

# Cuticle Deposition Duration In The Uterus Is One Of The Major Factors Affecting Eggshell Cuticle Quality In White Leghorn Laying Hens

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## Research

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# Abstract

**Background:** The cuticle formed in the uterus is the outermost layer as the first defense line of eggshell against microbial invasions in most avian species, and analyzing its genetic regulation and influencing factors are of great importance to egg biosecurity in poultry production worldwide. The current study compared the uterine transcriptome and proteome of laying hens producing eggs with good and poor cuticle quality (GC and PC, the top and tail of the cuticle quality distribution), and identified several genes involved with eggshell cuticle quality (ESCQ).

**Results:** Overall, transcriptomic analysis identified 53 differentially expressed genes (DEGs) between PC vs GC group hens, among which 25 were up-regulated and 28 were down-regulated. No differences were found in the uterine proteome. Several DEGs, including *PTGDS*, *PLCG2*, *ADM* and *PRLR* related to uterine functions and reproductive hormones, were validated by qPCR analysis. Egg quality measurements between GC and PC hens showed GC hens had longer laying interval between two consecutive ovipositions ( $25.64 \pm 1.23$  vs  $24.94 \pm 1.12$  h) and thicker eggshell thickness ( $352.01 \pm 23.04$  vs  $316.20 \pm 30.58$   $\mu\text{m}$ ) ( $P < 0.05$ ). Apart from eggshell traits, other egg quality traits didn't differ. The results above suggested the expression patterns of *PTGDS*, *PLCG2*, *ADM* and *PRLR* genes in the uterus regulated the muscular activity rhythm and secretion function, which ensured the eggshell and cuticle deposition duration, and lead to the good ESCQ of the GC hens.

**Conclusions:** The present study demonstrated eggshell and cuticle deposition duration in the uterus is one of the major factors affecting ESCQ in laying hens. *PTGDS*, *PLCG2*, *ADM* and *PRLR* genes were discovered and might play crucial roles in cuticle deposition by regulating the uterine muscular activities and secretion function. The findings in the present study provide new insights into the genetic regulation of cuticle deposition in laying hens and establish a foundation for further investigations.

## Background

The annual *Global Report on Food Crises 2020* by the Food Security Information Network (FSIN) indicates that the world is facing an unprecedented food crisis [1]. As a source of low-cost and high-quality animal protein, poultry production not only meets the basic nutritional needs of humans, but also is an important contributor to the economies and cultures, and thus the demand for poultry egg and meat is increasing [2]. Egg safety is absolutely pivotal to the success of today's poultry industry, however, egg contamination with pathogenic bacteria is considered one of the leading causes of economic loss in the poultry industry worldwide and represents a threat to public health [3, 4]. Furthermore, faced with current environmental pressures and animal welfare requirements, the modern poultry industry, which relies on the extensive use of artificial incubation, is more dependent on strict biosecurity of the egg [5].

Avian eggshells have evolved multiple physical and chemical barriers in response to microbial challenges, and these barriers are essential for the successful reproduction of avian species as well as to maintain safe and nutritious table eggs for human consumption [6, 7]. The eggshell is a complex with

several highly ordered and distinct layers (i.e., mammillae, palisades, vertical crystal layer, and cuticle) and the cuticle is the outermost layer as the first defense line of the eggshell against microbial invasions in most avian species [8–10]. The cuticle has been a relatively neglected structure while recent researches on the properties, composition, functions, physiology and genetics of the cuticle have highlighted its important role in ensuring egg biosecurity and quality in poultry production. The cuticle is deposited during the final hr of egg formation in the uterus (shell gland pouch), covering the shell surface and filling the external openings of the gas pores so that effectively exerting waterproof and antibacterial effects [11, 12]. The cuticle mainly consists of hydroxyapatite crystals, glycoprotein, polysaccharides, lipids, and pigment [13, 14], and abundant antibacterial proteins (e.g., lysozyme C, ovotransferrin, ovocalyxin-32, ovocleidin-17) constitute the molecular basis for the antimicrobial function of the cuticle [15, 16]. It has been demonstrated that good eggshell cuticle quality (ESCQ) can significantly reduce the opportunity of pathogen invasion [5, 17, 18], and the quantity of cuticle is a heritable trait that genetic selection to this trait can be an effective strategy to reduce transmission of microorganisms in poultry production [8, 19]. Therefore, how to improve ESCQ to ensure egg biosecurity and quality and further promote poultry production has drawn a worldwide interest.

Avian birds are oviparous and produce an egg at intervals precisely controlled by the hormonal secretion and gene expression of the hypothalamo-pituitary-gonadal-oviduct axis [10, 12, 20–22]. Previous transcriptomic and proteomic studies of hen (*Gallus gallus*) reproductive tract have well profiled the temporal and spatial transcriptome landscapes and important genes that regulate egg formation [22–28]. Cuticle deposition is also a well-defined and specific process that occurs in the uterus rather than the extension of mineralized eggshell [12]. It's reported that there is a significant association between *ovocleidin-116*, *ovocalyxin-32*, *ovalbumin*, and estrogen receptor (*ESR1*) genes and ESCQ [8, 29]. Transcriptome analysis of the uterus of hens laying eggs with good or poor cuticle quality suggested that clock genes and immediate early genes are prime candidates for the control of cuticle deposition and the uterus transcriptome did not differ that the genetic effects of cuticle deposition might not be expressed in the uterus [30]. However, there is few duration for the deposition of the cuticle compared with other components of the egg, making it difficult to exactly obtain the uterine tissue and the transcriptomic and proteomic data during cuticle deposition. Thus, specific pathways and genes that regulate cuticle deposition still remain unclear.

In the present study, transcriptomic and proteomic status of uterus from White Leghorn laying hens that produced eggs with good and poor cuticle quality (GC and PC, the top and tail of the cuticle quality distribution) were analyzed to identify unknown candidate regulatory genes involved in eggshell cuticle deposition and provide deeper insights into the biological basis of cuticle deposition. This is also an accurate study to describe the uterus transcriptome and proteome during the process of chicken eggshell cuticle deposition, providing a reference for further improving the ESCQ in laying hens.

## Methods

### Ethical statement

All the experimental procedures were conducted in accordance with the Guidelines for Experimental Animals established by the Animal Care and Use Committee of China Agricultural University (permit number: AW08059102-1).

## **Experimental design**

Two 28-week-old White Leghorn laying hen flocks were used in the present study for omics analysis and phenotypic measurements, respectively. There was no genetic selection for the cuticle trait of the two flocks. Hens that stably produced eggs with good and poor cuticle (GC and PC, the top and tail of the cuticle quality distribution) were obtained by evaluating the ESCQ. Transcriptomic, proteomic and qPCR analysis of GC and PC hen uterus tissues during cuticle deposition were designed for identifying genes involved with eggshell cuticle deposition. Subsequently, phenotypic measurements (ESCQ, laying interval, laying performance, serum hormone level, egg quality) of GC and PC group hens were carried out to further verify the results above and explore factors that affect ESCQ.

## **Animals and uterine tissue collection**

Two hundred and eight White Leghorn hens at 28-week-old were selected from the National poultry Testing Center, China Agricultural University. All hens were caged individually under standard conditions with a photoperiod of 16L:8D, and the diet and water were provided ad libitum.

To obtain individuals that stably produced eggs with good or poor cuticle (GC or PC) for subsequent sampling, the eggs were collected for 14 consecutive days to measure the ESCQ within 24 h after oviposition. The ESCQ evaluation was conducted according to the staining method proposed by Chen et al. previously [17]. Briefly, the ESCQ ( $\alpha$  value) was measured based on differences in cuticle staining before and after staining with a dye solution of MST cuticle blue (MS Technologies Ltd, UK) using a spectrophotometer (CM-2600d; Konica Minolta, Japan) with the XYZ color space system. A higher  $\alpha$  value represents to more cuticle deposition, that is better ESCQ. Each egg was measured at 3 points: the large end, equator, and small end. ESCQ per egg was determined from the mean value of these points and at least 8 eggs were collected for ESCQ measurement per hen.

Oviposition time of each hen was accurately recorded from the eighth day of egg collection. Then the oviposition time of the GC and PC group hens were estimated and the hens were euthanized 1 h before oviposition (the period for cuticle deposition) on the fifteenth day according to the Animal Care and Use Committee of China Agricultural University protocol. The uterus was aseptically retracted through an abdominal incision and a small incision was then made in the centre of the uterus, and the egg in the uterus was removed for staining by MST cuticle blue within 30 min to determine whether the cuticle is deposited in the uterus during sampling. Two approximately 500 mg sample tissues was collected from the centre of the uterus and transferred directly to RNeasy (Sigma-Aldrich, Shanghai, China), quickly frozen in liquid nitrogen and stored at -85 °C prior to total RNA extraction.

## **RNA isolation, library construction, and sequencing**

Total RNA was extracted from frozen uterus tissue sample using TRIzol reagent (TransGen Biotech, Beijing, China). The quality and quantity of RNA were evaluated by 1% agarose gels, NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA), respectively. The RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA). 3 µg RNA per sample was prepared to generate individual bar-coded sequencing libraries using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) following manufacturer's recommendations. Sequencing of these libraries was performed on an Illumina HiSeq 4000 sequencing system (Illumina, San Diego, CA, USA) using the 150-bp pair-end sequencing strategy, following the manufacturer's instructions.

### **Transcriptomic data analysis**

Raw data was quality controlled using the FastQC package (Babraham bioinformatics, Cambridgeshire, England). Clean reads were obtained by removing reads containing adapter, reads containing ploy-N, empty reads and low quality reads from raw data. Trimmed reads were mapped on the reference chicken genome *Gallus\_gallus\_ncbi\_GCF\_000002315.6\_GRCg6a* ([https://www.ncbi.nlm.nih.gov/assembly/GCF\\_000002315.6](https://www.ncbi.nlm.nih.gov/assembly/GCF_000002315.6)) using HiSAT2.0 [31, 32]. The clean reads of each sample were assembled and finally merged to a transcriptome using Stringtie (<http://ccb.jhu.edu/software/stringtie/>) [31] with Ensembl *Gallus gallus.v92* as the reference. Expression levels of the transcripts were quantified as RPKM (reads per kilobase per million) for gene expression analysis.

DEGs were identified using DESeq2 [33] according to the criteria of adjusted *P*-value < 0.05. Ensembl gene IDs from each group were uploaded to the DAVID Functional Annotation Tool and analysed for gene ontology (GO) and Kyoto Encyclopaedia of Genes and Genomes (KEGG) enrichment [34, 35]. The transcriptome data (raw mRNA-seq reads) have been deposited with the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database under accession number PRJNA664894 (<https://www.ncbi.nlm.nih.gov/sra/PRJNA664894>).

### **Sample processing and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS)**

Each frozen sample (50 mg) from GC and PC group hens (n=4 and 3, respectively) was ground in liquid nitrogen and suspended in lysis buffer consisting of 8 M urea (Sigma Aldrich, MO, USA), 10 mM dithiothreitol (DTT), and proteinase inhibitors (Merck Millipore, MA, USA). The digest was centrifuged at 12,000 × g for 20 min at 4 °C, and the supernatant was collected. The concentration was determined with a BCA kit (Thermo Fisher Scientific, Wilmington, DE, USA) according to the manufacturer's protocols. After trypsin digestion, the peptides were desalted with buffer A [10 mM KH<sub>2</sub>PO<sub>4</sub> in 25% acetonitrile (ACN), pH 3.0] and buffer B (10 mM KH<sub>2</sub>PO<sub>4</sub>, 500 mM KCl, in 25% ACN, pH 3.0) at a low rate of 1,000 µL/min, lyophilised in a centrifugal speed vacuum concentrator, reconstituted in 0.5 M TEAB and processed according to the manufacturer's instructions for the TMT kit (Thermo Fischer Scientific, MA, USA). The tryptic peptides were separated by high-pH reverse-phase HPLC using an Agilent 300 Extend C 18 column

(5 µm particle size, 4.6 mm inner diameter, 250 mm length), dissolved in 0.1% formic acid (Sigma Aldrich, MO, USA) and fractionated using EASY-Nano-LC 1000 ultra-high performance liquid phase system. Finally, fractionated peptides were exposed to an NSI source followed by tandem mass spectrometry (MS/MS) with a Q Exactive HF-X spectrometer (Thermo Fischer Scientific, MA, USA) coupled online to the UPLC system.

The MS/MS data were searched against the *Gallus gallus* database (<http://www.uniprot.org/proteomes/UP000000539>, chicken proteome ID: UP000000539) for peptide identification and quantification using the Maxquant search engine (v.1.6.6.0). All proteins with at least one unique peptide and false discovery rate (FDR) < 0.01 were qualified for further quantification data analysis. Protein abundance was quantified by hi-flyer through loading (SL), internal reference scaling (IRS) and trimmed mean of M values (TMM) corrections in the Maxquant search engine system. DEPs between GC and PC group were identified using limma [36] and  $P < 0.05$  was used as the cut-off for significance by FDR correction.

### **Quantitative real-time PCR (qPCR) analysis**

Expression of mRNA was verified by qPCR with cDNA from 11 uterine tissue samples. *GAPDH* gene served as a housekeeping gene. Primer Primer (v. 5.0) was used with default parameters to design primers on exon-exon spans for selected genes (Additional file 4: Table S3). Total RNA was extracted from uterine tissue using TRIzol reagent as described previously. Synthesis of the cDNA was performed using a PrimeScript RT reagent kit with 1 µg of the RNA pretreated with gDNA Eraser (TaKaRa, Dalian, China) following the manufacturer's instructions. The qPCR of the mRNA expression level of *ADM*, *PLCG2*, *PRLR* and *PTGDS* was performed using the Agilent Brilliant III Ultra-Fast SYBR Green qPCR Mix and MX3005P real time system (Agilent; Santa Clara, CA USA). The experiments were carried out in triplicate. The cycling conditions were 50°C for 2 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min by 42 cycles, and annealing and extension at 60 °C for 15 s. The melting curves were obtained for each sample amplified. The  $2^{-\Delta\Delta CT}$  method was used [37] to quantify the relative changes in gene expression versus those of *GAPDH* from the qPCR experiments and *t*-test was used for the statistical analysis by R Language (v. 3.4.0).

### **Phenotypic measurements and experimental details**

Five hundred and seventy four White Leghorn hens at 28-week-old were selected from the Poultry Genetic Resources and Breeding Experimental Base, College of Animal Science and Technology, China Agricultural University for subsequent verification analysis. The animal handling and housing conditions were the same as described above.

All eggs produced by this flock were collected, and ESCQ measurement and oviposition time recording were the same as described above for seven successive laying days. On the sixth day, hens that stably produced eggs with good and poor cuticle quality (GC and PC) were selected and their oviposition time on the seventh day were estimated. On the seventh day, whole blood samples were collected from the

brachial veins of the GC and PC group hens at 1 h before oviposition, and the blood samples were also collected from the GC hens at 0.5 h after oviposition (laid) (n=7). All the eggs were also collected after oviposition for validation by ESCQ measurement. The blood sample was collected only once per chicken and the ESCQ and oviposition time were in line with expectations.

Laying interval between two consecutive ovipositions was calculated (at least 4 ovipositions per hen). Laying recording sheet during the 27 consecutive laying days from 28-week-old age was obtained to evaluate laying performance (i.e., total laying days, total sequences, mean sequence length and intersequence pause length). Concentrations of prostaglandin D2 and oxytocin of the blood serum were subsequently determined by chicken prostaglandin D2 (PGD2) and oxytocin (OT) ELISA kits (mlbio, Shanghai, China) according to the manufacturer's protocols.

After ESCQ measurement, other egg quality traits of GC and PC group hens (n=30) including egg weight, eggshell thickness (EST, mean of the large end, equator, and small end without eggshell membranes), eggshell strength (ESS), eggshell weight (ESW), yolk weight, albumen height, yolk colour, Haugh unit and egg shape index were determined using an egg multimeter (EMT-5200, robotmation, Japan), an Eggshell Strength Tester (EFG-0503, robotmation, Japan) and a digital display micrometer gauge (Mitutoyo, Kawasaki, Japan) following the manufacturer's instructions. Eggshell pieces (about 1 cm<sup>2</sup>) of the GC and PC group hens (n=30) were taken around the equator of the egg and the pieces were mounted on an aluminum stub and gold sputter-coated using an EIKO IB-3 (EIKO Engineering CO., Ltd, Japan) for about 15 min. Thereafter, they were subjected to the SEM (JSM-7401, JEOL Ltd., Japan) for observations, photographing, and measurements of the eggshell cross-section ultrastructure. The eggshell cross-section ultrastructure in the present study refers to EST, effective layer thickness (combined thickness of the palisade, vertical crystal and cuticle layer) and mammillary layer thickness, and the determination were consistent with previous studies [29, 38].

## Statistical analysis

Statistically significant differences among descriptive statistics were determined by the Student's *t* test. All statistical analysis and figure plotting were performed with the statistical software RStudio (v. 3.4.0). The results of basic descriptive statistics are shown in paragraphs and tables using the mean and standard deviation (mean ± SD).

## Results

### ESCQ of eggs from GC and PC hens for transcriptomic and proteomic analysis

Earlier observations of the present study showed that the ESCQ ( $\alpha$  value) may have great variability in the same individual. Therefore, ESCQ of eggs produced by the experimental flock was monitored for the 14 consecutive laying days to obtain individuals that stably produced eggs with good or poor cuticle quality (GC or PC) for subsequent sampling and analysis. The ESCQ was extremely better in the GC group versus the PC group as indicated by the significant difference in  $\alpha$  value (GC  $30.18 \pm 7.62$  vs PC  $10.34 \pm 3.67$   $\alpha$

value;  $n = 7$  and  $4$ , respectively;  $F_{1,9} < 0.001$ ). In other words, the GC group hens had a strong ability for cuticle deposition whilst the PC groups hens had almost no deposition of cuticle. MST cuticle blue dye results of eggs taken from uterus of GC and PC hens during sampling demonstrated that the uterus of GC group had secreted a remarkable thicker cuticle layer than PC group (Additional file 1: Figure S1). This significant difference of the ESCQ phenotype was in line with expectations that the tissue samples obtained in the present study could be used for subsequent analysis.

### **Analysis of uterus Transcriptomic data of GC and PC group**

Seven and four biological replicate samples of uterus from GC and PC group hens during cuticle formation were analysed. A total of 590,111,516 clean reads were generated from the eleven libraries of GC and PC group (Additional file 2: Table S1). The reads feature summary indicated that the percentage of reads mapped to *Gallus gallus* genome was  $\geq 85\%$  (Additional file 3: Table S2). Differential gene expression analysis showed 53 differentially expressed genes (DEGs) (with 26 genes  $|\log_2$  fold change  $> 1$ ) between the GC and PC group during cuticle formation. Among the 53 DEGs, there were 25 up-regulated and 28 down-regulated genes at the PC group relative to the GC group. A full list of the 53 DEGs was shown in Table 1.

The heatmap of the 53 DEGs between GC and PC group was plotted and the pattern of expression for the 53 DEGs was visualised in Fig. 1, showing there were significant differences in the expression patterns of the 53 DEGs between GC and PC group.

**Table 1** DEGs of PC vs GC hen uterus during cuticle deposition from the transcriptomic analysis<sup>1</sup>

Gene ID	Gene name	padj	log <sub>2</sub> FoldChange
MSTRG.9297	LOC112532778	0.000107	-3.38665
MSTRG.4468	LOC112532092	0.012042	-2.55302
MSTRG.19172	novel.522	0.006783	-2.46577
MSTRG.14500	XLOC_018691	0.031623	-2.25265
MSTRG.374243	WNT2B	0.006028	-1.51444
MSTRG.15111	LOC107054937	0.031252	-1.34327
MSTRG.6687	XLOC_008333	0.041516	-1.28973
MSTRG.1320	LOC112530330	0.047795	-1.26421
MSTRG.7221	LOC112532581	0.031623	-1.2446
MSTRG.10347	LOC107054090	0.005377	-1.1694
MSTRG.11839	LOC112533379	0.007998	-1.07028
MSTRG.492	LOC112533329	0.047795	-0.97969
MSTRG.2992	ABCB5	0.013734	-0.9499
MSTRG.12238	XLOC_015835	0.00254	-0.83726
MSTRG.15139	C1orf167	0.019222	-0.83628
MSTRG.2723	RNF32	0.031623	-0.59485
MSTRG.12443	LOC107054608	0.030626	-0.55132
MSTRG.2160	RCBTB1	0.031623	-0.52116
MSTRG.8764	LOC112532722	0.007866	-2.24E-05
MSTRG.8709	LOC112532731	0.041516	-1.71E-05
MSTRG.6076	SLC25A43	0.027852	-1.66E-05
MSTRG.2635	OBSCN	0.041516	-1.41E-05
MSTRG.4968	LOC112532084	0.005377	-1.26E-05
MSTRG.5952	LOC112532329	0.031623	-1.50E-06
MSTRG.12943	RAD9B	0.037819	-1.31E-06
MSTRG.1636	LOC112532721	0.01454	-1.24E-06
MSTRG.8539	XLOC_011557	6.01E-09	-2.64E-07
MSTRG.7568	LOC112532547	0.043812	7.44E-06

MSTRG.18849	LOC101747670	0.021437	0.394261
MSTRG.11340	PLCG2	0.009689	0.477929
MSTRG.7243	ADM	0.021437	0.660846
MSTRG.2552	TAF10	0.041516	0.667103
MSTRG.13459	PTGDS	0.006028	0.728479
MSTRG.4035	BAALC	0.031252	0.76145
MSTRG.10742	BCL2L10	0.02381	0.805203
MSTRG.430	LOC101752108	0.002939	0.866194
MSTRG.17498	PRLR	0.000107	0.90763
MSTRG.14751	ID1	0.006641	0.939962
MSTRG.16737	TNS4	0.049612	1.06834
MSTRG.16730	LOC112530344	0.00254	1.133301
MSTRG.8092	XLOC_010789	0.009494	1.161744
MSTRG.5903	FGF13	0.041516	1.167194
MSTRG.4978	LRP11	0.016463	1.19355
MSTRG.12672	LOC101749583	0.000618	1.21108
MSTRG.8621	INA	0.016688	1.484377
MSTRG.4238	LOC107050437	0.043077	1.577259
MSTRG.11542	TCTA	0.000501	1.614429
MSTRG.2443	THRSPB	0.036456	1.677864
MSTRG.2098	novel.32	0.004284	1.780021
MSTRG.11804	novel.277	0.007203	1.802508
MSTRG.4206	ARC	0.036456	1.943186
MSTRG.10115	NEU4	0.007866	2.080434
MSTRG.5983	LOC100858332	0.000273	2.450145

<sup>1</sup>GC, good cuticle; PC, poor cuticle.

### GO functional annotation and pathway enrichment analysis of the DEGs between GC and PC group

Functional enrichment of Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of the 53 DEGs showed that there was no significant enriched category of GO

functional annotation or KEGG pathway when subject to analysis by the DAVID Functional Annotation Tool. This may be due to the small amount of DEGs as only functions of 32 genes from the DEGs are known in the DAVID database.

### **Verification of gene expression differences by quantitative real-time PCR (qPCR) analysis**

To validate the transcriptomics results, the relative expression of four genes [*ADM* (Adrenomedullin), *PLCG2* (Phospholipase C gamma 2), *PRLR* (Prolactin receptor) and *PTGDS* (Prostaglandin D2 synthase)] to *GAPDH* were determined in GC and PC group by qPCR on the basis of their known or potential influence on the hen uterus functions during cuticle deposition. The four genes were all up-regulated in PC group relative to GC group (Table 1). The qPCR results showed a highly similar expression pattern compared with the RNA-Seq analysis for the genes being validated though there was slight difference in the magnitude of changes in gene expression analyzed by RNA-Seq and qPCR (Table 1; Fig. 2). The qPCR results suggested that the RNA-Seq data obtained in the present study provided a good reference for the study of gene expression differences in the uterus of GC and PC group during cuticle deposition.

### **Proteomic analysis of uterus of GC and PC group**

TMT labeling based proteomic analysis of uterus samples of GC and PC group hens (n=4 and 3, respectively) during cuticle formation was further performed to identify differentially expressed proteins (DEPs). The peptide detection results of the TMT sequencing showed the length of the peptides is in a partial normal distribution with a mean value of 11, and the main length is between 8-13, which is reasonable for subsequent proteomic analysis. The proteomic analysis showed no DEPs were detected between the uterus samples from the GC and PC group hens.

### **Laying interval, laying performance and serum hormone levels of GC and PC group hens**

In order to further verify the results above and explore factors that affect ESCQ, laying interval between two consecutive ovipositions, laying performance during the twenty-seven days and concentrations of two serum hormones related to the RNA-Seq and qPCR results were determined in another larger White Leghorn laying hen flock. Significant differences were found in the laying interval and laying performance between GC and PC group that the GC group hens had a longer laying interval and better laying performance compared to those of PC group ( $P < 0.05$ ) (Fig.3, Table 2). The concentrations of prostaglandin D2 and oxytocin of PC group hen serum at 1 h before oviposition were higher than those of GC group, which were consistent with the RNA-Seq and qPCR results, and then the concentrations of the two hormones decreased after oviposition, but the statistical differences were not significant ( $P > 0.05$ ) (Fig. 4).

**Table 2** The laying performance during the 27-days of GC and PC group hens<sup>1</sup>

Traits	GC	PC
n	60	60
$\alpha^2$	38.14 ± 4.93 <sup>a</sup>	4.28 ± 1.68 <sup>b</sup>
Total laying days	20.67 ± 2.58 <sup>a</sup>	18.48 ± 2.46 <sup>b</sup>
Total sequences	6.02 ± 1.31	6.48 ± 1.45
Mean sequence length (days)	3.49 ± 1.13 <sup>a</sup>	3.06 ± 1.05 <sup>b</sup>
Intersequence pause length (days)	1.09 ± 0.26 <sup>b</sup>	1.54 ± 0.58 <sup>a</sup>

<sup>a,b</sup> Means within a row that do not share a common superscript differ significantly ( $P < 0.05$ ).

<sup>1</sup>GC, good cuticle; PC, poor cuticle.

<sup>2</sup> $\alpha$ , eggshell cuticle quality (%), measured in the seven-laying-days of the 28-week-old hen flock.

### Egg quality between GC and PC group hens

Egg quality between GC and PC group hens were further measured to investigate possible reasons for the differences in laying interval and laying performance between the two groups. The results of egg quality indicated that there were significant differences in EST, ESS and ESW between GC and PC group hens ( $P < 0.05$ ), and other egg quality traits were not significantly different, suggesting the differences between GC and PC eggs were mainly reflected in the eggshell quality (Table 3). Observations and measurements of the eggshell ultrastructure by scanning electron microscope (SEM) also visually showed the huge difference in the cuticle phenotype of GC and PC group hens (Fig. 5). Further, the thicker EST of GC group was largely derived from the increase in thickness of the effective layer (including the cuticle layer) ( $P < 0.05$ ) (Fig. 5; Table 4).

### Table 3 Egg quality between GC and PC eggs<sup>1</sup>

Traits	GC	PC
n <sup>2</sup>	30	30
α <sup>3</sup>	40.26 ± 5.24 <sup>a</sup>	13.0 ± 4.03 <sup>b</sup>
Eggshell thickness (μm) <sup>4</sup>	368.68 ± 23.95 <sup>a</sup>	342.95 ± 41.28 <sup>b</sup>
Eggshell weight (g)	5.88 ± 0.46 <sup>a</sup>	5.53 ± 0.75 <sup>b</sup>
Eggshell strength (kg/cm <sup>2</sup> )	3.65 ± 0.78 <sup>a</sup>	3.02 ± 0.74 <sup>b</sup>
Egg weight (g)	54.60 ± 3.62	55.01 ± 3.10
Egg yolk weight (g)	16.07 ± 3.30	16.60 ± 1.34
Egg albumen height (mm)	6.43 ± 1.16	6.33 ± 1.77
Egg yolk color	9.32 ± 0.59	9.31 ± 0.78
Haugh unit	81.15 ± 7.75	79.22 ± 12.30
Egg shape index	1.35 ± 0.08	1.35 ± 0.06

<sup>a,b</sup> Means within a row that do not share a common superscript differ significantly ( $P < 0.05$ ).

<sup>1</sup>GC, good cuticle; PC, poor cuticle.

<sup>2</sup>n, 30 eggs produced by 30 different individuals in each group.

<sup>3</sup>α, eggshell cuticle quality (%).

<sup>4</sup>Eggshell thickness without eggshell membranes.

**Table 4** Eggshell ultrastructure thickness of GC and PC eggs<sup>1</sup>

Traits	GC	PC
N <sup>2</sup>	30	30
α <sup>3</sup>	50.55 ± 9.53 <sup>a</sup>	0.29 ± 2.15 <sup>b</sup>
Cuticle thickness (μm)	8.18 ± 0.83 <sup>a</sup>	1.52 ± 0.21 <sup>b</sup>
Effective layer thickness (μm) <sup>4</sup>	281.09 ± 24.57 <sup>a</sup>	254.48 ± 32.18 <sup>b</sup>
Mammillary layer thickness (μm)	66.04 ± 14.97	60.70 ± 14.11
Eggshell thickness (μm)	352.01 ± 23.04 <sup>a</sup>	316.20 ± 30.58 <sup>b</sup>

<sup>a,b</sup> Means within a row that do not share a common superscript differ significantly ( $P < 0.05$ ).

<sup>1</sup>GC, good cuticle; PC, poor cuticle.

<sup>2</sup>n, 30 eggs produced by 30 different individuals in each group.

<sup>3</sup> $\alpha$ , eggshell cuticle quality (%).

<sup>4</sup>Effective layer thickness is the combined thickness of the palisade, vertical crystal and cuticle layer.

## Discussion

Egg formation in avian reproductive tract is strictly regulated by the hormones and gene expression [20, 22], and the cuticle layer is the last process of egg formation in most avian species [12], however, the genetic regulation of cuticle deposition is still poorly understood. The present study were conducted by analyzing the DEGs and DEPs of the uterus between the GC and PC group hens to elucidate potential genes and networks that regulate cuticle deposition. Besides, results of qPCR and a series of phenotypic measurements validated the RNA-Seq data. The current work not only picturised the differential expression profile of the uterus of GC vs PC group hens during eggshell cuticle deposition, but also revealed important genes affecting ESCQ.

Based on the results of DEGs, multiple genes (such as *PTGDS*, *PLCG2*, *ADM* and *PRLR*) related to uterine functions and reproductive hormones were found. The qPCR results further validated the DEGs that the gene expression levels of *PTGDS*, *PLCG2*, *ADM* and *PRLR* in the uterus of GC group hens were all lower than those of PC group, suggesting their vital roles during cuticle formation.

*PTGDS* is one of the prostaglandin synthases [39], and it's also an important catalytic enzyme for the synthesis of prostaglandins [40, 41]. *PLCG2*, which is a type of the phospholipase C (PLC), is an important mediator of oxytocin to regulate uterine contractions [42]. Oxytocin increases the synthesis of prostaglandin by stimulating the activity of endometrial prostaglandin synthetase, and then the synergetic effect of prostaglandin and oxytocin enhances the activity of myometrium, thereby inducing labor or oviposition [43-46]. Moreover, *ADM* could cooperate with oxytocin and prostaglandin to participate in the rhythmical contraction and relaxation of the myometrium [44, 47, 48]. The contractile responses of the uterus to oxytocin and prostaglandins play an important role in inducing oviposition [49]. Oxytocin and prostaglandin is thought to mediate the brain to ovary signalling of oviposition timing being involved in cuticle deposition, and the premature oviposition induced by oxytocin and prostaglandin significantly reduce the EST and ESCQ [12]. Previous study suggested clock gene expression in the uterus during shell formation may be responsible for controlling the cuticle deposition, and clock genes (*PER2*, *CRY2*, *CRY1*, *CLOCK* and *BMAL1*) were differentially expressed when cuticle deposition was prevented by oxytocin [30]. Therefore, genes related to uterine timing mechanism and muscular events constitute the components of cuticle deposition regulation, but the regulatory networks need further research.

Prolactin (PRL), secreted from the anterior pituitary, plays a series of roles in osmoregulation, corpus luteum formation and maintenance of broody behaviour in laying hens, and its receptor, PRLR, plays an important role in the PRL signal transduction cascade and cell growth and differentiation [50]. The *PLR* and *PRLR* genes are expressed in many tissues including the hypothalamus, ovary and oviduct [51, 52], and mediate the formation of egg quality [53]. It has been well established that the elevated plasma PRL inhibits gonadotropin release, ovum development and ovulation, resulting poor laying performance and even complete cessation of egg production in laying hens [54-56]. Previous study has also shown the inhibitory effects of excessive PRL on eggshell formation [57]. Besides, it's clear that the elevation of PRL can significantly inhibit the cuticle deposition [12, 50, 58]. Therefore, the significant difference in eggshell quality (ESCQ, EST, ESS, and ESW) and egg production performance between GC and PC group hens may be related to the different expression patterns of *PRLR* gene in the uterus. However, the effects of PRL and PRLR on hen uterine function are still unclear.

Summarizing the functions of *PLCG2*, *PTGDS*, *ADM* and *PRLR* expressed in the uterus, their express patterns in the uterus regulate the muscular activities and secretion function, which may lead to the difference in egg-laying rhythm between GC and PC group hens. The relatively high expressions of *PLCG2*, *PTGDS* and *ADM* genes could increase the frequency and intensity of contraction and relaxation activities of the myometrium that might negatively affect uterine functions and eventually facilitate oviposition accompanied by a reduced duration for the egg staying in the uterus. What's more, in the process of estimating the oviposition time of the hens, it was found the laying interval between two consecutive ovipositions of the GC group was longer. The difference in laying interval between GC and PC group hens was further confirmed by the larger experimental flock, namely the laying interval between two consecutive ovipositions of GC group hens was significantly longer than that of PC group by 0.7 h (Fig. 3). At the same time, the concentrations of prostaglandin D2 and oxytocin of PC hen serums at 1 h before oviposition were higher than those of GC group hens though the statistical differences were not significant ( $P > 0.05$ ). There was also a tendency that the concentrations of prostaglandin D2 and oxytocin of hen serum after oviposition were decreased, implying their important role in regulating uterine functions. The statistically insignificant differences may be due to the large difference in physiological status between individuals and the short biological half-life of oxytocin and prostaglandin [59, 60], however, the serum hormone levels was consistent with the RNA-Seq and qPCR results. The results above indicated that during the cuticle deposition period in the uterus, the frequency and intensity of myometrium contraction and relaxation in GC group hens might be lower than that of PC group hens, which created a more stable internal environment for the uterus and extend the duration of the cuticle deposition, and then lead to the good ESCQ.

There were no DEPs between the uterus samples of GC vs PC group hens analyzed by the proteomic analysis, implying there was no significant difference in the protein composition and content to a large extent. Alternative hypothesis is that undetected DEPs may be due to the extensive posttranslational modification regulation in the biological process [61, 62]. On the other hand, the extremely short half-life of the hormones (e.g. oxytocin) may be the reason why the DEPs were not found [59, 60, 63]. The expression characteristics of the transcriptome and proteome between GC and PC group are highly

similar (53 DEGs and no DEPs), suggesting that the biological processes of cuticle deposition of the two groups may be the same, but mainly the difference in the duration of cuticle deposition.

Therefore, the duration of cuticle deposition in the uterus may be one of the major factors affecting ESCQ. The longer laying interval in GC group hens might be partly derived from the extended cuticle deposition time. Further, the analysis of the egg quality of the GC and PC group hens confirmed this conjecture. From ovulation to oviposition, it takes about 24 hours for the formation of a complete egg in chicken [10, 20]. A complete egg includes egg yolk, egg white, eggshell membranes, calcified eggshell and cuticle formation. The forming egg stays in different segments of the oviduct (i.e., infundibulum, magnum, isthmus and uterus) for different duration, and the egg remains in the uterus for the longest period during shell and cuticle formation, for a duration over 18 h in laying hens [10, 20, 64]. The egg quality measurement results showed there were no significant differences in the egg weight, yolk weight, yolk color, albumen height, Haugh unit and egg shape index between GC and PC group. Surprisingly, the eggshell quality (ESCQ, EST, ESS and ESW) of GC group were significantly increased compared with PC group, suggesting that the longer laying interval in GC group was mainly due to the extended formation duration of the eggshell in the uterus. Eggshell ultrastructure of GC and PC group eggs by SEM shows the significantly increased EST of GC group was largely due to the increase in the thickness of the effective layer (palisade, vertical crystal layer and cuticle), demonstrating the extended laying interval of GC group hens both positively affects the calcified shell and cuticle deposition. Moreover, the laying interval between ovipositions were positively correlated with the duration of eggshell formation, eggshell deposition rate and eggshell quality, and in detail, a difference of 0.7 hours between egg laying intervals could significantly affect ESW by about 30% [64]. Consequently, in the present study, the prolonged laying interval by 0.7 h of GC group hens could explain the significant increase in the effective layer and cuticle layer thickness.

The cuticle as part of the eggshell structure, the qualities of eggshell and cuticle are both affected by the physiological state of the uterus. Previous studies have shown the ESCQ is positive correlated with the EST/ESS in several commercial laying hen flocks [17, 65]. The results of the present study directly prove that eggs with good or poor cuticle generally embody thicker or thinner EST. Eggshell calcification is precisely regulated by the interaction processes between mineral and organic precursors under gene expressions, including transcellular and paracellular transport of minerals and the secretion of different matrix proteins, and the processes establish the eggshell texture and ultrastructure, and then influence eggshell quality traits [66-68]. Eggshell matrix proteins (e.g., ovocleidin-17, ovocleidin-116 and ovalbumin) present in both the calcified eggshell and the cuticle, and eggshell matrix proteins constitutes the main components of the cuticle [68-70]. Previous transcriptomic study in the uterus of laying hens that produce eggs with good or poor eggshell quality (EST/ESS) demonstrated the most of identified DEGs were involved in eggshell calcification (ion transport) and cuticularization (matrix proteins) pathways [71]. It was also found that the expression of *FGF13* (fibroblast growth factor 13) is up-regulated in the uterus of PC group as compared to GC group (Table 1). *FGF13* is a well-known growth factor belonging to the *FGF* family and is the modulator voltage-gated  $\text{Na}^+$ ,  $\text{Ca}^{2+}$  and  $\text{K}^+$  channels [72-74]. Simultaneously, there is

also a significant association between *ovocleidin-116*, *ovocalyxin-32* and *ovalbumin* genes with EST and ESCQ [8, 29]. Thus, the poor eggshell and cuticle qualities are likely mediated by dysregulation of both ion channels and matrix proteins during eggshell formation, which may also be caused by the high expressions of *PTGDS*, *PLCG2*, *ADM* and *PRLR* genes that negatively influenced the uterine functions through the voltage-gated ion channels [43, 45].

The domestic laying chicken has been intensely selected to be a persistent ovulator and many strains of laying hens produce an egg on almost every day [75]. In the past several decades, intensive selection to egg number and egg laying rate traits in commercial layer hen breeds compromised the qualities of the eggshell and cuticle [8, 19, 53]. In order to achieve such excellent egg production performance, the residence duration of the egg in the uterus is shortened to synchronize with the hen's ovulation cycle, at the expense of the eggshell quality (e.g., ESCQ, EST, ESS.). This study also further confirmed the cuticle deposition duration in the hen uterus is an important factor affecting ESCQ.

## Conclusions

The physiological state of the uterus regulates the formation of eggshell ultrastructure and quality. By analyzing the transcriptome and proteome of the uterus from hens that produced eggs with good or poor cuticle quality, *PTGDS*, *PLCG2*, *ADM* and *PRLR* genes were discovered and might play crucial roles in cuticle deposition by affecting uterine secretion rhythm and function. Compared with the PC hens, the relatively low expression of these genes in the uterus ensured the eggshell and cuticle deposition duration, and lead to the good eggshell and cuticle quality of the GC hens. Moreover, eggshell quality and laying performance of hens that produce eggs with good cuticle are better, implying the importance of genetic selection to the cuticle trait in laying hens. This study also hints at the relationship between the ESCQ and the endogenous circadian clock, which might provide a basis for subsequent poultry production improving the cuticle quality.

## Abbreviations

*ADM*: Adrenomedullin; DEGs: Differentially expressed genes; DEPs: Differentially expressed proteins; ESCQ: Eggshell cuticle quality; EST: Eggshell thickness; ESS: Eggshell strength; *ESR1*: Estrogen receptor; FDR: False discovery rate; *FGF13*: fibroblast growth factor 13; FSIN: Food Security Information Network; *GAPDH*, Glyceraldehyde-3-phosphate dehydrogenase; GnRH: Gonadotropin-releasing hormone; GC: good cuticle; GO: Gene Ontology; KEGG: Kyoto Encyclopaedia of Genes and Genomes; PC: poor cuticle; PLC: phospholipase C; *PLCG2*: Phospholipase C gamma 2; PRL: Prolactin; *PRLR*: Prolactin receptor; *PTGDS*: Prostaglandin D2 synthase; qPCR: Quantitative real-time PCR.

## Declarations

### Ethics approval and consent to participate

All the experimental procedures were conducted in accordance with the Guidelines for Experimental Animals established by the Animal Care and Use Committee of China Agricultural University (permit number: AW08059102-1).

### **Consent for publication**

No applicable.

### **Availability of data and materials**

The transcriptome data (raw mRNA-seq reads) of present article data are available in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database under accession number PRJNA664894 (<https://www.ncbi.nlm.nih.gov/sra/PRJNA664894>).

### **Competing interests**

The authors declare that they have no competing interests.

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### **Authors' contributions**

JXZ, XC, ZXH and XZL developed the hypothesis and designed the overall experimental plan. XC, ZXH, JLS, MYH, XFS and XYL obtained the samples and measurement data. XC, ZXH and XZL participated in data analyses. JXZ, NY, GYX and JYL provide the necessary experimental resources for the development of this research. XC, ZXH, GYX, NY and JXZ drafted and revised this manuscript. All authors reviewed and approved the manuscript for publication.

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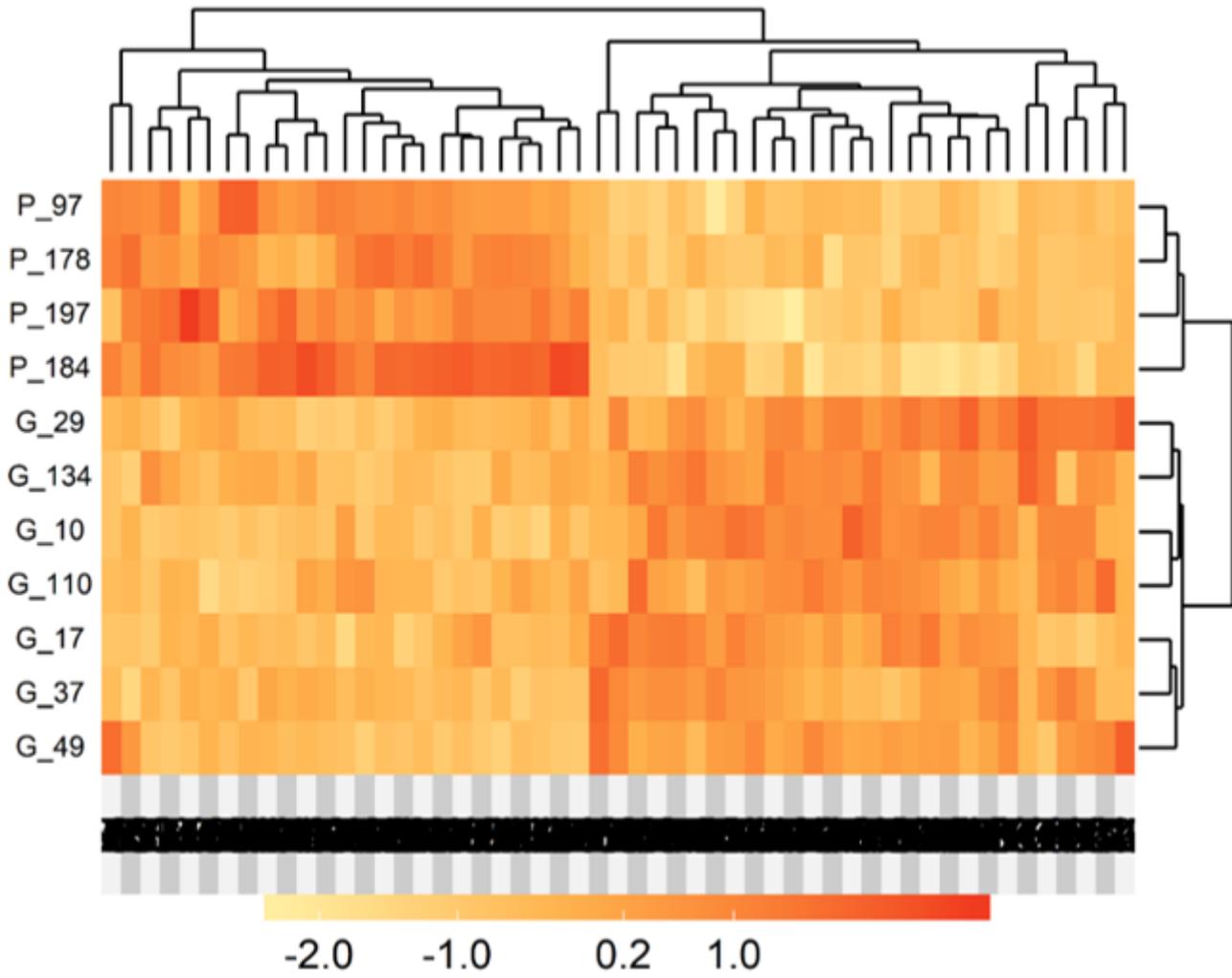
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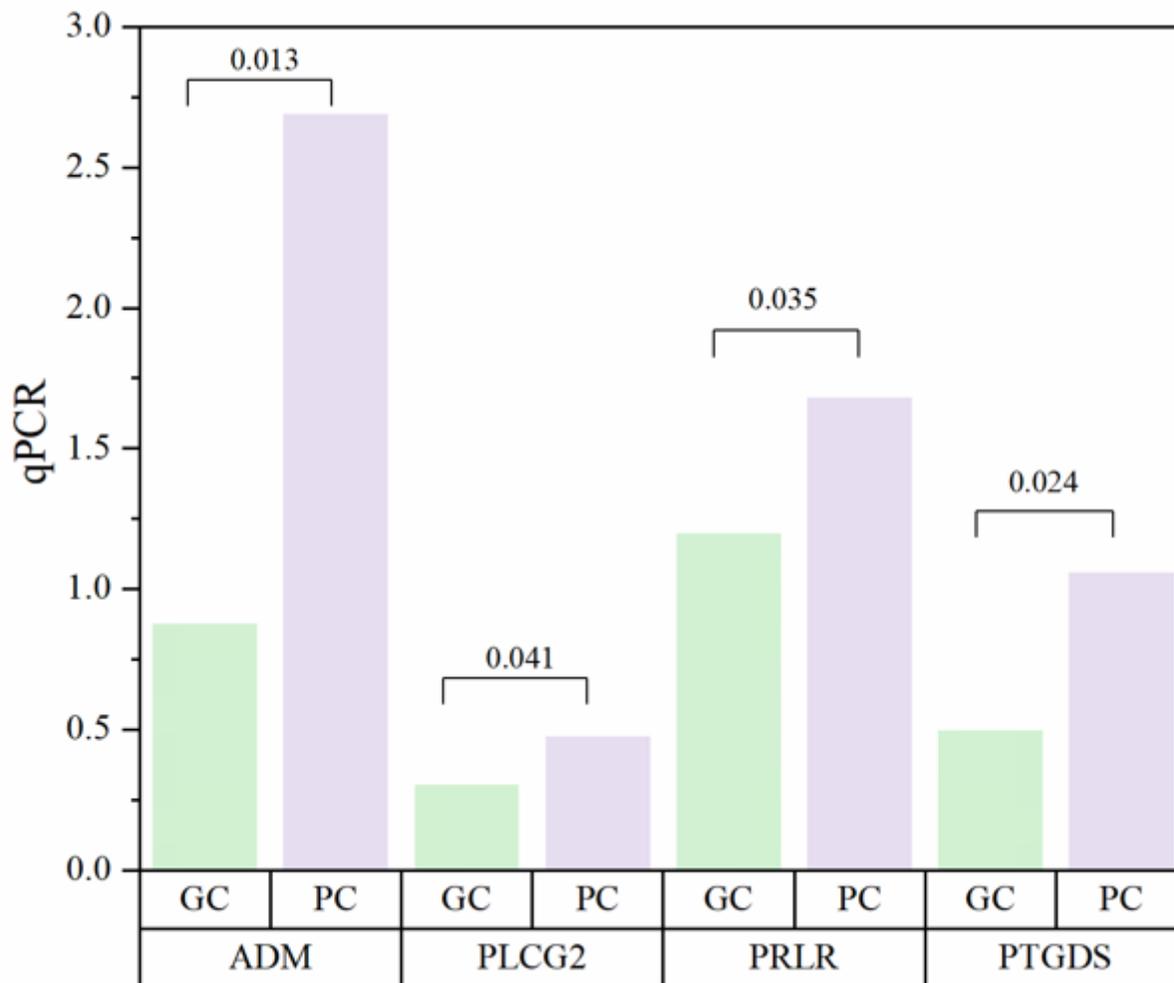
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## Figures



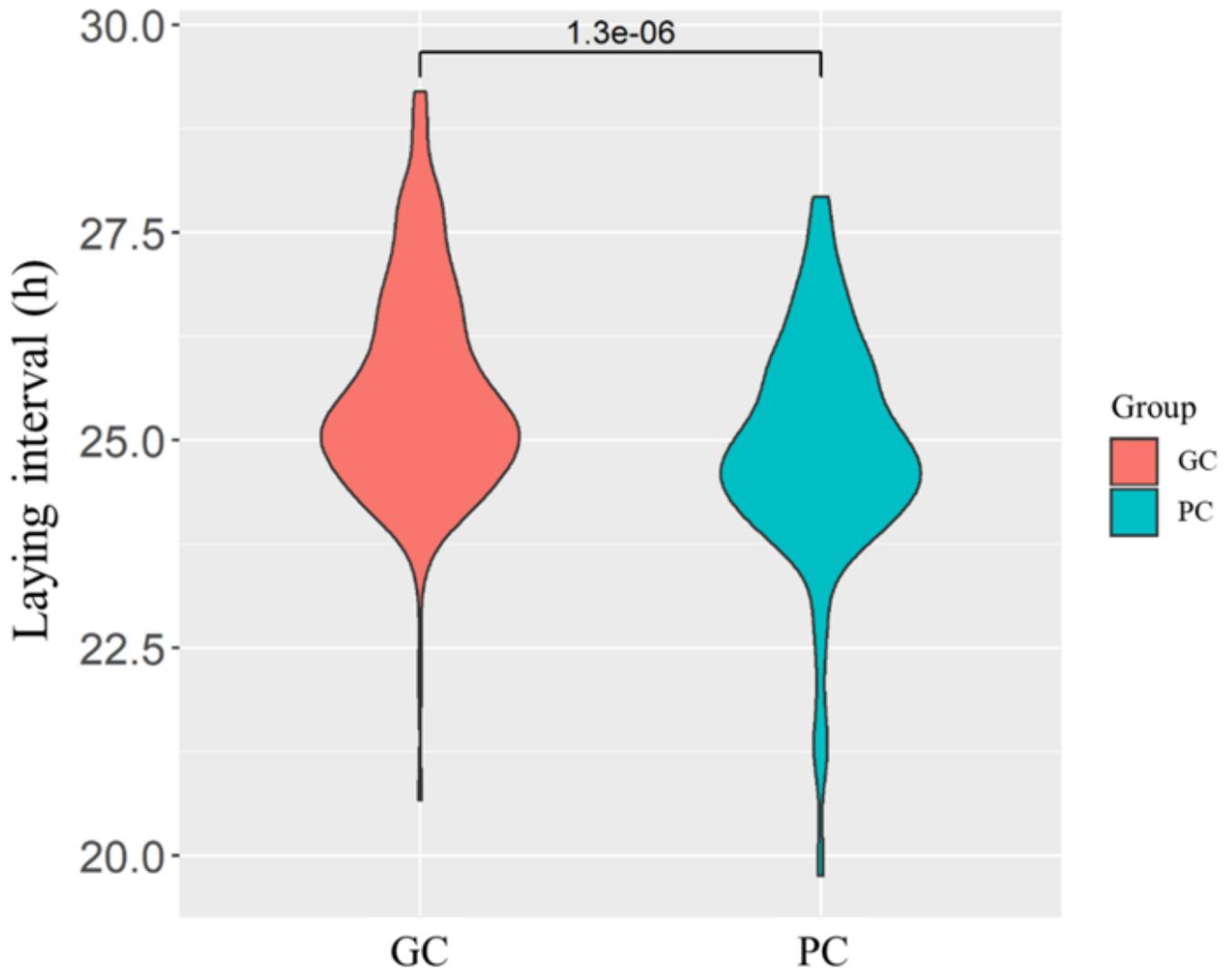
**Figure 1**

Heatmap of the DEGs between GC and PC hen uterus during cuticle deposition from the transcriptomic analysis. GC, good cuticle; PC, poor cuticle.



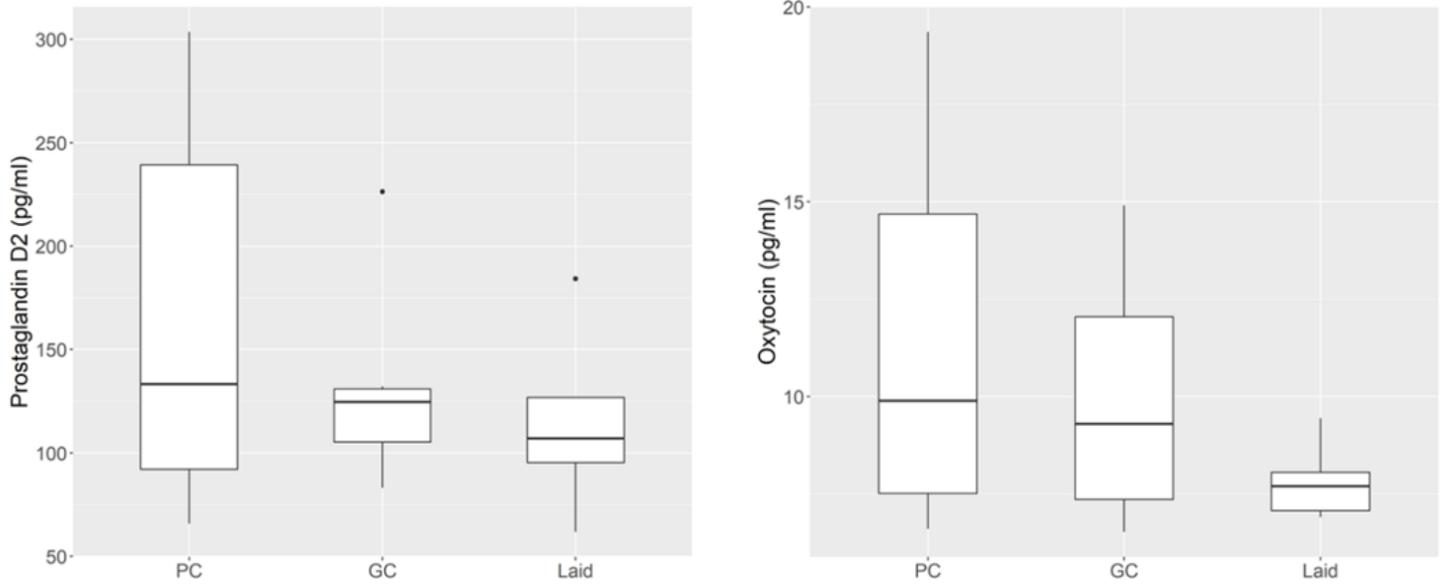
**Figure 2**

Validation of the DEGs of GC vs PC hen uterus during cuticle deposition from the transcriptomic analysis. The qPCR was performed to quantify the the relative gene expression level based on the  $2^{-\Delta\Delta CT}$  method. For gene expression data normalization, GAPDH was used as a reference gene. Fold changes between the GC (good cuticle) and PC (poor cuticle) group were calculated for the genes ADM, PLCG2, PRLR and PTGDS. The Y axis shows the fold changes.



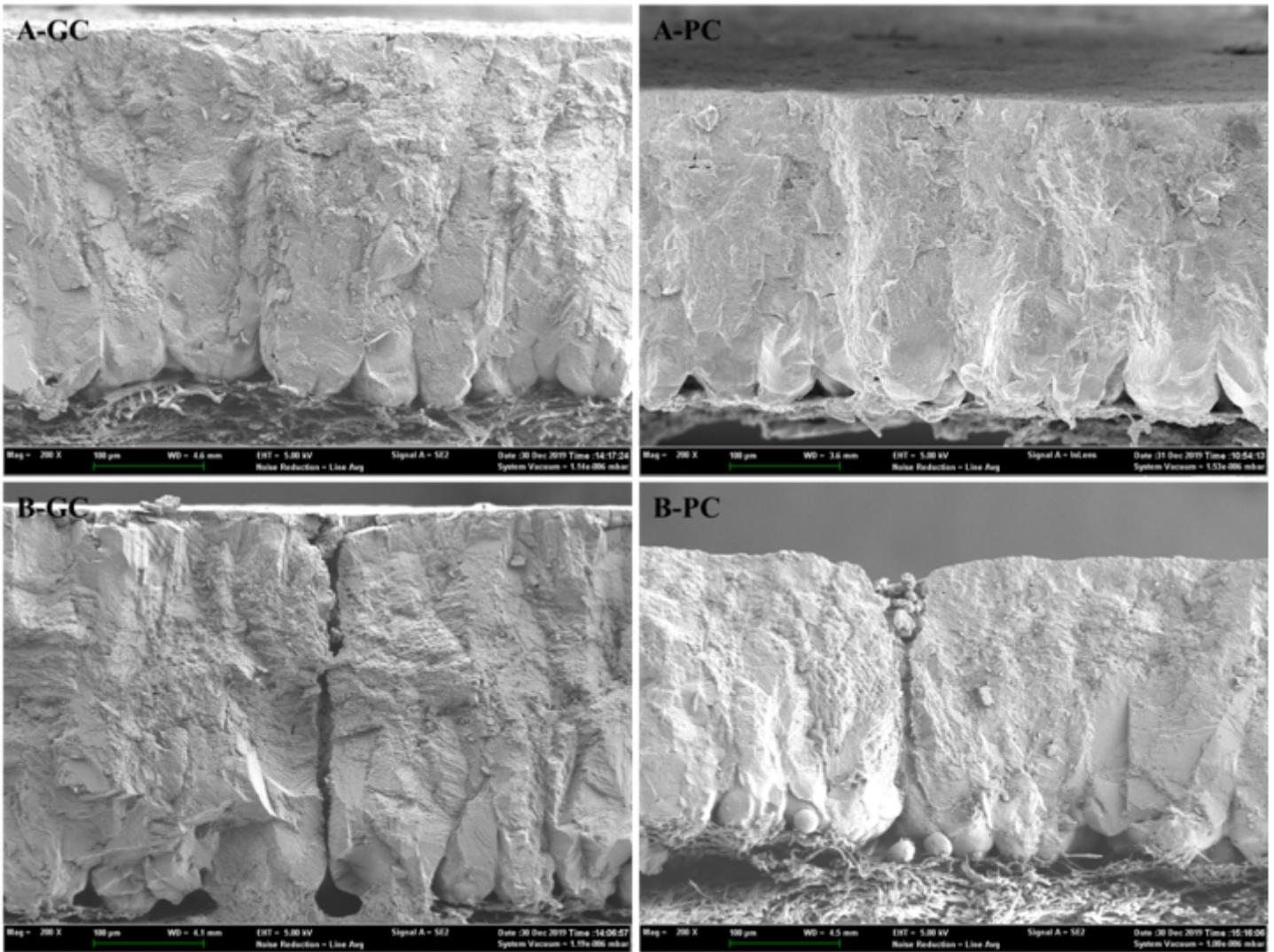
**Figure 3**

Laying interval of GC and PC group hens. The laying interval between two consecutive ovipositions was determined (at least 4 ovipositions per hen) for GC and PC group hens at 28-week old (GC  $25.64 \pm 1.23$  vs PC  $24.94 \pm 1.12$ , h;  $n=60$ ;  $P < 0.01$ ).



**Figure 4**

The concentrations of prostaglandin D2 and oxytocin in hen serum. GC, good cuticle quality hen 1 h before oviposition; PC, poor cuticle quality hen 1 h before oviposition; Laid, good cuticle quality hen 0.5 h after oviposition.



**Figure 5**

Eggshell ultrastructure of GC and PC group eggs by scanning electron microscope (SEM) (200 ×). GC, eggshells with good cuticle; PC, eggshells with poor cuticle; A and B, eggshell ultrastructure without and with a gas pore, respectively.

## Supplementary Files

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