

Subfertility of Offspring and Transgenerational Transmission of Splenic Transcriptome Induced by Lipopolysaccharide in Hens

Lei Liu

China Agricultural University

Di Wang

China Agricultural University

Xingzheng Li

Chinese Academy of Agricultural Sciences

Adeyinka Abiola Adetula

Chinese Academy of Agricultural Sciences

Adnan Khan

Chinese Academy of Agricultural Sciences

Guiyun Xu

China Agricultural University

Ning Yang

China Agricultural University

Ying Yu (✉ yuying@cau.edu.cn)

China Agricultural University

Research

Keywords: laying hens, LPS, maternal stimulation, immunity, egg-laying rate, transcriptome, multi-generational transmission

Posted Date: May 21st, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-522082/v1>

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Abstract

Background:

Transgenerational phenotypes are linked to genetic background, environmental factors, and diseases. Maternal stimulation is one of the suspected contributing factors to transgenerational phenotypes, and recent evidence from human studies suggests that maternal immune stimulation affects the phenotype and gene expression of the offspring, but few similar studies were reported in poultry. Here, we used laying hens as a model organism to investigate the effects of maternal stimulation on hens' immunity and reproductive performance, as well as the regulatory role of the splenic transcriptome in hens induced by lipopolysaccharide (LPS).

Methods:

To investigate the effect of maternal stimulation on egg-laying rate of hens and their offspring, laying hens were first intravenously injected with LPS. RNA-seq from the spleens of F0, F1, and F2 generations of hens was used to identify differentially expressed genes (DEGs) between the LPS group and controls. We investigated the effect of LPS maternal stimulation on the transcriptome of laying hens and its multi-generational transmission effect by analyzing shared genes, enrichment pathways, and protein-protein interaction networks across generations. We concentrated on immune and reproduction-related pathways and genes that existed across generations. Subsequently, we performed a correlation analysis between the DEGs associated with the multi-generational transmission effect and egg-laying rate of hens.

Results:

We found that the LPS maternal stimulation could reduce the egg-laying rate of hens and their offspring, especially during the early and late laying stages. The transcriptome study of the spleen in F0, F1 and F2 generations showed that the maternal stimulation of the LPS affects the patterns of gene expression in laying hens, and this change has the effect of transgenerational transmission. Further analysis of DEGs and their enrichment pathways found that the LPS maternal stimulation mainly affects the reproduction and immunity of laying hens and their offspring. The DEGs such as *AVD*, *HPS5*, *CATHL2*, *S100A12*, *EXFABP*, *RSFR*, *LY86*, *PKD4*, *XCL1*, *FOS*, *TREM2* and *MST1* may play an essential role in the regulation of the immunity and egg-laying rate of the hens. Furthermore, the *MMR1L3*, *C3*, *F13A1*, *LY86* and *GDPD2* genes with transgenerational transmission effects are highly correlated with the egg-laying rate. The DEGs mentioned above have an important reference value for research on the multi-generational transmission of maternal immune stimulation.

Conclusions:

In the current study, we discovered that maternal stimulation could reduce the immunity of laying hens and their offspring, resulting in a decrease in egg-laying rate. These effects could be regulated by the transcriptome's transgenerational transmission. Overall, our study is an important reference for future

research into the multi-generational transmission of maternal stimulation, and the selected marker genes are of great significance to the breeding of laying hens.

Background

Prenatal exposure to infectious or inflammatory environmental stimulating factors can cause mental illnesses in offspring, such as schizophrenia, autism and bipolar psychosis [1], as well as retarded growth and low fertility in offspring [2-5]. These environmental exogenous stimulus are being referred to as maternal stimulants. Lipopolysaccharide (LPS) is the main component of the outer membrane in gram-negative bacteria and can be used to simulate bacteria. It is a common immune-related maternal stimulant, which can be used to study the impact of maternal stimulation on animals and their offspring [6, 7]. LPS was reported to cause a strong immune response in animals, thereby causing immune stress. It can bind to the TLR4, CD14 and MD2 receptors of many types of cells, such as macrophages, dendritic cells, monocytes and B cells, and further promote the secretion of inflammatory cytokines, NO and eicosanoids [8]. Therefore, LPS stimulation could affect the immune system of laying hens and their offspring.

Many studies have shown that maternal immunostimulation could affect the reproductive function of animals [9, 10], and these effects can be transmitted across generations [11]. Yet, the impact of maternal stimulation on the reproductive performance of livestock and poultry offspring is rarely reported. The current environmental impact on livestock and poultry is large and there are many pathogenic microorganisms such as bacteria and viruses, which easily affect the immune systems of livestock and poultry, resulting in the decline of the animals' feed intake, production efficiency and reproductive performance. These adverse effects may also be passed on to the offspring, bringing huge economic losses to animal husbandry. Since the bacterial mimic LPS may affect the immune system, we presumed that the LPS could affect the immune system and reproductive performance of laying hens and their offspring.

Spleen is a vital immune organ in laying hens, and transcriptomes allow inferring gene expression patterns and phenotypic transmission. In this study, we investigated the effect of maternal stimulation on layers immunity and reproduction and the underlying mechanism regulating the changes in the splenic transcriptome across the F0, F1 and F2 generations. It was found that the maternal stimulation by LPS caused a decrease in the immunity and egg-laying rate of laying hens and their unchallenged offspring. This effect may be regulated by the intergenerational transmission effect of the transcriptome. Our results will provide important reference materials for animal breeding, future multigenerational transmission of maternal immune stimulation and immune stress-related research.

Materials And Methods

Ethics Statement

All the following animal works were performed in strict accordance with guidelines and regulations established by the Animal Welfare Committee of China Agricultural University in Beijing, China (permit number: DK996). All the procedures involving animals have been previously approved by the committee. We made all efforts to minimize the hen's suffering during the entire experiment.

Animal and LPS challenge

A total of 58 Rhode Island White hens were randomly selected from the nuclear brood of a poultry breeding farm. Hens were then divided into two groups, which were separately challenged with a single-dose intravenous injection of lipopolysaccharide (LPS, 0.2 mg/kg; *Escherichia coli*; MilliporeSigma, Burlington, MA, USA) (group L, n = 29) or equivalent sterile saline (group C, n = 29) under the wing of the hens at 53 weeks of age. Artificial insemination was performed and generated 60 LPS-exposed (L) and 75 control (saline, C) F1 hens. The hens were reared in wire cages and had free access to water and feed. There was no LPS challenge in F1 and F2 hens. The overall framework of this study was shown in Fig. 1.

Egg-laying rate testing

Eggs were collected and recorded for two weeks before and three weeks post the LPS challenge (52-56 weeks of age) in the F0 generation. In the F1 generation, the eggs collection was performed from 21-66 weeks of age. First of all, we calculated the total egg-laying rate before (two weeks) and after (three weeks) the maternal stimulation and studied its changes in the LPS group. The difference in egg-laying rate between LPS group and the controls was examined. Furthermore, we investigated the effect of maternal stimulation on the daily egg-laying rate of the chicken flock in different conditions in both F0 and F1 generations. Finally, the daily egg-laying rate of the F1 generation was divided into early, middle and late laying periods for further study.

Sampling

For tissue collection, six hens (group L, n = 3; group C, n = 3) were randomly sampled at 24 h post-injection at 53 weeks of age in the F0 generation. Spleens were immediately snap-frozen in liquid nitrogen and then stored at -80 °C for RNA extraction. To investigate the transgenerational transmission effect of transcriptome resulted from the maternal stimuli, we also sampled six spleens (group L, n = 3; group C, n = 3) with the same operation to perform RNA-seq at 53 weeks of age in the F1 generation and 1 day of age in the F2 generation, respectively.

RNA preparation, library construction, and RNA-seq

The total RNA of chicken spleens were isolated using Trizol (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's protocol after grinding the frozen spleen sample into powder in the liquid nitrogen environment. The quality of the RNA sample was assessed by 1% agarose gel electrophoresis and quantified using Qubit RNA Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) and NanoPhotometer spectrophotometer (Implen, Germany). Bioanalyzer 2100 System (Agilent Technologies, Santa Clara, CA, USA) was used to monitor the RNA integrity number (RIN, a score from 0 to 10). All RNA samples were of high quality (RIN > 7, OD₂₆₀/OD₂₈₀ > 1.96, Table S1), and 2 µg of total RNA for each sample were used to construct cDNA libraries using NEBNext® Ultra™ RNA library Prep Kit for Illumina® (NEB, Ipswich, MA, USA). At last, the cDNA libraries were clustered with TruSeq Rapid PE Cluster Kit (Illumina, USA) and sequenced on an Illumina HiSeq 2500 platform (Illumina, USA) and 125 paired-end reads were generated.

Sequencing data quality control and differentially expressed genes (DEGs) identification

FastQC v.0.11.5 was used to assess the quality of the raw data. To get the high-quality data, Trimmomatic-0.2.38 [12] was used to trim the raw data with the default parameters. In detail, the clean reads were obtained by removed adapters, leading and trailing low quality, N bases, reads that below the 36 bases long and reads with the average quality below 15. Before the downstream analyses, FastQC v.0.11.5 was used again to examine the quality of clean data. The GRCg6a reference genome (FASTA format) and gene annotation files (GTF format) were downloaded from the Ensembl database (<ftp://ftp.ensembl.org/pub/release-99/>). The clean data was aligned to the reference genome using HISAT2 [13] with basic options. The output files of HISAT2 were sam format. Then Samtools was used to convert the sam format to bam format. Cuffdiff v2.2.1 [14] was used to compute the DEGs with the bam files between group L vs. group C (L vs. C) in F0, F1 and F2 generations. The DEGs were filtered with criteria of $P < 0.05$ and fold change (FC) > 1.5 .

Functional enrichment and annotation

The DEGs in F0 and F1 generations were used to annotate biological functions, respectively. Gene ontology (GO) term of Biological Process analysis predicted with DEGs was conducted using the WEB-based Gene SeT AnaLysis Toolkit (WebGestalt, <http://www.webgestalt.org>) [15]. Ingenuity Pathway Analysis (IPA, Qiagen, Redwood City, CA, USA) was applied to do Ingenuity Canonical Pathways, Upstream Regulator analysis and Diseases or Functions Annotation analyses [16]. The database STRING v11.0 (https://string-db.org/cgi/input.pl?sessionId=LAxoF5nEd5U4&input_page_show_search=on) was used to analyze Protein-Protein Interaction (PPI) network [17].

Validation of gene expression with reverse transcription quantitative real-time PCR (RT-qPCR)

To verify the results of RNA-seq, three genes were randomly selected in this study, and RT-qPCR experiments were performed on the sequencing samples of F0 and F1 generations. The primer information of the selected genes was shown in Table S2. A total of 1 µg RNA sample were reverse transcribed into cDNA using PrimeScript RT kit (Takara Bio, Dalian, China) according to the manufacturer's instructions. RT-qPCR was then performed on LightCycler 480 II (Thermo Fisher Scientific) with Sybr Green I Master Mix Kit (Roche Diagnostics, Mannheim, Germany) refer to the manufacturer's instructions. The thermal cycle steps are as follows: pre-denaturation: 95 °C, 10 minutes; amplification (45 cycles): 95 °C denaturation 10 sec, 60°C annealing 10 s, 72 °C extension 10 s, and two technical replicates per reaction. Finally, the housekeeping gene *GAPDH* was used as an internal reference gene, and the relative expression of genes was calculated using the $2^{-\Delta\Delta CT}$ method.

Statistical analyses

Significance *chi-square* test was used to analyze the difference in egg-laying rate. In RT-qPCR, $2^{-\Delta\Delta CT}$ was used as the relative quantitative value, and the significant difference between different conditions was tested by the student's *t*-test. In order to evaluate the consistency of gene expression level between RNA-seq data and RT-qPCR results, $\log_2 FC$ was calculated with $2^{-\Delta\Delta CT}$ in different conditions. Then regression analysis was carried out with linear model fitting function in R software (v4.0.2). In addition, Pearson correlation analysis between DEGs and the egg-laying rate was performed in R. $P < 0.05$ was considered to be statistically significant.

Results

LPS maternal stimulation reduces the reproductive performance of laying hens and their offsprings

To explore the possible intergenerational transmission effects caused by the LPS maternal stimulation, we first measured the egg-laying rate of F0 hens. The results showed that the total egg-laying rate of the three weeks post the LPS treatment was 17.17% lower than that of the two weeks before the treatment. Also, the total egg-laying rate of two weeks before the LPS treatment was 3.20% higher than that of the control group with non-significant differences (Fig. 2A, $P > 0.05$). However, the egg-laying rate of LPS group became 8.31% significantly lower than that of the controls in the three weeks after the treatment (Fig. 2A, $P < 0.05$). The difference between the two groups widened to 11.51% after the stimulation, indicating that LPS stimulation could significantly reduce the reproductive performance of laying hens. Furthermore, the daily egg-laying rate of the LPS group was significantly lower than that of the control

group (Fig. 2B, $P < 0.05$), which proved that LPS treatment could significantly reduce the reproductive performance of the laying hens. We further examined the two groups of hens as a whole union and calculated the differences in egg-laying rate between the two groups for 22 consecutive days and found that the daily egg-laying rate of the LPS group that was lower than the control group accounted for 72.73% of the total statistics (Fig. 2C), which was extremely significantly higher than the probability of the LPS group that was higher than that of the controls (18.18%, $P < 0.001$). These findings support the notion that LPS stimulation could reduce the egg-laying rate of hens.

For the aim of studying the effect of LPS maternal stimulation on the egg-laying rate of the corresponding untreated offspring, we calculated the daily egg-laying rate of F1 hens for 304 consecutive days (21-66W, except 39-40W), and found that the daily egg-laying rate of the F1 hens was significantly lower than that of the control group (Fig. 2D, $P < 0.001$). Furthermore, we compared the daily egg-laying rate between the two groups during the 304 days, and found that the egg-laying rate of the LPS group was lower than that of the control group almost during the entire laying period in the F1 generation (Fig. 2E), especially in the early and late laying stages. We also found that 90.79% (276/304) of the daily egg-laying rate in the LPS group was lower than that of the control, of which 30.07% were significantly different (Fig. 2E and 2F, $P < 0.05$). The daily egg-laying rate of the LPS group was significantly lower than that of the control group accounted for 28%, 18.25% and 54.76% in early, middle and late laying stages, respectively (Fig. 2F). These results showed that the LPS maternal stimulation has a greater negative impact on the early and late laying stages of F1 hens.

Intergenerational transmission of the transcriptome caused by LPS maternal stimuli

To reveal the molecular mechanism of LPS maternal stimulation that regulates the decreased egg-laying rate of the hens and their offspring from the perspective of immunity, we examined the transcriptome changes in the spleen using 53-week-old laying hens in F0 and F1 generations. It was found that LPS maternal stimulation caused changes in the F0 and F1 transcriptomes (Fig. 3A, B). Using the screening criteria of $P < 0.05$ and $FC > 1.5$, 634 and 577 differentially expressed genes (DEGs) were obtained in F0 and F1, respectively, of which up-regulated genes in the LPS group accounted for 48.58% and 66.90%, respectively. With the criteria of $P < 0.05$, $FC > 2$, 282 and 272 DEGs were separately detected in F0 and F1, of which up-regulated genes accounted for 50.35% and 59.56% (Fig. 3C). It can be seen that LPS activates the expression of some genes. The follow-up studies are based on $P < 0.05$, $FC > 1.5$.

To investigate the transcriptome's intergenerational transmission of maternal effect on laying hens, we examined the changes in the expression of common DEGs in the two generations of laying hens. The results showed that a total of 1077 DEGs were involved in the F0 and F1 generations, including 134 common DEGs (coDEGs), of which 63 coDEGs (47.01%, 42 up-regulated, 21 down-regulated) showed the same expression trend between the two generations (Fig. 3D). Furthermore, we used the FPKM values of the above 1077 DEGs to perform the correlation analysis between the two generations in each group. The

results showed that the gene expression between the two generations of the same group had a very high positive correlation, and the correlation coefficients reached 0.9 (Fig. 3E, $P < 2.2\text{E-}16$), indicating that the effect of maternal stimulation on the transcriptome of laying hens has a transmission effect. We lists the 134 coDEGs between the two generations, including 63 coDEGs with the same expression trend, which can be used as marker genes for further studies, such as *APLNR*, *BFSP1*, *C3*, *CCL1*, *CATHL2*, *CEP162*, *EXFABP*, *F13A1*, and *FOS* (Fig. 3F).

The RNA-seq data used in this study was verified by RT-qPCR by randomly selecting three genes in the F0 and F1 generations. We compared the $\log_2\text{FC}$ predicted by RNA-seq with the actual $\log_2\text{FC}$ obtained by RT-qPCR. These results showed that all genes have the same expression trend (Fig. 4A). In addition, the regression analysis results showed that $\log_2\text{FC}$ obtained by RNA-Seq is highly correlated with $\log_2\text{FC}$ obtained by RT-qPCR ($r = 0.92$, $P < 0.01$), confirming the reliability of the current RNA-seq data (Fig. 4B).

LPS could influence the immune response of hens in F0 and F1 generations

To analyze the biological functions separately involved in the DEGs of F0 and F1 generations, we used Ingenuity Pathway Analysis (IPA) to predict Ingenuity Canonical Pathways using the DEGs of F0 and F1 generations. A total of 103 and 44 significantly enriched pathways were identified in F0 and F1 generations, respectively (Table S3 and S4, $P < 0.05$). Then 33 and 18 Ingenuity Canonical Pathways were separately retained with the screening criteria of $P < 0.01$, of which 8 pathways were shared by both F0 and F1 generations. All of the eight pathways are directly or indirectly related to immune response. The eight shared pathways of F0 and F1 contained 34 and 23 genes, respectively, of which 15 genes were shared between the two generations, such as *ALB*, *AMBP*, *C3*, *EGR1*, *F13A1*, *F2*, *FGA*, *FGB*, *FOS*, *GC*, *HPX*, *MST1*, *RBP4*, *SERPINC1*, *VTN* (Table 1). These results indicated that the treatment of LPS could influence the immune system of the F0 generation, and the effect could be transmitted to the next generation.

We also used IPA to study upstream regulators of the DEGs in F0 and F1 generations and found that there were 6 (30%) shared upstream regulators in the top 20 upstream regulators of F0 and F1 generations, of which, all were related to immune-inflammatory responses, such as lipopolysaccharide, TNF, IL1B, PPARA, dexamethasone and IL6 (Table 2), further indicated that the immune system of F0 and F1 was affected. The discovery of a common regulator such as lipopolysaccharide suggests that the maternal response to LPS affects the immune system and has a long-lasting intergenerational transmission effect.

In order to further understand the intergenerational transmission effect of the LPS maternal stimulus, the 63 coDEGs with the same expression trend in F0 and F1 generations were used to perform GO term enrichment analysis. We only focused on GO terms related to biological processes, and top30 significantly enriched terms are shown in Fig. 5A. The majority of the top terms are related to immune system (from the bottom up), such as defense response to bacterium, inflammatory response, response

to external stimulus, innate immune response, response to external biotic stimulus, immune response, humoral immune response, defense response to Gram-negative bacterium (Fig. 5A), suggesting that the LPS maternal stimulation could cause the immune-inflammatory response of the F0 generation, and the effect had been transmitted to the F1 generation. Further analysis of the seven terms (terms in red font) with $FDR < 0.05$ showed that these seven terms were related to immune-inflammatory response, including defense response to bacterium, response to external stimulus, antimicrobial humoral response, innate immune response and inflammatory response. In addition, we found that these seven terms were mainly associated with 10 DEGs such as *AVD*, *HPS5*, *CATHL2*, *S100A12*, *EXFABP*, *RSFR*, *LY86*, *PKD4*, *XCL1* and *FOS* (Fig. 5B). Among these 10 DEGs, only *XCL1* and *FOS* were significantly down-regulated in the LPS group ($P < 0.05$), while the remaining ones were significantly up-regulated ($P < 0.05$). These results indicate that the F1 hens produce a certain immune memory to the F0 LPS maternal stimulation, and the innate immunity and humoral immunity of the F1 hens are activated, which further autonomously stimulates the regulatory pathways to external stimuli. These genes could be used as genetic markers for the transmission of maternal immune stimulation between generations.

The decrease of egg-laying rate might result from the immune system deterioration of hens

It was reported that the diversity of LPS structures and the differential recognition of these structures by TLR4 had been associated with several bacterial diseases [18]. LPS could induce innate immunity with Alzheimer's disease [19] and airway disease[20]. It also increases the invasive ability of pancreatic cancer cells through the TLR4/MyD88 signaling pathway [21]. So the IPA was used to analyze the Diseases or Functions Annotation to study the effect of LPS maternal stimulation on the immunity of the challenged hens and their offspring. We extracted the top20 terms of both F0 and F1 generations and found that 90% (18) of the terms are shared in the two generations, and these terms were mostly involved in diseases such as cancer and tumors (Table 3, all terms are shown in Table S5 and S6), revealing that LPS could affect the health of F0 and the unchallenged F1 hens. We also found several terms related to reproduction in the F0 such as development of female reproductive tract, development of ovary, abnormal morphology of ovary and in the F1 such as development of reproductive system. Two common terms morphology of ovary and tumorigenesis of reproductive tract were also found in the two generations (Table S5 and S6). These terms indicated that the LPS maternal stimulation might negatively impact on the reproductive system of laying hens, and this influence has a succession effect, which might also explain the decline in egg-laying rate of F0 and F1 hens.

Moreover, the IPA was used to do interaction network analysis using the DEGs of F0 and F1 (Fig. 6A, B and Fig. S1), and found that the second network of F0 was related to Organismal Injury and Abnormalities (Fig. S1B) and the third network was related to Antimicrobial Response and Inflammatory Response (Fig. S1C), which indicates that maternal response to LPS caused an immune inflammatory response. The top3 networks in the F1 generation are mainly enriched in metabolism and development-

related pathways, such as drug metabolism, carbohydrate metabolism, lipid metabolism and embryonic development (Fig. 6A,B and Fig. S1D). It is worth noting that we found *TREM2* and *MST1* in the first and third networks of the F1 generation respectively (Fig. 6A,B). Our previous studies found these two genes to be significantly negatively correlated with the egg-laying rate [22]. At the same time, we also found *TREM2* and *MST1* were in the PPI network predicted with the shared DEGs of F0 and F1 (Fig. 6C), indicating the importance of these two genes in the intergenerational transmission effect of maternal stimulation. Moreover, *MST1* gene is highly negatively correlated with the egg-laying rate of F1 53-week-old hens ($r = -0.97$, $P = 0.001$, Fig. 6D). The significantly up-regulation of *MST1* in the LPS group of F0 and F1 generations (Fig. 6E) may be closely related to the decrease in the egg-laying rate of the two generations hens. These findings suggested that the LPS could induce subfertility and influence the immunity of the unchallenged offspring via the transmissible transcriptome. Additionally, the decline in the immunity may compromise the laying rate.

Transgenerational transmission of transcriptome induced by the LPS maternal stimulation

Although the F2 generation RNA-seq data comes from the spleen of 1-day-old chicken, the common DEGs obtained from the spleen tissues of F0, F1, and F2 chickens are still of reference value for the study of multi-generational transmission effects. The splenic transcriptome of the F2 generation was further analyzed to investigate the transgenerational transmission effect of transcriptome changes induced by the maternal stimulation. The results showed that the LPS maternal stimulus induced 2791 DEGs in the F2 generation, including 1741 up-regulated DEGs and 1050 down-regulated DEGs (Fig. 7A, $P < 0.05$, $FC > 1.5$). A total of 37 common DEGs were found among the F0, F1 and F2 generations, in which 10 DEGs have the same expression trend, including *ENSGALG00000045832*, *ENSGALG00000036119*, *C3*, *CEP162*, *F13A1*, *GDPD2*, *MMR1L3*, *LY86*, *PDK4* and *PPEF2* (Fig. 7B, C), revealing that the effect of maternal stimulation on the transcriptome of laying hens has a transgenerational transmission effect. The PPI networks showed that *MMR1L3* and *LY86* proteins are related to *TLR4* (Fig. 7D, E), which is the main receptor of the LPS [18], indicating the transgenerational transmission effect of transcriptome may result from the LPS maternal stimulation.

Regulation of transgenerational transmission of transcriptome on egg-laying rate of hens

In addition, we extracted the FPKM of the F1 hens of the above 10 DEGs with transgenerational transmission effect and conducted a Pearson correlation analysis with the egg-laying rate of the F1 hens at 53 weeks of age. Five genes (50%) were found to have a correlation of more than 0.5 with egg-laying rate, including *MMR1L3*, *C3*, *F13A1*, *LY86* and *GDPD2* (Fig. 8). Among them, *MMR1L3*, *C3*, *F13A1* and *LY86* genes were negatively correlated with egg-laying rate, depicting that these genes inhibit the

reproductive performance of laying hens. The *GDPD2* gene was positively correlated with the egg-laying rate, indicating that this gene can moderately promote the reproductive performance of hens (Fig. 8). The significantly up-regulation of *MMR1L3*, *C3*, *F13A1* and *LY86* genes and the significantly down-regulation of *GDPD2* in the LPS group of the three generations of hens indicate that the LPS maternal stimulation may promote the expression of *MMR1L3*, *C3*, *F13A1*, *LY86* genes while inhibiting the expression of *GDPD2* gene to reduce the egg-laying rate of hens, and this effect may have a transgenerational transmission effect.

Discussion

Stimulation from the maternal source can cause the transmission of phenotypes across generations[1], and this phenomenon is often controlled by epigenetics [23]. Research on this phenomenon mainly focuses on the epigenetic multi-generational transmission and reprogramming events during gamete formation, differentiation, and development [24, 25]. As the interface between the environment and gene expression, epigenetics can regulate gene expression after being influenced by environmental factors, which in turn influences the phenotype [26]. The transcriptome of an organism can be regarded as the intermediate phenotype of the final phenotype of the organism, and it often plays an important role in the transmission of maternal stimulation across generations. However, in the study of maternal stimulation of multi-generational transmission, the multi-generational transmission of the transcriptome directly related to the phenotype is often ignored. In this study, laying hens were used as model organisms to study the multi-generational transmission effect of the bacterial mimic LPS maternal stimulation. It was observed that the maternal stimulation had an impact on the egg-laying rate, immunity and transcriptome of the challenged hens and their offspring. The results of this experiment will be discussed in three points:

The first point is that the LPS maternal stimulation could significantly decrease the egg-laying rate of F0 and F1 generation hens. More and more studies have shown that prenatal exposure to immune stress can inhibit the development and reproductive performance of mice [27, 28]. As a result, this negative effect can be transferred from parents to the next generations [29-32]. The current study found that LPS treatment significantly reduced the egg-laying rate of hens. The overall egg-laying rate in three weeks after treatment decreased by 17.17% compared with the previous two weeks (Fig. 2A). This negative impact was passed over to the F1 generation. In the F1 generation (Fig. 2C), the overall egg-laying rate of 304 days in LPS group was significantly lower than that of the controls, and the daily egg-laying rate of 276 (90.79%) days was lower than that of the control group (Fig. 2E). In addition, we discovered that the negative effect of the LPS maternal stimulation on the egg-laying rate of F1 generation hens was severe in the early and late laying stages (Fig. 2E and 2F). Studies have reported that toxic substances such as disinfectants and pesticides in the environment can lead to ovarian diseases in female animals and affect the DNA methylation and transcriptome levels of offspring across generations [33]. Our results suggest that LPS maternal stimulation has a negative effect on the hens' ovaries and thus their offspring; it inhibits follicle maturation in the early stage and promotes follicle senescence in the late stage, resulting in a decrease in the egg-laying rate of F1 generations.

The second point is that LPS maternal stimulation leads to the transgenerational transmission of splenic transcriptome and affects the immunity of hens and their offspring. The successive generational transmission includes intergenerational and transgenerational transmission. Intergenerational transmission means that the maternal stimulus has the same or similar effects on the treated parents and the offspring that are directly stimulated, and transgenerational transmission means that the offspring are not directly affected by the stimulus factor, but have the same or similar phenotype or gene expression to the treated parents [34]. Using the poultry maternal stimulation model, intergenerational and transgenerational transmission effects can be observed in F1 and F2 generation, respectively [22]. In this study, we found that LPS maternal stimulation induced 134 common DEGs in F0 and F1 generations, of which 47.01% of the DEGs shared the same expression trend (Fig. 3D and 3F). The correlation of the splenic transcriptome levels in the same condition between F0 and F1 hens reached 0.9 (Fig. 3E, $P < 2.2 \times 10^{-16}$). Furthermore, 37 shared genes were found in F0, F1, and F2 generations, in which 10 DEGs had the same expression trend among the three generations (Fig. 7B and 7C), indicating that the LPS maternal stimulation could result in transcriptome changes, and this alteration has a transgenerational transmission effect.

External immune stimulation given to the mother prior to childbirth will result in an immune response in the offspring [35]. We also found that LPS could affect the immunity of laying hens and their offspring. Using the DEGs shared by F0 and F1 generations, we have discovered many canonical pathways and biological processes related to immune response, such as IL-12 signaling and production in macrophages, defense response to bacterium, humoral immune response, innate immune response, inflammatory response (Table 1 and Fig. 5). Meanwhile, we discovered that lipopolysaccharide (LPS) is a common upstream regulator of F0 and F1 generation DEGs (Table 2), indicating that the F1 generation generates immune memory to the F0 generation's LPS maternal stimulation, and the innate immunity and humoral immunity are activated, which further autonomously stimulates the regulatory pathways to external stimuli. These findings suggest that maternal LPS stimulation induced an intergenerational transmission effect of the immune response in laying hens, and this effect may be regulated by the transcriptome and the related pathways. The immune-related DEGs inherited between generations can be used as genetic markers for the intergenerational transmission effects of maternal immune stimulation, such as *AVD*, *HPS5*, *CATHL2*, *S100A12*, *EXFABP*, *RSFR*, *LY86*, *PKD4*, *XCL1* and *FOS*.

Thirdly, the intergenerational transmission of reduced egg-laying rate may be affected by immunity and transcriptome. Maternal exposure to infectious agents or maternal stimuli can increase disease risk in the offspring through epigenetic regulation [27-30, 36, 37]. In the present study, we found that DEGs in F0 and F1 generations were enriched in tumorigenesis of reproductive tract, development of female reproductive tract, morphology of ovary, development of ovary, abnormal morphology of ovary, development of reproductive system and other reproduction-related disease pathways (Table S5 and S6), verifying that toxic substances in the environment can cause ovarian diseases in female animals [33, 38]. It indicates that the LPS maternal stimulation may have a negative impact on the reproductive system of laying hens, and this influence has the effect of intergenerational transmission, which may explain the decline of the egg-laying rate of F0 and F1 hens. Our previous study found that all of the genes that were significantly

negatively related to egg-laying rate ($P < 0.05$) were up-regulated in the LPS group [22]. The expression of the *MST1* gene in the F1 generation was highly negatively correlated with the egg-laying rate of 53-week-old hens, and the correlation coefficient reached -0.97 (Fig. 6D, $P = 0.001$). *MST1* is an immune-related gene that is significantly enriched in IL-12 signaling and production in macrophages in this study, indicating that the intergenerational transmission of reduced egg-laying rate may be caused by immunity decline and transcriptional regulation.

In the current study, among the 10 shared DEGs transmitted across the three generations, the correlation coefficients between five genes and the egg-laying rate of F1 hens at 53 weeks of age were above 0.5, of which *MMR1L3*, *C3*, *F13A1*, *LY86* were up-regulated in the LPS group and negatively correlated with the egg-laying rate (Fig. 8). *GDPD2* was down-regulated in the LPS group and positively correlated with egg-laying rate (Fig. 8). These genes may be the key regulatory genes for the intergenerational transmission of the drop in egg-laying rate of hens. Among these genes, *LY86* (lymphocyte antigen 86) and *MMR1L3* (macrophage mannose receptor 1-like 3, macrophage mannose receptor 1-like 3) are two important genes. *LY86* and *MMR1L3* expressions in the LPS group of the three generations were consistently significantly up-regulated, and both of them are linked to TLR4 (Fig. 7D and 7E), which is the receptor of LPS [39], suggesting that these two genes may play an important role in the transgenerational transmission effect of LPS maternal stimulation. *LY86* gene is closely related to the innate immune system and inflammatory response [40], suggesting that maternal stimulation may continue to affect the innate immunity of the unchallenged offspring. In addition, the PPI shows that the proteins *MMR1L3* and *TREM2* are also directly related (Fig. 7D). Our previous study has shown that the *TREM2* gene is significantly negatively correlated with the egg-laying rate [22], indicating that the decline of the egg-laying rate in F1 hens may also be related to the significant up-regulation of the *MMR1L3* gene. And the significant up-regulation of *MMR1L3* in the F2 generation suggests that the egg-laying rate of F2 hens may also be reduced.

Conclusions

In summary, the current study is the first to explore the transgenerational transmission effect of the transcriptome caused by maternal stimulation in laying hens. With the chicken model, our study reveals the profound implications of maternal stimulation on the immunity and reproduction of offspring, implying that the environment of parents' lives has an important impact on offspring. Our research elaborated on the impact of immune alteration on the intergenerational transmission of egg-laying rates, emphasizing the regulatory role of intergenerational transmission of the transcriptome. This study adds to the body of knowledge about intergenerational transmission in domestic animals and has important reference significance for livestock and poultry production management and breeding.

Abbreviations

LPS: lipopolysaccharide

DEGs: Differentially expressed genes

L: group L, treated with LPS

C: group C, control, treated with saline

FC: Fold change

GO: Gene ontology

IPA: Ingenuity Pathway Analysis

PPI: Protein-Protein Interaction

RT-qPCR: Reverse transcription quantitative real-time PCR

coDEGs: Common DEGs

Declarations

Ethics approval and consent to participate:

All animal management and experimental procedures followed the animal care protocols approved by the China Agricultural University Animal Care and Use Ethics Committee.

Consent for publication:

Not applicable.

Availability of data and material:

All genomic annotation data defining gene regions are available for download (<ftp://ftp.ensembl.org/pub/release-99/>). RNA-seq data from China Agricultural University is available upon the agreement of China Agricultural University and should be requested directly from the authors.

Competing interests:

The authors have declared that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Funding:

This research was financially supported by the National Natural Science Foundation of Major International Cooperation Project (31320103905); the Program for Changjiang Scholar and Innovation Research Team in University Grant (IRT-15R62); and Modern Agro-Industry Technology Research System Grant (CARS-40).

Authors' contributions:

Ying Yu and Lei Liu conceived the study. Lei Liu and Di Wang collected the samples and analyzed the data. Lei Liu, Di Wang, Xingzheng Li, Adeyinka Abiola Adetula, Adnan Khan, Guiyun Xu, Ning Yang, and Ying Yu wrote and prepared the manuscript.

All authors reviewed and contributed to the manuscript.

Acknowledgments:

The authors thank Jingwei yuan, Shuang Yang, Zhongyi Duan, Changsheng Nie, Ting Yang (all from China Agricultural University) for sample collection; Novogene Technology (Beijing, China) for sequencing services.

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Tables

Table 1. Overlapped pathways in F0 and F1 generations by IPA with the threshold of $P < 0.01$

	Canonical Pathways	-log(P-value)	Genes
F0 generation	Acute Phase Response Signaling	6.21	<i>JUN,C3,SERPINF1,FGA,RBP4,C1R,PLG,FOS,IL1R1, FGB,C1S,HPX,AMBP,F2,ALB</i>
	Coagulation System	5.64	<i>F7,FGB,FGA,F13A1,SERPINC1,PLG,F2</i>
	Extrinsic Prothrombin Activation Pathway	6.69	<i>F7,FGB,FGA,F13A1,SERPINC1,F2</i>
	FXR/RXR Activation	6.41	<i>GC,APOA4,C3,SERPINF1,CETP,FGA,RBP4,APOB, VTN,LPL,HPX,AMBP,ALB</i>
	IL-12 Signaling and Production in Macrophages	4.22	<i>FOS,FGFR3,JUN,APOA4,APOB,STAT1,IL12RB2, PRKCH,RBP4,ALB,MST1</i>
	Intrinsic Prothrombin Activation Pathway	3.07	<i>FGB,FGA,F13A1,SERPINC1,F2</i>
	LXR/RXR Activation	9.34	<i>GC,APOA4,C3,SERPINF1,CETP,FGA,IL1R2, RBP4,APOB,VTN,IL1R1,PTGS2,LPL,HPX,AMBP,ALB</i>
	Role of Tissue Factor in Cancer	2.65	<i>FGFR3,CYR61,F7,FGB,CTGF,FGA,EGR1,F2</i>
F1 generation	Acute Phase Response Signaling	6.93	<i>FOS,ALB,HPX,FN1,C3,APOH,AMBP,FGB,MAPK13,FGA, F2,FGG,RBP4</i>
	Coagulation System	5.60	<i>SERPINC1,F13A1,FGB,FGA,F2,FGG</i>
	Extrinsic Prothrombin Activation Pathway	7.83	<i>SERPINC1,F13A1,FGB,FGA,F2,FGG</i>
	FXR/RXR Activation	5.74	<i>HPX,ALB,C3,APOH,VTN,AMBP,ABCB11,GC,FGA,RBP4</i>
	IL-12 Signaling and Production in Macrophages	2.84	<i>TLR2,FOS,ALB,MST1,MST1R,MAPK13,RBP4</i>
	Intrinsic Prothrombin Activation Pathway	5.12	<i>SERPINC1,F13A1,FGB,FGA,F2,FGG</i>
	LXR/RXR Activation	4.99	<i>HPX,ALB,C3,APOH,VTN,AMBP,GC,FGA,RBP4</i>
	Role of Tissue Factor in Cancer	3.14	<i>EGR1,HBEGF,FGB,MAPK13,FGA,F2,FGG</i>

Table 2. Top 20 upstream regulator analysis with IPA in F0 and F1 generations

	Upstream Regulator	Molecule Type	P-value
F0 generation	lipopolysaccharide	chemical drug	3.87E-16
	TGFB1	growth factor	5.16E-16
	TNF	cytokine	1.37E-14
	leukotriene D4	chemical - endogenous mammalian	3.29E-14
	ethanol	chemical - endogenous mammalian	4.96E-13
	IL1B	cytokine	3.82E-12
	beta-estradiol	chemical - endogenous mammalian	6.88E-12
	deferoxamine	chemical drug	1.18E-11
	STAT3	transcription regulator	2.49E-11
	PPARA	ligand-dependent nuclear receptor	6.24E-11
	phorbol myristate acetate	chemical drug	9.70E-11
	CREB1	transcription regulator	2.73E-10
	dexamethasone	chemical drug	3.27E-10
	IL6	cytokine	5.56E-10
	TLR3	transmembrane receptor	2.13E-09
	SP3	transcription regulator	2.63E-09
	Raf	group	2.94E-09
F1 generation	F2	peptidase	3.50E-09
	EGF	growth factor	4.31E-09
	rosiglitazone	chemical drug	4.58E-09
	Upstream Regulator	Molecule Type	P-value
	IL1B	cytokine	4.92E-12
	TNF	cytokine	1.77E-11
	dexamethasone	chemical drug	1.40E-10
	IL6	cytokine	1.62E-10
	LIF	cytokine	4.45E-09
	lipopolysaccharide	chemical drug	6.13E-09
	nitrofurantoin	chemical drug	1.02E-08

IL4	cytokine	1.22E-08
CEBPB	transcription regulator	2.49E-08
actinonin	chemical reagent	2.69E-08
OSM	cytokine	8.88E-08
Growth hormone	group	9.53E-08
PPARA	ligand-dependent nuclear receptor	1.05E-07
tretinoin	chemical - endogenous mammalian	1.36E-07
progesterone	chemical - endogenous mammalian	1.75E-07
CREBBP	transcription regulator	2.06E-07
DAP3	other	2.51E-07
diethylstilbestrol	chemical drug	2.62E-07
HNF1A	transcription regulator	3.26E-07
CD38	enzyme	4.52E-07

Note: Upstream Regulators in red font are commonly shared by F0 and F1 generation.

Table 3. Top20 diseases or functions annotation predicted by IPA in F0 and F1 generations

	Diseases or Functions Annotation	P-Value	Gene number
F0 generation	Cancer	2.70E-15	367
	Solid tumor	2.56E-12	365
	Malignant solid tumor	1.84E-14	364
	Non-hematological solid tumor	1.97E-16	363
	Extracranial solid tumor	1.01E-12	363
	Nonhematologic malignant neoplasm	1.08E-16	362
	Non-melanoma solid tumor	1.28E-14	354
	Tumorigenesis of tissue	4.87E-15	353
	Epithelial neoplasm	3.57E-15	352
	Abdominal neoplasm	1.51E-12	352
	Carcinoma	4.68E-15	351
	Abdominal cancer	2.55E-13	344
	Digestive organ tumor	7.03E-13	344
	Abdominal carcinoma	4.07E-13	336
	Adenocarcinoma	1.83E-15	332
	Digestive system cancer	4.45E-14	332
	Abdominal adenocarcinoma	1.98E-13	323
	Gastrointestinal tract cancer	8.53E-16	316
	Gastrointestinal carcinoma	4.35E-15	306
	Malignant neoplasm of large intestine	3.02E-13	300
	Diseases or Functions Annotation	P-Value	Gene number
F1 generation	Solid tumor	5.59E-09	235
	Cancer	5.73E-09	233
	Extracranial solid tumor	7.95E-09	233
	Malignant solid tumor	1.74E-08	231
	Non-hematological solid tumor	4.26E-07	225
	Tumorigenesis of tissue	1.50E-08	223
	Abdominal neoplasm	2.26E-07	223

Nonhematologic malignant neoplasm	1.04E-06	223
Non-melanoma solid tumor	1.43E-07	222
Epithelial neoplasm	1.27E-07	220
Carcinoma	1.16E-06	217
Abdominal cancer	3.59E-07	216
Digestive organ tumor	6.38E-07	216
Abdominal carcinoma	7.67E-06	207
Digestive system cancer	1.40E-06	205
Adenocarcinoma	5.78E-07	204
Abdominal adenocarcinoma	5.61E-06	198
Gastrointestinal tumor	2.42E-05	188
Gastrointestinal tract cancer	2.35E-05	187
Large intestine neoplasm	2.32E-05	181

Note: Diseases or Functions Annotation terms in red font are commonly shared by F0 and F1 generation.

Figures

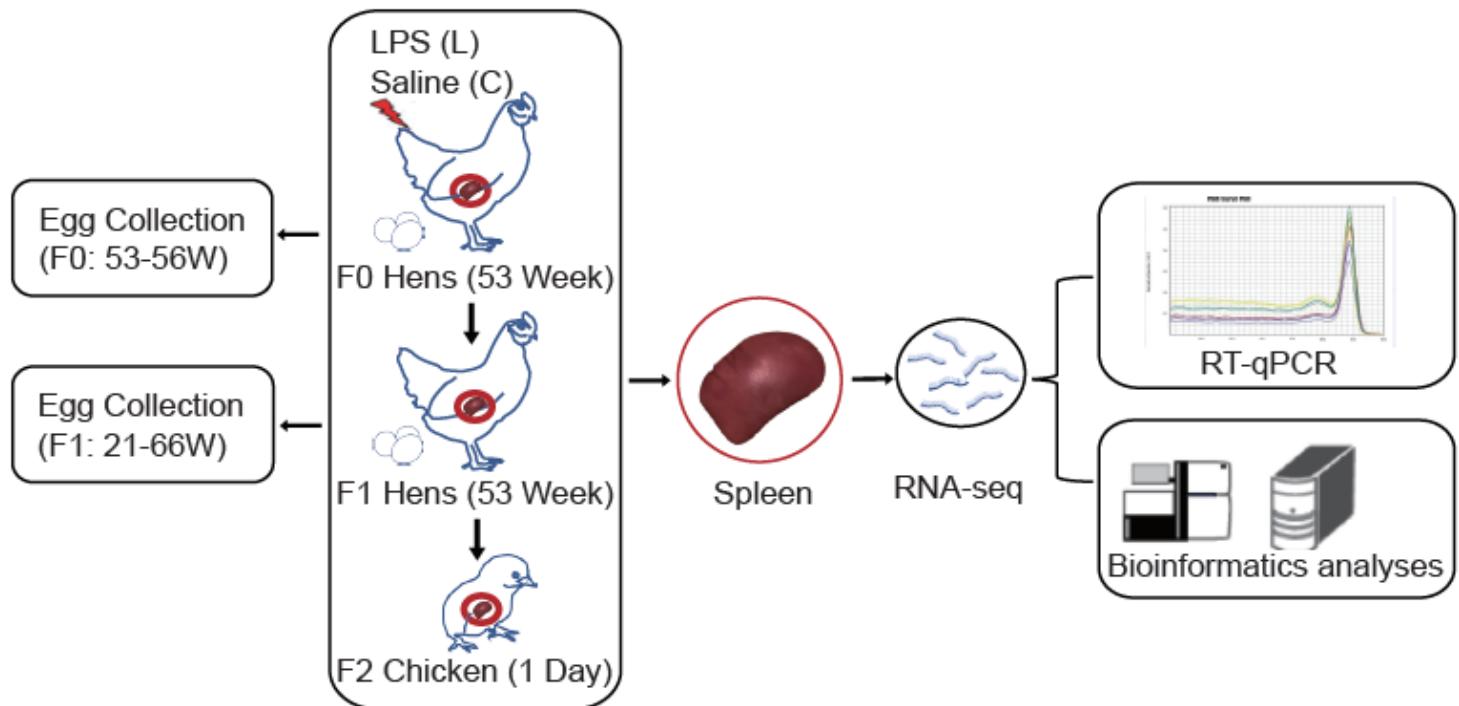


Figure 1

The technical route of the study caused by the LPS maternal stimulation.

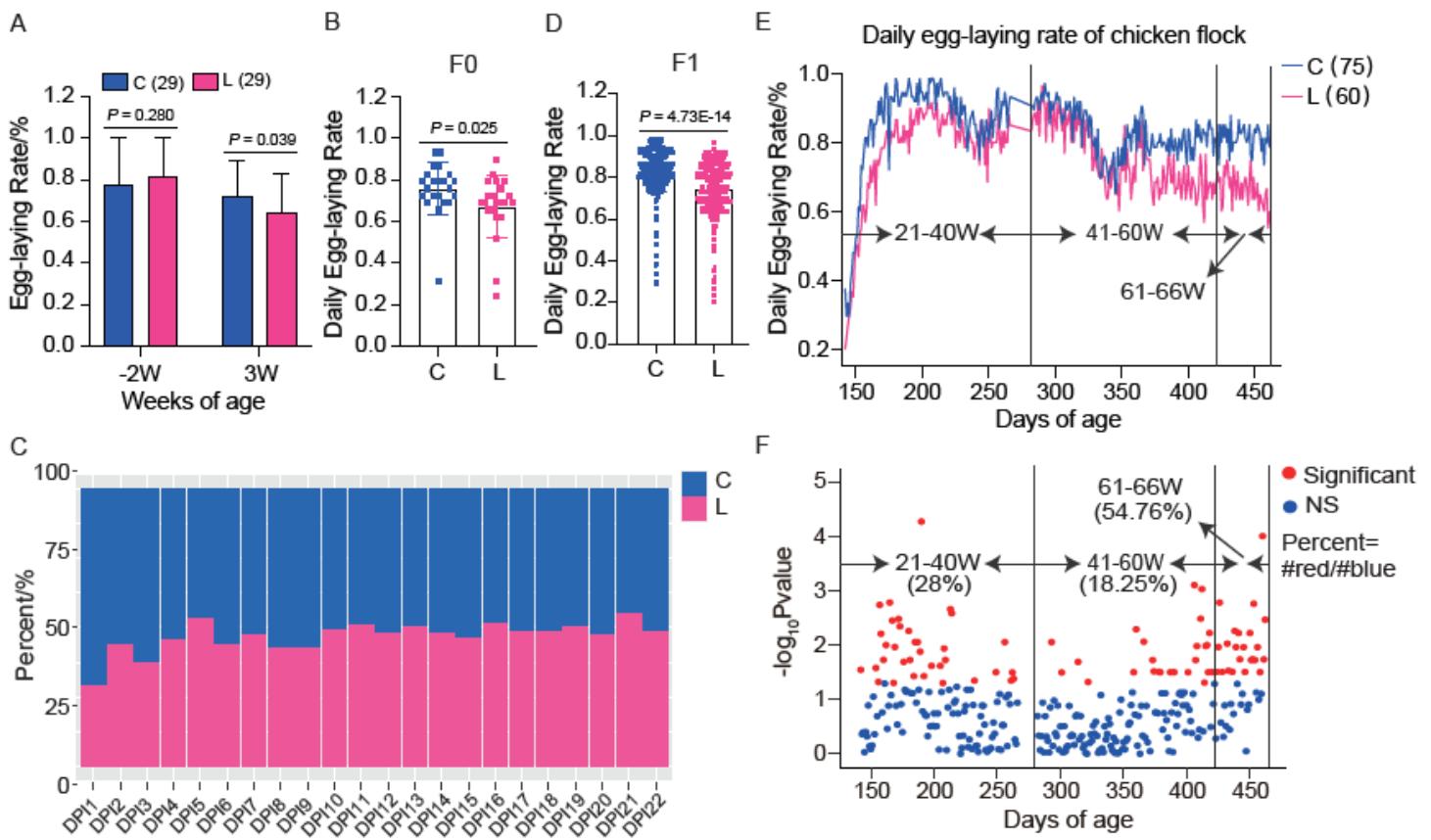


Figure 2

Egg-laying rate of hens in F0 and F1 generations. (A) Total egg-laying rate of F0 hens before (-2W) and post (3W) LPS treatment. -2W represents 2 weeks before LPS stimulation. 3W represents 3 weeks after LPS stimulation. The egg-laying rate (Y-axis) was obtained by dividing the total number of eggs laid by the theoretical total number of eggs. (B) Daily egg-laying rate of F0 hens population. The dots denote the daily egg-laying rate of the population in each group. A total of 22 consecutive days are investigated post the injection of F0 hens. (C) Percentage stacked bar of the daily egg-laying rate of F0 hens. The longer the bar, the higher the egg-laying rate of the group. (D) Daily egg-laying rate of F1 hens population. The dots denote the daily egg-laying rate of the population in each group. (E) Change trend of daily egg-laying rate of F1 hens population. (F) Significance chi-square test of daily egg-laying rate between group L and group C. Red dots represent significant difference ($P < 0.05$). The percentage in the bracket indicates the number of red dots divided by the number of blue dots. 21-40W: Early laying period, 41-60W: Middle laying period, 61-66W: Late laying period.

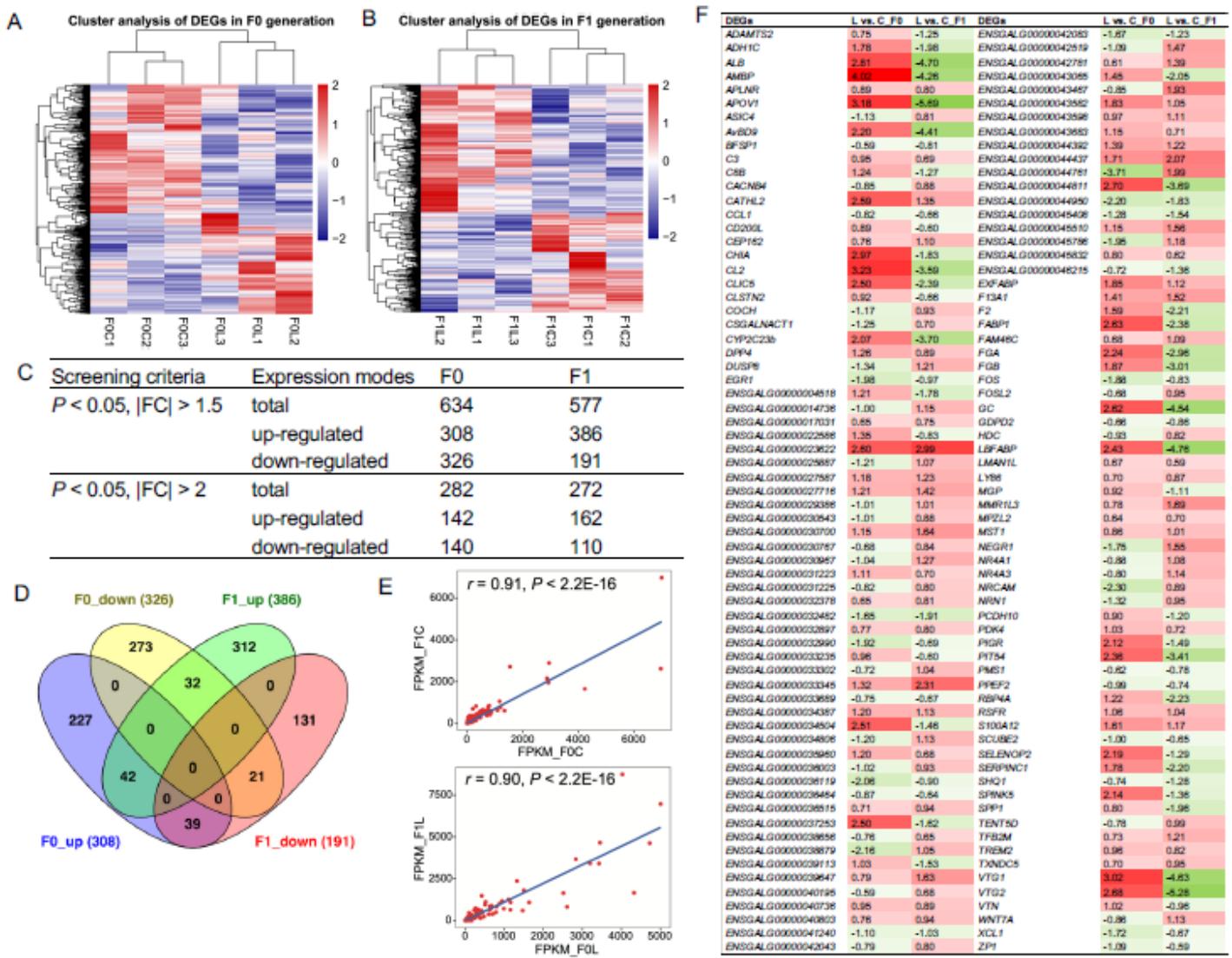


Figure 3

Comparative transcriptomic profiles of spleen tissue between LPS treated F0 hens and untreated F1 hens. (A,B) Cluster analysis of DEGs in F0(A) and F1(B) generations. (C) Statistics of the number of DEGs. (D) Venn diagrams of DEGs. (E) Regression analysis of the FPKM between F0 and F1 generations of different conditions. (F) Expression trend of DEGs in F0 and F1 generations. The number in the table represents log2FC, red and green numbers indicate the DEGs that were up-regulated and down-regulated, respectively, in the LPS group.

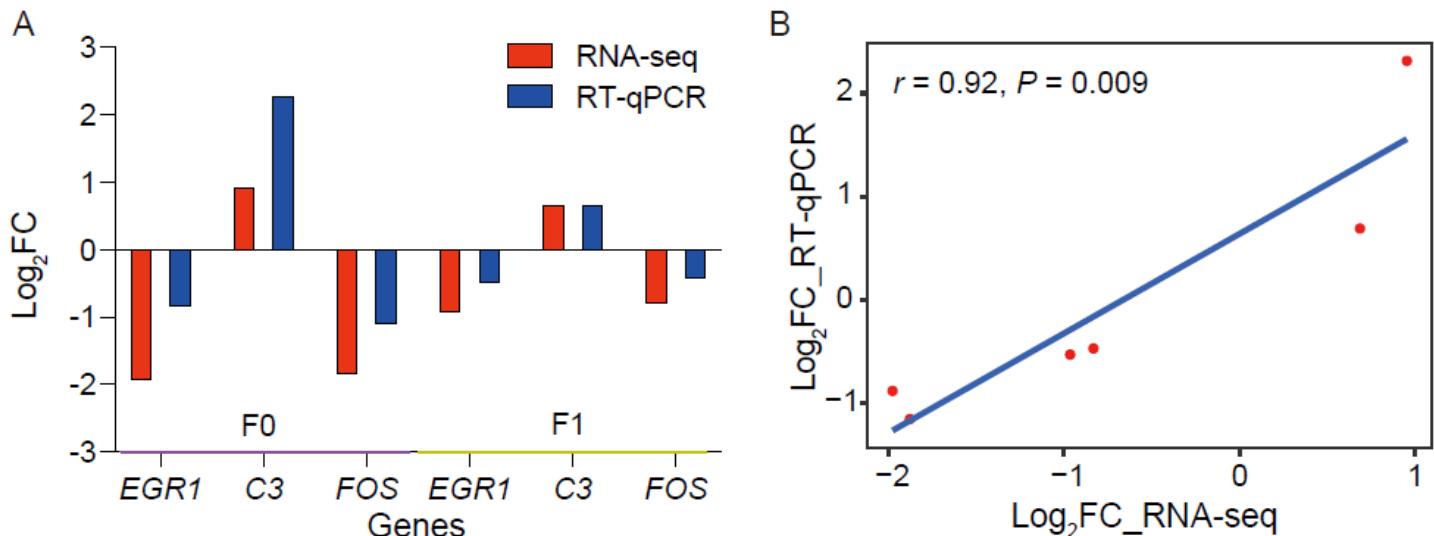


Figure 4

Validation of the accuracy of RNA-seq data by RT-qPCR. (A) Comparison of the result between RNA-seq and RT-qPCR with three randomly selected genes. (B) Regression analysis of the log₂FC values between RNA-seq and RT-qPCR validation of sequencing data.

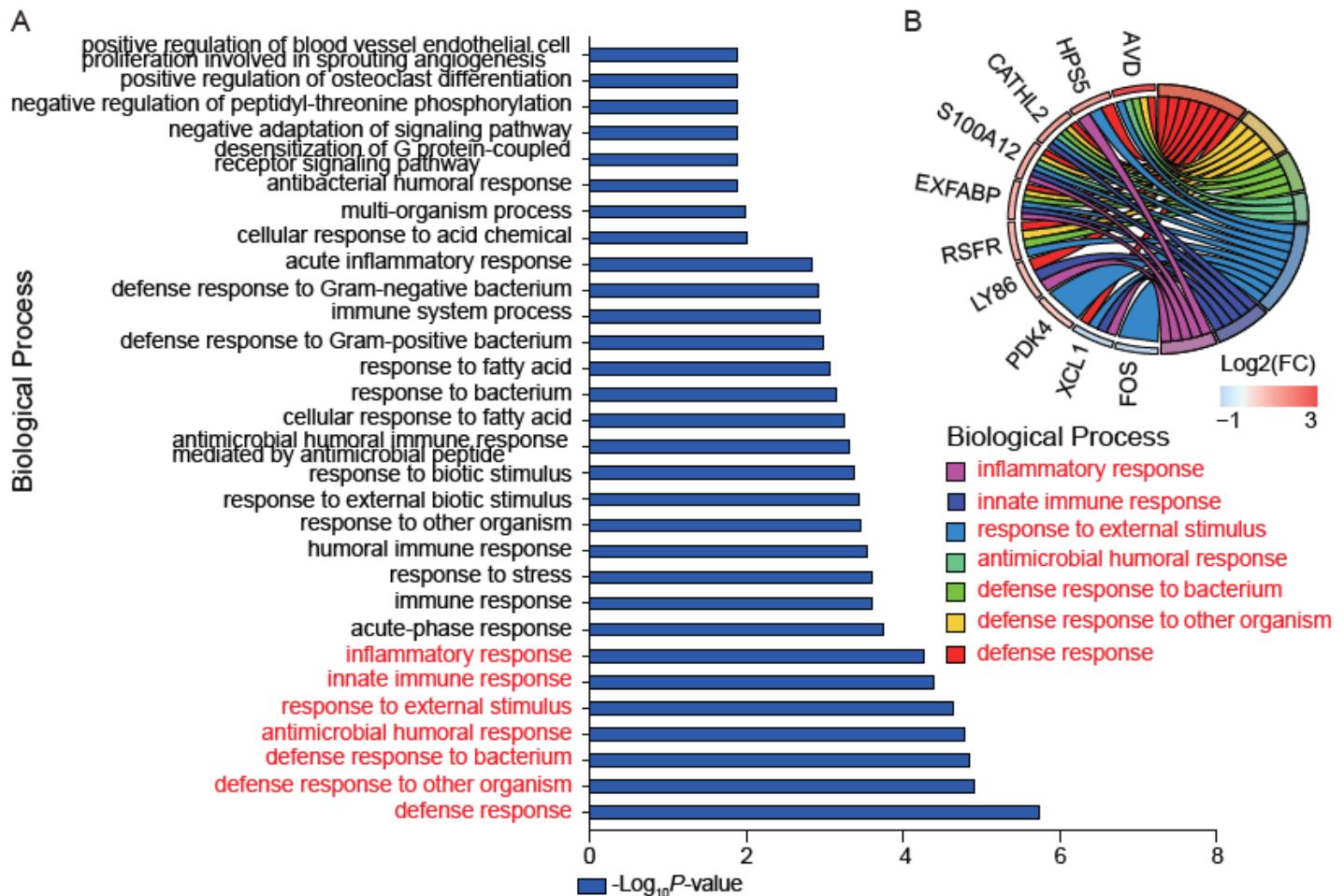


Figure 5

GO terms of biological process predicted with the 63 coDEGs showing the same expression trend in F0 and F1 generations. (A) Top30 significant GO terms of biological process. The terms in red font are the biological process with $FDR < 0.05$. (B) Goplot of the seven terms that were significantly associated with immune response ($FDR < 0.05$). The 10 DEGs listed around the left side of the circle represents DEGs that were mainly associated with these seven terms; red and blue denote the up-and down-regulated genes in the LPS group, respectively. The right side of the circle represents the terms of the biological process.

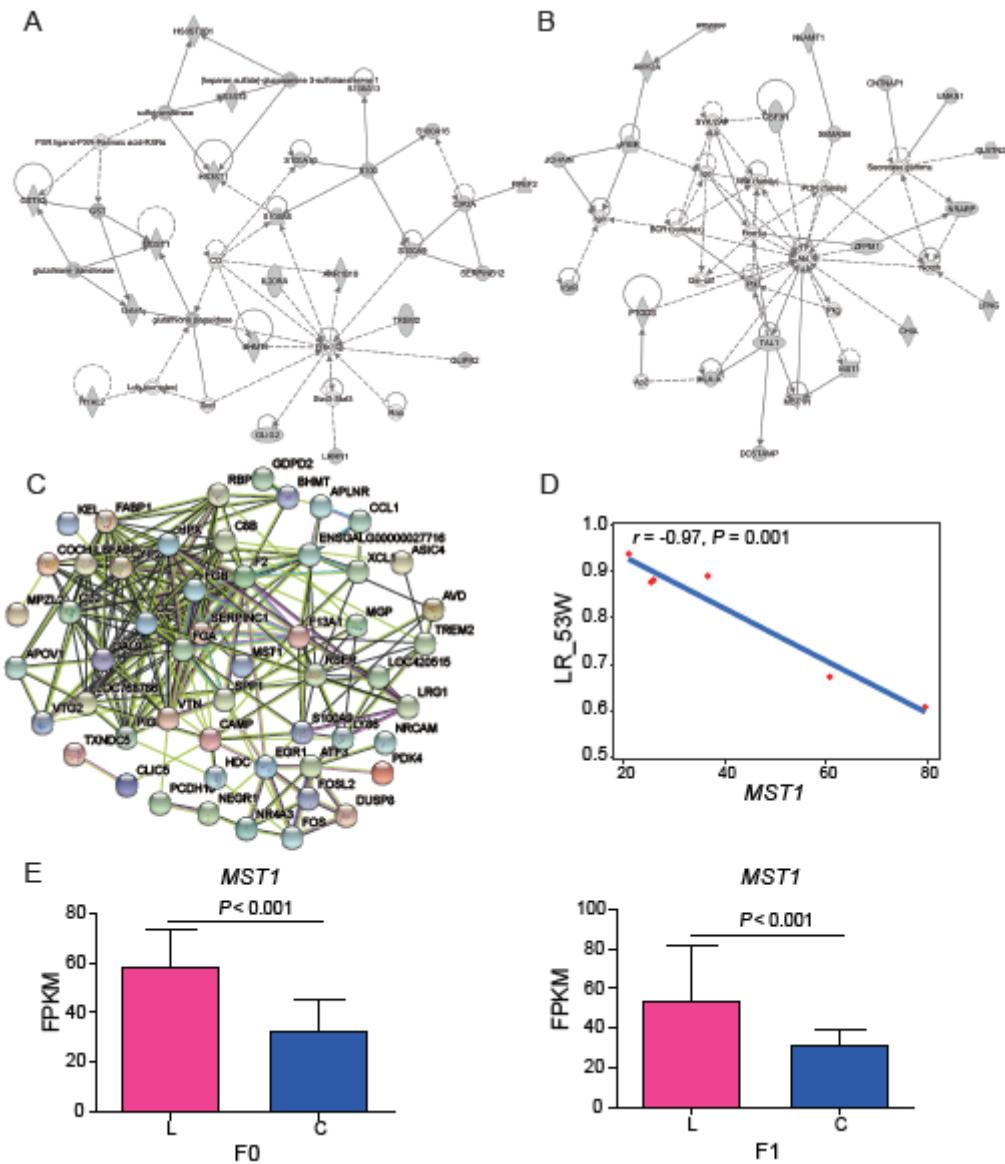
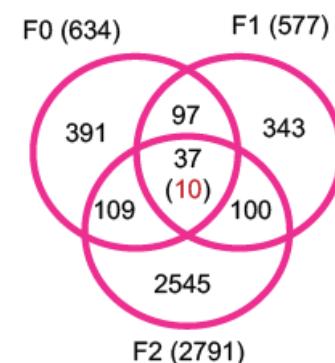
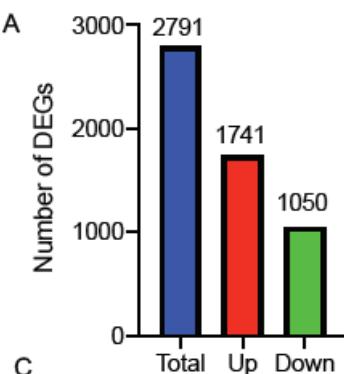


Figure 6

Interaction networks of differentially expressed genes in the F1 generation. (A,B) The first and third networks predicted with DEGs of the F1 generation. The top disease and functions enriched with the molecules in the first network are drug metabolism, glutathione depletion in liver and cell-to-cell signaling and interaction (A). The third network enriched in cell morphology, embryonic development and cellular development (B). (C) The PPI of common DEGs between the F0 and F1 generations. (D) Regression

analysis between FPKM of the gene MST1 with egg-laying rate at the age of 53 weeks in F1 generation.

(E) The expression level of MST1 in F0 and F1 generations.



C

L vs. C	F0	F1	F2	L vs. C	F0	F1	F2
<i>ADH1C</i>	1.78	-1.98	-1.40	<i>GDPD2</i>	-0.66	-0.86	-1.25
<i>AVD</i>	2.60	2.99	-2.41	<i>HDC</i>	-0.93	0.82	0.95
<i>C3</i>	0.95	0.69	0.99	<i>HPX</i>	1.35	-0.83	-2.50
<i>CEP162</i>	0.76	1.10	0.99	<i>KIAA0040</i>	-1.01	0.88	0.98
<i>CLIC5</i>	2.50	-2.39	1.53	<i>LOC100858647</i>	2.70	-3.69	-3.00
<i>ENSGALG00000030700</i>	1.15	1.64	-0.89	<i>LY6CLEL</i>	1.83	1.05	-3.99
<i>ENSGALG00000031223</i>	1.11	0.70	-1.46	<i>MANBAL7</i>	-1.09	1.47	0.84
<i>ENSGALG00000031225</i>	-0.82	0.80	1.07	<i>MMR1L3</i>	0.78	1.69	1.18
<i>ENSGALG00000032378</i>	0.65	0.81	-1.27	<i>NEGR1</i>	-1.75	1.55	-1.69
<i>ENSGALG00000034387</i>	1.20	1.13	-1.37	<i>NRN1</i>	-1.32	0.95	-0.90
<i>ENSGALG00000036119</i>	-2.06	-0.90	-2.47	<i>PDK4</i>	1.03	0.72	0.76
<i>ENSGALG00000036515</i>	0.71	0.94	-1.09	<i>PIGR</i>	2.12	-1.49	-4.91
<i>ENSGALG00000040736</i>	0.95	0.89	-1.03	<i>PPEF2</i>	-0.99	-0.74	-1.30
<i>ENSGALG00000040803</i>	0.76	0.94	-1.55	<i>SELENOP2</i>	2.19	-1.29	-1.50
<i>ENSGALG00000043683</i>	1.15	0.71	-1.36	<i>SPIK5</i>	2.14	-1.36	-7.01
<i>ENSGALG00000045832</i>	0.80	0.62	1.12	<i>VTN</i>	1.02	-0.96	-1.60
<i>EXFABP</i>	1.85	1.12	-0.59	<i>WNT7A</i>	-0.86	1.13	2.02
<i>F13A1</i>	1.41	1.52	1.42				
<i>FOS</i>	-1.88	-0.83	1.08				

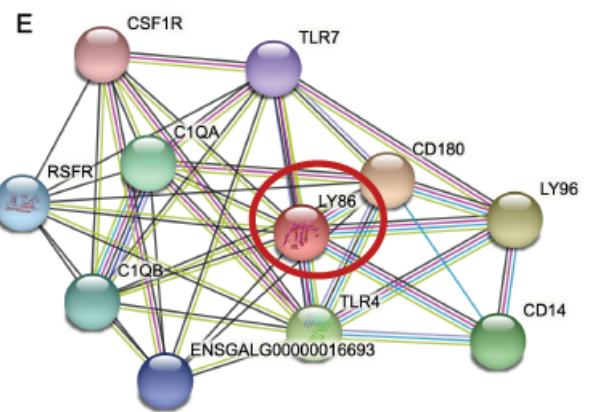
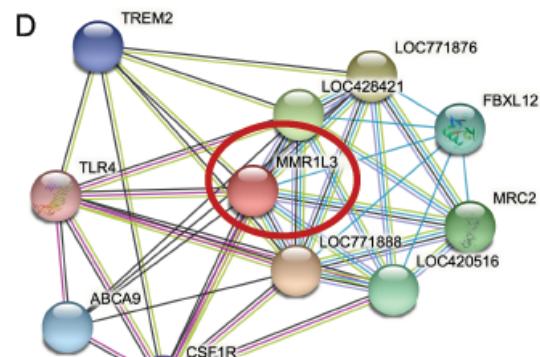


Figure 7

Common DEGs in the transcriptome of three generations of hens caused by maternal stimulation. (A) The number of DEGs in the F2 generation. (B) Venn diagram of the DEGs across three generations. (C) Common DEGs of the three generations of hens induced by maternal stimulation. The numbers indicate log2FC, positive values indicate up-regulated DEGs in the LPS group (the larger, the redder), negative values indicate down-regulated DEGs (the smaller, the greener), caused by maternal stimulation. The DEGs in the same color in the F0, F1 and F2 indicate the same expression trend across the three generations. (D,E) The PPI of MMR1L3 (D) and LY86 (E) predicted with STRING.

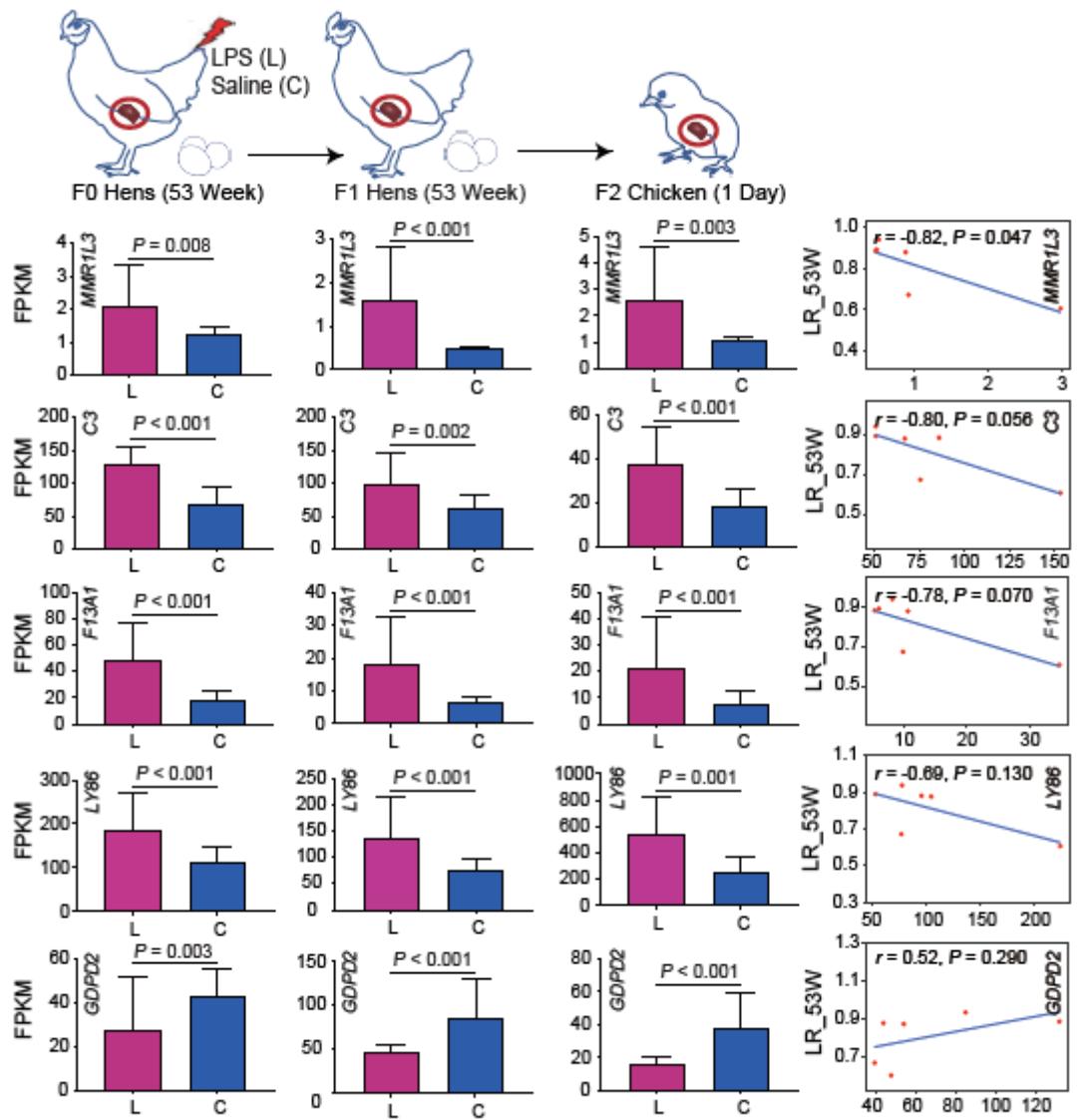


Figure 8

Correlation analysis between transgenerational heritable DEGs and egg-laying rate.

Supplementary Files

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- [FigureS1.pdf](#)
- [TableS1.xlsx](#)
- [TableS2.xlsx](#)
- [TableS3.xlsx](#)
- [TableS4.xlsx](#)
- [TableS5.xlsx](#)

- TableS6.xlsx