

# Transcriptomic and metabolomic insights into the variety of sperm storage in oviduct of egg layers

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**ABSTRACT** In birds, the sperm storage tubules (SST) are dispersed in uterovaginal junction (UVJ) and highly correlated with differential capacity of sperm storage (SS) in and among species with unspecified mechanisms. Here, the SS duration of 252 egg layer breeders was evaluated in 5 rounds with 3 phenotypic traits to screen high- and low-SS individuals, respectively, followed with transcriptome of UVJ tissues and metabolome of serum (high-SS vs. low-SS) to decipher the candidate genes and biochemical markers correlated with differential SS capacity. Histological characterization suggested slightly higher density of SST in UVJ (high-SS vs. low-SS). Transcriptome analyses identified 596 differentially expressed genes (336 upregulated vs. 260 downregulated), which were mainly enriched in gene ontology terms of homeostasis, steroid and

lipid metabolism and hormone activity, and 12 significant pathways ( $P < 0.05$ ) represented by calcium, steroid, and lipid metabolism. Immunohistochemical staining of GNAQ, ST6GAL1, ADFP, and PCNA showed similar distribution in UVJ tissues between 2 groups. Several candidates (*HSD11B2*, *DIO2*, *AQP3*, *GNAQ*, *NANS*, *ST6GAL1*) combined with 4 (11 $\beta$ -prostaglandin F2 $\alpha$ , prostaglandin B1, 7 $\alpha$ -hydroxytestosterone, and N-acetylneuraminic acid) of 40 differential metabolites enriched in serum metabolome were considered as regulators and biomarkers of SS duration in egg layer breeders. The integrated transcriptome and metabolome analyses of chicken breeder hens will provide novel insights for exploration and improvement of differential SS capacity in birds.

**Key words:** transcriptome, metabolome, sperm storage, uterovaginal junction, chicken

2021 Poultry Science 100:101087  
<https://doi.org/10.1016/j.psj.2021.101087>

## INTRODUCTION

Chicken is the most popular domesticated animal in the world which provides cheap and valuable proteins and nutrients from eggs and meat to nourish the human bodies. Many traits in association with egg performance and fertilization are applied to assess the reproductive capacity of hens. After natural mating or artificial insemination, the migration of spermatozoa in oviduct is an interesting and dynamic process including the sperm entering into the reproductive tract, resisting

the microenvironmental immunoreactions in the tract, temporarily maintaining in the sperm storage (SS) reservoir, sperm activation, and combination with oocytes after reaching the infundibulum (Publicover et al., 2007; Huang et al., 2011; Yang et al., 2020). Sperm storage is defined as temporary preservation of spermatozoa in female reproductive tract which is a crucial part in the complex reproductive process (Orr and Zuk, 2012). The prolonged SS is one of the key points to cut down frequency of artificial insemination and rooster usage for better profits in poultry breeding industry, including layer and broiler breeding. However, the hen's SS capacity is difficult to measure and usually described by some phenotypic traits instead (Supplementary Table 1). Among these, the days post-insemination until the last fertile egg (DN), the number of fertile eggs after insemination (FN), and fertility rate (FE) are widely used to delineate the SS efficiency of hens since 1990s (Brillard

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Received June 2, 2020.

Accepted February 26, 2021.

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and Antoine, 1990; Beaumont et al., 1992; Liu et al., 2008; Liu et al., 2015; Gu et al., 2017).

The phenomenon of long-term SS in female genital tract emerges extensively across a variety of vertebrate species (Holt and Fazeli, 2016), in association with either particular characteristics of spermatozoa or unique storage microenvironment, or both (Crichton et al., 1994). In birds, the occurrence of SS is highly dependent on a special tubular gland termed as sperm storage tubule (SST), which consists of tight collection of simple columnar epithelial cells (Bakst, 1987). The SST are small and short single-layered epithelial glands which are dispersed in the uterovaginal junction (UVJ) tissue, regarded as the primary site to temporarily keep the spermatozoa in hen's oviduct and highly associated with fertility (Degen and Hawes, 1972). Since the identification of SST in UVJ in 1960s, there are studies clarifying the mechanisms of long-term sperm maintenance in poultry oviduct (Froman, 2003). For example, researchers have found that the differential number of SST contributes to the varied duration of SS between broilers and turkeys (Bakst et al., 2010). The amount of sperm-filled SST in UVJ folds in breeding season of birds is associated with discrepant SS capacity among various species (Matsuzaki and Sasanami, 2017).

In addition to the morphological differences, several proteins and enzyme-coding genes are possibly involved in the SS in SST. Carbonic anhydrases (CA) are pH regulators to maintain spermatozoa quiescence by regulating carbonic acid buffer and acid-base balance (Holm and Ridderstrale, 1998; Swietach et al., 2007). CA may control the production and transportation of  $\text{HCO}_3^-$  with the cooperation of the solute carrier transporters to achieve the acidification of the epididymal fluid because the acidulous pH is requisite for sperm survival in rat epididymis (Levine and Kelly, 1978; Bernardino et al., 2019). It is supposed that CA are potentially involved in long-term SS in SST with similar mechanisms. Aquaporins (AQP) are possibly in association with the exchange of substances between sperm and SST epithelial cells (Zaniboni and Bakst, 2004). For instance, AQP8 is responsible for regulating sperm volume through water transfer in mice (Yeung et al., 2009), whereas AQP5 may upregulate and maintain cell volume in rat submandibular acinar cells (Hansen and Galtung, 2007). Consequently, AQP may facilitate SS via altering sperm volume through water transport between sperm and epithelium. Besides, recent investigations suggest the antiapoptotic proteins to be favored in long-term SS by regulating apoptosis (Urhausen et al., 2011; Le et al., 2015). The heat shock protein 70 is involved in spermatozoa migration toward the infundibulum after the release of sperm from SST in Japanese quail (Hiyama et al., 2014). The strong alkaline phosphatase activity in SST epithelia indicates the process for transferring lipids from SST to sperm (Bakst and Akuffo, 2007). Furthermore, L-lactic acid is reported to inactivate spermatozoa in the SST of Japanese quail for prolonged storage (Matsuzaki and Sasanami, 2017). The enriched metal elements, including calcium, zinc,

and potassium, in the lumens of SST are regulators associated with the sperm remaining in or releasing from SST (Holm et al., 2000).

The immune responses correlated with transforming growth factor  $\beta$  (TGF $\beta$ ), Toll-like receptors, antimicrobial peptide, and sialic acid are also potential factors to influence SS in SST. After insemination, the TGF $\beta$  (TGF $\beta$ 2, TGF $\beta$ 3, and TGF $\beta$ 4) and receptors in UVJ tissues present an increasing expression trend, which may assist the sperm survival in SST by inhibiting the anti-sperm immune responses (Das et al., 2006). The Toll-like receptor cascades potentially regulate the spermatozoa against the immunoreactions in oviduct of *Pelodiscus sinensis* (Liu et al., 2016b). The avian  $\beta$ -defensin-3 is supposed to have a positive effect on preventing spermatozoa from microbial infections in the female oviduct (Shimizu et al., 2008). Sialic acid is shown as a component of sperm glycocalyx in chicken and turkey, distributing widely on the surface of sperm (Pelaez and Long, 2008). Furthermore, sialic acid can conceal the antigenic sites of the sperm surface during the traveling of sperm in female vagina, which is necessary for providing sperm protection against vaginal immunoreactions in hen (Schauer, 1985; Steele and Wishart, 1996).

Hormones are suggested to be another regulator for the long-term SS (Roy and Krishna, 2011). The secretion of prostaglandin  $F_{2\alpha}$  from the cloacal gland leads to the contraction of vagina, which unfolds the access of SST and facilitates the spermatozoa to move into the SST in quail (Sasanami et al., 2015). Meanwhile progesterone is a pivotal factor in the sperm releasing from SST (Ito et al., 2011). It is suggested that androgens and androgen receptors may support the SS in *P. sinensis* oviduct owing to epithelial cilia (Liu et al., 2016a). The high level of androgen generated by the ovary and adrenal during SS in *Scotophilus heathi* implies potential roles of androgen in prolonging SS (Roy and Krishna, 2010). Consequently, the hormones, particularly steroid hormones, are important to support sperm survival during storage in female oviduct.

Sufficient energy, comprising carbohydrates, fat, and amino acids is essential to support all cellular events, including SS in female oviduct (Heifetz and Rivlin, 2010). In particular, lipid metabolism is vital for long-term sperm maintenance in SST. The lipid droplets containing triacylglycerol are regarded as energy storage center as well as resources for cell membrane formation in vertebrates (Brasaemle, 2007). The fatty acids are generated via the degradation of lipid droplets catalyzed by adipose triglyceride lipase in SST cells (Smirnova et al., 2006; Chandak et al., 2010), and released into SST lumen to prolong the longevity of spermatozoa (Huang et al., 2016).

The capacity of long-term SS in female reproductive tract provides a natural basis for the reproduction in asynchronous copulation and ovulation animals (Liu et al., 2016a). The improvement of SS duration could extend the interval of artificial insemination, which decreases the frequency of repeated mating, increases the animal welfare, saves the labor cost, reduces the usage

of roosters, and makes more profits for breeding farming of egg layers or broilers.

The assessment of differential capacity of SS duration currently has no settled criteria. With the integrated analyses of transcriptome and metabolome, our present study suggest to combine 3 traits (DN, FN, and FE) with genetic markers and biochemical indicators to evaluate the egg layers and select the outmost individuals with long SS duration as good breeders for better performance in commercial chicken breeder farming.

## MATERIALS AND METHODS

### *Experimental Animals and Ethics Statement*

A total of 300 of experimental egg layer breeders (commercial Jinghong egg layer, Beijing, China) aged 34 wk were raised one hen per cage in chicken farm until 56 wk. The experiment was designed 30 d as a cycle and repeated 5 times during the approximately 5-month experimental period. The layers were artificially inseminated with approximately 30  $\mu$ L of mixed semen, followed with recording of the egg production and egg fertility to evaluate the potentially high or low SS capacity each cycle (30 d per cycle). A number of 48 egg layers were excluded from the statistical analysis due to unexpected loss (35 layers) and poor egg production (13 layers) from the starting age to closing age. The statistics of traits of SS duration were performed and visualized using software GraphPad Prism 7.0. A total of 12 hens of high or low SS capacity, respectively, were artificially inseminated, sacrificed 3 d later and dissected to collect the UVJ tissues. The tissues were separated into 3 parts for immediate fixation in 4% paraformaldehyde (PFA) in PBS, frozen in liquid nitrogen, and embedded in OCT, respectively. The blood samples were collected to prepare the serum for further use. All the animals were kept under the standard conditions at approximately 18°C to 22°C (thermos-neutral temperature) and relative humidity (65–85%), and under 12 h light program. All the procedures were approved by the Standing Committee of Hubei People's Congress and the ethics committee of Huazhong Agricultural University.

### *Hematoxylin and Eosin Staining and Statistics*

The UVJ tissue samples fixed in PFA or embedded in OCT were processed for wax section or frozen section, respectively, in accordance with the standard procedures. The sections were stained with hematoxylin and eosin (H&E) to view the potential differences of UVJ tissues between high and low SS capacity individual egg layer. The sections were examined under microscopy and imaging system (Olympus, Japan). The number of SST was calculated per  $\text{mm}^2$  by ImageJ and graphed using software GraphPad Prism 7.0. The statistical analysis was performed using the Student t-test ( $n = 7$ ).

### *RNA Isolation and cDNA Synthesis*

The total RNA was extracted from UVJ samples with TRIzol reagent (Invitrogen) following the manufacturer's instructions and prepared for the transcriptome sequencing. The detailed procedures to assess the quality and quantity of RNA followed with the construction of cDNA library were described previously (Li et al., 2018; Nie et al., 2018). Briefly, the qualified RNA derived from 6 independent UVJ samples were applied to construct the cDNA library using NEBNext Ultra Directional RNA Library Prep Kit for Illumina (NEB) and assessed the quality on the Agilent Bioanalyzer 2100 system. The index-coded samples were clustered on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumina) and sequenced on the Illumina NovaSeq 6000 platform with 150 bp paired-end reads (Novogene, China).

### *Transcriptome Sequencing Assembly and Mapping*

The RNA sequencing reads were processed for quality assessment by removing the adapter and low-quality sequences to gain the clean reads followed with mapping to chicken reference genome (version: galGal6.0, [ftp://ftp.ensembl.org/pub/release-102/fasta/gallus\\_gallus/dna/Gallus\\_gallus.GRCg6a.dna.toplevel.fa.gz](ftp://ftp.ensembl.org/pub/release-102/fasta/gallus_gallus/dna/Gallus_gallus.GRCg6a.dna.toplevel.fa.gz)) and assembling transcripts using TopHat2 (version: V2.0.9), Scripture (beta2), and Cufflinks (v2.1.1) software. The expression level of each mapped transcript was estimated fragments per kilobase of transcript per million fragments mapped in each sample to obtain differentially expressed genes (DEG) (Trapnell et al., 2010). The differential gene expression analysis was performed with R package DESeq. The threshold of differentially expressed transcripts with significant differences in 3 biological replicates was set as  $P$ -value  $< 0.05$  and  $|\log_2(\text{fold change})| > 1$  (Supplementary Table 2).

### *Bioinformatics Analyses*

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses of DEG were implemented by KOBAS 3.0 (<http://kobas.cbi.pku.edu.cn/kobas3/?t=1>) and visualized by R (version 3.6.3). Gene Ontology terms and KEGG pathways with  $P$ -value  $< 0.05$  were considered as significant enrichment (Supplementary Tables 3 and 4). The STRING database (<https://string-db.org/>) was used to construct mRNA-mRNA interaction network of DEG with the visualization of the interaction network by Cytoscape software (version 3.6.1).

### *Immunohistochemistry*

Immunohistochemistry was conducted to localize the expression of genes of interest in UVJ tissue samples. The UVJ tissues fixed in PFA were dehydrated, embedded with wax, and processed sections with 5  $\mu$ m

thickness. The sections were gone through dewaxing with xylene, antigen retrieval with sodium citrate buffer (pH 6.0  $\pm$  0.1), subsequently incubated with specific primary antibodies (GNAQ, rabbit, Thermo Fisher, PA5-79318, 1:2000; ST6GAL1, mouse, Abcam, ab77676, 1:200, UK; proliferating cell nuclear antigen [PCNA], rabbit, Servicebio, GB11010, 1:2000, China; adipocyte differentiation-related protein [ADFP], rabbit, Huabio, ET1704-17, 1:1000, China) overnight at 4°C. The sections were then incubated with secondary antibody (Proteintech, China) for 1 h at room temperature and stained by using DAB (1:50) followed with hematoxylin counterstain. The stained sections were sealed and recorded as images with microscopy and imaging system (Olympus, Japan). The experiments were performed at least twice in 2 individuals each group per antibody staining.

### **Oil Red O Staining**

The UVJ tissues were embedded in OCT and cut into 10  $\mu$ m frozen sections in accordance with the standard procedures. The sections were stained with Oil Red O to detect lipids in UVJ tissues. Sequentially, the sections were processed into drying, 4% PFA fixation, dehydration with absolute propylene glycol, staining in pre-warmed Oil Red O solution, differentiation in 85% propylene glycol solution, rinsing, and counterstaining in hematoxylin solution. The sections were photographed under microscopy and imaging system (Olympus, Japan).

### **Blood Serum Metabolome**

The serum collected from the egg layers were labeled and sent for metabolome analyses (Novogene, China) following the standard procedures. LC-MS/MS analyses were processed using a Vanquish UHPLC system (Thermo Fisher) coupled with an Orbitrap Q Exactive series mass spectrometer (Thermo Fisher). The accurate qualitative and relative quantitative results of each metabolite were performed using the Compound Discoverer 3.1 (CD3.1; Thermo Fisher) and matched with the mzCloud (<https://www.mzcloud.org/>). Metabolites were annotated using the KEGG database (<http://www.genome.jp/kegg/>), Human Metabolome database (<http://www.hmdb.ca/>), and LIPID MAPS database (<http://www.lipidmaps.org/>). Principal components analysis and partial least squares discriminant analysis (PLS-DA) were performed using metaX software. The threshold of differential metabolites was set as *P*-value of < 0.05, fold change >1.2 or fold change <0.833, and variable importance in the projection (VIP) > 1.0. The cluster and correlation analyses of differential metabolites were calculated and plotted in R language. The metabolites were applied for routine enrichment analyses using the KEGG database.

### **Statistical Analysis**

All statistical analyses were carried out by using IBM SPSS Statistics21.0 (International Business Machines Corp., Armonk, NY). *P*-value of < 0.05 was considered to be statistically different.

## **RESULTS**

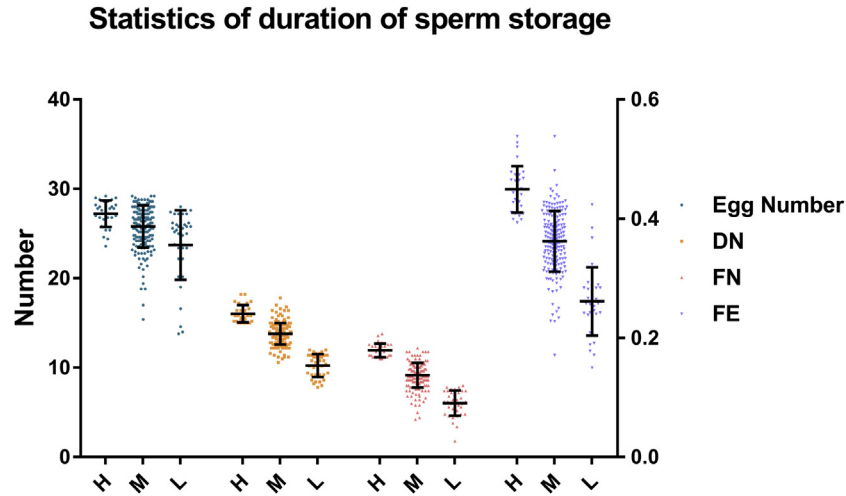
### **Evaluation of Sperm Storage Capacity and Morphological Characterization of UVJ Tissues of Layer Breeders**

Three parameters including i) the days after insemination until the last fertile (DN), ii) the number of fertile eggs laid after artificial insemination (FN), and iii) fertility rate (FE) which are highly related to performances of egg production and fertility were applied to evaluate the differential capacity of SS of layer breeders. A total of 252 experimental layer breeders were categorized into high, medium, and low performance groups in association with SS capacity based on the mean of DN and FN of 5 repetitions. In accordance with the intersection of the foremost and last of DN and FN, the layer breeders with DN  $\geq$  15 and FN  $\geq$  11 were classified as individuals with high SS capacity (high-SS group, *n* = 30), whereas those with DN  $\leq$  12 and FN  $\leq$  8 were regarded as individuals with low SS capacity (low-SS group, *n* = 36). The rest of the layer breeders were classified into medium-SS group (*n* = 186) as presented in Figure 1. The DN, FN, and FE traits of each layer breeder were categorized, grouped and compared with show the significantly decreasing (*P* < 0.05) performance from high to low groups in these 3 traits, respectively (Figure 1). The records showed that DN, FN, and FE were approximately 6 d longer (16.04 vs. 10.24), 6 eggs more (11.93 vs. 6.03), and 19% higher (44.94 vs. 26.14), respectively, in egg layers of high-SS group compared with low-SS group (Table 1). The high and low groups were the main collection used to further address the potential morphological and molecular differences in association with the differential duration of SS among individual hens.

The UVJ tissues which were collected from individuals with high and low SS capacity were subsequently sectioned and stained with H&E procedures. The morphology on the sectioned UVJ tissues did not display clear differences between the high and low individuals, whereas the density of SST showed higher trend (*P* = 0.044) in UVJ tissues of high SS individuals than those of low individuals (Figure 2).

### **Exploration of Candidate Genes Associated With the Differential Sperm Storage Capacity in Layer Breeders**

**Screening the Differentially Expressed Genes** To further address the molecular differences regulating the potential SS capacity, the UVJ tissue samples which



**Figure 1.** Scatter plot of duration of sperm storage in experimental hen population. Scatter plot was based on the mean of 5 rounds of experiments. Abbreviations: DN, the days after insemination until the last fertile egg; Egg number, total number of eggs; FE, fertility rate; FN, the number of fertile eggs after insemination. Data are presented as mean  $\pm$  SD; H, high-SS group (n = 30); L, low-SS group (n = 36); M, medium-SS group (n = 186); SS, sperm storage.

were collected from the high-SS and low-SS individual hens were applied for RNA-sequencing program (n = 3). The generated sequencing data were gone through routine bioinformatics analyses to gain 24,356 transcripts and 596 DEG including 336 upregulated and 260 downregulated transcripts compared the high-SS group with the low-SS group (high vs. low) with the thresholds of  $P < 0.05$  and  $|\log_2(\text{fold change})| > 1$  (Figure 3A and Supplementary Table 2).

**Gene Ontology Terms and KEGG Pathway Analyses of the DEG** The 596 DEG were applied for GO term and KEGG analyses. The top 30 GO terms among 297 significantly enriched terms ( $P < 0.05$ ) were classified into biological process, molecular function, and cellular components (Figure 3B). The functional analyses of DEG revealed that the GO terms were mainly enriched into 5 parts in association with homeostatic process and pH regulation, hormone secretion and transport, component of membrane, cell junction and adhesion, steroid and lipid metabolism, which were represented by chemical homeostasis (14 genes), hormone activity (8 genes), integral component of membrane (62 genes), cell adhesion (22 genes), steroid metabolic process (6 genes), and lipid metabolic process (17 genes), respectively (Table 2 and Supplementary Table 3). The enriched GO terms of steroid, lipid, and homeostasis suggest the important impacts of varied

microenvironment in UVJ tissues on the differential SS of hens, whereas the GO terms of membrane components, and cell junction and adhesion indicate the morphological or cellular differences in the epithelium of SST and UVJ tissues between the high-SS and low-SS egg layers.

The KEGG analyses were performed to gain insight into potential signaling pathways associated with SS capacity. A total of 12 pathways were statistically significant with  $P < 0.05$ . The foremost 20 of 109 pathways were displayed in Figure 3C. The pathways are consistent with the results of GO enrichment and mainly involved in steroid and fatty acid metabolism, hormone, receptor interaction, and ion signaling pathway (Table 3). For instance, the steroid biosynthesis (9 genes), steroid hormone biosynthesis (3 genes), biosynthesis of unsaturated fatty acids (3 genes), GnRH signaling pathway (2 genes), calcium signaling pathway (9 genes), neuroactive ligand-receptor interaction (14 genes), and amino sugar and nucleotide sugar metabolism (2 genes) were considered as promising candidates related to differences of SS capacity between high-SS and low-SS hens.

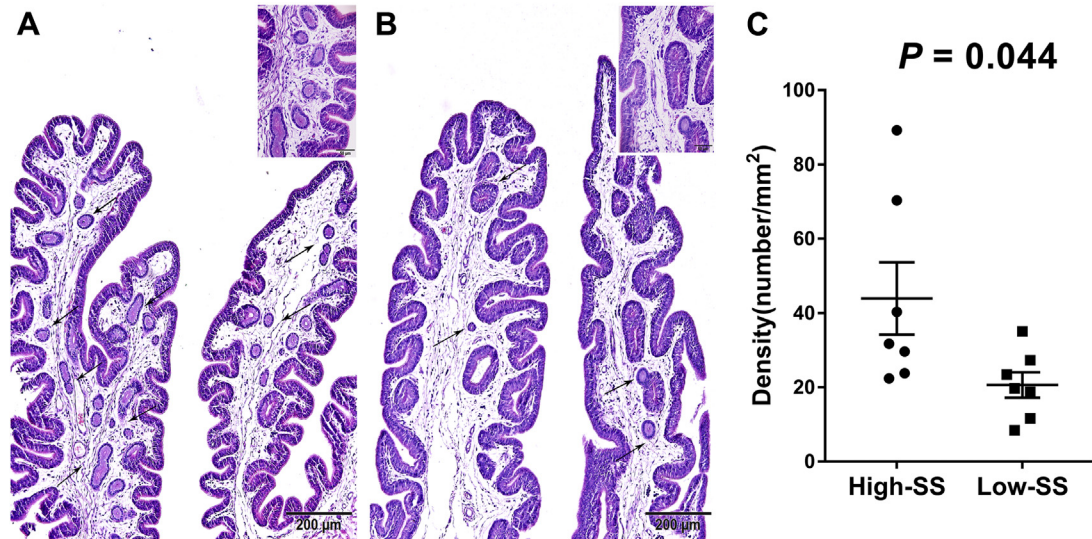
**Interaction Network Construction of DEG** To explore the connections among enriched core genes, an mRNA-mRNA interaction network of DEG was constructed and visualized in Cytoscape (Figure 3D). The genes including *SQLE*, *LSS*, *NSDHL*, *MOMS1*, *CYP51A1*, *FDPS*, *MVD*, *HMGCS1*, *INSIG1*, *FADS2*, *SCD*, *HSD17B7*, *DHCR24*, and *FDFT1* were grouped into a highly concentrated network which was involved in steroid and lipid metabolism, and steroid hormone biosynthesis. Besides, other genes such as *GNAQ*, *NPY*, and *FOS* were centered, respectively, to construct several minor networks correlated to regulation of ionic homeostasis, hormone activity, neuroactive interaction and immune in SST of UVJ folds. In addition to establish the complex networks in different pathways, the

**Table 1.** Measurement of the traits for sperm storage duration of egg layer breeders.

Traits	DN (days)	FN (eggs)	Fertility rate (%)
High-SS	16.04 $\pm$ 0.97	11.93 $\pm$ 0.77	44.94 $\pm$ 3.89
Low-SS	10.24 $\pm$ 1.29	6.03 $\pm$ 1.42	26.14 $\pm$ 5.73

The statistic analysis was based on the mean of 5 rounds of experiments. Data are shown by mean  $\pm$  SD,  $P < 0.05$ .

Abbreviations: DN, the days after insemination until the last fertile egg; FN, the number of fertile eggs after insemination; SS, sperm storage.



**Figure 2.** The H&E-stained UVJ tissue sections of egg layer breeders with high and low sperm storage capacity. Histological observation of UVJ tissues containing SST in (A) high-SS layer and (B) low-SS layer. (C) Descriptive statistics of SST density between high-SS and low-SS groups. The arrows point to SST. Abbreviations: H&E, hematoxylin and eosin; UVJ, uterovaginal junction; SS, sperm storage; SST, sperm storage tubule; A-B, Bar, 200  $\mu$ m. Data are showed by mean  $\pm$  SD ( $n = 7$ ),  $P < 0.05$ .

interconnection of these genes established the crosstalk among different pathways as well. For example, *PLA2G10L* has connection with *GNAQ* and *FADS2*. Combined with GO terms, KEGG pathways and interaction network, several DEG (*HSD11B2*, *NPY*, *AQP3*, *GNAQ*, and *NANS*) were picked up as candidate genes involved in regulating the different performances between the high- and low-SS groups.

### Histological Localization in UVJ

**Immunohistochemistry in UVJ of High-SS and Low-SS Layer Breeders** The enriched 2 DEG (ST6GAL1 and GNAQ) and 2 molecular markers (ADFP and PCNA) were further used to detect the protein distribution in UVJ tissues of high-SS and low-SS egg layer breeders.

ST6  $\beta$ -galactoside  $\alpha$ -2,6-sialyltransferase 1 (ST6GAL1) is a member of glycosyltransferase family 29. Using immunohistochemistry, ST6GAL1 protein was primarily localized at the microvillus border and in epithelial cytoplasm in both SST and UVJ (Figures 4A and 4B). However, no apparent expression differences of ST6GAL1 were found in UVJ between 2 groups.

*GNAQ* with the high expression fold change has been supposed as an important candidate gene. Abundant positive signals of G-protein subunit Galphaq (GNAQ) were localized at the epithelial cell membranes of SST and UVJ (Figures 4C and 4D). The immunohistochemical staining manifested strong and dispersive expression pattern of GNAQ in UVJ tissues. GNAQ was used to detect the potential differences in membrane components of UVJ between high-SS and low-SS hens. Unexpectedly, we did not find apparent differences between high-SS and low-SS groups. It indicates that the molecular cascades regulate the membrane parts of UVJ at the

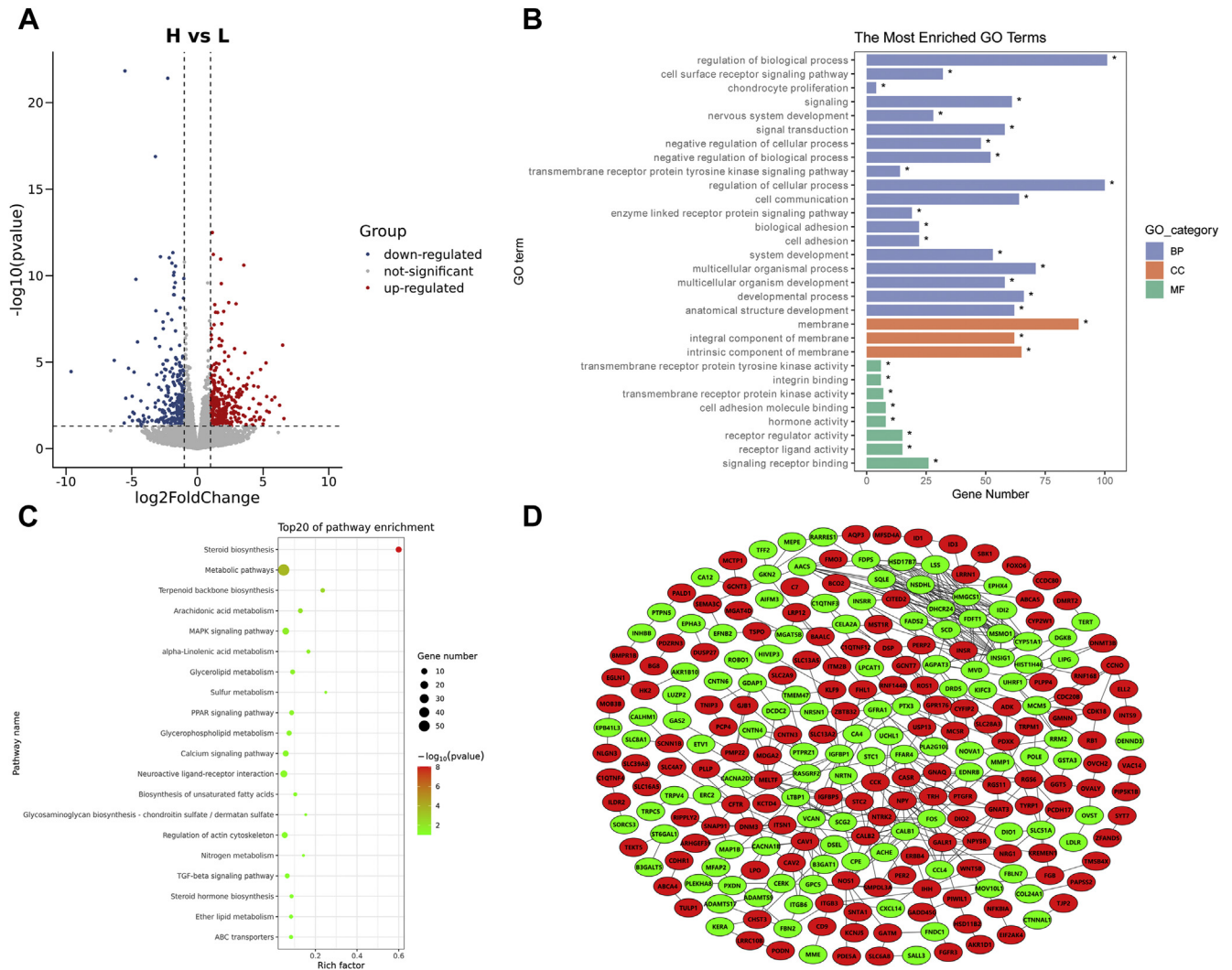
RNA level, not at protein level. This point needs further validation.

Immunohistochemical staining of the UVJ tissue sections derived from high-SS or low-SS hens showed that the ADFP signals were specifically positive in the SST luminal epithelial cells on both high-SS and low-SS UVJ sections (Figures 4E and 4F). Adipocyte differentiation-related protein, also known as PLIN2, is a specific and sensitive indicator for lipid accumulation. Such strong ADFP positive signals in SST lumen indicate that plenty of lipids are concentrated in or around the SST.

Next, the UVJ tissue sections of high-SS and low-SS were examined by immunohistochemistry for the detection of PCNA, a marker of cell proliferation state. The immunohistochemistry revealed the presence of PCNA in almost all epithelial cells including UVJ and SST (Figures 4G and 4H). These results show no clear differences in cell proliferation in UVJ tissues between high-SS and low-SS hens.

### Oil Red O Staining of UVJ Tissues of High-SS and Low-SS Layer Breeders

The frozen sections (10  $\mu$ m) of UVJ tissues that were embedded in OCT from the individuals of high-SS and low-SS groups were progressed for the Oil Red O staining. The red positive signals representing lipid droplets were observed in SST epithelial cells and UVJ tissues (Figures 5A and 5B). Abundant lipid droplets were localized at SST cells or surrounded the SST. Some lipid droplets were observed to diffuse in UVJ epithelial cells. The results of Oil Red O staining are consistent with the immunohistochemical localization of ADFP antibody (Figures 4E and 4F). The positive signals of lipid droplets exhibit higher trend in UVJ tissues of high-SS layer breeders compared with those of low-SS group.



**Figure 3.** The outcome of bioinformatics analyses of RNA sequencing of UVJ tissues from egg layer breeders with high and low sperm storage capacity. (A) Volcano plot depicted DEG enriched in UVJ tissues between high-SS and low-SS hens (H vs. L). The x-axis and y-axis represented the log<sub>2</sub> fold change and statistical significance ( $-\log_{10}$  of  $P$ -value), respectively. There were 596 DEG, including 336 upregulated (red, right) and 260 down-regulated (blue, left) between 2 groups. Transcripts with an absolute log<sub>2</sub> fold change  $>1$  and  $P < 0.05$  were set up as DEG. (B) The top 30 GO terms were presented in the enrichment analyses of DEG of UVJ between high-SS and low-SS hens. The blue, green, and orange columns represented the categories termed biological process, molecular function and cellular components, respectively. (C) The top 20 KEGG pathways were shown with the enrichment analyses of DEG of UVJ between high-SS and low-SS hens. Red indicates significant pathways and bubble size represented the number of DEG. (D) The mRNA-mRNA interaction networks were constructed by analyzing the DEG with String Database and Cytoscape. The red and green nodes represented upregulated and downregulated genes, respectively. Abbreviations: DEG, differentially expressed genes; GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; SS, sperm storage; UVJ, uterovaginal junction.

### Identification of Candidate Metabolites Related to the Differential Sperm Storage Capacity in Layer Breeders

**Quality Control, Principal Component Analyses of Serum Metabolome** To profoundly understand the molecular mechanism of SS in hen, the serum metabolome of the high-SS and low-SS individual hen was conducted to detect the whole biochemical variation in present study ( $n = 6$ ). As shown in [Supplementary Figure 1](#), quality control of the samples formed a cluster, which suggests a reliable detection system and data quality. The two-dimensional principal components analysis score plots displayed different overall trends of the metabolite distribution between high-SS and low-SS samples in positive ([Supplementary Figure 1C](#)) and

negative models ([Supplementary Figure 1D](#)). Besides, the clear separation of the metabolite between high-SS and low-SS groups was observed by performing the PLS-DA score plots in positive ([Supplementary Figure 1E](#)) and negative models ([Supplementary Figure 1F](#)), with the permutation test confirming the accuracy of PLS-DA models ([Supplementary Figure 2](#)). **Analyses of Differential Metabolites** To filter differential metabolites contributing to the SS between high-SS and low-SS groups, the volcano plots of metabolites in positive and negative models were generated ([Figures 6A and 6B](#)). Each spot in volcano plots represented an identified metabolite. The VIP value represented variable importance in the projection of the first principal component in PLS-DA models, indicating the importance of variance in different groups. A total of 40 metabolites (31 in positive

**Table 2.** Gene Ontology terms highly related to sperm storage enriched in uterovaginal junction of egg layer breeders with high and low sperm storage capacity.

Terms	Number	Gene
Calcium ion binding	13	SLC24A2 LRP8 IHH VCAN CALB2 FBN2 MYL10 CALB1 OVA CDHR1 PCDH17 MMP1 PLA2G10 L
Homeostatic process	17	SLC24A2 ANGPTL4 C1QTNF12 GALR1 CITED2 AQP3 STC1 MELTF CALCR VSIG1 FHL1 CALB1 STC2 CELA2A NOV CALB2 IGFBP5
Chemical homeostasis	14	SLC24A2 ANGPTL4 C1QTNF12 GALR1 AQP3 STC1 MELTF CALCR FHL1 CALB1 STC2 CELA2A CALB2 IGFBP5 CELA2A AQP3
Multicellular organismal water homeostasis	2	
Regulation of anion transport	3	STC1 TRH ROS1
Steroid metabolic process	6	INSIG1 NSDHL FGFR4 DHCR24 MSMO1 HMGCS1
Lipid metabolic process	17	FDPS PLPPR1 INSIG1 PLA2G10 L FADS2 VAC14 FGFR3 FGFR4 BCO2 DHCR24 MSMO1 NSDHL PIP5K1B CYP2C23b HMGCS1 TYRP1 AACCS ID3 PTGFR
Cellular response to fatty acid	2	VAC14 FGFR3 PIP5K1B
Lipid phosphorylation	3	ITGB6 CCN3 EPHA3 CNTN4 VCAN
Cell adhesion	22	CAV1 CDHR1 MELTF PERP2 NOV CITED2 MADCAM1 ITGB3 EFNB2 CTNNAL1 COL14A1 IL12 B NUA1 CD9 TMEM47 PCDH17 CNTN3
Hormone activity	8	C1QTNF12 STC1 CCK TRH STC2 ENSGALG00000043381 NPY INHBB
Secretory vesicle	7	CPE SYPL1 MME CALCR TRH CA4 ATP8B3
Positive regulation of hormone secretion	4	TRH C1QTNF12 GALR1 INHBB

model, 9 in negative model) were considered as differential metabolites compared the high-SS group with the low-SS group with selection criteria of  $P < 0.05$ , fold change  $> 1.2$  or fold change  $< 0.833$ , and VIP  $> 1.0$ .

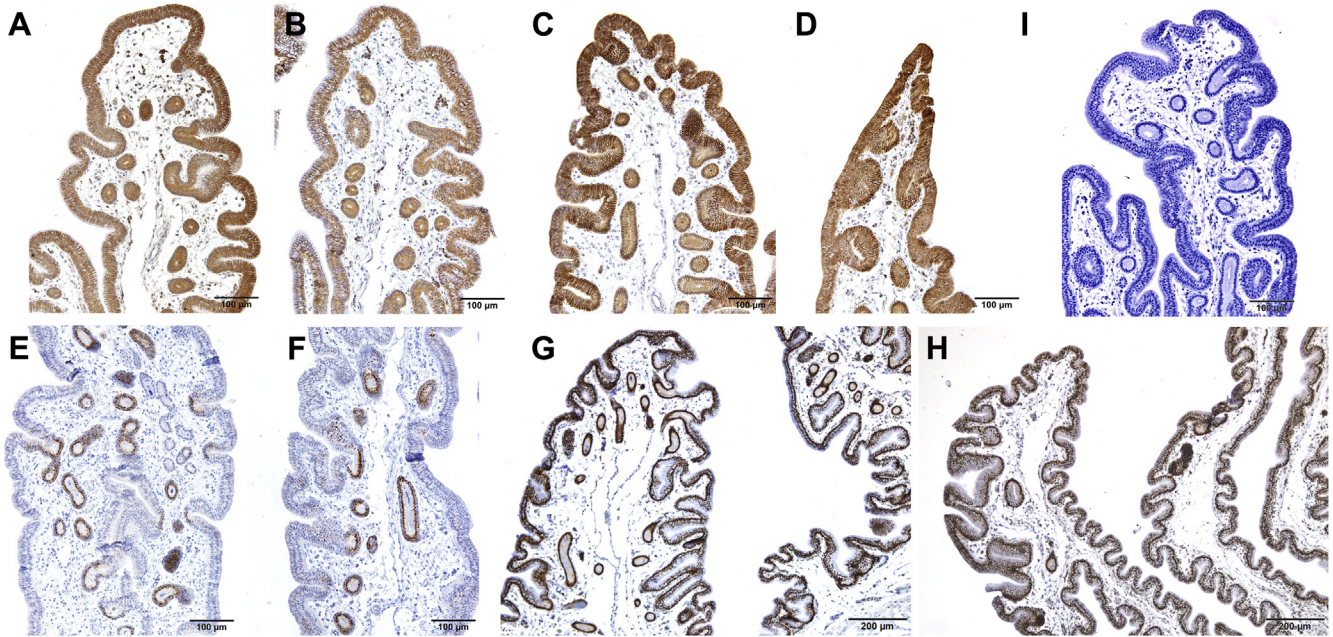
The heatmap analyses of the differential metabolites were used to visually observe the different expression pattern of compounds. [Figures 6C](#) and [6D](#) show that the expression level of serum metabolites in high-SS

**Table 3.** The prospective KEGG pathways in association with sperm storage enriched in uterovaginal junction of egg layer breeders with high and low sperm storage capacity.

Pathway	Number	Gene
Steroid biosynthesis	9	MSMO1 CYP2R1 LSS NSDHL CYP51A1 DHCR24 FDFT1 SOLE HSD17B7
Steroid hormone biosynthesis	3	AKR1D1 HSD11B2 HSD17B7
GnRH signaling pathway	2	PLA2G4F GNAQ
Arachidonic acid metabolism	5	TBXAS1 CBR3 PLA2G10L PLA2G4F GGT5
Alpha-linolenic acid metabolism	3	PLA2G10L FADS2 PLA2G4F
PPAR signaling pathway	5	MMP1 SCD ANGPTL4 FADS2 HMGCS1
Biosynthesis of unsaturated fatty acids	3	SCD FADS2 FADS1
Linoleic acid metabolism	2	PLA2G10L PLA2G4F
Fatty acid metabolism	3	SCD FADS2 FADS1
Calcium signaling pathway	9	DRD5 EDNRB GNAQ PTGFR ERBB4 NOS1 SLC8A1 ENSGALG00000015176 CACNA1B
Amino sugar and nucleotide sugar metabolism	2	NANS HK2
Glycerolipid metabolism	5	AKR1B10 AGPAT3 PLPP4 LIPG DGKB
Glycerophospholipid metabolism	6	PLA2G10L PLA2G4F DGKB PLPP4 AGPAT3 LPCAT1
Neuroactive ligand-receptor interaction	14	EDNRB GALR1 PTGFR TSPO CALCR MCHR2 NPY5R MC5R ENSGALG00000028895 CCK DRD5 NPY ENSGALG00000043381 TRH
Adipocytokine signaling pathway	2	NFKBIA NPY
Tight junction	3	TJP2 RAPGEF2 CFTR
Lysosome	2	LAMP3 ATP6V0A4

Abbreviations: KEGG, Kyoto Encyclopedia of Genes and Genomes; GnRH, gonadotropin-releasing hormone; PPAR, peroxisome proliferators-activated receptors.





**Figure 4.** Immunohistochemistry stained localization of ST6GAL1, GNAQ, ADFP, and PCNA in UVJ of egg layer breeders with high and low sperm storage capacity. (A-B) ST6GAL1 proteins were localized at epithelial cytoplasm in (A) high-SS and (B) low-SS hen UVJ. (C-D) The positive signals of GANQ antibody were localized at epithelial cell membranes in (C) high-SS and (D) low-SS hen UVJ. (E-F) The strong positive signals of ADFP antibody staining were converged on SST in (E) high-SS and (F) low-SS hen UVJ. (G-H) Positive PCNA signals appeared in all epithelial cells in (G) high-SS and (H) low-SS hen UVJ. (I) The negative control without incubating the primary antibody in immunohistochemistry. Abbreviations: SS, sperm storage; SST, sperm storage tubules; UVJ, uterovaginal junction; A-F and I, Bar, 100  $\mu\text{m}$ . G and H, Bar, 200  $\mu\text{m}$ .

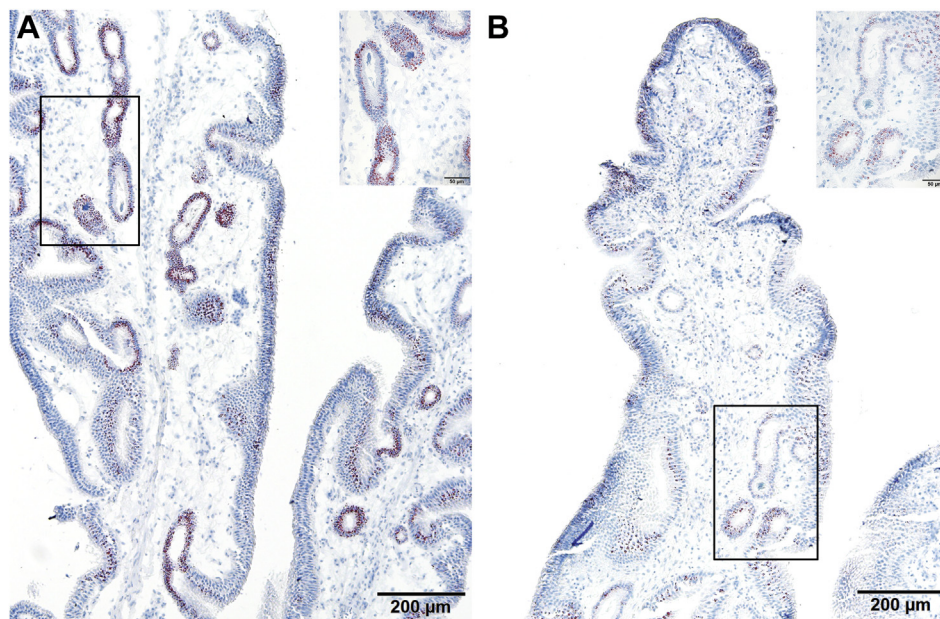
egg layer breeders was significantly different from low-SS group, particularly in hormones.

There are synergistic or exclusive relationships between different metabolites in serum. The correlation analyses of differential metabolites were conducted via Pearson correlation coefficient between metabolites (Figures 6E and 6F, Supplementary Table 5). Particularly, strong correlations were observed in

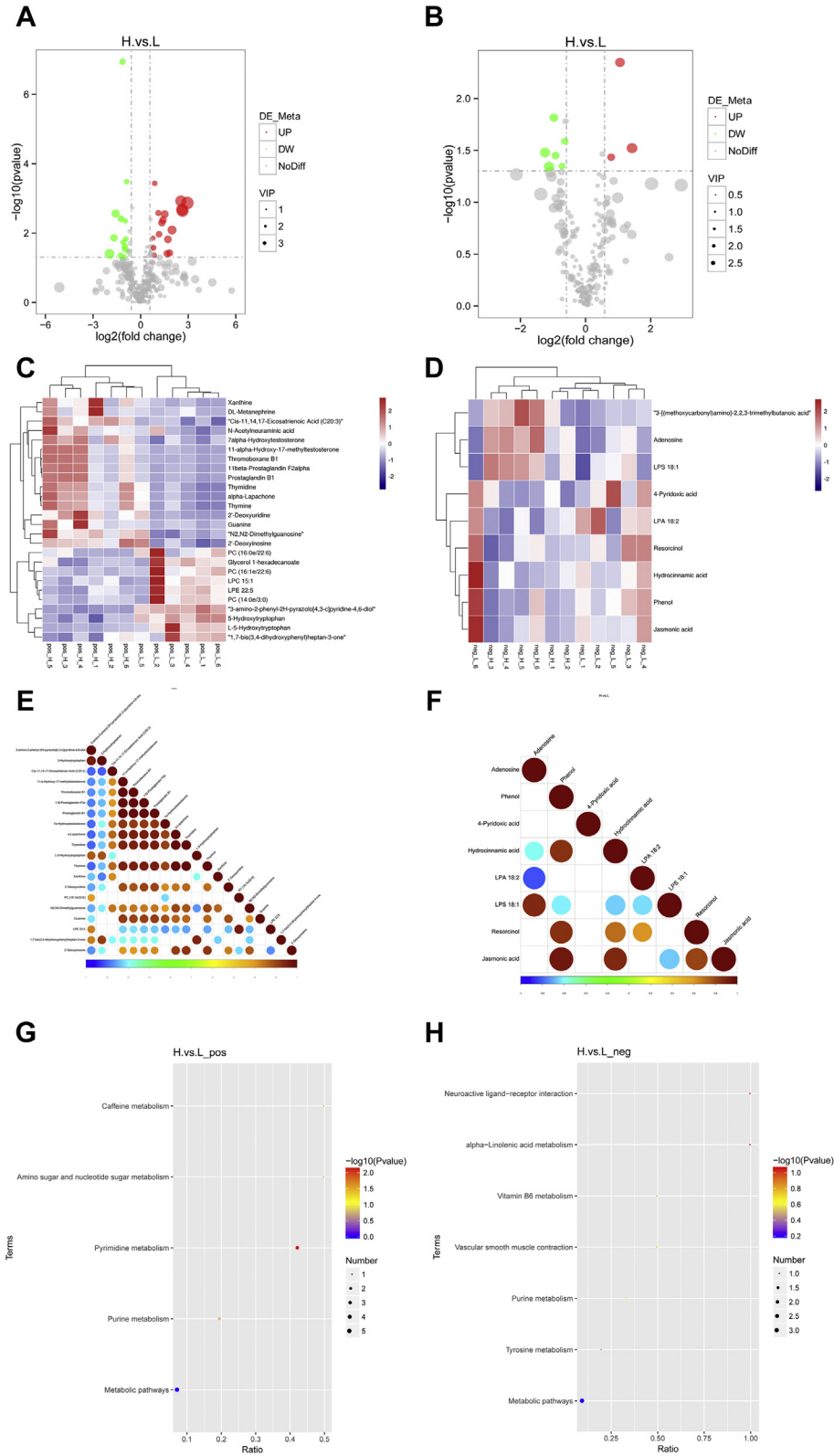
hormones (Figure 6E), including 11- $\alpha$ -hydroxy-17-methyltestosterone, thromboxane B1, 11 $\beta$ -prostaglandin F2 $\alpha$ , prostaglandin B1, and 7 $\alpha$ -hydroxytestosterone.

#### **Kyoto Encyclopedia of Genes and Genomes Enrichment Analyses of Differential Metabolites**

Functional and category annotation were performed for all identified metabolites based on KEGG, Human



**Figure 5.** Oil Red O stained UVJ tissues of egg layer breeders with high and low sperm storage capacity. The red positive signals which indicated lipid droplets were detected in UVJ containing mucosal SST. More lipid droplets were observed in UVJ tissues of (A) high-SS than that in (B) low-SS hens. Abbreviations: SST, sperm storage tubule; SS, sperm storage; UVJ, uterovaginal junction; Bar, 200 $\mu\text{m}$ .



**Figure 6.** The outcome of bioinformatics analyses of differential metabolites from serum of egg layer breeders with high and low sperm storage capacity. (A-B) Volcano plots depicted differential metabolites identified in high-SS and low-SS hens' serum. The x-axis and y-axis represented the fold change and statistical significance ( $-\log_{10}$  of  $P$ -value), respectively. There were 31 differential metabolites including 17 upregulated (red, right) and 14 downregulated (green, left) in (A) positive model, and 9 differential metabolites including 3 upregulated (red, right) and 6 downregulated (green, left) in (B) negative model between 2 groups. (C-D) Heat maps of differential metabolite clusters in the (C) positive and (D) negative models. The vertical clusters represented different samples, and the horizontal clusters represented different metabolites ranging from  $-2$  (blue) to  $2$  (red). (E-F) Correlation matrix of differential metabolites in the high-SS and low-SS hens' serum in the (E) positive and (F) negative models. Correlations are Pearson correlation coefficient ranging from  $-1$  (blue) to  $1$  (red). (G-H) KEGG enrichment analyses of differential metabolites between high-SS and low-SS hens. A total of 5 terms in (G) positive model and 7 terms in (H) negative model were enriched. Abbreviations: KEGG, Kyoto Encyclopedia of Genes and Genomes; SS, sperm storage; VIP, variable importance in the projection.

Metabolome, and LIPID MAPS databases (Supplementary Figure 3). The KEGG database annotation was set up as background to enrich the functional pathways for the abovementioned 40 differential compounds. A total of 5 and 7 pathways were identified in the positive model (Figure 6G) and negative model (Figure 6H), respectively. The KEGG enrichment analyses revealed that differential metabolites between high-SS and low-SS egg layer breeders were mainly involved in elementary endogenous molecules metabolic process, that were roughly coincided with transcriptomic results, like alpha-linolenic acid metabolism (including Jasmonic acid) and amino sugar and nucleotide sugar metabolism (including N-acetylneuraminic acid), which were considered to be associated with SS capacity.

## DISCUSSION

The proper evaluation of egg production of hens assures the selection of outmost individuals in breeding farming and industry of egg layers. The criterion to assess the production ability of hens remains uncertain. The duration of SS which is closely associated with the fertility is increasingly considered as a representative production index of egg layers, particularly of egg layer breeders.

The female genital tract plays an important role in regulating sperm motility and sperm survival after mating or artificial insemination. Uterovaginal junction is referred to the primary SS site and highly correlated with fertility in birds. Although the UVJ is observed in many birds, the molecular basis underlying the differential capacity of SS remains largely unexplored. In present study, the egg layer breeders were assessed for the egg production and fertility rate for 5 rounds with approximately 30 d per round. The layers were categorized into high, medium, and low groups based on 3 traits (DN, FN, and FE). The transcriptome of UVJ tissues containing SST and metabolome of serum derived from high-SS and low-SS groups were performed to further investigate the potential effectors for SS capacity. The enrichment of DEG in steroid, lipid, and hormone metabolic processes, combined with the identification of several metabolites in hen's serum including hormones (11 $\beta$ -prostaglandin F2 $\alpha$ , prostaglandin B1, and 7 $\alpha$ -hydroxytestosterone), lipids (Jasmonic acid), and N-acetylneuraminic acid, highly suggests that differential capacity of SS in egg layer breeders is partially regulated via steroids, hormones, lipids, and pH variation.

### **Influences of Hormone Activity for Duration of Sperm Storage**

The steroid hormones and receptors are expressed in SST cells (Yoshimura et al., 2000). The steroidogenic enzyme-related genes including *LSS*, *NSDHL*, *CYP51A1*, *MSMO1*, *DHCR24*, *FDFT1*, *FDPS*, *IDI2*, *MVD*, and *SQLE* were significantly enriched in GO terms and KEGG pathways, and highly clustered in the interaction networks (Figures 3B–3D), indicating

the important roles of steroid and subsequent hormone biosynthesis during female SS. Previous studies suggest that hormones are possibly one of the molecular factors in association with long-term SS (Roy and Krishna, 2011). Progesterone is the final product of steroid biosynthesis process (Shen et al., 2016) and regarded as one of the most reliable factors for assessing the SS in SST (Khillare et al., 2018). Progesterone is correlated with the structural development of SST via the activation of the receptors, and progesterone stimulates effusion of the stored spermatozoa from SST in Japanese quail (Yoshimura et al., 2000; Ito et al., 2011). Hence, high level of progesterone may lead to poor SS capacity, whereas inadequate level of progesterone possibly causes lower fertilization competence in laying hens. Interestingly, *FKBP5* gene, encoding nuclear receptor cochaperone which regulates the activity of steroid hormone receptors including progesterone receptors (Lussier et al., 2017), was over ten-fold in UVJ tissues containing mucosal SST in high-SS hens than low-SS hens (Supplementary Table 2). High level of *FKBP5* is supposed as a contributor of progesterone resistance in squirrel monkey (Hubler et al., 2003). The increased expression of *FKBP5* may occur via induction of various hormones including progesterone or androgens and have an important role in the dynamic process of the sperm uptaking into, maintenance within, and releasing from the SST (Hahle et al., 2019).

Besides progesterone, SS also depends on androgen. Many studies in bat fertilization event reveal a high level of androgen during female spermatozoa store (Abhilasha and Krishna, 1996; Roy and Krishna, 2010). An elevated concentration of testosterone agrees with SS stage in bats, indicating the androgen dependence of female SS (Roy and Krishna, 2011). Moreover, a distinct microenvironment with abundant androgen may support spermatozoa survival through maintaining cellular integrity (Jones, 2004; Roy and Krishna, 2011). We found that 2 derivatives of testosterone, 11- $\alpha$ -hydroxy-17-methyltestosterone and 7 $\alpha$ -hydroxytestosterone were upregulated in high-SS hens' serum than in low-SS group (Figure 6C), indicating pathways related to steroid metabolism are highly active. In addition, 3 key enzyme-encoding genes (*HSD11B2*, *HSD17B7*, and *AKR1D1*) were enriched in steroid hormone biosynthesis pathway (Table 3). *HSD11B2* encodes hydroxysteroid 11-beta dehydrogenase 2, which is regarded as a crucial enzyme responsible for conversion among testosterone derivatives (Kusakabe et al., 2003; Lee et al., 2017), and facilitates androgen synthesis in fish under temperature-induced condition (Baroiller and D'Cotta, 2016). The increased expression of *HSD11B2* in high-SS group suggests that the androgen as well as derivatives may have a positive impact during female SS (Supplementary Table 2). In addition, *NPY* encodes a 36-amino acid neuropeptide, which was enriched in the neuroactive ligand-receptor interaction pathway and partial GO terms involved in hormone activity (Tables 2 and 3), may be associated with female SS because of its important role in reproduction event

(Kalra and Kalra, 2004). *NPY* has a positive effect on androgen production in fish testis and can influence the secretion of luteinizing hormone in accordance with the ovarian steroid environment (Kalra and Crowley, 1984; Singh Nee Priyadarshini and Lal, 2018). Furthermore, *NPY* is supposed to suppress secretion of luteinizing hormone and follicle-stimulating hormone with the effects of Y5 receptor subtype (Raposinho et al., 1999). From the results of transcriptome, we found the significantly higher expression of *NPY* and *NPY5R* in UVJ tissues of high-SS hens than in those of low-SS hens (Supplementary Table 2), indicating that *NPY* may regulate the secretion of gonadal hormones (including androgen, progesterone, estrogen) through gonadotropic axis with involvement of *NPY5R*, which possibly contributes to discrepant SS capacity between 2 group hens.

Prostaglandins are candidate factors to regulate spermatozoa motility and penetration into SST through varying amount of ATP in sperm (Gottlieb et al., 1988). The live sperm facilitates prostaglandin synthesis and secretion in the epithelia of bovine reproductive tract (Kodithuwakku et al., 2007). Prostaglandin F<sub>2α</sub>, which exists in cloacal gland secretion and boosts fertilization in Japanese quail (Sasanami et al., 2015), may be in favor of sperm movement into SST effectively (Matsuzaki and Sasanami, 2017). The content of prostaglandins in tissues or serum depends on prostaglandin synthases and reductases by regulating the synthesis and degradation (Orr et al., 2015). The observation that *PTGR1*, prostaglandin reductase 1 encoding gene, displayed approximately 10 times lower expression in UVJ tissues of high-SS group compared with low-SS group (Supplementary Table 2), combined with over six-fold levels of 11β-prostaglandin F<sub>2α</sub> and prostaglandin B1 in serum of high-SS hens than low-SS hens in the current study (Figure 6C), suggests prostaglandins being a crucial biochemical marker causing differences of SS capacity.

In addition to sex steroid hormones and prostaglandins, thyroxines may be an important hormone for regulating reproductive function and sperm feature in domestic fowl (Kirby et al., 1996). Sperm penetration rate and period of duration both decline in hens' oviduct with treatment with thyroxine (T<sub>4</sub>) (Saemi et al., 2018). *DIO2*, an iodothyronine deiodinase 2 encoding gene, is responsible for T<sub>4</sub> to 3,5,3'-triiodothyronine (T<sub>3</sub>) conversion and regional synthesis of T<sub>3</sub> (Williams and Bassett, 2011), which regulates seasonal reproduction in birds (Ubuka et al., 2013). The reproductive function is severely impaired in zebrafish with *dio2* deficiency including a striking decrease of the duration of reproductive period and fertilization rate (Houbrechts et al., 2016), and upregulated steroid signaling in gonad (Houbrechts et al., 2019). Interestingly, the fertility defect in *dio2*-deficient individuals can be rescued partly through T<sub>3</sub> supplementation (Houbrechts et al., 2019), suggesting the positive impact of T<sub>3</sub> activity on reproduction. The observation of upregulated expression of *DIO2* with approximately 20 times in UVJ tissues of high-SS compared with that of low-SS hens in the

current study (Supplementary Table 2), indicating *DIO2* being an important candidate gene highly correlated with differential SS capacity via regulating thyroid hormone between high-SS and low-SS hens.

In short, the DEG enriched in hormone activity (represented by *NPY*), regulation of hormone secretion, steroid hormone biosynthesis (represented by *HSD11B2* and *HSD17B7*), and GnRH signaling pathway (represented by *GNAQ*) may influence the biosynthesis and secretion of hormones. The dramatic changes of interactions of steroidogenic enzyme genes and hormone regulatory genes in UVJ may lead to variation of steroid hormones, prostaglandins, and thyroxines, which are in association with the differential SS between high-SS and low-SS hens.

### **Balance Between Energy Supply and Peroxidation of Lipid Homeostatic State of Sperm Storage**

Another important factor supporting SS in SST is the lipid environment of UVJ tissue. Semen extenders contained fatty acids are used to facilitate long-term liquid store of boar semen (Waberski et al., 1994). Similar to semen supplements, the cellular secretions also provide fatty acids to sperm in female oviduct (Jakop et al., 2019). In *S. heathi*, a vespertilionid bat, the periods are approximately consistent between SS and body fat exhaustion (Roy and Krishna, 2012), indicating that SS in bat reproductive tract is possibly associated with fat consumption.

The metabolism of intracellular lipids is important for providing endogenous energy for resident sperm (Chen et al., 2019). The lipid droplets are found in the cytoplasm of epithelial cells in UVJ and SST (Fujii, 1963). Various lipid receptors are identified in SST cells, including very-low-density lipoprotein receptor and low-density lipoprotein receptor (Huang et al., 2016), which may be related to transportation of fatty acids to surrounding tissues (Tacke et al., 2001). Previous studies state the importance of unsaturated fatty acids in spermatogenesis (Stoffel et al., 2008), sperm transportation (Abi Nahed et al., 2014), and sperm capacitation (Murase et al., 2016), which coincide with the highly enriched unsaturated fatty acid metabolism in transcriptome and metabolome outcome of our study.

Apart from providing energy for sperm survival, the continuous synthesis and repair of phosphatidylcholine also results from fatty acid supply (Hossain et al., 2007). Recent study states that synthesis of phosphatidylcholine and phospholipid has positive impact on keeping sperm integrity during SS in female oviduct (Jakop et al., 2019). Therefore, lipids can not only provide metabolic energy for resident sperm but also serve as material components to hold plasmalemma stability of spermatozoa (Bakst, 1993). Interestingly, several phospholipids and relevant pathways were identified in the metabolomic profiling (Figure 6), and in the transcriptome analyses, including phosphatidylinositol

signaling system, and glycerophospholipid metabolism (Table 3).

However, another point of long-term SS is that sperm metabolism should be inhibited, thereby decreasing the requirement for substrates such as fatty acids (Roy and Krishna, 2011). Our results of lipid localization by immunohistochemistry of ADFP antibody and Oil Red O staining showed largely distributed lipids in the UVJ tissues containing SST (Figures 4E, 4F, 5A and 5B). The presence of abundant lipids in UVJ suggests the impacts of lipids on SS between high-SS and low-SS groups. In addition, the localization of ADFP is not limited to adipocytes but expressed in various cultured cell lines, such as endothelial and epithelial cells (Heid et al., 1998), which coincides with the mild ADFP positive signals in UVJ epithelium. Therefore, one possibility is that excessive lipids provide sufficient energy for sperm, which facilitates sperm metabolism and influences the quiescent state of spermatozoa during storage in SST. What is more, recent report suggests high level of polyunsaturated fatty acids may cause lipid peroxidation in avian sperm membrane (Wen et al., 2020). Exorbitant peroxidation may produce abundant reactive oxygen species, which is harmful for SS and fertility (Tremellen, 2008). Interestingly, several DEG associated with generation of fatty acids at the transcription level and lipids in serum presented a higher expression in low-SS group. It follows that lipid metabolism has an enormous effect on long-term SS in SST. However, whether and how the amount and type of lipids in UVJ containing SST influence female SS needs further investigation.

### **Regulation of Homeostatic Process and Subacid pH Facilitates Sperm Storage**

In general, the vitality of cells such as sperm is intimately bound up with the peripheral microenvironment, the exchange balance of various substances is also essential for SS. Aquaporins have a vital impact on maintaining osmolarity of sperm via adjusting fluid homeostasis in female reproductive tract during SS (Heifetz and Rivlin, 2010). Moreover, the movement of water between UVJ and SST lumens may boost sperm migration into SST (Froman, 2003). Up to now, a total of 13 diverse isoforms of AQP have been found in mammals (Carrageta et al., 2020), including 11 AQP isoforms identified in the reproductive system (Huang et al., 2006). As water channels, AQP 2, 3, and 9 present immunocytochemically positive staining in SST epithelia in turkeys (Zaniboni and Bakst, 2004). Here, we found that *AQP3*, which facilitates water homeostasis between sperm and local tissues, was enriched with higher mRNA expression level in the UVJ of high-SS group compared with low-SS group and potentially in favor of SS in SST (Supplementary Table 2).

Similarly, ionic homeostasis is related to maintain normal sperm function. The ion and proton exchange would change pH in a rapid and precise way between outside and inside of a cell (Pang et al., 2001). The alkaline pH can drastically raise sperm motility (Holm and

Wishart, 1998), whereas subacid pH in sperm reservoirs of the sow inhibits sperm motility (Rodriguez-Martinez, 2007). Thus, the ion exchange may effectively affect SS in SST, mainly by keeping sperm quiescence through variation in pH. Several pH regulatory genes such as *CA4*, *GNAQ*, *CFTR*, and *ATP6V0A4* were enriched in the present study. Carbonic anhydrases are the zinc metalloenzymes contributing to the  $\text{HCO}_3^-$  secretion in a variety of epithelia (Kaunisto et al., 1995), which play an important role in the regulation of acid-base balance.  $\text{HCO}_3^-$  is reported to have effects on stimulating sperm capacitation (Harrison and Gadella, 2005), whereas other studies indicate that delayed capacitation of sperm facilitates long-term SS in female reproductive tract (Krutzschnig et al., 1982). Slightly less signals of CA are localized at SST and vaginal epithelia compared with infundibulum (Holm et al., 1996), implying possibly negative effect of CA on SS. CA4, one of the 14 CA isozymes, participating in re-absorption and transport of  $\text{HCO}_3^-$  (Ekstedt et al., 2004), was expressed in lower level in UVJ of high-SS than low-SS group (Supplementary Table 2), which may lead to differences of SS capacity between 2 groups.

Besides the CA, the pH alteration of female reproductive tract also depends on the roles of various ion channels on epithelium and sperm membrane. As an important anion channel, cystic fibrosis transmembrane conductance regulator (*CFTR*) is widely expressed in female reproductive tract among species and involved in the transportation of  $\text{HCO}_3^-$ , which achieves through conducting  $\text{HCO}_3^-$  directly or exchange of  $\text{Cl}^-$  and  $\text{HCO}_3^-$  (Reddy and Quinton, 2003; Wang et al., 2003; Chan et al., 2006). It suggests that *CFTR* plays an important role in the secretion of  $\text{HCO}_3^-$ , which changes the lumen pH and sperm homeostasis subsequently. In fact, it is certain that transportation of ions between intra and extracellular spaces is vital for movement of fluid. Besides taking effect in  $\text{HCO}_3^-$  transport, *CFTR* is supposed to interact with AQP to maintain water homeostasis in rat testis (Jesus et al., 2014). Therefore, *CFTR*, a chloride channel encoding gene, plays an important role in pH homeostasis in reproductive system by fluid reabsorption (Chen et al., 2012). Furthermore, *CFTR* is considered as an essential factor for fertility in female and male. The mutation of *CFTR* induces cystic fibrosis, and infertility (Cutting, 2015). Notably, the *Gnaq* knockout mice suggest that Gq activity and Gq-PKC cascade are important for mediating *CFTR* and ADGRG2 coupling, which regulates  $\text{Cl}^-$  and pH homeostatic process for fluid reabsorption and male fertility (Zhang et al., 2018). An important member of G protein-coupled receptor, *GNAQ* is not only involved in gonad development, but also in spermatogenesis and maturation event in animals (Li et al., 2016). The aforementioned researches combined with increased expression levels of *CFTR* and *GNAQ* in high-SS group imply potentially collective roles of *CFTR* and *GNAQ* in sperm maintenance and movement.

In addition, the vacuolar-type  $\text{H}^+$ -ATPases also have an important effect on acid-base balance of eukaryotic cells. The V-ATPases, as a proton pump, mainly mediate

acidification of intracellular compartments by H<sup>+</sup> secretion and reabsorption (Stehberger et al., 2003), which is necessary for constructing an acidic pH environment in the epididymis and vas deferens (Pietrement et al., 2006). Notably, the ATP6V0A4, V-ATPase a4 subunit, has an important role in the maintenance of systemic pH because *Atp6v0a4* knockout mice present serious metabolic acidosis (Norgett et al., 2012). Besides, upregulated *Atp6v0a4* is detected along the entire uterine luminal epithelium in mouse, suggesting the underlying roles in female fertility (Xiao et al., 2014). Here, we found *ATP6V0A4* was increased at the transcription level in the high-SS group than that in low-SS group (Supplementary Table 2), suggesting *ATP6V0A4* may contribute to differences of SS capacity due to V-ATPase activity.

Interestingly, the expression level of AQP and V-ATPases present significantly reduction in epididymal tubules of *Cftr* deficiency mice (Ruan et al., 2014). It is now believed that pH maintenance through ion equilibrium, including HCO<sub>3</sub><sup>-</sup>, Cl<sup>-</sup>, and H<sup>+</sup>, is crucial for SS in the female reproductive tract. For example, HCO<sub>3</sub><sup>-</sup> with an overly high concentration may establish alkaline microenvironment which is adverse to sperm quiescence, whereas insufficient HCO<sub>3</sub><sup>-</sup> would result in lower sperm capacitation and impair sperm function. The slight change of oviductal pH would influence physiological state of sperm, which depends on the periods either sperm moving toward the SST, storing in SST or releasing from SST for fertilization. Therefore, we propose that a proper acid-base equilibrium and fluid movement in reproductive tract, which caused by ion and water homeostasis via cooperation of candidate genes including *AQP3*, *CA4*, *CFTR*, *GNAQ*, and *ATP6V0A4*, is a crucial factor contributing to different SS capacity between high-SS and low-SS hens.

### **Inhibition of Anti-sperm Immune Responses by N-Acetylneuraminic Acid**

It is reported that a barrier system may be built in SST for protection of sperm against immune reaction in female genital tract, including the sialic acid in potential anti-immune response process (Schauer, 1985; Steele and Wishart, 1996). The sialic acid, also named N-acetylneuraminic acid, was identified in our metabolome results, displaying a higher level in serum of the high-SS than in low-SS group (Figure 6C). Furthermore, as an amino sugar, N-acetylneuraminic acid was enriched into amino sugar and nucleotide sugar metabolism in KEGG analyses of metabolome (Supplementary Table 6), as well as the pathway in transcriptomic results represented by *NANS* and *HK2* (Table 3). N-acetylneuraminic acid is encoded by *NANS* gene and mediates the synthesis of sialic acid (van Karnebeek et al., 2016). The approximately two-fold expression level of *NANS* in high-SS hens contributes to more output of sialic acid (Supplementary Table 2), which coincides with the results of nearly 2 times of N-acetylneuraminic acid in serum of high-SS by metabolomic detection. In addition,

another candidate gene *ST6GAL1* encodes ST6 β-galactoside α-2,6-sialyltransferase 1 which is related to the level of sialic acid acidification via catalyzing the transfer of sialic acid residues (Noel et al., 2017). Establishment of balance of sialylation and desialylation is pivotal for maintaining sperm survival during SS in female reproductive tract (Ma et al., 2016). During SS in female oviduct, *NANS* and *ST6GAL1* potentially function in the event of sialic acid biosynthesis and acidification. However, it is uncertain that whether sialic acid plays a role in vagina or in UVJ. Further investigation is essential to speculate this point.

In summary, the DEG and metabolites which participate in steroid and subsequent hormone synthesis, lipid metabolism, chemical homeostasis, immune-modulation, and membrane components may contribute to the dynamic process of the female SS. Particularly, the hormones including testosterone class, prostaglandins, and thyroid hormones and regulatory genes (*DIO2* and *HSD11B2*) are assumed as important regulators which result in differential duration of SS for hens. Moreover, the DEG such as *AQP3* and *GNAQ*, which are in relation to pH regulation through water and ion homeostasis, may be other crucial candidates influencing the SS capacity of SST in UVJ. The N-acetylneuraminic acid and regulatory genes (*NANS*, *ST6GAL1*) are also regarded as considerable factors for supporting sperm survival in reproductive tract of egg layer breeders. The potentially varied membrane components in SST and UVJ are essential physiological structure in long-term SS and subsequently in fertility rate of layer breeders. The present assessment of transcriptome and metabolome contributes to further understanding of the mechanism of SS and provide new clue for prolonging SS duration in egg layer breeders.

### **ACKNOWLEDGMENTS**

This research was funded by the grants from National Key R&D Program of China (2018YFD0501301) and National Natural Science Foundation of China (No. 31972548). The authors are thankful to Huadu Yukou Poultry Industry Co. Ltd, China for providing the experimental chickens.

### **DISCLOSURES**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

### **SUPPLEMENTARY DATA**

Supplementary data associated with this article can be found in the online version at <https://doi.org/10.1016/j.psj.2021.101087>.

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