

Regulation of sperm storage capability in aged hens - a comparison using integrative multi-omics analysis

Running title: Multi-omics reveals sperm storage in aged hens

Jun-Jie Xu^{1,3}, Xin-Xin Li¹, Yan-Hui Li¹, Si-Han Guo⁶, Yi-Hao Fan¹, Xing-Lin Wu¹, Yi-Chun Yao¹, Rong-Cai Zhang¹, Xiao-Yu Zhao², Dan Su⁴, Er-Dong Sun⁵, De-He Wang¹, Yi-Fan Chen¹, Er-Ying Hao¹, Hui Chen^{1,*}, Lei Shi^{1,*}

¹College of Animal Science and Technology, Hebei Agricultural University, Baoding, Hebei 071001, China

²Baoding Xingrui Animal Husbandry and Agriculture Development Co., Ltd. Baoding Hebei 071001, China

³College of Animal Science and Technology, Yangzhou University, Yangzhou, Jiangsu 225009, China

⁴BeijingHuadu Yukou Poultry Industry Co., Ltd. Beijing 101200, China

⁵Hebei Taomu Geda Agricultural Science and Technology Co., Ltd. Baoding Hebei 071001, China

⁶College of Animal Science and Veterinary Medicine, Shenyang Agricultural University, Shenyang, Liaoning 110866, China

Funds: This work was supported by the General Program of the National Natural Foundation of China (32202731), the Hebei Provincial Higher Education Scientific Research Project (BJ2026029), the Baoding Municipal Science and Technology Plan "Jie Bang Gua Shuai" Project (2024 Agri 101), and the Modern Agricultural Industry Technology System Construction Project of Hebei Province (HBCT2024260201).

*Corresponding author (Hui Chen, email: 13930259580@163.com & Lei Shi, email: shilei2017@foxmail.com)

Tel: +86-0312-7528453

Fax: +86-0312-7528453

Address: College of Animal Science and Technology, Hebei Agricultural University

Article type: Original Article

SECTION: Zoology and Animal Science

Funds: This work was supported by the General Program of the National Natural Foundation of China (32202731), the Hebei Provincial Higher Education Scientific Research Project (BJ2026029), the Baoding Municipal Science and Technology Plan "Jie Bang Gua Shuai" Project (2024 Agri 101), and the Modern Agricultural Industry Technology System Construction Project of Hebei Province (HBCT2024260201)

The authors declare that they have no conflict of interest

*Corresponding author, E-mail: 13930259580@163.com; Lei Shi, email: shilei2017@foxmail.com

ABSTRACT

Sperm storage capability is the female reproductive tract's ability to maintain sperm viability and fertility over prolonged periods. This trait declines with age, especially during the late laying period, reducing production efficiency. However, the underlying mechanisms remain unclear. This study assessed sperm storage traits, hormone profiles, uterovaginal junction (UVJ) histomorphology, and performed multi-omics (single-cell transcriptomics, metabolomics, metagenomics) in 90-week White Leghorn hens. Compared to the low sperm storage capability group, hens with high capability showed higher serum levels of prostaglandin $F_{2\alpha}$, testosterone, and triiodothyronine, as well as a larger cross-sectional area of sperm storage tubules (SST). Single-cell transcriptomic profiling of the UVJ revealed nine major cell types. Pseudotime trajectory analysis identified SST cells as a terminally differentiated cell type, originating from ciliated epithelial precursors via a transitional stage of proliferating epithelial cells. SST cells from the high group showed upregulation of hypoxia-responsive genes (*HIF1A* and *EIF2AK3*) and glycolysis. Meanwhile, immune cells exhibited downregulation of immune-related genes, indicating a local immunosuppressive microenvironment that may facilitate sperm survival. Integrating metabolomic and metagenomic data further suggested a possible link between *Ligilactobacillus aviarius* (*L. aviarius*) activity and enhanced glycolysis, potentially contributes to lactate accumulation. Collectively, we propose that a hypoxic SST niche drives host anaerobic glycolysis, and may be reinforced by *L. aviarius*. This synergistic lactate accumulation may induce cytoplasmic acidification to suppress sperm activation,

thereby contributing to long-term storage. These findings provide a theoretical foundation for targeted manipulation of the UVJ microenvironment to enhance reproductive efficiency in aging hen populations.

Keywords: Hens; Sperm storage capability; Single-cell transcriptome; Hypoxia microenvironment; *Ligilactobacillus aviarius*; Glycolysis

INTRODUCTION

As the world's most abundant poultry species, the domestic chicken (*Gallus gallus*) has an annual population of approximately 65 billion and accounts for roughly 13% of the global animal protein supply. Under natural conditions, the asynchrony between ovulation and copulation poses a significant reproductive challenge (Yang et al., 2021). To overcome this, the female uterovaginal junction (UVJ) has evolved specialized tubular invaginations known as sperm storage tubules (SST). With an inner diameter of only 10–20 μm , SST serve as the primary reservoir for sperm, endowing hens with the capability to store viable sperm and produce fertilized eggs for 7 to 21 days post-mating (Mero & Ogasawara, 1970; Chai et al., 2024). The poultry industry has utilized this trait for artificial insemination, which supports large-scale standardized production and maintains global avian genetic diversity (Aklilu, 2025). However, the natural decline in sperm storage capability during the late laying period necessitates shorter insemination intervals to maintain high fertility (Blackburn et al., 2009; Liu & Gao, 2023). This practice not only escalates labor costs but also exacerbates hen stress and increases the risk of mechanical trauma to the reproductive tract (Das et al., 2005; Aklilu, 2025). Therefore, elucidating the regulatory mechanisms governing sperm storage capability

in aging hens is critical for improving production efficiency and animal welfare.

Sperm storage capability in birds is regulated by a complex interplay of various reproductive hormones. Research utilizing the quail model has demonstrated that prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), progesterone (P_4), and androgens regulate sperm storage and release within SST (Sasanami et al., 2013; Matsuzaki & Sasanami, 2017). Notably, $PGF_{2\alpha}$ facilitates dilation of SST orifices by modulating vaginal smooth muscle contractility, thereby enhancing sperm filling rates (Matsuzaki & Sasanami, 2017). Similarly, studies in peak laying hens have linked divergent sperm storage phenotypes to alterations in hormone-mediated pathways, with significant variations observed in P_4 , $PGF_{2\alpha}$, and testosterone (T) levels (Fan, 2020; Yang et al., 2021; Yang et al., 2023). These findings suggest conserved hormonal regulatory mechanisms in birds. However, whether this regulatory network undergoes remodeling during reproductive senescence in hens remains unclear.

In addition to hormonal regulation, the genomic and transcriptomic basis of sperm storage capability has been extensively investigated. Candidate genes involved in calcium signaling, such as *HIP1* and *PED1C*, have been implicated in long-term sperm maintenance (Yang et al., 2020). Meanwhile, the dysregulation of immune-related genes, including the downregulation of *TFAP* and *PLD1*, and upregulation of *IL15* has been associated with the age-related decline in sperm storage capability (Yang et al., 2021). The deep embedding of SST within the epithelial folds of the UVJ has rendered traditional isolation methods challenging. Therefore, prior research has largely been confined to whole-tissue analyses (Bakst, 1998; Yang et al., 2021). Yang et al. (2023)

provided the first single-cell transcriptomic atlas of the hen UVJ, resolving its cellular heterogeneity and identifying SST as the functional unit responsible for sperm storage. However, that previous study focused on intra-individual cellular heterogeneity, without integrating inter-individual phenotypic variation in sperm storage capability. Consequently, the functional heterogeneity among SST across hens with divergent sperm storage capacities remains a critical knowledge gap.

Beyond host genetics and hormonal milieu, the reproductive tract microbiome is increasingly recognized as a key regulator of the local microenvironment and a potential modulator of sperm storage dynamics (Zhu et al., 2022). In mammals, *Lactobacillus* species dominate the vaginal microbiota and maintain homeostasis through lactic acid production, secretion of antimicrobial compounds, and competitive exclusion of pathogens (Zhu et al., 2022). Dysbiosis of this community is strongly associated with conditions such as bacterial vaginosis and endometriosis (Ma et al., 2012; Chen et al., 2017). In poultry, the reproductive tract microbiota is intimately linked to egg quality and shell formation. This microbiota exhibits distinct segment-specificity, with diversity gradually increasing from the infundibulum to the cloaca (Wen et al., 2021). Previous studies have shown that the SST lumen constitutes a localized hypoxic microenvironment in which high lactate concentrations suppress sperm motility (Sasanami et al., 2015; Matsuzaki et al., 2015). However, the composition of its resident microbial community and its specific regulatory role in sperm storage capability remain unexplored.

Therefore, this study integrated single-cell transcriptomics, metabolomics, and

metagenomics to systematically dissect the molecular and cellular crosstalk between SST and the surrounding microenvironment. Based on the results, we propose that the hypoxic SST microenvironment drives host anaerobic glycolysis, which is reinforced by anaerobic respiration of *L. aviarius*. This synergy promotes lactate accumulation, induces sperm quiescence, and ultimately enhances sperm storage capability. These findings provide a theoretical framework for the targeted manipulation of the UVJ microenvironment to enhance reproductive efficiency in aging hen populations.

MATERIALS AND METHODS

Ethics approval

The use of research animals and the associated care methods adhered to national and institutional requirements, as approved by the Hebei Agriculture University Animal Care and Use Committee (approval No. 2022118).

Experimental animals

A total of 1 200 healthy White Leghorn hens (90 weeks) and 50 healthy roosters (30 weeks) were used. The hens had not been artificially inseminated for the month before the experiment and were housed individually. Throughout the experimental period, room conditions were maintained at 21°C, 50%–70% humidity, and a 16 h photoperiod. Feed and water were provided ad libitum according to the chicken feeding standards “Ministry of Agriculture, 2004”. All hens were artificially inseminated on two consecutive afternoons with 40 µL of diluted pooled semen. To minimize variation among roosters, semen concentration and motility were assessed by light microscopy.

Semen inclusion criteria were as follows: ejaculate exceeding 0.4 mL, a milky white appearance, and sperm concentration surpassing 2.15×10^9 /mL. Ejaculates from five roosters were pooled and diluted 1:1 with glutamate diluent to ensure insemination with equal sperm numbers per hen (Lake, 1960; Wen et al., 2020).

Measurement of sperm storage capability

Eggs were collected and marked daily for a period of 21 days, beginning from the day after the final insemination. All eggs were candled on day 7 of incubation to determine fertility. Based on the incubation results, the following traits were used to reflect the sperm storage capability of each hen: Fertilization rate, defined as the ratio of the total fertile eggs to the total incubated eggs within 21 days post-insemination; Duration of fertility (DF), defined as the number of days from the first to the last fertilized egg post-insemination; Days of longest fertilization (DN), defined as the number of days post-insemination until the last fertilized egg; Intermittent fertilization intensity (IFI), defined as the percentage of infertile eggs produced during the DF relative to total egg production; Day of first infertile egg (DE), defined as the number of days post-insemination until the first unfertilized egg; Maximum consecutive days producing fertilized eggs (MCD), defined as the number of days fertilized eggs are continuously laid post-insemination. A total of 274 hens with a laying rate $\geq 75\%$ and DF ≥ 14 days were selected. Based on the rankings, the 10 hens with a fertilization rate $\geq 75\%$ were assigned to the high sperm storage capability (High) group, and 10 hens with a fertilization rate $\leq 50\%$ to the low sperm storage capability (Low) group (Fan, 2020; Wen et al., 2020; Yang, 2021).

Serum reproductive hormone measurement

At 93 weeks, blood was collected from the wing vein of 10 hens per group at a fixed time. After 30 min at room temperature, samples were centrifuged at $3\,000\times g$ for 10 min at 4°C , and serum was separated and stored at -20°C . Serum P_4 , T, $\text{PGF}_{2\alpha}$, estradiol (E_2), thyroxine (T_4), and triiodothyronine (T_3) levels were measured using ELISA kits (COIBO BIO, China) according to the manufacturer's instructions. Absorbance at 450 nm was read on a Mindray BS-420 analyzer (Mindray, China), and hormone levels were calculated from standard curves. These hormones have been reported to be closely associated with sperm motility and oviductal function.

UVJ histological morphology

At 93 weeks, 10 hens per group were sacrificed by cervical dislocation at a fixed time. The reproductive tract was exposed via midline laparotomy, and the UVJ was identified and dissected free of surrounding adipose tissue. UVJ tissue was divided into three equal portions: two were stored at -80°C for subsequent analysis (detailed sample allocation table is provided in Supplementary Table S1), and one was fixed in 4% paraformaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). Sections were examined using an microscope and imaging system (Olympus, Japan). Five random mucosal fields per section were analyzed. Total mucosal area, SST number, SST cross-sectional area, and SST density were measured using Digimizer (v.6.5.0).

Effects of lactic acid on sperm motility in vitro

Semen from 3–4 healthy roosters was pooled, diluted with extender (33.3 mmol/L glucose, 4.24 mmol/L potassium citrate, 89.9 mmol/L sodium L-glutamate, 3.73 mmol/L magnesium acetate, buffered with 143 mmol/L BES), and aliquoted into petri dishes. Exogenous lactate was added at concentrations of 3×10^{-3} mol/L, 6×10^{-3} mol/L, 9×10^{-3} mol/L, 1.2×10^{-2} mol/L, and 1.5×10^{-2} mol/L (The physiological concentration of lactate in SST is 0.014 ± 0.0034 mol/L) to assess changes in motility capacity. At 2 h intervals, sperm motility was scored (0–9) under stereomicroscopy. Viability was evaluated by eosin-nigrosin staining (20 μ L 5% eosin, 80 μ L 1% nigrosin, and 3 μ L semen) and counting live sperm under light microscopy. For morphology, 2 μ L semen was diluted in 100 μ L saline; 20 μ L of this dilution was fixed with 95% ethanol, stained with 0.5% gentian violet for 3 min, rinsed and examined for abnormal. All parameters were measured synchronously at each time point.

Effects of *L. aviarius* on sperm motility in vitro

Semen from 3–4 healthy roosters was pooled, diluted with 1 $\times$ PBS, and aliquoted into petri dishes. To evaluate changes in sperm motility, *L. aviarius* suspensions with concentrations of 2.46×10^7 CFU/mL, 2.46×10^6 CFU/mL, 2.46×10^5 CFU/mL, and 2.46×10^4 CFU/mL (Bacterial starter culture was purchased from Huizao Biotechnology) were added separately. Observe sperm motility, viability, and abnormal sperm rate every 2 hours. Follow the procedure described above.

Reverse transcription and quantitative real-time PCR analysis

Using the PrimeScript RT Reagent Kit (TaKaRa, China) and the manufacturer's instructions, total RNA from UVJ tissue samples of hens was reverse transcribed into

cDNA. qRT-PCR was carried out on the ABI QuantStudio 7 Flex Real-Time Detection System (Life Technologies, USA) using the KAPA SYBR Fast universal qRT-PCR kit (TaKaRa, China). Six gene' relative expression levels were measured using the $2^{-\Delta\Delta Ct}$ method, with β -actin serving as a reference. Primers were designed using NCBI Primer-BLAST and verified via BLAST. Sequences are listed in Supplementary Table S2.

Single-cell suspension preparation, library construction, and sequencing

UVJ samples from three hens per group were submitted to Shanghai Personal Biotechnology Co., Ltd. for single-cell RNA sequencing. Samples were minced into approximately 0.5 mm² fragments and washed repeatedly with 1×PBS. After adding dissociation solution for 20 min, the cell suspension was filtered through a 7–30 μ m sieve and centrifuged at 300×g for 5 min at 4°C; the supernatant was discarded. The cells were then purified with erythrocyte lysis buffer and microbeads to remove dead cells. Cellular viability was assessed by trypan blue staining, and cells were counted using a hemocytometer or automated cell counter. The qualified cell suspension was loaded into a Chromium Controller (10x Genomics, USA), and library construction was performed using the Chromium Next GEM Single-Cell 3' Reagent Kit. The library was sequenced on a NovaSeq 6000 system (Illumina, USA). The sequencing data were then aligned and quantified using Cell Ranger (v.6.0) to obtain the cell × gene expression matrix.

Single-cell dimensionality reduction and clustering

Seurat (v.4.1.1) was used for data filtering. Cells were retained if they met the following criteria: genes detected in at least 3 cells; 400–7 000 detected genes per cell;

and cells with $\geq 5\ 000$ UMIs. Samples were then integrated and clustered using Seurat.

The clustering process included the following steps: (1) normalization of expression using the LogNormalize method of the Normalization function; (2) Principal component analysis (PCA), with the first 10 principal components used for clustering (20 components for integrated data); (3) the normalized data were clustered using the Louvain algorithm; and (4) dimensionality reduction was performed using the UMAP and t-SNE methods. Marker genes were identified using the Wilcoxon rank-sum test and were annotated using the CellMarker 2.0 database, previously published data (Holm et al., 1996; Holm & Ridderstrale, 1998; Yoshimura et al., 2000; Das et al., 2006; Yang et al., 2023; Zhang et al., 2025), and turkey SST transcriptome data (Brady et al., 2022).

Single-cell pseudotime trajectory and communication analysis

The developmental trajectory of UVJ epithelial cells was analyzed using the pseudotime algorithm in Monocle3 (v.1.3.4) to elucidate the sequential molecular changes underlying cellular differentiation (Cao et al., 2019). Orthologous genes between human and chicken were identified using BabelGene (v.22.9). These genes were used for cell-cell communication analysis with CellChat (v.2.1.0), based on the CellChatDB database (Jin et al., 2021).

Differential expression analysis

Differentially expressed genes (DEGs) were identified to investigate transcriptional variations between comparative groups within the same cell cluster. The FindMarkers function in Seurat was employed with the following thresholds: an

absolute $|\log_2(\text{fold change})| > 1.5$ and an adjusted $P\text{-value} < 0.05$ (Benjamini-Hochberg false discovery rate, FDR). Subsequent Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed on the identified DEGs using annotations from the Ensembl database for the chicken genome (*Gallus gallus*, assembly GRCg6a).

Metabolomics analysis

UVJ samples from six hens per group were collected for metabolomics analysis. Each 20 mg tissue sample was homogenized in 400 μL methanol/acetonitrile/water (4:4:2, v/v) and incubated at -20°C for 60 min to precipitate proteins. All samples were then centrifuged at $14\,000\times g$ for 20 min at 4°C . The vacuum-dried residue was reconstituted in 100 μL acetonitrile/water (1:1, v/v), vortexed, centrifuged at $14\,000\times g$ for 15 min at 4°C , and 2 μL of the supernatant was analyzed by UHPLC-MS/MS. An Agilent 1290 Infinity LC system (Santa Clara, USA) with AB SCIEX Triple TOF 6600 mass spectrometer (Boston, USA) was used in both positive and negative ion modes. Raw data were converted to mzXML format using ProteoWizard and processed with the XCMS program for peak detection, extraction, alignment, and integration. Metabolites were annotated using the Human Metabolome Database 4.0 and BiotreeDB (v.2.1) with a cutoff score of 0.3. Quality control (QC) analysis evaluated sample quality, methodological consistency, and system stability. Overall metabolic differences between groups were assessed using PCA and Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA). Model reliability was assessed using the criteria of R^2_Y , Q^2 , and variable importance in projection (VIP) values. Differentially expressed

metabolites (DEMs) were identified utilizing the criteria of adjusted P -value<0.05, VIP>1, and $|\log_2(\text{fold change})|>1.5$.

Metagenomic analysis

Six hens from each of the above groups were sacrificed by cervical dislocation at a fixed time. The UVJ tissue was then exposed by dissecting along the oviduct. Microbial samples were collected by swabbing the mucosal surface of the UVJ with sterile cotton swabs and stored at -80°C . DNA extraction and metagenomic shotgun sequencing were outsourced to Nanjing Personalbio Technology Co., Ltd. Using the DNeasy PowerSoil Kit (Qiagen, USA), genomic DNA was extracted from -80°C frozen samples. DNA quality and quantity were assessed using a NanoDrop ND-1000 and 1 % agarose gel electrophoresis. Metagenomic shotgun sequencing libraries (insert size, 400 bp) were prepared with a TruSeq Nano DNA LT Library Preparation Kit (Illumina, USA), and sequenced on an Illumina NovaSeq platform using the PE150 strategy. Raw sequencing data were output in FASTQ format. QC was performed using FastQC. Clean reads were assembled into contigs using MEGAHIT (v.1.2.9). Open reading frames (ORFs) were then predicted from scaffolds (≥ 300 bp) using MetaGeneMark. Functional annotation and relative abundance tables against KEGG, EggNOG, and CAZy databases were generated using QIIME 2 (v.2024.10). Taxonomic annotation was performed by aligning scaffolds against the NCBI-NT database using BLASTN (E -value<0.001). Linear discriminant analysis effect size (LEfSe) was employed to identify differentially abundant microbial taxa, using LDA score>2 and adjusted P -value<0.05 as the significance thresholds, results of the Mann-Whitney U

Nonparametric Test.

Statistical analysis

All statistical analyses were performed using SPSS 22.0. The Shapiro-Wilk test was used to assess normality, and an independent samples. Student's *t*-tests were applied to normally distributed data with FDR correction for multiple testing, adjusted *P*-value<0.05 was considered statistically significant. Origin 2023 was used to fit the daily fertilization rate curve over one month using $y(x)=\gamma/(1+e^{-(\beta(\tau-x))})$ model (Fan, 2020). Joint pathway analysis of DEGs and DEMs was performed using MetaboAnalyst 6.0, combining *P*-values via the weighted *z*-test and generating KEGG enrichment maps. Pearson correlation analysis was conducted between DEMs and differential microbial taxa. Data visualizations were performed using GraphPad Prism 9.5 and R (v.4.3.3).

RESULTS

Phenotypic characteristics between the high and low groups

To compare sperm storage capability between the high and low groups, daily fertilization rate over one month was analyzed using curve-fitting functions. The fertilization rate in the high group remained stable for the first two weeks, whereas the low group exhibited a decline beginning in the first week (Figure 1A). Significant differences between the two groups were observed for all sperm storage indicators, including fertilization rate, IFI, DF, DN, DE, and MCD ($P<0.01$, Figure 1B). Serum analysis revealed significantly elevated levels of T, $\text{PGF}_{2\alpha}$, and T_3 in the high group

($P<0.05$), while E_2 , T_4 , and P_4 levels showed no significant differences between groups (Figure 1C). Histological analysis of the UVJ section revealed a significantly larger SST cross-sectional area in the high group ($P<0.05$), whereas the number of SST was slightly higher in the high group, though not substantially (Figure 1D).

Constructing UVJ single-cell transcriptomic atlas

To investigate the cellular composition and cell-specific transcriptional profiles of the UVJ in hens with high and low sperm storage capability, single-cell transcriptome sequencing was performed on UVJ tissues from three hens per group. After data processing, quality control, and batch-effect correction, 31 817 and 30 648 high-quality cells were obtained from the high and low groups, respectively. Nine cell types were identified based on canonical marker genes: T cells, fibroblasts, epithelial cells, mural cells, erythrocytes, plasma cells, macrophages, proliferating cells, and endothelial cells (Figure 2A–C). Among these, epithelial cells (59.50%), immune cells (17.15%), and fibroblasts (14.39%) constituted the majority. Analysis of cell proportions revealed no significant differences between the two groups for any cell type (Figure 2D). Further differential expression analysis identified 137, 54, and 146 DEGs in the epithelial cells, immune cells, and fibroblasts, respectively (Figure 2E). Enrichment analysis revealed that epithelial cells were predominantly associated with lipid and energy metabolism pathways, including glycerolipid metabolism, oxidative phosphorylation, and folate biosynthesis. Immune cells were primarily enriched in cell adhesion molecule pathways, while fibroblasts were linked to cell adhesion molecules, cytoskeleton in muscle cells, and TGF-beta signaling (Figure 2F).

Epithelial cells subpopulation analysis

To further characterize the biological features of epithelial cell populations in the UVJ, epithelial cells were subdivided into 11 clusters (Figure 3A), and five cell subtypes were identified: epithelial cells, endothelial cells, ciliated epithelial cells, SST cells, and proliferating epithelial cells (Figure 3B). No significant differences in the number of any cell subtype were observed between the high and low groups (Figure 3C). Pseudotime trajectory analysis inferred a potential differentiation trajectory from ciliated epithelial cells to terminally differentiated SST cells, with proliferative epithelial cells as an intermediate stage (Figure 3D). To further investigate the functional transitions during SST development, differential expression comparisons were performed between cluster 4 (SST cell) and two distinct stages: the initial stage cluster 0 (ciliated epithelial cell) and the transitional stage cluster 3 (proliferating epithelial cell). The analysis identified 2 141 and 2 205 DEGs, respectively (Figure 3E). Functional enrichment analysis of these DEGs showed that the differences between cluster 4 and cluster 0 were mainly related to signal transduction mechanisms, extracellular matrix organization, protein phosphorylation, and energy metabolism pathways (Figure 3F). In contrast, DEGs between cluster 4 and cluster 3 were primarily enriched in pathways associated with cellular signal transduction, cell motility, tube development, and tube morphogenesis (Figure 3G).

SST cells subpopulation analysis

To investigate the functional characteristics of SST cells, the key site for sperm storage, differential expression analysis of SST cells was performed between the high

and low groups. A total of 271 DEGs were identified, with 127 significantly upregulated and 144 downregulated in the high group (Figure 4A, B). GO enrichment analysis showed that these DEGs were notably enriched in terms related to ribosomal small subunit biogenesis, ribosome assembly, and cytosolic ribosome (Figure 4C). KEGG enrichment analysis similarly revealed significant enrichment in ribosome metabolism, as well as pathways associated with cellular stress and signal transduction, including Notch, autophagy, FoxO, and MAPK signaling (Figure 4D). Candidate genes potentially related to sperm storage capability were further identified, including *HIF1A*, *EIF2AK3*, *SOD2*, and *TGFB2*, all of which were significantly upregulated in the high group (Figure 4E). Cell-cell communication analysis revealed that SST cells interacted with other epithelial subtypes primarily through pathways involved in signal transduction, proliferation, differentiation, and apoptosis. Notably, the DHEA signaling pathway (involved in sex hormone synthesis) was specifically enriched in the high group (Figure 4F).

Immune cells subpopulation analysis

To investigate whether an immune privilege mechanism exists in the UVJ, immune cells were further analyzed. Five major immune cell types were classified, including T cells, B cells, plasma cells, macrophages, and neutrophils (Figure 5A). Although no significant differences in immune cell numbers were observed between the high and low groups (Figure 5B). Differential expression analysis identified 920 DEGs across immune cells (Figure 5C), including 120 DEGs that were shared among multiple immune cell types (Figure 5D). GO analysis revealed that these DEGs were

markedly associated with humoral and cellular immune response (Figure 5E). KEGG analysis further indicated enrichment in pathways related to Salmonella infection, Apoptosis, and Phagosome (Figure 5F). Notably, several immune-related genes, including *IGLL1*, *LY6E*, *LY86*, *CCR5*, *CFLAR*, *FASLG*, *AVD*, and *ANXA1* were markedly downregulated in the high group (Figure 5G). Cell-cell communication analysis further demonstrated that immune-related signaling pathways, such as FASLG, CADM, VCAM, THBS, and SEMA6, exhibited weaker interaction intensities in the high group (Figure 5H), indicating a reduced immune activation state that may favor local immunosuppression.

Metabolomic analysis

PCA of the UVJ tissue metabolome revealed a clear separation between the high and low groups (Figure 6A). 47 DEMs were identified, with 23 upregulated and 24 downregulated in the high group. These DEMs included reproductive hormone-related metabolites, such as 5 α -dihydrotestosterone (DHT) and 13,14-dihydro-15-keto PGF_{2 α} , as well as glycolysis-related metabolites, such as S-lactoylglutathione, β -D-fructose-6-phosphate, and glycerol 3-phosphate (Figure 6B). KEGG analysis demonstrated that these DEMs were substantially enriched in energy and lipid metabolism pathways, including nicotinate and nicotinamide metabolism, pyruvate metabolism, and glycerophospholipid metabolism (Figure 6C). To further explore the associations between DEMs and DEGs in different cell types, integrated pathway analysis was performed. These DEMs and DEGs were jointly enriched in energy metabolism pathways, particularly nicotinate and nicotinamide metabolism (Figure 6D). Notably,

SST cells in the high group also showed expression of energy metabolism related genes including *ENPP3*, and pyruvate metabolism genes *PDHB* and *GLO1* (Figure 6E). To validate the scRNA-seq results, qRT-PCR analysis was conducted on a subset of DEGs, which confirmed the expression changes observed in the transcriptomic data (Figure 6F).

Microbiota metagenomic analysis

At the phylum level, the UVJ microbial community mainly consisted of *Firmicutes* (54.32±19.00%), *Bacteroidota* (17.46±7.93%), *Actinobacteria* (5.80±1.53%), and *Proteobacteria* (3.72±3.05%, Figure 7A). At the genus level, *Megamonas* (8.96±0.10%), *Ligilactobacillus* (8.57±6.41%), *Phocaeicola* (7.78±0.69%), and *Mediterraneibacter* (6.38±0.17%) were the most abundant taxa (Figure 7B). Notably, the relative abundances of *Firmicutes* and *Ligilactobacillus* were significantly higher in the high group ($P<0.05$). Alpha-diversity analysis showed that the Chao 1, Shannon, and Simpson indices were markedly higher in the high group ($P<0.05$, Figure 7C). Furthermore, LEfSe identified microbial taxa that differed significantly between groups from phylum to species levels. *Bacilli*, *Firmicutes*, *Lactobacillales*, *Ligilactobacillus*, and *L. aviarius* were key differential microorganisms (Figure 7D), with a significantly higher relative abundance in the high group ($P<0.05$, Figure 7E). Additionally, correlation analysis between these key differential microorganisms and DEMs revealed that *Firmicutes*, *Ligilactobacillus*, and *L. aviarius* were highly positively correlated with glycerol 3-phosphate ($P<0.01$, Figure 7F).

Sperm motility analysis

The effects of exogenous lactic acid on sperm motility were investigated in vitro. Sperm motility decreased in response to exposure to lactic acid in a dose-dependent manner (Figure 8A), while there were no markedly changes in sperm viability or abnormal sperm rate (Figure 8B, C), suggesting that lactic acid may play a role in inhibiting sperm motility. The effects of *L. aviarius* suspensions on sperm motility were then investigated in vitro. Sperm motility remained high across suspensions of *L. aviarius* at various concentrations, whereas in the blank control, sperm motility gradually decreased over time (Figure 8D). Sperm viability followed a similar trend to sperm motility (Figure 8E). However, no clear trend was observed in abnormal sperm rate (Figure 8F).

DISCUSSION

Sperm storage capability is widespread among vertebrates but highly variable: most mammals store sperm for only days, while bats store for months, birds for weeks, and some reptiles and fish for years (Holt & Fazeli, 2016; Matsuzaki & Sasanami, 2017). In laying hens, this trait dictates optimal artificial insemination intervals, yet its cryptic nature causes it to be frequently overlooked in breeding (Wolc et al., 2009). Research has largely focused on the peak laying period, while the late laying period has received comparatively little attention. Consequently, no uniform standard currently exists for grouping hens by sperm storage capacity across production stages, and an appropriate model is needed to optimize selection in the future. Fertility declines after 55 weeks

(Han et al., 2022). By 90 weeks, individual variation becomes more pronounced, providing a unique window to uncover the physiological mechanisms that sustain sperm storage and to identify their key regulators. To elucidate the molecular regulatory mechanisms underlying sperm storage capability in late laying hens, this study integrated phenotypic assessments with multi-omics analyses. We propose a mechanistic framework in which the hypoxic microenvironment within SST may drive host anaerobic glycolysis. Our data further suggest a possible reinforcement of this metabolic shift by *L. aviarius* activity. The resulting lactate accumulation suppresses sperm activation, thereby enhancing storage capability.

Phenotypic divergence in sperm storage capability

Given that sperm storage capability is challenging to assess directly under production conditions, this study employed multiple assessment metrics, including the 21-day fertilization rate, DF, and DN. The results demonstrated significant differences in all sperm storage indices between the high and low groups, accompanied by a pronounced divergence in fertilization stability. Specifically, the high group maintained a stable output of fertilized eggs through the second week post-insemination, whereas the low group exhibited a sharp decline within the first week. Avian sperm storage capability generally correlated with SST morphology; hens with stronger sperm storage capability typically display larger SST cross-sectional areas (Bakst & Bauchan, 2016; Ma et al., 2020; Chai et al., 2024). Consistent with these reports, UVJ histomorphological analysis in this study confirmed a significantly larger SST cross-sectional area in the high group, although the total number of SST did not differ

significantly between the groups. Given that 80% of inseminated sperm are expelled with vaginal secretions and fewer than 1% successfully enter the SST (Bakst, 1994; Bakst et al., 2010), a larger cross-sectional area implies a greater sperm storage reservoir. Therefore, the cross-sectional area of SST may be an important morphological correlate of sperm storage capability. However, the cross-sectional nature of this study does not allow us to determine whether this morphological difference is the cause or result of the enhanced storage capacity.

Potential biomarkers for sperm storage capability

Multiple reproductive hormones have been implicated in regulating sperm storage and release (Sasanami et al., 2013; Matsuzaki & Sasanami, 2017). Our findings further characterized the hormonal regulation, revealing significantly elevated serum levels of $\text{PGF}_{2\alpha}$, T, and T_3 in the high group, alongside increased expression of their derivatives 13,14-dihydro-15-keto $\text{PGF}_{2\alpha}$ and DHT in the UVJ. $\text{PGF}_{2\alpha}$ has been shown to dilate SST orifices by stimulating smooth muscle contractions in the quail reproductive tract, thereby enhancing sperm filling efficiency (Matsuzaki & Sasanami, 2017). This suggests that $\text{PGF}_{2\alpha}$ may contribute to regulating SST cross-sectional area. T has been identified as a negative regulator of apoptosis in bat sperm storage tissues, promoting Bcl-2 expression to establish a microenvironment conducive to sperm survival (Roy & Krishna, 2010; Roy & Krishna 2011). T, as a precursor to DHT, can be converted to DHT by 5 α -reductase (Lee et al., 2022). The simultaneous elevation of serum T and UVJ DHT levels in the high group suggests enhanced local conversion of T to DHT. Consistent with this role, previous studies have detected elevated expression of T-

related receptors, the derivative 7α -hydroxytestosterone, and the key conversion enzyme *HSD11B* in the serum and UVJ of high sperm storage capability hens (Ma et al., 2020; Yang et al., 2021). Moreover, cell communication analysis in this study revealed a significant enrichment of the DHEA signaling pathway in the high group. As a precursor for sex hormones, DHEA provides a mechanistic basis for sustained local T synthesis (McNelis et al., 2013; Ali et al., 2024), and may actively regulate the SST microenvironment. T_3 has been shown to improve reproductive function in zebrafish, and its deficiency causes reproductive dysfunction and reduced fertilized egg production (Houbrechts et al., 2016), but its specific role in avian sperm storage capability remains unclear. Collectively, these findings suggest that certain hormonal mechanisms may be conserved across birds and mammals.

Cellular composition and functional characteristics of the UVJ

The avian UVJ is located at the terminal end of the oviduct and consists of pseudostratified ciliated epithelium comprising both ciliated and non-ciliated secretory cells (Yang, 2021). The single-cell transcriptomic atlas constructed in this study confirmed that the UVJ is a multicellular tissue, predominantly composed of epithelial cells (59.50%), immune cells (17.15%), and fibroblasts (14.39%), along with blood cells, endothelial cells, SST cells, and proliferating cells. This composition is consistent with previous reports (Yang et al., 2023). Within the UVJ, epithelial cells are primarily responsible for directional sperm transport and maintenance of cell polarity; fibroblasts provide structural support and tissue maintenance; and immune cells execute pathogen surveillance and clearance functions (Yang, 2021; Yang et al., 2023). Notably, no

significant differences in cell numbers were observed between groups, suggesting that functional divergence, rather than cellular abundance, underpins the differences in storage capability. Further analysis revealed that epithelial cells from the high group showed significant enrichment in lipid and energy metabolism pathways. This implies heightened metabolic and biosynthetic activity that may support sperm viability and microenvironmental regulation. Fibroblasts showed marked enrichment in pathways related to extracellular matrix organization and cytoskeleton structure, underscoring their crucial role in tissue repair and remodeling. Remarkably, immune cells from the high group, although enriched in immune response pathways, displayed an overall attenuated reactive profile. This was evidenced by downregulation of immune-related genes, including *IGLL1*, *LY86*, *LY6E*, and *CCR5*, or diminished signaling intensity within immune-related networks such as FASLG and CADM. Since sperm are foreign bodies in the female reproductive tract and can trigger an immune rejection response, such localized immunosuppression is critical for long-term sperm storage (Das et al., 2008). Prior studies have shown that post-mating elevation of *TGFB2* levels in SST suppresses lymphocyte activity and local immune responses, thereby protecting sperm from clearance (Das et al., 2005; Das et al., 2006). The present study further reveals elevated *TGFB2* expression in SST of the high group, underscoring its pivotal role in sustaining long-term sperm storage. In addition, our study revealed that *ENPP3* was downregulated in SST cells of the high group. *ENPP3* is a cell surface marker that regulates inflammation in immune cells by hydrolyzing extracellular nucleotides, and its expression levels reflect the local immune and inflammatory status (Shih et al., 2015;

Borza et al., 2022). Importantly, the local immunosuppression at the UVJ is an active adaptive state induced by sperm storage (Das et al., 2005; Das et al., 2006), this immunosuppression is localized rather than systemic, thereby preserving the overall immune function of the reproductive tract. At the same time, studies have shown that the physiological lactate concentration in the SST of poultry is 0.014 ± 0.0034 mol/L (Matsuzaki et al., 2015), corresponding to a pH range of 2.83–2.94; this level of acidity likely serves as a potent antibacterial barrier. Together, these findings indicate that the UVJ is a heterogeneous tissue in which hens with high sperm storage capability establish a more effective local immunosuppressive environment for sperm survival.

The hypoxic environment within SST maintains sperm quiescence

As the functional units directly responsible for sperm storage, SST originate as tubular outpouchings of the epithelial layer, driven by differential proliferation between UVJ epithelial cells and fibroblasts (Yang et al., 2023). This morphogenetic process is analogous to the formation of epithelial folds in the mouse oviduct (Koyama et al., 2016). Further, Yang et al. (2021) characterized UVJ development from 16.5 to 30 weeks of age, demonstrating that SSTs originate from ciliated epithelial cells and that the major morphogenetic phase occurs between 18.5 and 21 weeks. Pseudotime trajectory analysis delineated the potential differentiation trajectory of SST cells, revealing that they originate from ciliated epithelial cells and transition through a proliferative epithelial stage before terminal differentiation. The enrichment of numerous DEGs in pathways such as tube development and tube morphogenesis during this transitional stage further substantiates the tubular origin of SST. Differential

analysis revealed that SST cells from the high group exhibited a transcriptional signature indicative of a hypoxic microenvironment, marked by the coordinate upregulation of the hypoxia-responsive transcription factor *HIF1A*, the hypoxia-induced autophagy kinase *EIF2AK3*, and the antioxidant stress factor *SOD2*. *HIF1A* serves as a master transcriptional regulator of the cellular response to hypoxia (Ros & Schulze, 2013; Qiu et al., 2023), while *EIF2AK3* is a key kinase mediating hypoxia-induced autophagy (Fu et al., 2025); both are transcriptionally induced under low oxygen conditions. This pattern aligns with hypoxia conditions reported in quail SST; while prior studies confirmed SST hypoxia using hypoxyprobe and immunohistochemistry (Matsuzaki & Sasanami, 2017), the present findings provide transcriptional evidence consistent with a hypoxic environment. Notably, hypoxia is often accompanied by excessive mitochondrial reactive oxygen species (ROS) production, which can induce peroxidative damage to sperm (Hernansanz-Agustin et al., 2020). Elevated *SOD2* expression in SST of the high group suggests that the hypoxic environment concurrently triggers host antioxidant defenses, mitigating peroxidative injury to stored sperm.

Lactate accumulation within the SST lumen is a critical suppressor of sperm motility, acting by acidifying the cytoplasm (Bobr et al., 1964; Matsuzaki et al., 2015). As an end product of anaerobic glycolysis, lactate accumulation within SST has been directly linked to the glycolysis pathway (Matsuzaki & Sasanami, 2017). The present study provides correlative evidence for enhanced glycolytic activity in the SST of the high group. Metabolomic analysis revealed differential expression of glycolytic

intermediates such as β -D-fructose-6-phosphate and glycerol 3-phosphate. Fructose-6-phosphate is a key glycolytic intermediate whose phosphorylation by phosphofructokinase represents a critical rate-limiting step (Ros & Schulze, 2013; Wang et al., 2024; You et al., 2026). The lower level of fructose-6-phosphate, coupled with elevated levels of the downstream product glycerol 3-phosphate in the high group, collectively indicates enhanced glycolytic flux, although direct flux measurements would be required for confirmation. Furthermore, integrated pathway analysis highlighted significant enrichment of pyruvate metabolism within SST, accompanied by elevated expression of *PDHB*, *GLO1*, and increased levels of S-lactoylglutathione. Pyruvate, as the terminal product of glycolysis is the primary precursor for lactate conversion under hypoxic conditions (Prochownik & Wang, 2021). *PDHB* encodes a crucial subunit of the pyruvate dehydrogenase complex, which channels pyruvate into downstream metabolic pathways (Rong et al., 2024). *GLO1* catalyzes the conjugation of the cytotoxic glycolytic byproduct methylglyoxal with glutathione to form S-lactoylglutathione, which is subsequently hydrolyzed to lactate (Zhao et al., 2025). This pathway not only detoxifies methylglyoxal but may also serve as a direct lactate source within SST. qRT-PCR results further confirmed the transcriptional upregulation of the pyruvate metabolism-related genes *PDHB* and *GLO1* in the high group. At the same time, in vitro experiments showed that sperm motility decreased in a dose-dependent manner as lactate concentration increased. These findings indicate that lactate is a key regulatory factor mediating sperm storage arrest. Taken together, this study suggest that the hypoxic environment within avian SST drives anaerobic glycolysis, and the

resulting lactate accumulation induces sperm quiescence, thereby prolonging storage duration.

***L. aviarius* promotes sperm storage through lactate production via glycolysis under hypoxic conditions**

The hypoxic, lactate-rich microenvironment within the SST lumen is likely sustained not only by host cell metabolism but also through synergistic contributions from the resident microbiota. Metagenomic analysis revealed that the UVJ microbiota is dominated by *Firmicutes* and *Bacteroidota* at the phylum level, with *Megamonas* and *Ligilactobacillus* predominating at the genus level. Notably, the high group exhibited markedly higher alpha diversity, indicating a more stable microbial ecosystem. Given that lactate is a characteristic metabolite of lactic acid bacteria, its accumulation likely correlates with the abundance of these species. We further demonstrated that the relative abundances of *Firmicutes*, *Ligilactobacillus*, and *L. aviarius* were markedly higher in the high group. This finding aligns with cecal microbial profiles reported in hens with high sperm storage capability (Elokil et al., 2020), suggesting that *Lactobacillus* species may represent a core microbial group influencing sperm storage capability. Given that the cloaca serves as the common exit for the intestinal, urinary, and reproductive tracts in hens, significant overlap likely exists between the gut and oviductal microbiota (Lee et al., 2019; Shterzer et al., 2020).

L. aviarius is a stable colonizer of the poultry gut, with reported relative abundances ranging from 0.5% to 40% in intestinal and fecal samples (Rios et al., 2023).

The present study identified *L. aviarius* as a key differential microorganism, exhibiting

a more than 10-fold difference in relative abundance between the high (4.14%) and low (0.32%) groups. To explore whether this microbial enrichment had metabolic relevance to the host, we identified a strong positive correlation between *L. aviarius* abundance and glycerol 3-phosphate. While correlation does not establish causality, this observation is consistent with the known genomic capacity of *L. aviarius*; this species universally encodes lactate dehydrogenase and is a facultative anaerobe capable of fermenting carbohydrates to lactate via glycolysis (Rios et al., 2023). Thus, *L. aviarius* populations may contribute to anaerobic lactate production within the hypoxic SST lumen. Vitro experiments showed that exogenous lactic acid inhibited sperm motility in hens in a dose-dependent manner, consistent with the aforementioned functional mechanism, suggesting that lactic acid is a key factor in inhibiting sperm motility. Given the difficulty in precisely controlling anaerobic conditions, this study observed that *L. aviarius* can sustain sperm motility and viability for extended periods under aerobic conditions. In an aerobic environment, lactic acid bacteria primarily rely on oxidative metabolism, resulting in significantly reduced lactic acid production and improved ATP generation efficiency; higher ATP levels may be key to sustaining sperm motility over time. This provides preliminary experimental evidence supporting the addition of *L. aviarius* suspensions to diluents to maintain sperm motility. However, we acknowledge that the functional contribution of *L. aviarius* to SST lactate levels is inferred from its genomic capacity for anaerobic lactate production, and its abundance positively correlated with glycolytic intermediates. These findings are correlative in nature; causality remains to be established through targeted experiments.

Implications and future perspectives

Translating these findings into practical applications is a key goal of this study. The upregulated genes (*HIF1A*, *EIF2AK3*, *TGFB2*, *SOD2*, etc.) in SST cells of the high group may serve as candidate molecular markers for sperm storage capability. Previous studies have identified SNPs associated with fertility traits, suggesting that marker assisted selection for storage capability is technically feasible. Following further validation, these genes could be incorporated into SNP based breeding programs. *L. aviarius* was significantly enriched in the UVJ of the high group, indicating its potential as a candidate probiotic. However, transplantation experiments in low capacity hens are needed to evaluate its causal effects on sperm storage. Additionally, serum $\text{PGF}_{2\alpha}$, T, and T_3 were significantly elevated in the high group, suggesting their potential as non-invasive biomarkers for optimizing the timing of artificial insemination, though exogenous supplementation studies are required to confirm their functional roles.

Several limitations of this study should be acknowledged. First, we acknowledge that using a consistent sample size in multi-omics studies can improve detection efficiency. Due to the limited sample size in this study, we selected three representative individuals per group from the metabolomic and metagenomic samples for scRNA analysis. This selection method and sample size have been reported in the relevant literature (Liu et al., 2025; Dai et al., 2026; Ge et al., 2026). However, larger sample sizes will still be needed in future studies to validate our findings and detect smaller effects. Second, the cross-sectional design does not distinguish whether *L. aviarius* enrichment drives or results from the SST microenvironment; functional studies

(probiotic supplementation) are required to establish causality. Addressing these limitations will help translate our hypothesis generating findings into practical strategies for improving reproductive efficiency in aging hens.

CONCLUSION

In summary, our findings suggest that SST from hens with high sperm storage capability exhibit a hypoxic, glycolysis-associated microenvironment. This state is potentially maintained by both host metabolic adaptations and the lactate-producing activity of *L. aviarius*. The resultant lactate accumulation suppresses sperm activation, thereby prolonging sperm storage and enhancing storage capability.

DATA AVAILABILITY

The data presented in this study were available upon reasonable request from the corresponding author.

SUPPLEMENTARY DATA

Supplementary data will be made available on request.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

J.J.X. Conceptualization, Methodology, Software, Validation, Formal analysis, Writing—original draft preparation, Writing—review and editing, Visualization. X.X.L. Investigation, Data curation. Y.H.L. Investigation, Data curation. S.H.G. Investigation, Data curation. Y.H.F. Software, Formal analysis, Investigation. X.L.W. Software,

Formal analysis, Investigation. Y.C.Y. Software, Formal analysis, Investigation. R.C.Z. Software, Formal analysis, Investigation. X.Y.Z. Methodology, Validation, Project administration. D.S. Methodology, Validation, Project administration. E.D.S. Methodology, Validation, Project administration. D.H.W. Methodology, Validation, Resources. Y.F.C. Methodology, Validation, Resources. E.Y.H. Methodology, Validation, Project administration. H.C. Methodology, Supervision, Funding acquisition. L.S. Conceptualization, Methodology, Validation, Resources, Writing—review and editing, Visualization, Supervision, Project administration. All authors read and approved the final version of the manuscript.

ACKNOWLEDGEMENTS

The authors sincerely thank the members of the poultry team at Hebei Agricultural University and Baoding Xingrui Agriculture and Animal Husbandry Development Co., Ltd.

References

- Aklilu H. 2015. Significance of artificial insemination over natural mating in poultry and the risks of post-insemination physical trauma. *American Journal of Biomedical and Life Sciences*, **13**(1): 1–4.
- Ali A, Motaleb A, Alam MT, et al. 2024. Synthesis and pharmacological properties of modified a- and d-ring in dehydroepiandrosterone (DHEA): A Review. *ACS Omega*, **9**(30): 32287–32327.
- Bakst MR, Bauchan G. 2016. Lectin staining of the uterovaginal junction and sperm-storage tubule epithelia in broiler hens. *Poultry Science*, **95**(4): 948–955.
- Bakst MR, Donoghue AM, Yoho DE, et al. 2010. Comparisons of sperm storage tubule distribution and number in 4 strains of mature broiler breeders and in turkey hens before and after the onset of photostimulation. *Poultry Science*, **89**(5): 986–992.
- Bakst MR, Wishart G, Brillard JP. 1994. Oviducal sperm selection, transport, and storage in poultry. *Poultry Science Reviews*, **5**: 117–143.
- Bakst MR. 1998. Structure of the avian oviduct with emphasis on sperm storage in poultry. *Journal of Experimental Zoology*, **282**(4-5): 618–626.
- Blackburn HD, Silversides F, Purdy PH. 2009. Inseminating fresh or cryopreserved semen for maximum efficiency: implications for gene banks and industry. *Poultry Science*, **88**(10): 2192–2198.

- Bobr LW, Lorenz FW, Ogasawara FX. 1964. Distribution of spermatozoa in the oviduct and fertility in domestic birds. *Journal of Reproduction and Fertility*, **8**(1): 39–47.
- Borza R, Salgado-Polo F, Mouter HM, et al. 2022. Structure and function of the ecto-nucleotide pyrophosphatase/phosphodiesterase (ENPP) family: Tidying up diversity. *Journal of Biological Chemistry*, **298**(2): 101526.
- Brady K, Krasnec K, Long JA. 2022. Transcriptome analysis of inseminated sperm storage tubules throughout the duration of fertility in the domestic turkey, *Meleagris gallopavo*. *Poultry Science*, **101**(4): 101704.
- Cao J, Spielmann M, Qiu X, et al. 2019. The single-cell transcriptional landscape of mammalian organogenesis. *Nature*, **566**(7745): 496–502.
- Chai R, Yang Z, Lv K, et al. 2024. The sperm storage capacity in hens was correlated with the morphological differences of the oviduct and uterus-vagina junction. *Poultry Science*, **103**(8): 103861.
- Chen C, Song X, Wei W, et al. 2017. The microbiota continuum along the female reproductive tract and its relation to uterine-related diseases. *Nature Communications*, **8**(1): 875.
- Dai D, Wang P, Zhang H, et al. 2026. Temporal landscapes of the gut microbiota-host axis reveal mechanisms of age-related eggshell quality decline in laying hens. *npj Biofilms Microbiomes*. <https://doi.org/10.1038/s41522-026-01079-4>.
- Das SC, Isobe N, Nishibori M, et al. 2006. Expression of transforming growth factor-beta isoforms and their receptors in utero-vaginal junction of hen oviduct in presence or absence of resident sperm with reference to sperm storage. *Reproduction*, **132**(5): 781–790.
- Das SC, Isobe N, Yoshimura Y. 2008. Mechanism of prolonged sperm storage and sperm survivability in hen oviduct: a review. *American Journal of Reproductive Immunology*, **60**(6): 477–481.
- Das SC, Nagasaka N, Yoshimura Y. 2005. Effects of repeated artificial insemination on the structure and function of oviducal sperm storage tubules in hens. *Journal of Poultry Science*, **42**(1): 39–47.
- Elokil AA, Abouelezz K, Adetula AA, et al. 2020. Investigation of the impact of gut microbiotas on fertility of stored sperm by types of hens. *Poultry Science*, **99**(2): 1174–1184.
- Fan J. 2020. Analysis of sperm storage ability related traits of chicken and the preliminary mechanism study based on transcriptome sequencing. Master's Thesis, Hebei University of Engineering, Handan, China. 10.27104/d.cnki.ghbjy.2020.000237. (in Chinese)
- Fu J, Xu W, Wang G, et al. 2025. Hypoxia-triggered autophagy modulates cisplatin resistance in non-small cell lung Cancer via *EIF2AK3*-dependent PI3K/AKT signaling and mTOR-independent mechanisms. *Cell Death Discovery*, **12**(1): 40.
- Ge QQ, Wang JJ, Li J, et al. 2026. Single-cell atlas of *Exopalaemon carinicauda* gills provides insights into pillar cell-mediated responses to low-salinity stress in crustaceans. *Zoological Research*, **47**(2): 315–332.
- Han X, Zeng D, Liu L, et al. 2022. Factors that reduce the fertilization rate of egg breeds in the late laying period. *China Poultry Science*. **10**: 10–13. (in Chinese)
- Hernansanz-Agustín P, Choya-Foces C, Carregal-Romero S, et al. 2020. Na(+) controls hypoxic signalling by the mitochondrial respiratory chain. *Nature*, **586**(7828): 287–291.
- Holm L, Ridderstrale Y, Knutsson PG. 1996. Localisation of carbonic anhydrase in the sperm storing regions of the domestic hen oviduct. *Acta Anatomica (Basel)*, **156**(4): 253–260.
- Holm L, Ridderstrale Y. 1998. Localization of carbonic anhydrase in the sperm-storing regions of the turkey and quail oviduct. *Journal of Histochemistry & Cytochemistry*, **30**(7): 481–488.

- Holt WV, Fazeli A. 2016. Sperm storage in the female reproductive tract. *Annual Review of Animal Biosciences*, **4**: 291–310.
- Houbrechts AM, Delarue J, Gabriëls IJ, et al. 2016. Permanent deiodinase type 2 deficiency strongly perturbs zebrafish development, growth, and fertility. *Endocrinology*, **157**(9): 3668–3681.
- Jin S, Guerrero-Juarez CF, Zhang L, et al. 2021. Inference and analysis of cell-cell communication using CellChat. *Nature Communications*, **12**(1): 1088.
- Koyama H, Shi D, Suzuki M, et al. 2016. Mechanical regulation of three-dimensional epithelial fold pattern formation in the mouse oviduct. *Biophysical Journal*, **111**(3): 650–665.
- Lake PE. 1960. Studies on the dilution and storage of fowl semen. *Journal of Reproduction and Fertility*, **1**: 30–35.
- Lee HG, Kim CJ. 2022. Classic and backdoor pathways of androgen biosynthesis in human sexual development. *Annals of Pediatric Endocrinology & Metabolism*, **27**(2): 83–89.
- Lee S, La TM, Lee HJ, et al. 2019. Characterization of microbial communities in the chicken oviduct and the origin of chicken embryo gut microbiota. *Scientific Reports*, **9**(1): 6838.
- Liu J, Liu SF, Mao HR, et al. 2025. Ribosome profiling and single-cell RNA sequencing identify the unfolded protein response as a key regulator of pigeon lactation. *Zoological Research*, **46**(1): 54–74.
- Liu Y, Gao J. 2023. Reproductive aging: biological pathways and potential interventive strategies. *Journal of Genetics and Genomics*, **50**(3): 141–150.
- Ma B, Forney LJ, Ravel J. 2012. Vaginal microbiome: rethinking health and disease. *Annual Review of Microbiology*, **66**: 371–389.
- Ma T, Fan J, Wang P, et al. 2020. The sperm storage tubules morphology, hormones concentration, and hormone receptor genes expression of laying hens with divergent sperm storage ability. *Chinese Journal of Animal and Veterinary Sciences*, **51**(8): 1845–1852. (in Chinese)
- Matsuzaki M, Mizushima S, Hiyama G, et al. 2015. Lactic acid is a sperm motility inactivation factor in the sperm storage tubules. *Scientific Reports*, **5**: 17643.
- Matsuzaki M, Sasanami T. 2017. Sperm storage in the female reproductive tract: A conserved reproductive strategy for better fertilization success. In: Sasanami, T. (eds) *Avian Reproduction. Advances in Experimental Medicine and Biology*. Singapore: Springer, 173–196.
- McNelis JC, Manolopoulos KN, Gathercole LL, et al. 2013. Dehydroepiandrosterone exerts antigluco-corticoid action on human preadipocyte proliferation, differentiation, and glucose uptake. *American Journal of Physiology-endocrinology and Metabolism*, **305**(9): E1134–E1144.
- Mero KN, Ogasawara FX. 1970. Dimensions of uterovaginal sperm-storage tubules of the chicken and their possible significance in sperm release. *Poultry Science*, **49**(5): 1304–1308.
- Prochownik EV, Wang H. 2021. The metabolic fates of pyruvate in normal and neoplastic cells. *Cells*, **10**(4): 762.
- Qiu B, Yuan P, Du X, et al. 2023. Hypoxia inducible factor-1 α is an important regulator of macrophage biology. *Heliyon*, **9**(6): e17167.
- Rios-Galicia B, Saenz JS, Yergaliyev T, et al. 2023. Host specific adaptations of *Ligilactobacillus aviarius* to poultry. *Current Research in Microbial Sciences*, **5**: 100199.
- Rong Y, Liu SH, Tang M, et al. 2024. Analysis of the potential biological value of pyruvate dehydrogenase E1 subunit β in human cancer. *World Journal of Gastrointestinal Oncology*, **16**(1): 144–181.
- Ros S, Schulze A. 2013. Balancing glycolytic flux: the role of 6-phosphofructo-2-kinase/fructose 2,6-

- bisphosphatases in cancer metabolism. *Cancer & Metabolism*, **1**(1): 8.
- Roy VK, Krishna A. 2010. Evidence of androgen-dependent sperm storage in female reproductive tract of *Scotophilus heathi*. *General and Comparative Endocrinology*, **165**(1): 120–126.
- Roy VK, Krishna A. 2011. Sperm storage in the female reproductive tract of *Scotophilus heathii*: role of androgen. *Molecular Reproduction and Development*, **78**(7): 477–487.
- Sasanami T, Izumi S, Sakurai N, et al. 2015. A unique mechanism of successful fertilization in a domestic bird. *Scientific Reports*, **5**: 7700.
- Sasanami T, Matsuzaki M, Mizushima S, et al. 2013. Sperm storage in the female reproductive tract in birds. *Journal of Reproduction and Development*, **59**(4): 334–338.
- Shih HT, Makoto K, Takashi K, et al. 2015. The ectoenzyme *E-NPP3* negatively regulates atp-dependent chronic allergic responses by basophils and mast cells. *Immunity*, **42**(2): 279–293.
- Shterzer N, Rothschild N, Sbehat Y, et al. 2020. Large overlap between the intestinal and reproductive tract microbiomes of chickens. *Frontiers in Microbiology*, **11**: 1508.
- Wang G, Zhu Y, Feng D, et al. 2024. Hepatic gluconeogenesis and regulatory mechanisms in lactating ruminants: A literature review. *Animal Research and One Health*, **3**: 230–239.
- Wen C, Li Q, Lan F, et al. 2021. Microbiota continuum along the chicken oviduct and its association with host genetics and egg formation. *Poultry Science*, **100**(7): 101104.
- Wen C, Mai C, Wang B, et al. 2020. Detrimental effects of excessive fatty acid secretion on female sperm storage in chickens. *Journal of Animal Science and Biotechnology*, **11**: 26.
- Wolc A, White IM, Olori VE, et al. 2009. Inheritance of fertility in broiler chickens. *Genetics Selection Evolution*, **41**(1): 47.
- Yang G, Li S, Zhao Q, et al. 2021. Transcriptomic and metabolomic insights into the variety of sperm storage in oviduct of egg layers. *Poultry Science*, **100**(6): 101087.
- Yang G. 2021. Transcriptomic and metabolomic insights into sperm storage of egg layer breeder. Master's Thesis, Huazhong Agricultural University. Wuhan, China. 10.27158/d.cnki.ghznu.2021.000662. (in Chinese)
- Yang L, Cai J, Rong L, et al. 2023. Transcriptome identification of genes associated with uterus-vagina junction epithelial folds formation in chicken hens. *Poultry Science*, **102**(6): 102624.
- Yang L, Li S, Mo C, et al. 2021. Transcriptome analysis and identification of age-associated fertility decreased genes in hen uterovaginal junction. *Poultry Science*, **100**(3): 100892.
- Yang L, Zheng X, Mo C, et al. 2020. Transcriptome analysis and identification of genes associated with chicken sperm storage duration. *Poultry Science*, **99**(2): 1199–1208.
- Yang L. 2021. Identification of Important Genes involved in the Development of Uterus-Vagina Junction Epithelial Fold (Containing Sperm Storage Tubules) in White Leghorns Hens. Doctoral Dissertation, Huazhong Agricultural University. Wuhan, China. 10.27158/d.cnki.ghznu.2021.000039. (in Chinese)
- Yoshimura Y, Koike K, Okamoto T. 2020. Immunolocalization of testosterone and estrogen receptors in the sperm storage tubules of laying and diethylstilbestrol-injected immature hens. *Poultry Science*, **79**(1): 94–98.
- You R, Lv X, Zhang Y, et al. 2026. Biosynthesis of functional carbohydrates from glycolytic node precursors glucose-6-phosphate and fructose-6-phosphate: Advances and prospects. *Biotechnology Advances*, **87**: 108808.
- Zhao Q, Wang Q, Yao Q, et al. 2025. Nonenzymatic lysine D-lactylation induced by glyoxalase II substrate SLG dampens inflammatory immune responses. *Cell Research*, **35**(2): 97–116.

- Zhang XK, Chen JL, Sun YY, et al. 2025. Multi-omics reveals key cell types and gene families regulating eggshell strength in chicken uteri. *Zoological Research*, **46**(6): 1396–1410.
- Zhu B, Tao Z, Edupuganti L, et al. 2022. Roles of the microbiota of the female reproductive tract in gynecological and reproductive health. *Microbiology and Molecular Biology Reviews*, **86**(4): e0018121.

Accepted

Figure titles and legends

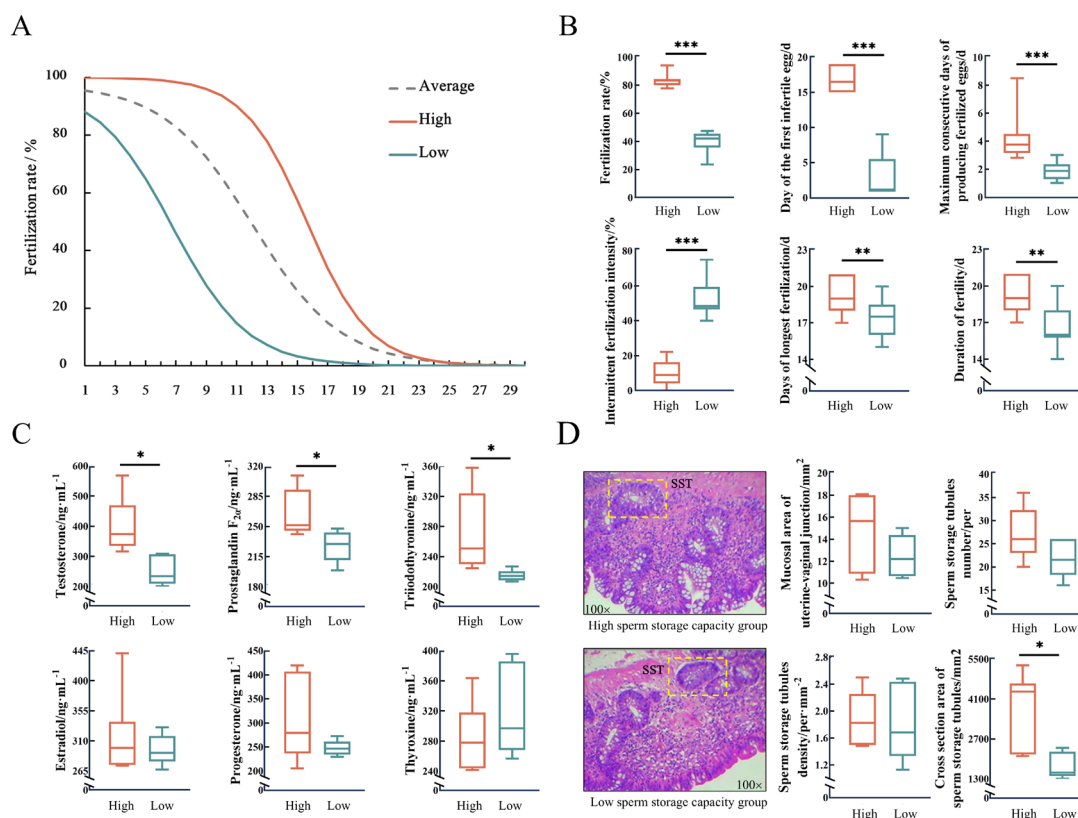


Figure 1 Phenotypic characteristics of the high and low sperm storage capability groups

A: Fertilization rate trends in High and Low groups. B: Statistical analysis of sperm storage capacity indicators. C: Serum reproductive hormone levels. D: Morphological observation of UVJ tissue, and yellow boxes indicate SST.

Note: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

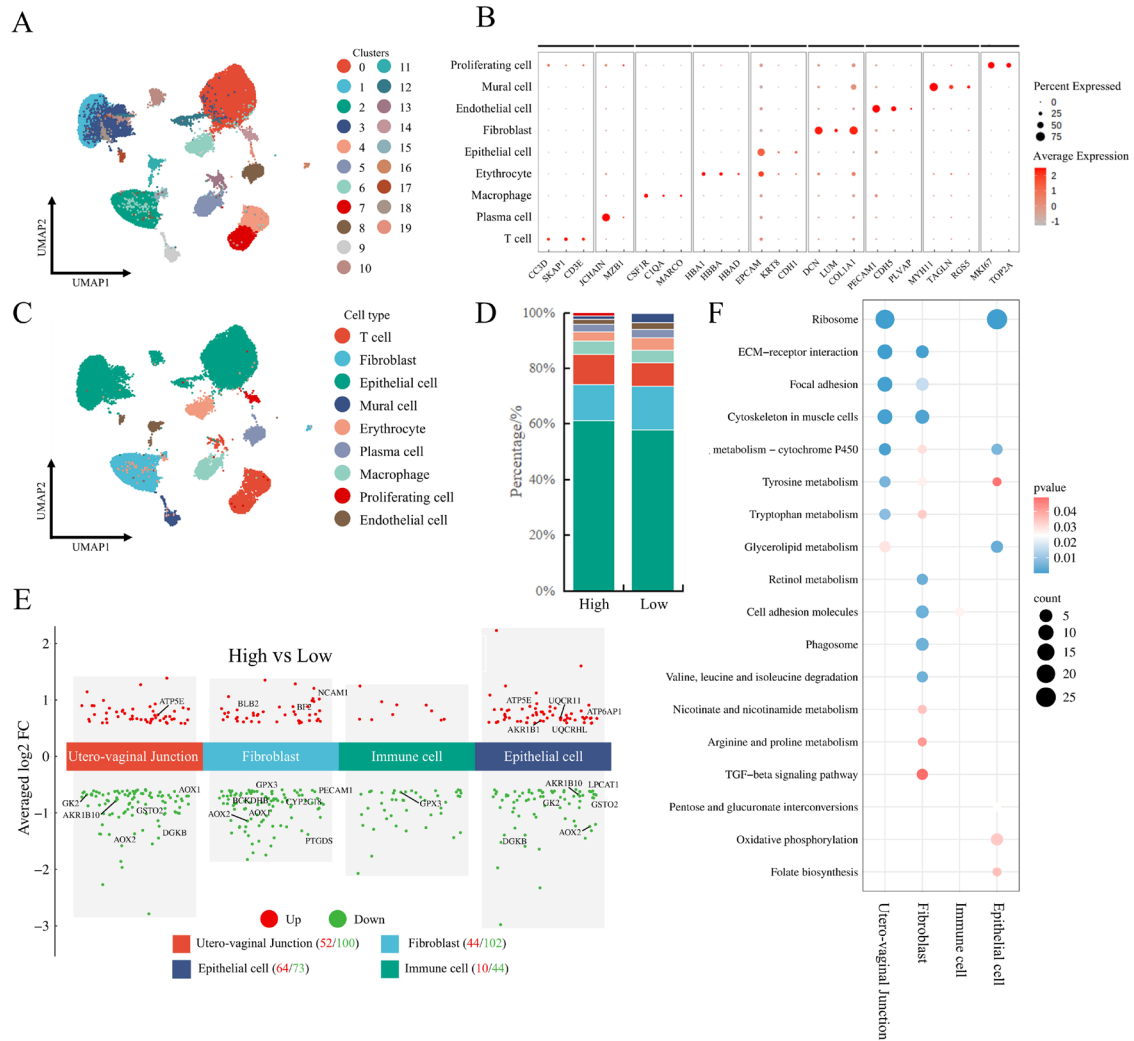


Figure 2 Construction of the UVJ single-cell transcriptomic atlas

A: Clusters identified by graph-based clustering of isolated single cell. B: Cell composition visualized by UMAP, colored by major type. C: Expression of marker genes in each cell type. D: Proportions of cell types in the high and low sperm storage capability groups. E: DEGs in the three major cell types between the high and low groups. F: KEGG enrichment analysis of the DEGs.

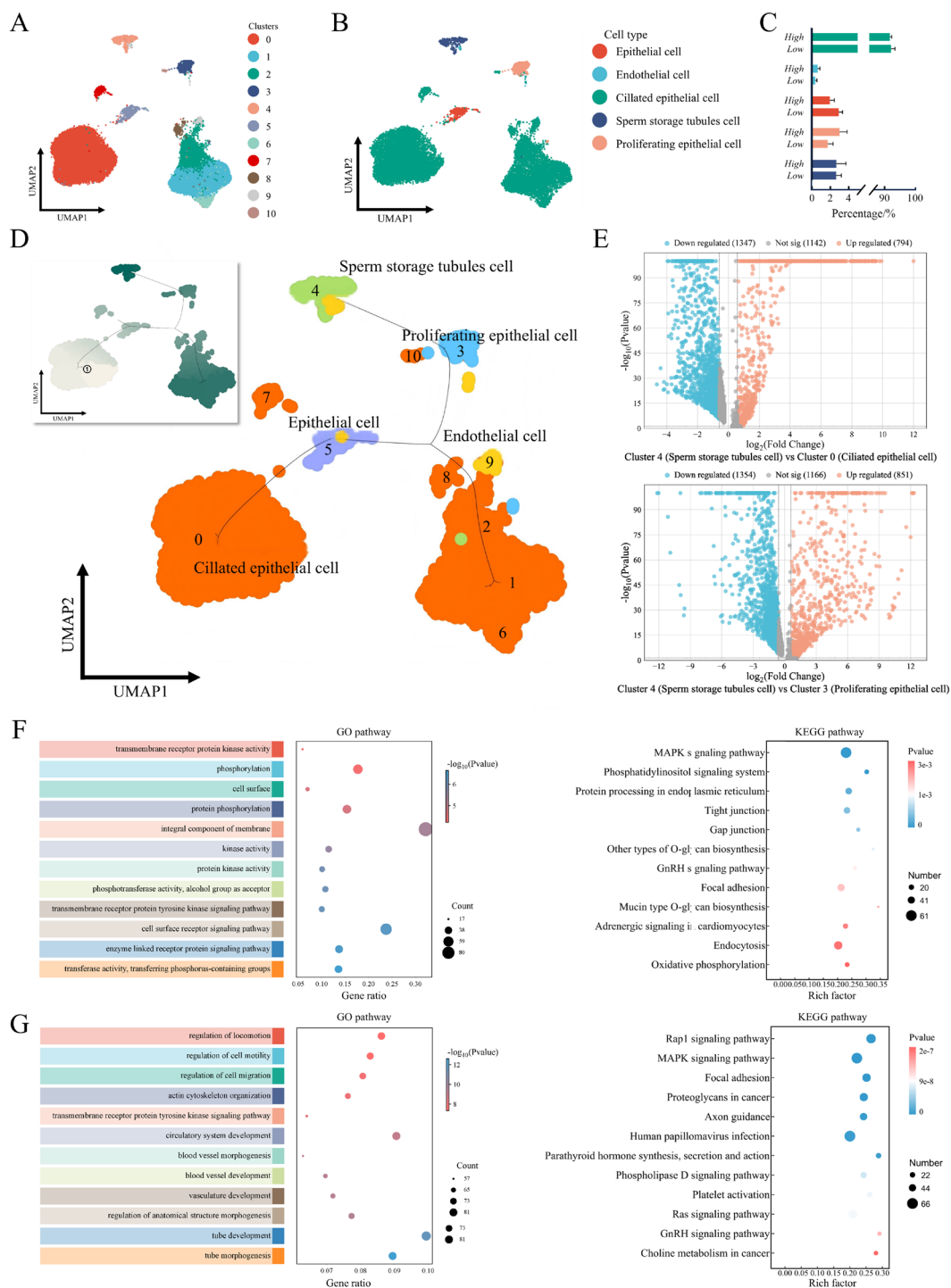


Figure 3 Subpopulation analysis of UVJ epithelial cells

A: Clusters identified by graph-based clustering of epithelial cell population. B:

Composition of epithelial cell subpopulations visualized by UMAP, colored by major

subtype. C: Proportions of cell subtypes in the high and low sperm storage capability groups, colored by major subtype, and results are expressed as mean $\pm$ SEM. D: Pseudotime single-cell trajectory of epithelial cells, pseudotime is indicated by color in a gradient from light to dark green, and the start of the pseudotime trajectory is denoted. E: Volcano plot of DEGs across epithelial cell developmental stages. F: Bubble chart of GO and KEGG enrichment analysis for DEGs between Cluster 4 (sperm storage tubules cell) and Cluster 0 (ciliated epithelial cell). G: Bubble chart of GO and KEGG enrichment analysis for DEGs between Cluster 4 (sperm storage tubules cell) and Cluster 3 (proliferating epithelial cell).

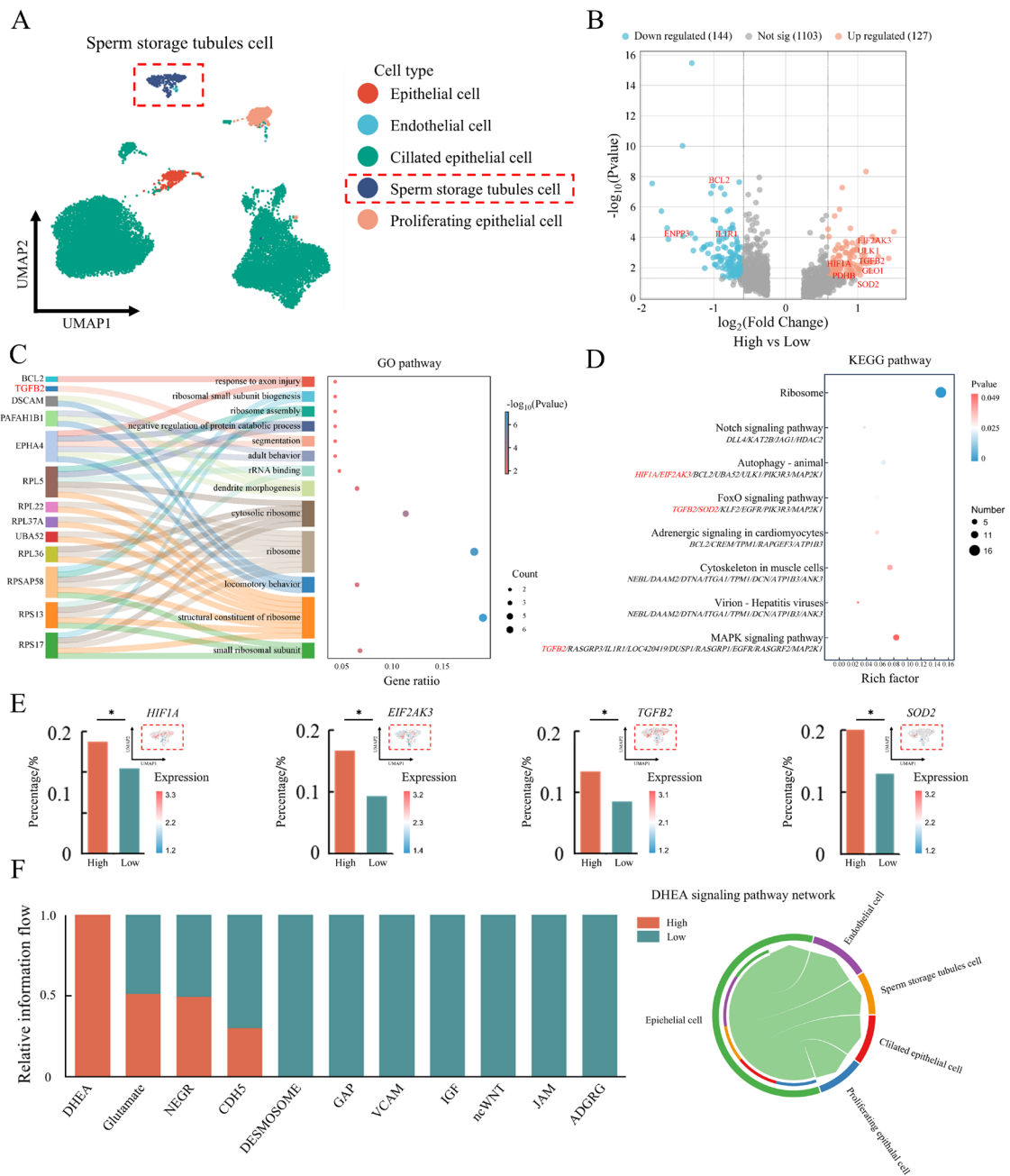


Figure 4 Subpopulation analysis of SST cell

A: SST cell within the UVJ epithelial cell subpopulation. B: Differential expression analysis of SST cell subpopulations between the high and low sperm storage capability groups. C: GO enrichment analysis of DEGs in SST cell. D: KEGG enrichment analysis of DEGs in SST cell. E: Visualization of key candidate gene expression (*HIF1A*,

EIF2AK3, *SOD2*, *TGFB2*) in SST cell. F: Cell-cell communication analysis, where the width of intercellular connections represents the strength of communication.

Accepted

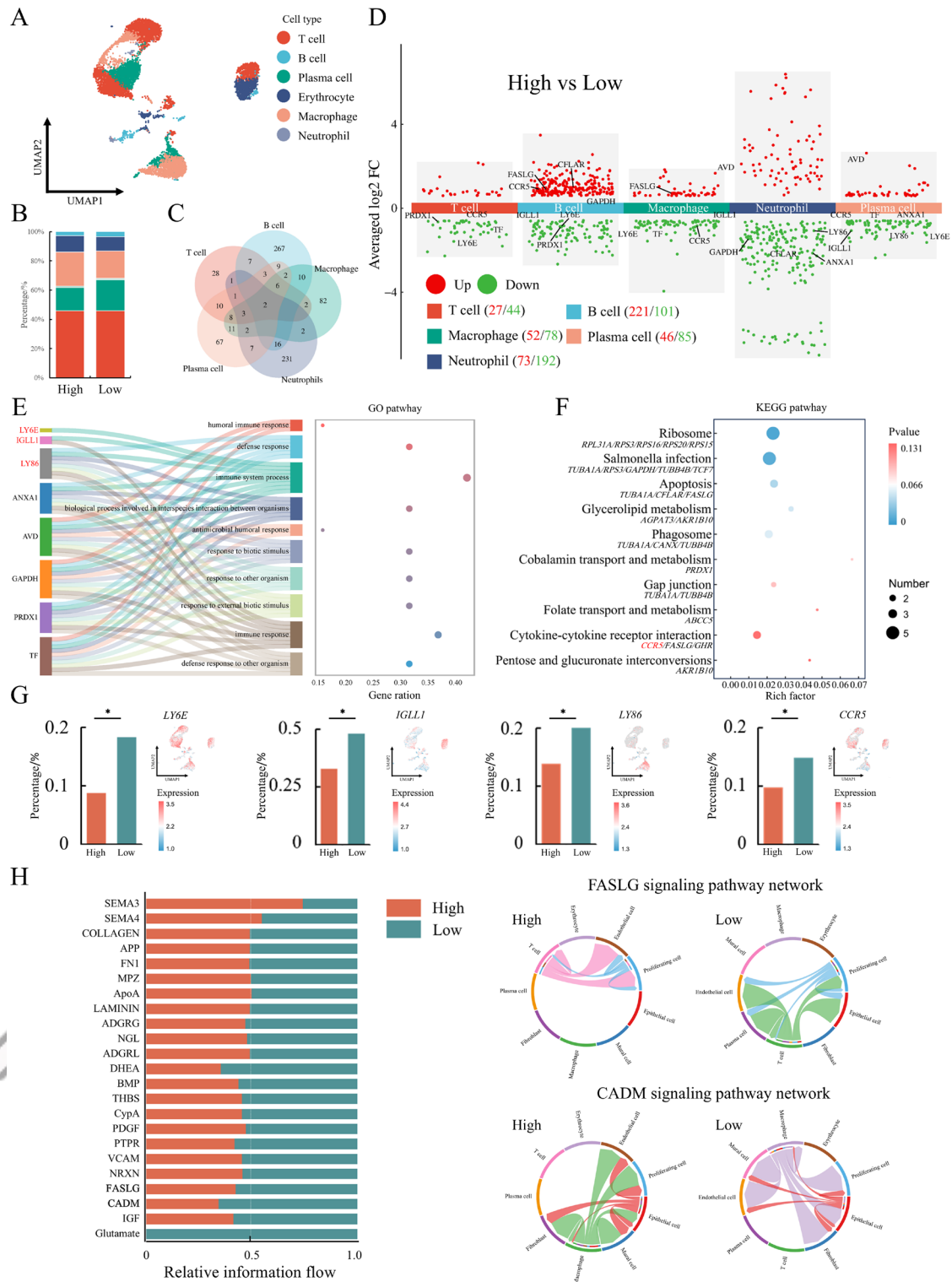


Figure 5 Subpopulation analysis of UVJ immune cell

A: Immune cell composition in the UVJ, visualized by UMAP and colored by major cell type. B: Proportions of immune cell types in the high and low sperm storage

capability groups. C: Venn diagram of DEGs in immune cell. D: Expression changes of DEGs in immune cell. E: GO enrichment analysis of DEGs in immune cell. F: KEGG enrichment analysis of DEGs in immune cell. G: Visualization of immune-related gene expression in immune cell. H: Cellular communication analysis associated with immune response, where the width of intercellular connections represents the strength of communication.

Accepted

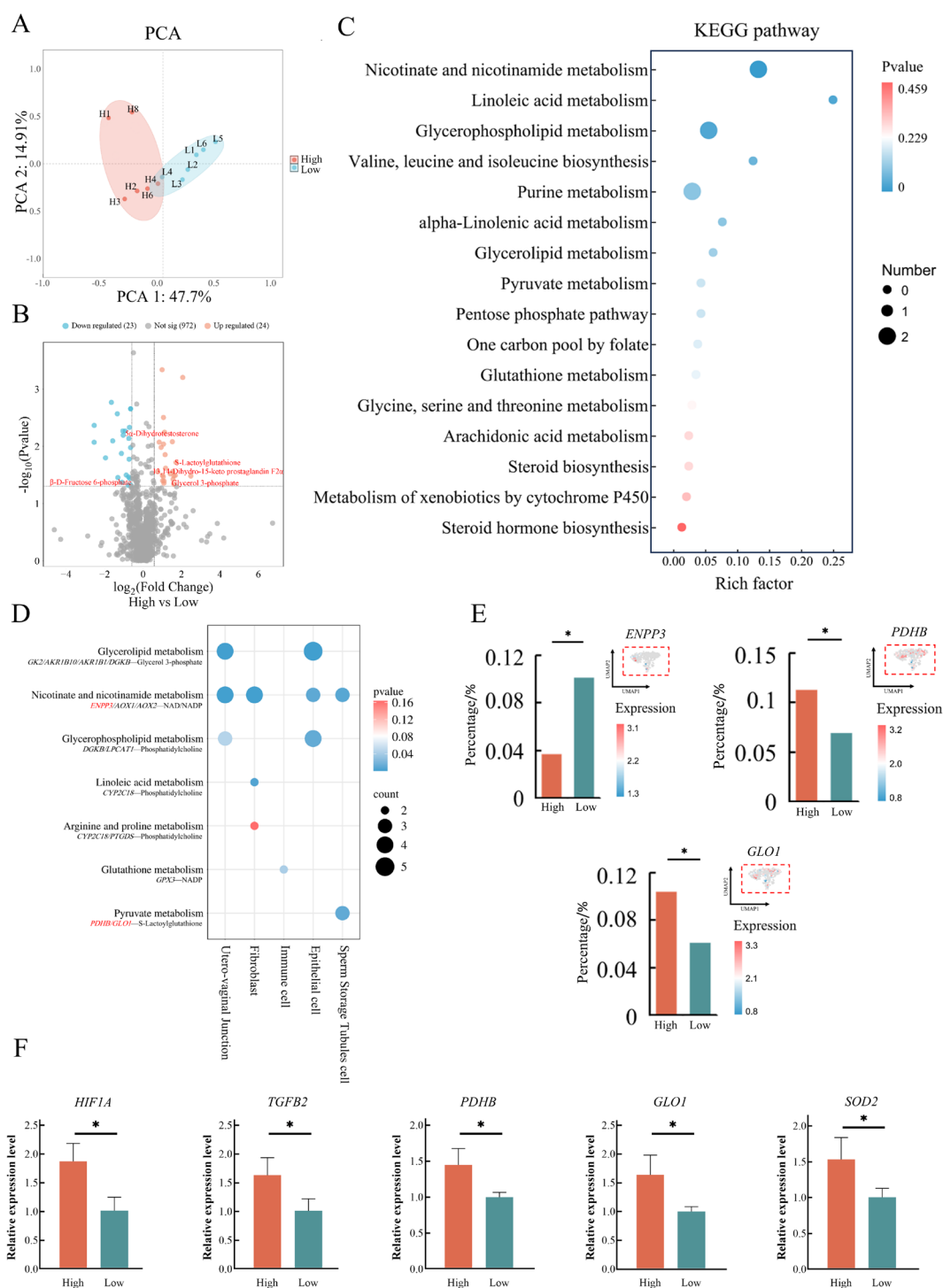


Figure 6 Metabolomic analysis of the UVJ

A: PCA for the high and low sperm storage capability groups. B: Volcano plot of DEMs

between the high and low sperm storage capability groups. C: KEGG enrichment

analysis of DEMs. D: Integrated pathway analysis of DEMs and DEGs in different cell types. E: Visualization of energy metabolism-related gene expression in SST cell. F: qRT-PCR validation of selected DEGs identified in the scRNA-seq analysis (*HIF1A*, *TGFB2*, *PDHB*, *GLO1*, *SOD2*).

Accepted



A: Relative abundance of microorganisms at the phylum level. B: Relative abundance of microorganisms at the genus level. C: Microbial α -diversity analysis. D: Differential

phylogenetic branch diagram and LDA bar plot of differential microorganisms. E: Differences in microbial relative abundance between the high and low sperm storage capability groups. F: Correlation analysis between key differential microorganisms and DEMs.

Accepted

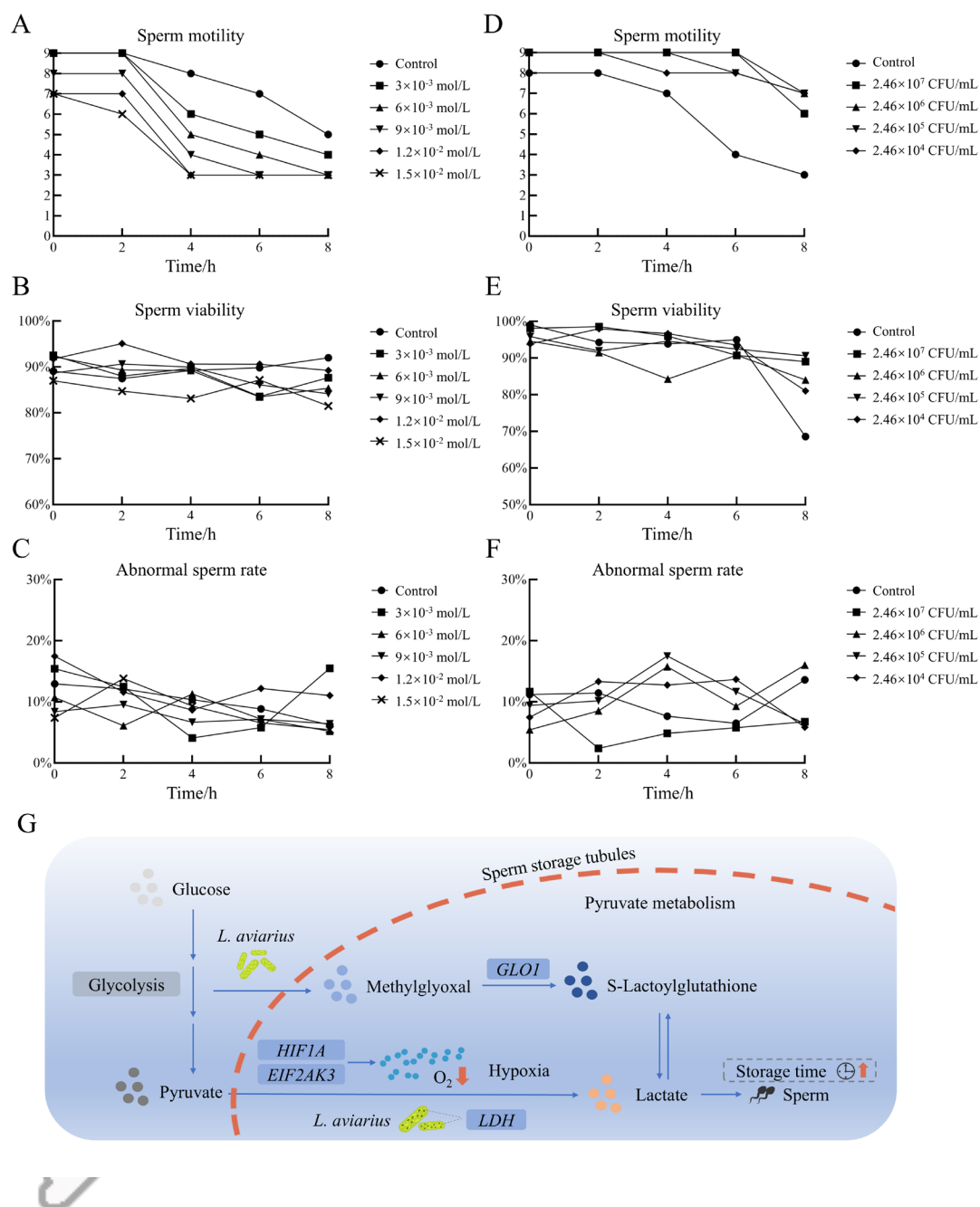


Figure 8 Sperm motility analysis

A: Effects of lactic acid on sperm motility in vitro, sperm motility was evaluated on a scale of 0 to 9. B: Effects of lactic acid on sperm viability in vitro. C: Effects of lactic acid on abnormal sperm rate in vitro. D: Effects of *L. aviarius* on sperm motility in vitro, sperm motility was evaluated on a scale of 0 to 9. E: Effects of *L. aviarius* on sperm

viability in vitro. F: Effects of *L. aviarius* on abnormal sperm rate in vitro. Control refers to the blank control group. G: Proposed model, an *L. aviarius*-reinforced, hypoxia-driven glycolytic program in SST cells promotes lactate production, which in turn suppresses sperm activation and prolongs sperm storage.

Accepted

Supplementary Tables

Supplementary Table S1. Sample source information

Group	Metabolomic	Metagenomic	scRNA-seq
High group	H1、H2、H3、H4、H5、H6	H1、H2、H3、H4、H5、H6	H1、H2、H3
Low group	L1、L2、L3、L4、L5、L6	L1、L2、L3、L4、L5、L6	L1、L2、L3

Accepted

Supplementary Table S2. List of primers used

Primer	Primer sequence (5'-3')
<i>HIF1A_F</i>	TACGGGACAATACAGAATGCTTGC
<i>HIF1A_R</i>	TCAAGTCCTTCTGAACAATTCCACTC
<i>SOD2_F</i>	GACCTGCCCTACGACTATGG
<i>SOD2_R</i>	TTGCCAGCGCCTCTTTGTAT
<i>TGFB2_F</i>	AGTGGGAAGACCCACATCT
<i>TGFB2_R</i>	GACGCAGGCAGCAATTATCC
<i>PDHB_F</i>	GTTGAGGCCGGTGTGTGAAT
<i>PDHB_R</i>	TCTTGGCGGCAGAGTTGATG
<i>GLO1_F</i>	AACTGACACACAACCTGGGGC
<i>GLO1_R</i>	AATGTGCCCAAATCCTCGGG
<i>ENPP3_F</i>	ACGTGCAAGGATGTGTCGAA
<i>ENPP3_R</i>	CAGCCCTTCTGGGTAGTTGG
<i>β-actin_F</i>	CCGCTCTATGAAGGCTACGC
<i>β-actin_R</i>	CTCTCGGCTGTGGTGGTGAA

老年母鸡贮精能力调控机制——基于整合多组学分析的比较研究

徐俊杰^{1,3}, 李欣欣¹, 李艳慧¹, 郭思含⁶, 范毅豪¹, 吴星霖¹, 姚懿纯¹, 张荣财¹, 赵晓钰², 苏丹⁴, 孙二东⁵, 王德贺¹, 陈一凡¹, 郝二英¹, 陈辉^{1,*}, 石雷^{1,*}

¹ 河北农业大学动物科技学院, 保定 071001, 中国

² 保定兴芮农牧发展有限公司, 保定 071001, 中国

³ 扬州大学动物科学与技术学院, 扬州 225009, 中国

⁴ 北京华都峪口禽业有限责任公司, 北京 101200, 中国

⁵ 河北桃木疙瘩农业科技有限公司, 保定 071001, 中国

⁶ 沈阳农业大学动物科学与医学学院, 沈阳 110866, 中国

通讯作者: 陈辉, 13930259580@163.com; 石雷, shilei2017@foxmail.com

摘要: 母鸡贮精能力是指雌性生殖道在较长时间内维持精子活性和受精能力的一种特性。该能力随周龄增长而下降, 尤其在产蛋后期表现明显, 从而降低生产效率, 但其潜在机制尚不清楚。本研究以 90 周龄白来航蛋鸡为对象, 评估了贮精能力性状、激素水平、子宫阴道结合部 (uterovaginal junction, UVJ) 组织形态学, 并开展了多组学分析 (单细胞转录组学、代谢组学、宏基因组学)。与低贮精能力组相比, 高贮精能力组母鸡血清中前列腺素 F_{2α}、睾酮和三碘甲状腺原氨酸水平更高, 贮精腺 (sperm storage tubules, SST) 横截面积也更大。UVJ 的单细胞转录组图谱揭示了 9 种主要细胞类型。拟时序轨迹分析显示, SST 细胞是一种终末分化细胞类型, 起源于纤毛上皮细胞, 并经过增殖上皮细胞的过渡阶段。高贮精能力组 SST 细胞中缺氧响应基因 (*HIF1A* 和 *EIF2AK3*) 及糖酵解相关基因 (*PDHB* 和 *GLO1*) 表达上调。同时, 免疫细胞中免疫相关基因表达下调, 表明存在局部免疫抑制微环境, 这可能有利于精子存活。整合代谢组和宏基因组数据进一步提示, 禽类乳杆菌 (*L. aviarius*) 活性与糖酵解增强之间可能存在关联, 并可能促进乳酸积累。综合而言, 我们提出缺氧的 SST 微环境驱动宿主无氧糖酵解, 并可能被 *L. aviarius* 所增强。这种协同性的乳酸积累可能诱导细胞质酸化, 从而抑制精子活化, 进而有助于长期储存。这些发现为通过靶向调控 UVJ 微环境以提高老龄母鸡群体繁殖效率提供了理论基础。

关键词: 母鸡; 贮精能力; 单细胞转录组; 缺氧微环境; 禽类乳杆菌; 糖酵解