995.

Solomon, S. E. 2010. The eggshell: strength, structure and function. Br. Poult. Sci. 51:52–59.

Stefanello, C., T. C. Santos, A. E. Murakami, E. N. Martins, and T. C. Carneiro. 2014. Productive performance, eggshell quality, and eggshell ultrastructure of laying hens fed diets supplemented with organic trace minerals. Poult. Sci. 93:104–113.

Truong, A. D., D. Renganayaj, Y. Hong, C. T. Hoang, Y. H. Hong, and H. S. Lillehoj. 2017. Analysis of JAK-STAT signaling pathway genes and their microRNAs in the intestinal mucosa of genetically disparate chicken lines induced with necrotic enteritis. Vet. Immunol. Immunopathol. 187:1–9.

Turinsky, J., and A. Damrau-Abney. 1998. Akt1 kinase and dynamics of insulin resistance in derenervated muscles in vivo. Am. J. Physiol. Regul. Integr. Comp. Physiol. 275:R1425–R1430.

Walzem, R. L., C. Simon, T. Morishita, L. Lowenstein, and R. J. Hansen. 1993. Fatty liver hemorrhagic syndrome in hens overfed a purified diet. Selected enzyme activities and liver histology in relation to liver hemorrhage and reproductive performance. Poult. Sci. 72:1479–1486.

Wan, Y., S. Jin, C. Ma, Z. Wang, Q. Fang, and R. Jiang. 2017. RNA-Seq reveals seven promising candidate genes affecting the proportion of thick egg albumen in layer-type chickens. Sci. Rep. 7:18083.

Wang, C., S. Yang, N. Zhang, Y. Mu, H. Ren, Y. Wang, and K. Li. 2014. Long-term supranutritional supplementation with selenium decreases hyperglycemia and promotes fatty liver degeneration by inducing hyperinsulinemia in diabetic db/db Mice. PLoS One 9:e101315.

Washburn, K. W. 1982. Incidence, cause, and prevention of eggshell fracture in commercial production. Poult. Sci. 61:2005–2012.

Watt, M. J., P. M. Miotto, W. D. Nardo, and M. K. Montgomery. 2019. The liver as an endocrine organ—linking NAFLD and insulin resistance. Endocur. Rev. 40:1367–1393.

Wei, Z., P. Li, S. Huang, P. Lkhagvagarav, M. Zhu, C. Liang, and C. Jia. 2019. Identification of key genes and molecular mechanisms associated with low egg production of broiler breeder hens in ad libitum. BMC Genomics 20:408.

Welskitchen, R., and F. Tacke. 2010. Liver fibrosis: from pathogenesis to novel therapies. Drug Discov. Today 15:410–422.

Whitehead, C. C. 2004. Overview of bone biology in the egg-laying hen. Poult. Sci. 83:193–199.

Yan, C., M. Zhao, S. Li, T. Liu, C. Xu, L. Liu, T. Geng, and D. Gong. 2020. Increase of E3 ubiquitin ligase NEDD4 expression leads to degradation of its target proteins PTEN/IGF1R during the formation of goose fatty liver. J. Anim. Sci. 98:eaka270.

Yang, L., T. He, F. Xiong, X. Chen, X. Fan, S. Jin, and Z. Geng. 2020. Identification of key genes and pathways associated with feed
efficiency of native chickens based on transcriptome data via bioinformatics analysis. BMC Genomics 21:292.
Yu, Q., H. Liu, K. Yang, X. Tang, S. Chen, K. M. Ajuwon, A. Degen, and R. Fang. 2020. Effect of the level and source of supplementary dietary zinc on egg production, quality, and zinc content and on serum antioxidant parameters and zinc concentration in laying hens. Poult. Sci. 99:6233-6238.
Zaefarian, F., M. R. Abdollahi, A. Cowieson, and V. Ravindran. 2019. Avian liver: the forgotten organ. Animals 9:63.
Zhang, H.-A., X.-Y. Yang, and Y.-F. Xiao. 2016. AMPKα1 overexpression alleviates the hepatocyte model of nonalcoholic fatty liver disease via inactivating p38MAPK pathway. Biochem. Biophys. Res. Commun. 474:364–370.
Zhang, J., Y. Wang, C. Zhang, M. Xiong, S. A. Rajput, Y. Liu, and D. Qi. 2019. The differences of gonadal hormones and uterine transcriptome during shell calcification of hens laying hard or weak-shelled eggs. BMC Genomics 20:707.
Zhang, L.-C., Z.-H. Ning, G.-Y. Xu, Z.-C. Hou, and N. Yang. 2005. Heritabilities and genetic and phenotypic correlations of egg quality traits in brown-egg dwarf layers. Poult. Sci. 84:1209–1213.
Zhang, Q., F. Zhu, L. Liu, C. W. Zheng, D. H. Wang, Z. C. Hou, and Z. H. Ning. 2015. Integrating transcriptome and genome reshuffling data to identify key genes and mutations affecting chicken eggshell qualities. PLoS One 10:e0125890.

Zhuang, Y. N., H. J. Zhang, S. G. Wu, J. Wang, and G. H. Qi. 2017. Dietary manganese supplementation modulated mechanical and ultrastructural changes during eggshell formation in laying hens. Poult. Sci. 96:2699–2707.
Zhong, W., K. Qian, J. Xiong, K. Ma, A. Wang, and Y. Zou. 2016. Curcumin alleviates lipopolysaccharide-induced sepsis and liver failure by suppression of oxidative stress-related inflammation via PI3K/AKT and NF-κB related signaling. Biomed. Pharmacother. 83:302–313.
Zhou, R., D. J. De Koning, H. McCormack, P. Wilson, and I. Dunn. 2019. Short tandem repeats and methylation in the promoter region affect expression of cystathionine beta-synthase gene in the laying hen. Gene 710:367–374.
Zhu, M., S. Hao, T. Liu, L. Yang, P. Zheng, L. Zhang, and G. Ji. 2017. Lingguizhugan decoction improves non-alcoholic fatty liver disease by altering insulin resistance and lipid metabolism related genes: a whole transcriptome study by RNA-Seq. Oncotarget 8:82621–82631.
Zhu, Y., H. Mao, G. Peng, Q. Zeng, Q. Wei, J. Ruan, and J. Huang. 2021. Effect of JAK-STAT pathway in regulation of fatty liver hemorrhagic syndrome in chickens. Anim. Biosci. 34:143–153.
Zhuang, Y., C. Xing, H. Cao, C. Zhang, J. Luo, X. Guo, and G. Hu. 2019. Insulin resistance and metabonomics analysis of fatty liver haemorrhagic syndrome in laying hens induced by a high-energy low-protein diet. Sci. Rep. 9:10141.