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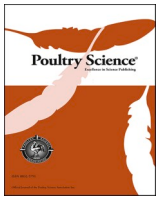

Full-Length Article

Molecular mechanisms underlying age-dependent effects of rearing system on the goose testicular development and semen quality

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ABSTRACT

As an important non-genetic factor, the rearing system has significant effects on male poultry reproductive system development. However, compared with other poultry such as chickens and ducks, less is known about the effects and mechanisms of rearing system on the gander reproductive organ development and semen quality. In the present study, the testicular morphological, histological, and transcriptomic responses of three goose breeds to the two dryland rearing systems (i.e., cage rearing system, CRS and net-floor mixed rearing system, MRS) were systematically analyzed and compared. Results from histomorphological analysis demonstrated that the effects of rearing system on the gander testicular development were age-dependent, and moreover, the CRS may be more conducive than MRS to the testicular development and semen quality during the period from post-hatch week 10 to week 43. At week 30, compared to Sichuan White goose (SW), the rearing system showed more pronounced effects on the testicular size, weight, and organ index of Gang goose (GE) and Landes goose (LD). However, such effects were mitigated in LD and even reversed in GE at week 43. Meanwhile, most testicular histological parameters of three goose breeds were higher under MRS than under CRS at week 30, while the converse was seen in some histological parameters of either GE or LD at week 43. Moreover, the semen quality was generally better under CRS than under MRS at week 43. Through comparative transcriptomics analysis, the Wnt signaling pathway together with several involved hub genes were identified to have important roles in mediating the effects of rearing system on the goose testicular development. Moreover, the metabolism-related, cell cycle, and Wnt signaling pathways could be partially responsible for differences in the goose breed-related testicular development and semen quality under CRS, where a number of genes involved in meiosis could have crucial roles. These results would not only provide novel insights into the effects and mechanisms of rearing system on male poultry reproductive performance, but they would also be helpful for the optimization and selection of dryland rearing systems in male geese.

Introduction

China is the largest producer of geese in the world, accounting for more than 90% of global goose production (Kozák, 2021), and the goose industry plays essential roles in Chinese agricultural economics and rural revitalization. Compared to male chickens and ducks, the reproductive performance of ganders is much lower, as primarily reflected by their high percentage of abnormal external genital development, late

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maturity, and poor semen quality (Akhtar, et al., 2021; Shi, et al., 2008; Zhou, et al., 2020). Recently, with the increasing demands for environmental protection, food security, and efficient and sustainable goose production, the Chinese goose rearing systems have been gradually transitioning from the traditional extensive free-range rearing ones near water to the modern intensive dryland rearing ones. In this context, there is an urgent and important need to improve gander reproductive efficacy, which is crucial for widespread applications of the artificial insemination techniques and thereby has a great potential in increasing the economic gains from commercial goose breeder stocks (Akhtar, et al., 2021).

The gander reproductive tract mainly comprises testis, epididymis, vas deferens, and external genitalia (Aire, 2014). Among them, testis is the site of spermatogenesis and sex steroid hormone secretion, hence the orderly progression of testicular development is essential for gander fertility (Vizcarra, et al., 2022). In general, genetics, age, and a variety of environmental factors (*e.g.,* rearing system, nutrition, climate, light, temperature, and health status) can have significant impacts on the testicular development of male poultry. In the past half century, a multitude of extensive and intensive rearing systems have been used in commercial poultry production, and the type of rearing system is widely recognized as a key factor influencing their physiological and reproductive responses and thereby productive performance including quantity and quality (Bogosavljević-Bošković, et al., 2012). With respect to the reproductive responses of male poultry, a recent study in layer breeder roosters reported that three cage rearing systems exerted differential effects on the testicular development, semen quality, and the apoptosis of spermatogonia and spermatocytes (Du, et al., 2021). Meanwhile, compared to natural mating systems in mixed-sex pens, a higher male fertility was observed in broiler breeders when housed individually and artificially inseminated, which could be attributed to male-male competition (Bilcik, et al., 2005). In Muscovy ducks, it was also shown that the rearing system significantly affected their semen quality, and the availability of swimming water seemed to be positively associated with sperm concentration and motility (Etuk, et al., 2006). By contrast, no significant differences were seen in semen quality indices of turkeys reared under intensive and semi-intensive management systems (Ezike, et al., 2021). Nevertheless, compared to survivability, immunity, growth, and female reproduction traits (Bogosavljević-Bošković, et al., 2012), less information is available about the effects and mechanisms of rearing systems on the poultry testicular development and functions.

As for the impacts of dryland rearing systems on goose production performance, there is emerging evidence that most economically important traits including body weight, carcass traits, meat quality, egg production, intestinal digestion, and immunity are significantly influenced by the rearing system type (Boz, et al., 2017a; Boz, et al., 2017b; Chen, et al., 2024; Li, et al., 2022a; Liu, et al., 2011). However, studies on the effects of different dryland rearing systems on the gander testicular development and semen quality are still scarce. In the present study, by taking two Chinese domestic goose breeds (Sichuan White goose, SW and Gang goose, GE) that originate from swan goose (*Anser cygnoides*) and one European domestic goose breed (Landes goose, LD) that originates from graylag goose (*Anser anser*) due to their excellent production performance and significant economic values as the experimental objects, we comprehensively addressed the age-dependent morphological, histological, and transcriptomic responses of gander testes to the two different dryland rearing systems, *i.e.,* net-floor mixed rearing system (MRS) and cage rearing system (CRS). Moreover, the key protein-coding genes, signaling pathways, and regulatory networks implicated in the rearing system-modulated gander testicular responses were also delineated. These data would provide novel insights into the effects and mechanisms of environmental management systems on reproductive performance of male poultry and be useful for optimizing the dryland rearing system of male breeder stocks in commercial goose breeding and production.

Materials and methods

Ethics statement

All experimental procedures involving the manipulation of geese in this study were conducted in concordance with the "Guidelines for Experimental Animals" of the Ministry of Science and Technology (Beijing, China). The animal use protocol has been reviewed and approved by the Sichuan Agricultural University Animal Ethical and Welfare Committee (Approval No: 20190035).

Experimental birds and sample collection

All experimental ganders of SW, GE, and LD were provided by the Waterfowl Breeding Experimental Farm of Sichuan Agricultural University (Ya'an, Sichuan, China). A total of 100 healthy 0-day-old male geese from each breed were hatched from respective fertilized eggs at the same batch, respectively, and were reared under the similar environmental and feeding conditions during the first 10 post-hatching weeks. Then, the ganders from each goose breed were randomly equally divided into two experimental groups, respectively, including the cage-rearing system (CRS) and net-floor mixed rearing system (MRS). Under CRS, each gander was kept in a single cage, with a size of 350 mm width × 550 mm length × 700 mm height. Under MRS, the ganders were reared in an indoor area with a size of 13 m length × 6 m width, which consisted of a 60 m^2 plastic net at a height of 1 m above the ground level and an 18 m^2 deep litter floor.

At 30 weeks of age, 12 male geese of similar body weights randomly selected from each experimental group per breed were sampled for testicular morphological and histological analysis, respectively. At 43 weeks of age, semen was firstly collected from at least 12 male geese of each experimental group per breed, respectively, for subsequent quality analysis. Then, twelve male geese of similar body weights from each experimental group per breed were used for sample collection, respectively. At each sampling time point, by recording the live body weights of ganders after a 12-hour fasting, they were euthanized by inhaling carbon dioxide, followed by cervical dislocation. After slaughter, both the left and right testes were weighed, measured, and photographed to reveal their morphological characteristics. The reproductive organ index was calculated using the following formula: organ index (%) = (organ weight (g)/body weight (kg)) × 100%. The long, short, and dorsoventral diameters of both left and right testis were also measured. Subsequently, the testes from 6 individuals per experimental group per breed were used for histological examination. Meanwhile, the testes sampled from the other 4 individuals of each experimental group per breed at 43 weeks of age were rapidly frozen in liquid nitrogen and stored at -80°C until RNA extraction.

Histological analysis

The freshly-collected goose testes were firstly fixed with 4% formaldehyde at room temperature for 72 h, then dehydrated through a series of different concentrations of ethanol, and finally transferred to xylene and embedded in paraffin wax. After being cut into about 5 μm thick slices, they were stained with hematoxylin and eosin (H&E). The H&E-stained slices were observed and photographed under a digital trinocular camera microscope BA410-Digital (Motic China Group Co. Ltd., Xiamen, China). The testicular histological parameters were measured using Image-Pro Plus 6.0 software (National Institutes of Health, Bethesda, MD, USA), including the testicular parenchymal-to-interstitial ratio, the thickness of seminiferous epithelium, the diameter and number of seminiferous tubules, the number and density of Leydig cells, the number of Sertoli cells, the number of spermatogonia, the number of spermatids, and the number of spermatozoa. Each testicular histological parameter was calculated as the mean of the observations of all examined individuals from each experimental group per

goose breed at each sampling time point.

Semen quality analysis

As for goose semen quality analysis, several parameters, including the ejaculate volume, sperm density, sperm viability, sperm motility, morphological abnormal sperm, and acrosomal integrity, were measured as follows. After semen collection, the ejaculate volume of each gander was measured by a 1 mL syringe, and sperm density (defined as the number of sperms per mL) was subsequently counted by a hemocytometer after an appropriate dilution with 0.9% sterile physiological saline solution. Next, sperm viability (%) was assessed using 1% trypan blue solution (Solarbio, Beijing, China) to determine the proportion of live and dead sperms at 400 × magnification under a phase contrast microscope (Olympus, Tokyo, Japan), where blue-stained sperms were considered dead and unstained sperms were considered live. Meanwhile, sperm motility (%) was calculated as the proportion of linearly moving sperms to the total sperm counts after appropriate dilutions under a phase contrast microscope (Olympus, Tokyo, Japan). Then, morphological abnormal sperm (%) was assessed using Gentian violet staining solution (Sangon Biotech, Shanghai, China) to calculate the proportion of deformed sperms (such as head, neck, midpiece, and tail defects) to the total sperm counts under a phase contrast microscope (Olympus, Tokyo, Japan). Besides, the Aniline blue staining solution (Sangon Biotech, Shanghai, China) was used to differentiate the sperms with intact or damaged acrosomes under a phase contrast microscope (Olympus, Tokyo, Japan), and sperm acrosomal integrity (%) was calculated as the proportion of sperms with intact acrosomes to the total sperm counts. Each of the above-mentioned semen quality parameters was tested in triplicates. Finally, the Semen Quality Factor (SQF) value of each gander was calculated as described in a previous study (Liu, et al., 2008) according to the following equation: SQF = ejaculate semen volume (mL) × sperm density (× 106/mL) × live and morphologically normal sperm (%).

Total RNA extraction, library preparation, and sequencing

Total RNA was extracted from the 24 testes (n = 4 per rearing system per breed) of three goose breeds at 43 weeks of age using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol, respectively. The RNA concentration and integrity were assessed by a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and Agilent 2100 Bioanalyzer system (Agilent Technologies, Palo Alto, CA, USA). The RNA-Seq libraries were prepared using the Illumina TruSeq mRNA Sample Preparation Kit (Illumina, San Diego, CA, USA) following the manufacturer's instruction and sequenced on an Illumina NovaSeq 6000 platform in PE150 mode (Beijing Glbizzia Biotechnology Co., Ltd, China). The sequencing data of this study have been archived in the GSA database with the accession number PRJCA028036 (https://ngdc.cncb.ac.cn/gsa).

Data filtering, mapping, and bioinformatic analysis

The quality of all raw reads was evaluated by Fastqc v0.11.9 software, and according to the filtering criteria, the short sequencing fragments with base quality values below 20, adaptor reads, poly-N contained reads (N% > 10%), and low-quality reads were removed. The obtained clean reads were subsequently mapped to our recently assembled high-quality chromosomal-level genome of SW using HISAT2 v2.2.1 software to obtain the sequence alignment map (SAM) files (Kim, et al., 2015). The SAM files were then converted and sorted into the binary alignment/map (BAM) files using SAMtools v1.6.0 software (Li, et al., 2009). The relative expression levels of each transcript were calculated using FeatureCounts v1.6.0 software (Liao, et al., 2014), and the obtained read counts were normalized as the fragments per kilobase of transcript per million reads mapped (FPKM) values. Differentially expressed genes (DEGs) between any two experimental groups were identified according to the criteria: adjusted P value < 0.05 and $\log_2|$ foldchange$| > 1$ using DESeq2 v1.34.0 package of R (Love, et al., 2014). Both the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) functional enrichment analyses were performed using KOBAS v3.0 software (Xie, et al., 2011). The top 5000 testicular genes of all sequencing samples filtered with the median absolute deviation (MAD) were used to identify key gene modules by weighted gene co-expression network analysis (WGCNA), with the parameters set as follows: power = 15, minModuleSize = 50, and mergeCutHeight = 0.25. Finally, the protein-protein interaction (PPI) networks of selected DEGs were analyzed using the STRING 10 database (Szklarczyk, et al., 2019), and the Cytoscape v3.9.1 software (Smoot, et al., 2011) was used to visualize their interaction networks.

Quantitative real-time PCR verification

A total of 8 testicular DEGs, including Wnt family member 3A (*WNT3A*), hypoxia inducible factor 1 subunit alpha (*HIF1A*), short transmembrane mitochondrial protein 1 (*STMP1*), mesoderm development LRP chaperone (*MESD*), CD59 molecule (*CD59*), potassium channel tetramerization domain containing 1 (*KCTD1*), MYC binding protein (*MYCBP*), and semaphorin 4D (*SEMA4D*), were randomly selected for validation of their expression profiles using quantitative real-time PCR (RT-qPCR). Equal amount of total RNA extracted from each sample was reversely transcribed into the cDNAs using the HiScript® RT SuperMix for qPCR (+gDNA wiper) (Vazyme, Nanjing, China) following the manufacturer's instruction. The Primer Priemer 5.0 software was used to design the RT-qPCR primer pairs, which are listed in Table 1. The RT-qPCR reactions were conducted in the Bio-Rad CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA, USA) with the following conditions: pre-denaturation at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5 s, and annealing/extension at the optimal temperature of each primer pair for 30 s. The no-template controls and negative controls without reverse transcriptase were also included in all RT-qPCR runs. Target specificity for each primer set was validated by melting curve analysis, and the identity of all amplicons was verified by sequencing. Each sample was run in triplicate, and *GAPDH* and *β-ACTIN* were used as the two housekeeping genes. The $2^{-\Delta\Delta CT}$ method was used for normalization of the relative expression levels of these selected DEGs (Schmittgen and Livak, 2008).

Table 1
The primer pairs used for RT-qPCR in this study.

Gene	Primer Sequence (5′→3′)	Size (bp)	Tm (°C)
WNT3A-F	CTGTGCCAGTTTTCTCACCAT	106	59
WNT3A-R	AAGGCAAAGTCATTCACAACAA		
HIF1A-F	TATAAAGTCTGCTACGTGGAAGG	151	58
HIF1A-R	AATGTTGGATGGGTGAGGAAT		
STMP1-F	CAATGTGGTTGGGATGTATCTG	116	60
STMP1-R	AGGACTTGTCACTGGGAGGTT		
MESD-F	AAGAAGGACATTCGGGACTACAACG	171	57
MESD-R	CGTCAGTTTCAGGATACTTTCAGGCT		
CD59-F	AACCGTCTGCATCGTTCTGGT	191	58
CD59-R	CATTCACGTTGCACTGGGACA		
KCTD1-F	CTCAAGCAGCATTATTTCATCG	224	57
KCTD1-R	ACCACCAGGCACTCACAAGAC		
MYCBP-F	TTGCAGTGTTGGTAGCCTTAT	111	57
MYCBP-R	GTGCCTCTATTTCTGGATTCTC		
SEMA4D-F	TTTGTGGAAGTGGCAGTAACAT	152	57
SEMA4D-R	CTGGCTCCTACATACAGAACAT		
**GAPDH*-F	CATGTTCGTGATGGGTGTG	239	59
**GAPDH*-R	CTGGGATAATGTTCTGGGC		
**β-ACTIN*-F	CAACGAGCGGTTCAGGTGT	99	60
**β-ACTIN*-R	TGGAGTTGAAGGTGGTCTCGT		

Note: F, forward primer. R, reverse primer. The asterisk indicates two housekeeping genes.

Statistical analysis

All data were expressed as the mean ± SEM. Statistical analysis of the obtained testicular histomorphological parameters and semen quality indicators was performed using a two-way analysis of variance (ANOVA) model in the General Linear Model (GLM) procedure of SAS 9.4 (SAS Institute, Cary, USA), and the statistical model included the main effects of breed (SW, GE or LD), rearing system (CRS or MRS), and their interaction. When a significant effect was observed, the Duncan's multiple range test was used to assess the significance of differences between different rearing systems or breeds. The *P* values below 0.05 and 0.01 were considered statistically significantly and extremely significantly different, respectively. Pearson correlation coefficients were calculated to analyze the correlations between testicular histomorphological parameters and semen quality indicators. The GraphPad Prism 8.0 software was used to visualize the results.

Results

Age-dependent effects of two rearing systems on testicular morphology of ganders from three goose breeds

At the morphological level, the bilateral testicular size and volume of either GE or LD under MRS appeared to be larger than those under CRS, while minor differences were observed in SW under the two rearing systems at week 30 (reaching sexual maturation). By comparison, the bilateral testicular size and volume of GE under CRS seemed to be larger than those under MRS, while minor differences were observed in either SW or LD under the two rearing systems at week 43 (reaching body maturation, Fig. 1A). Statistically, at 30 weeks of age, there were no significant differences ($P > 0.05$) in the testicular size, weight, and organ index of SW under the two rearing systems; however, the bilateral testicular long diameter, weight, and organ index, as well as the left testicular short diameter of either LD or GE under MRS were significantly higher than those under CRS (Fig. 1B; $P < 0.05$). At 43 weeks of age, the right testicular dorsoventral diameter of SW under CRS were significantly longer than those under MRS, and the bilateral testicular dorsoventral diameter, weight, and organ index, as well as the right testicular long and short diameter of GE under CRS were significantly higher than those under MRS (Fig. 1C; $P < 0.05$).

As for the breed-related comparison, at 30 weeks of age, the left testicular long and short diameter, the right testicular dorsoventral diameter, and the bilateral testicular weight and organ index were significantly higher in SW than in either GE or LD under CRS (N

Fig. 1A; $P < 0.05$). By comparison, the left testicular short diameter, the right testicular short diameter, and the left testicular weight were significantly lower in SW than in GE under MRS (Supplementary Fig. 1B; $P < 0.05$). At 43 weeks of age, the left testicular short and dorsoventral diameter, weight, and organ index were significantly higher in GE than in either SW or LD under CRS, while the right testicular dorsoventral diameter, weight, and organ index were significantly lower in LD than in either SW or GE under CRS (Supplementary Fig. 1C; $P < 0.05$). Besides, no significant differences were seen in the bilateral testicular size, weight, and organ index among SW, GE, and LD under MRS (Supplementary Fig. 1D; $P > 0.05$).

Age-dependent effects of two rearing systems on testicular histology of ganders from three goose breeds

At the histological level, as shown in Fig. 2, the testes isolated from all three goose breeds under the two rearing systems at week 30 or 43 exhibited similar histological components, including the seminiferous tubule, seminiferous epithelium, Leydig cell, Sertoli cell, spermatogonia, spermatid, and spermatozoon. However, some testicular histological parameters differed in the diameter and number between CRS and MRS depending on the developmental stage. Statistically, as shown in Fig. 3A, at 30 weeks of age, the testicular parenchymal-to-interstitial ratio and Sertoli cell number of SW under CRS were significantly lower than those under MRS ($P < 0.05$). The number of seminiferous tubules of GE under CRS was significantly higher than those under MRS ($P < 0.01$), while the number of Sertoli cells and spermatids of GE under CRS were significantly lower than those under MRS ($P < 0.05$). Meanwhile, the diameter of seminiferous tubules and the number of Sertoli cells, spermatogonia, spermatids, and spermatozoa of LD under CRS were significantly lower than those under MRS ($P < 0.05$); however, the number of seminiferous tubules of LD under CRS was significantly higher than those under MRS ($P < 0.01$). As shown in Fig. 3B, at 43 weeks of age, the seminiferous epithelium thickness and seminiferous tubule number of SW under CRS were significantly higher than those under MRS ($P < 0.05$). The testicular parenchymal-to-interstitial ratio, seminiferous epithelium thickness, and seminiferous tubule diameter of GE under CRS were significantly higher than those under MRS ($P < 0.05$), while the Sertoli cell number of GE under CRS was significantly lower than those under MRS ($P < 0.01$). However, no significant differences were observed in the measured testicular histological parameters of LD under the two rearing systems ($P > 0.05$).

As for the breed-related comparison, at 30 weeks of age, the seminiferous epithelium thickness was significantly lower in SW than in LD under CRS ($P < 0.05$), while the seminiferous tubule diameter was significantly higher in SW than in GE under CRS ($P < 0.05$). Meanwhile, the number of spermatids and spermatozoa was significantly higher in SW and GE than in LD under CRS (Supplementary Fig. 2A; $P < 0.05$). By comparison, the seminiferous epithelium thickness, seminiferous tubule diameter, and the number of spermatids and spermatozoa were significantly higher in LD than in SW and GE under MRS ($P < 0.05$); however, the Leydig cell density and seminiferous tubule number were extremely significantly lower in LD than in SW and GE under MRS (Supplementary Fig. 2B; $P < 0.01$). At 43 weeks of age, the testicular parenchymal-to-interstitial ratio was extremely significantly higher in GE than in SW and LD under CRS ($P < 0.01$), while the Leydig cell density and seminiferous tubule number were significantly higher in SW than in GE and LD under CRS ($P < 0.05$). Meanwhile, the number of Sertoli cells, spermatids, and spermatozoa were significantly higher in LD than in SW and GE under CRS (Supplementary Fig. 2C; $P < 0.05$). Besides, the testicular parenchymal-to-interstitial ratio was lower in SW than in GE and LD under MRS ($P < 0.01$), while the number of Sertoli cells was higher in GE than in SW and LD under MRS (Supplementary Fig. 2D; $P < 0.01$).

Effects of two rearing systems on semen quality of ganders from three goose breeds

As shown in Fig. 4, in general, the semen quality of all three goose breeds (SW, GE, and LD) under CRS were better than those under MRS at 43 weeks of age. Specifically, the sperm density and semen quality factor (SQF) of all three goose breeds under CRS were significantly higher than those under MRS ($P < 0.05$). The sperm motility and morphological abnormal sperm (MAS) percentages of GE under CRS were significantly lower than those under MRS ($P < 0.05$), while the sperm viability percentage of GE under CRS was extremely significantly higher than those under MRS ($P < 0.01$). Meanwhile, the sperm motility percentage of LD under CRS was significantly higher than those under MRS ($P < 0.01$), while the MAS of LD under CRS was significantly lower than those under MRS ($P < 0.05$).

Regarding the breed-related comparison, as shown in Supplementary Fig. 3, the semen quality of SW was generally better than that of GE and LD under either CRS or MRS. Specifically, the ejaculation volume, sperm density, and SQF were higher in SW than in GE and LD under CRS, and the ejaculation volume was extremely significantly lower in LD than in SW and GE under CRS ($P < 0.01$). Besides, the ejaculation volume was significantly higher in SW than in LD under MRS ($P < 0.05$), while the sperm motility and acrosomal integrity percentage were extremely

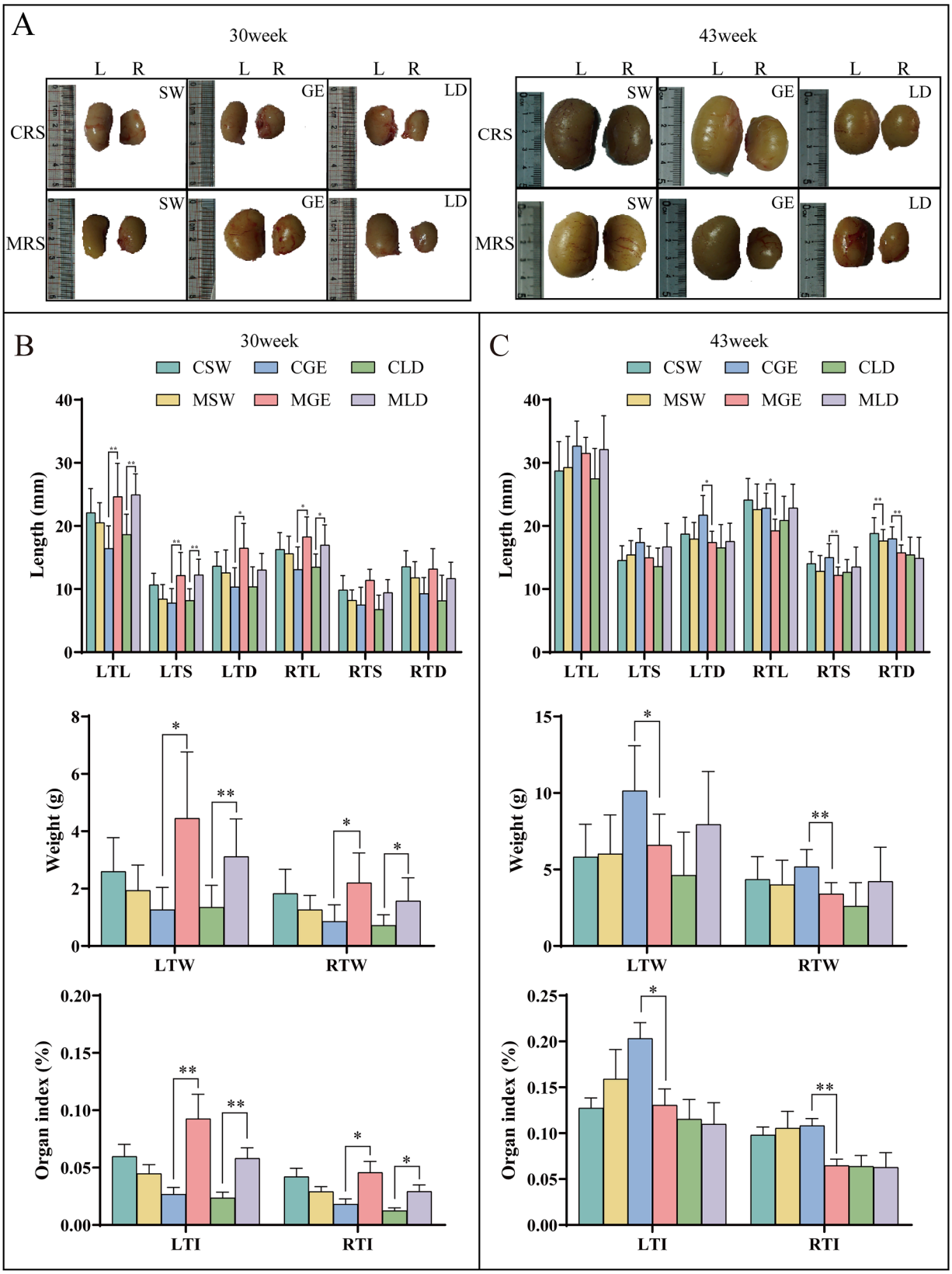

Fig. 1. Morphological differences in the testes of three goose breeds under two rearing systems at different weeks of age. (A) Representative testicular photos of three goose breeds at different weeks of age. SW, Sichuan White goose. GE, Gang goose. LD, Landes goose. CRS, cage-rearing system. MRS, net-floor mixed rearing system. L, left testis. R, right testis. (B-C) Comparison of testicular morphological parameters of three goose breeds under two rearing systems at 30 (B) and 43 weeks of age (C). CSW, SW under CRS. MSW, SW under MRS. CGE, GE under CRS. MGE, GE under MRS. CLD, LD under CRS. MLD, LD under MRS. LTL/RTL, the long diameter of left/right testis. LTS/RTS, the short diameter of left/right testis. LTD/RTD, the dorsoventral diameter of left/right testis. LTW/RTW, the left/right testicular weight. LTI/RTI, the left/right testicular organ index. "*" indicates a significant difference between the two designated groups ($P < 0.05$), while "**" indicates an extremely significant difference ($P < 0.01$).

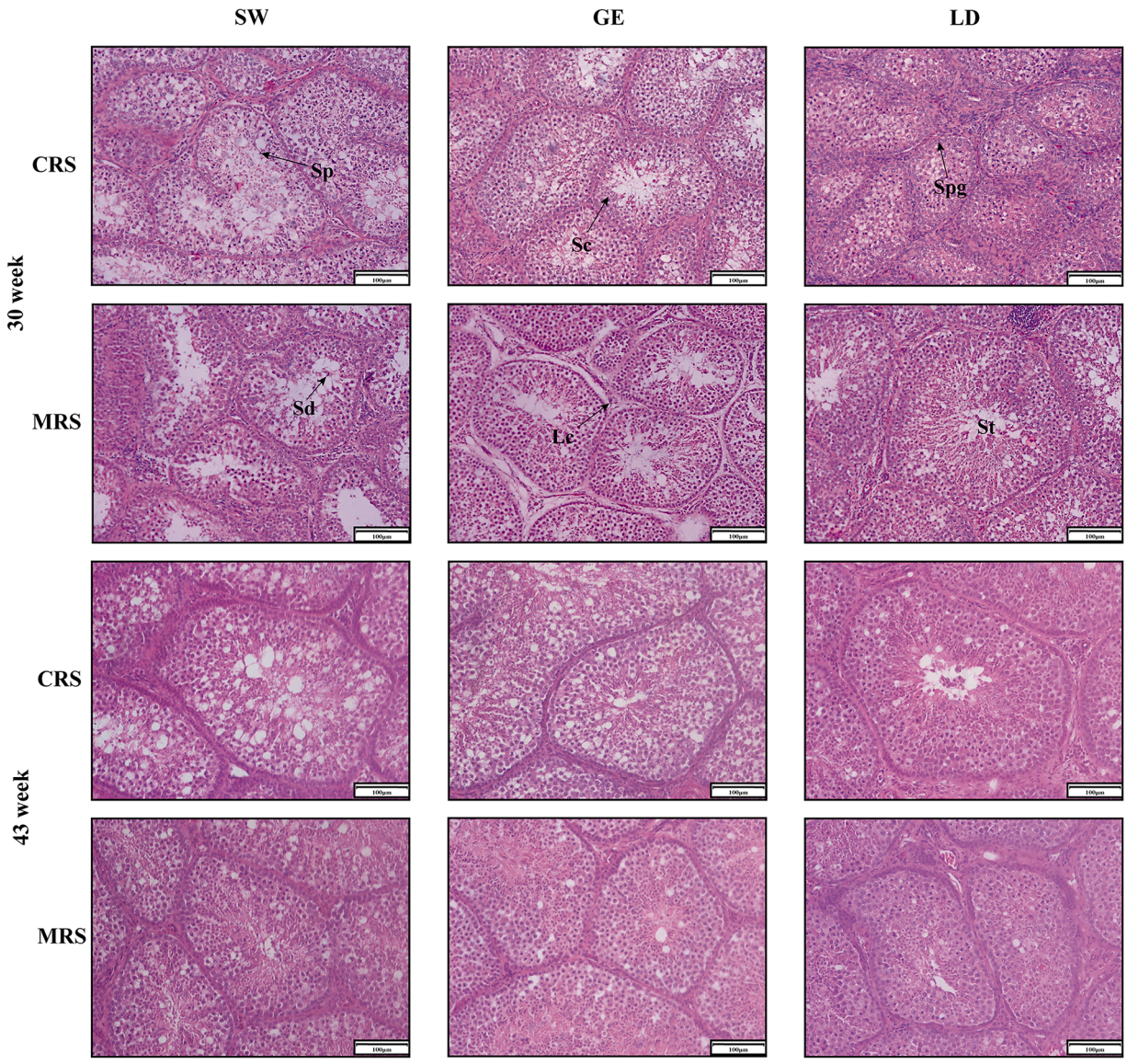

Fig. 2. Histological observation of the testes from three goose breeds under two rearing systems at different weeks of age. SW, Sichuan White goose. GE, Gang goose. LD, Landes goose. CRS, cage-rearing system. MRS, net-floor mixed rearing system. Sp, spermatozoon. Sc, sertoli cell. Spg, spermatogonia. Sd, spermatid. Lc, Leydig cell. St, seminiferous tubule.

significantly lower in LD than in either SW or GE ($P < 0.01$).

Correlation analysis between gander testicular development and semen quality

The correlations between the testicular histomorphological parameters and semen quality indicators of all three goose breeds (SW, GE, and LD) were analyzed and shown in Supplementary Fig. 4. Under CRS, the left testicular long diameter was negatively correlated with sperm density and SQF ($P < 0.05$), and its dorsoventral diameter was negatively correlated with sperm density ($P < 0.05$). Also, the weight of left testis was negatively correlated with sperm density and SQF ($P < 0.05$). The right testicular long diameter was negatively correlated with the number of spermatozoa ($P < 0.05$), while the number of seminiferous tubules was positively correlated with the right testicular long and dorsoventral diameter as well as ejaculation volume ($P < 0.05$). The testicular parenchymal-to-interstitial ratio was positively correlated with the weight of left testis ($P < 0.05$), but it was negatively correlated with sperm density and SQF ($P < 0.05$). Besides, the number of spermatids was positively correlated with sperm viability ($P < 0.05$), while the number of Sertoli cells was negatively correlated with sperm motility ($P < 0.05$). Under MRS, the left testicular short diameter was negatively correlated with acrosomal integrity ($P < 0.05$), and the right testicular dorsoventral diameter was negatively correlated with the number of spermatids ($P < 0.05$). The weight of left testis was negatively correlated with acrosomal integrity ($P < 0.05$). The number of seminiferous tubules was negatively correlated with ejaculation volume ($P < 0.05$). Besides, the testicular parenchymal-to-interstitial ratio was positively correlated with the morphological abnormal sperm percentage ($P < 0.05$), but it was negatively correlated with sperm density ($P < 0.05$).

Identification of testicular DEGs between two rearing systems in three goose breeds

To explore the molecular mechanisms of rearing system regulating the goose testicular development and semen quality, the testes collected from SW, GE, and LD reared under both CRS and MRS at 43 weeks of age were used for transcriptomic analysis. As shown in Supplementary Table 1, an average of 28,822,910 clean reads was obtained from each sample, and the Q30 values of all samples were more than 92.8%.

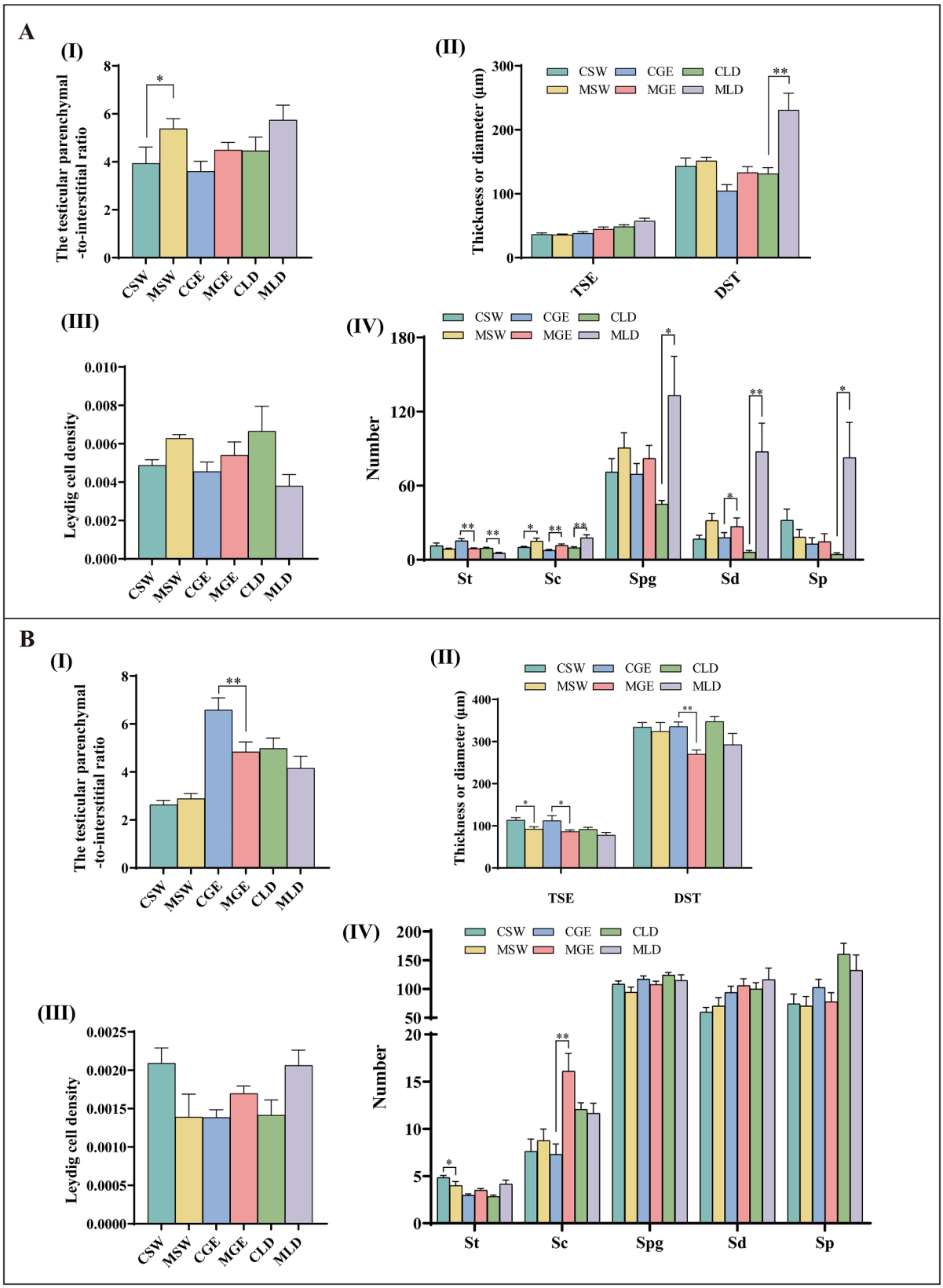

Fig. 3. Comparison of testicular histological parameters of three goose breeds under two rearing systems at 30 (A) and 43 weeks of age (B). CSW, Sichuan White goose (SW) under the cage-rearing system (CRS). MSW, SW under the net-floor mixed rearing system (MRS). CGE, Gang goose (GE) under CRS. MGE, GE under MRS. CLD, Landes goose (LD) under CRS. MLD, LD under MRS. TSE, the thickness of seminiferous epithelium. DST, the diameter of seminiferous tubule. St, seminiferous tubule. Sc, sertoli cell. Spg, spermatogonia. Sd, spermatid. Sp, spermatozoon. "*" indicates a significant difference between the two designated groups ($P < 0.05$), while "**" indicates an extremely significant difference ($P < 0.01$).

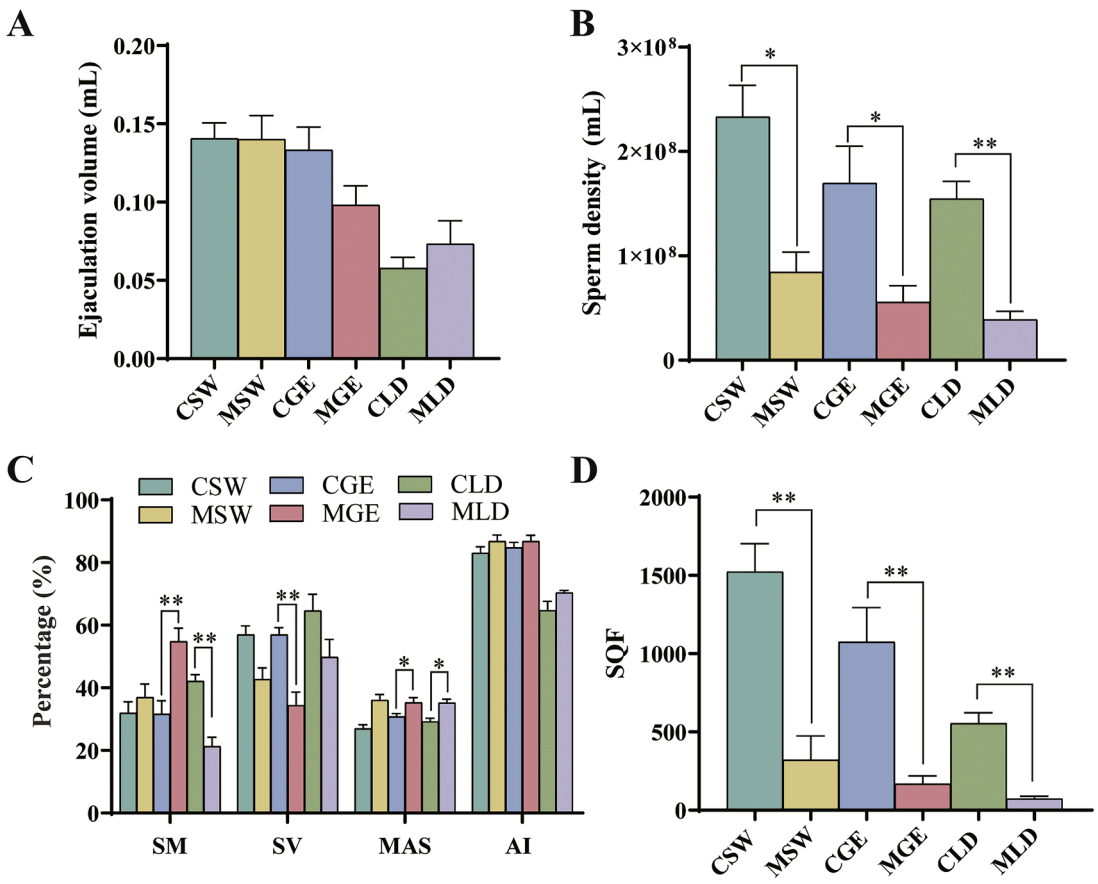

Fig. 4. Comparison of semen quality of three goose breeds under two rearing systems at 40 weeks of age. (A) Ejaculation volume. (B) Sperm density. (C) Other semen quality paramters. SM, sperm motility. SV, sperm viability. MAS, morphological abnormal sperm. AI, acrosomal integrity. (D) Semen quality factor (SQF). CSW, Sichuan White goose (SW) under the cage-rearing system (CRS). MSW, SW under the net-floor mixed rearing system (MRS). CGE, Gang goose (GE) under CRS. MGE, GE under MRS. CLD, Landes goose (LD) under CRS. MLD, LD under MRS. "*" indicates a significant difference between the two designated groups ($P < 0.05$), while "**" indicates an extremely significant difference ($P < 0.01$).

Meanwhile, a range from 92.56% to 94.52% clean reads were mapped to the goose genome. The results of principal component analysis showed that the biological samples in each experimental group were more tightly clustered and those in the same goose breed were clustered together according to the rearing system (Supplementary Fig. 5A-C). Taken together, these data demonstrated the high quality and reliability of our transcriptomic sequencing data, which can be used for subsequent bioinformatic analysis.

As shown in Supplementary Fig. 5D-E and Fig. 5A, by comparing the testicular transcriptomes of each goose breed between the two rearing systems, a total of 158 DEGs, including 73 upregulated- and 85 downregulated genes, were identified in SW, and a total of 73 upregulated- and 95 downregulated DEGs were identified in GE. Meanwhile, a total of 838 DEGs, including 200 upregulated- and 638 downregulated genes, were identified in LD. Of note, one DEG (deoxynucleotidyltransferase terminal interacting protein 1, *DNTTIP1*) was commonly identified in these three pairwise comparisons (Fig. 5B). The hierarchical clustering analysis of these identified DEGs unequivocally confirmed their differential expression patterns in the testes of SW, GE, and LD under different rearing systems (Fig. 5C-E).

Functional enrichment analysis of testicular DEGs

Next, we performed both GO and KEGG enrichment analyses for the identified testicular DEGs of all three goose breeds between the two rearing systems. As shown in Fig. 5F, the top 10 GO biological process (BP) terms significantly enriched by these testicular DEGs included clustering of voltage-gated sodium channels, mRNA stabilization, cell fate commitment, cell-cell adhesion, positive regulation of cell-substrate adhesion, positive regulation of transcription by RNA polymerase II, skeletal system development, enteric nervous system development, positive regulation of gene expression, and phosphatidylinositol-mediated signaling. Meanwhile, they were significantly enriched in 11 KEGG pathways, including melanogenesis, Wnt signaling pathway, neuroactive ligand-receptor interaction, RNA degradation, histidine metabolism, SNARE interactions in vesicular transport, cytokine-cytokine receptor interaction, metabolic pathways, thiamine metabolism, mTOR signaling pathway, and arginine and proline metabolism (Fig. 5G).

Identification of testicular gene modules through WGCNA and functional enrichment analyses

As shown in Fig. 6A-B, by constructing a weighted gene co-expression network using the top 5000 genes of these 24 sequenced goose testicular transcriptomes filtered by median absolute deviation, a total of 13 gene modules were identified to show correlations with different experimental treatments, *e.g.,* the goose breed or the rearing system. As shown in Fig. 6B, the MEtan gene module showed a significant positive correlation with GE under CRS ($P < 0.05$), the MEblack module showed a significant positive correlation with LD under CRS ($P < 0.05$), and the MEgreen module showed a significant positive correlation with SW under CRS ($P < 0.05$). Besides, the MEturquoise module showed a significant positive correlation with GE under MRS ($P < 0.05$), the MEpurple module showed an extremely significant positive correlation with LD under MRS ($P < 0.01$), and the MEgreenyellow module

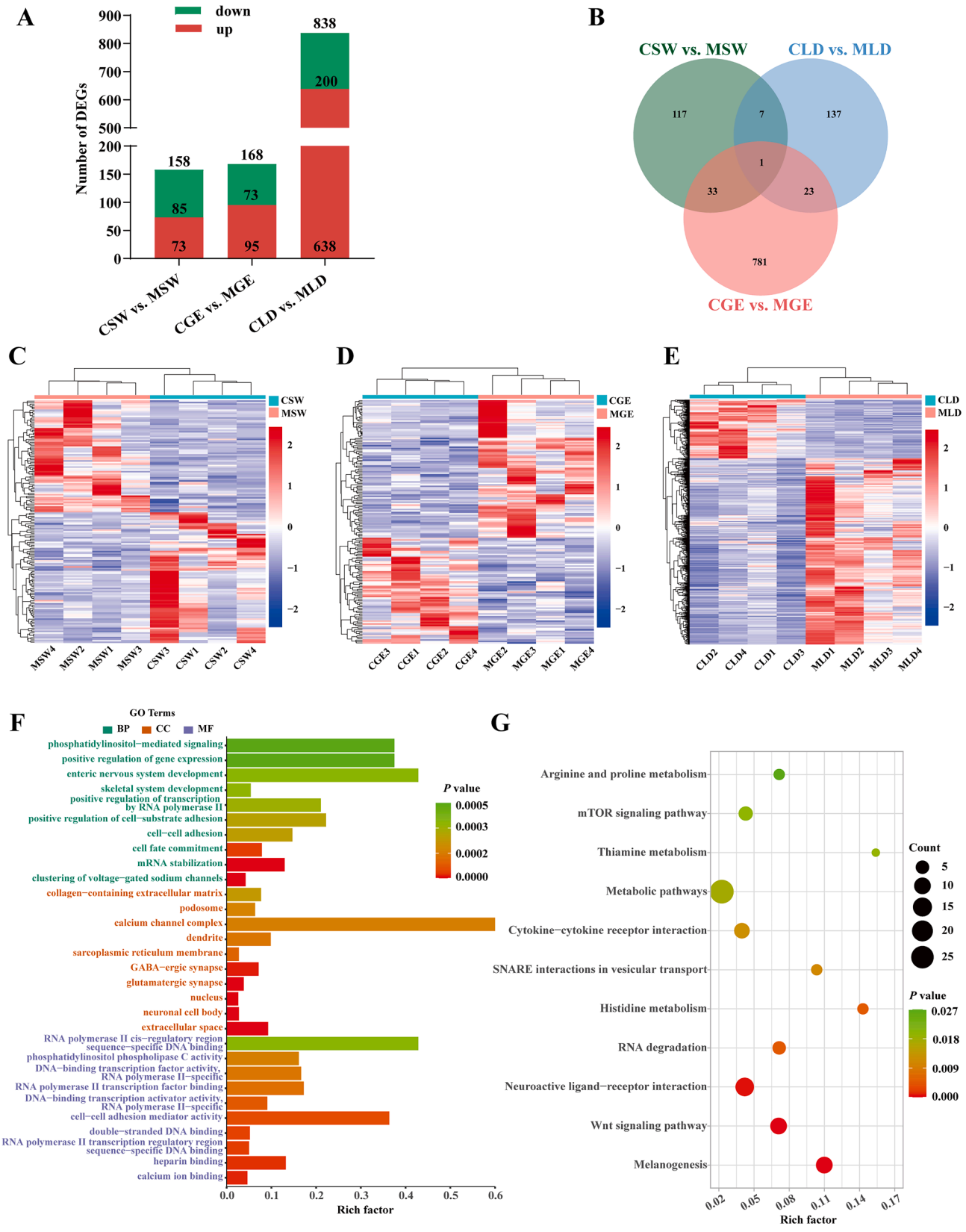

Fig. 5. Comparison of testicular transcriptomic profiles of three goose breeds under different rearing systems. (A) Identification of differentially expressed genes (DEGs) in the testes of three goose breeds between different rearing systems. CSW, Sichuan White goose (SW) under the cage-rearing system (CRS). MSW, SW under the net-floor mixed rearing system (MRS). CGE, Gang goose (GE) under CRS. MGE, GE under MRS. CLD, Landes goose (LD) under CRS. MLD, LD under MRS. (B) Venn plot showing the overlap of testicular DEGs of three goose breeds between CRS and MRS. (C-E) Hierarchical clustering analysis of testicular DEGs resulting from the comparisons of CSW vs. MSW (C), CGE vs. MGE (D), and CLD vs. MLD (E). (F) The GO terms significantly enriched by all testicular DEGs of three goose breeds. (G) The KEGG pathways significantly enriched by all testicular DEGs of three goose breeds.

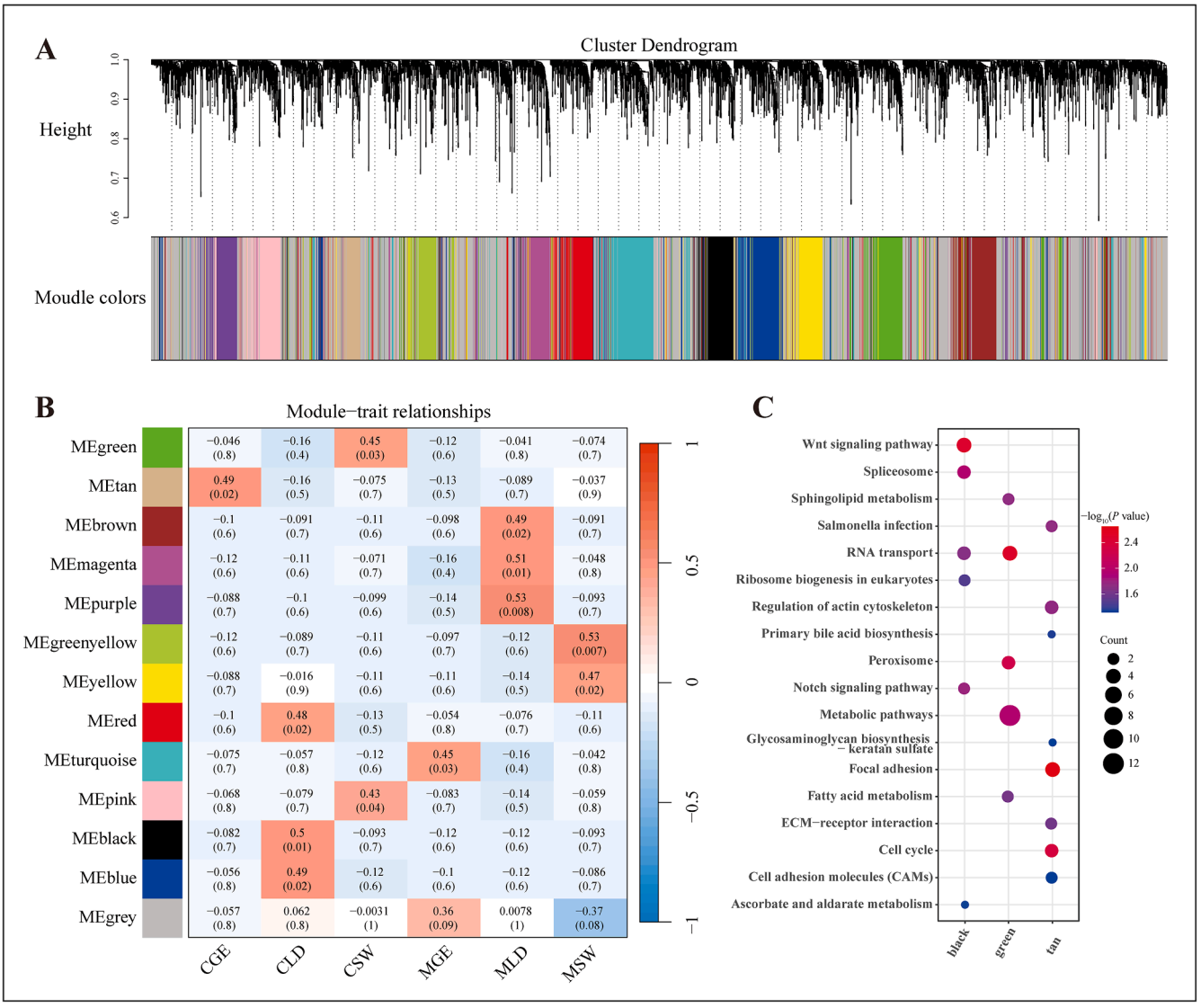

Fig. 6. Results of weighted gene co-expression network analysis (WGCNA). (A) Hierarchical clustering tree was constructed to idenfity gene expression modules. Different colors on the abscissa represent different clusters of genes. (B) Correlation analysis of the identified modules and traits. The abscissa represents different experimental groups, and the ordinate represents different clusters of genes. CSW, Sichuan White goose (SW) under the cage-rearing system (CRS). MSW, SW under the net-floor mixed rearing system (MRS). CGE, Gang goose (GE) under CRS. MGE, GE under MRS. CLD, Landes goose (LD) under CRS. MLD, LD under MRS. (C) The KEGG pathways significantly enriched by the goose breed-related module genes under CRS.

showed a significant positive correlation with SW under MRS ($P < 0.05$).

Furthermore, functional enrichment analysis of those genes identified in the goose breed-related modules under CRS was analyzed. As shown in Fig. 6C, those genes in the MEgreen module correlated with SW under CRS were significantly enriched in 5 KEGG pathways, including RNA transport, peroxisome, metabolic pathways, sphingolipid metabolism, and fatty acid metabolism. Those genes in the MEtan module correlated with GE under CRS were significantly enriched in 8 KEGG pathways, including focal adhesion, cell cycle, regulation of actin cytoskeleton, salmonella infection, ECM-receptor interaction, primary bile acid biosynthesis, glycosaminoglycan biosynthesis-keratan sulfate, and cell adhesion molecules (CAMs). Besides, those genes in the MEblack module correlated with LD under CRS were significantly enriched in 6 KEGG pathways, including Wnt signaling, spliceosome, Notch signaling, RNA transport, ribosome biogenesis in eukaryotes, and ascorbate and aldarate metabolism.

Screen of testicular hub genes through PPI analysis

To further identify hub genes responsible for differences observed in the rearing system-related testicular development and semen quality of three goose breeds, one PPI network was constructed with the testicular DEGs enriched in either the top 10 GO terms (Fig. 5F) or the top 10 KEGG pathways (Fig. 5G). As depicted in Fig. 7A, this network, which consisted of 148 nodes and 150 edges, displayed complex interactions among these selected rearing system-associated DEGs, and those DEGs within the nodes with darker color and bigger size might play more important roles. Furthermore, by using the CytoHubba plugin in Cytoscape, the top 10 hub DEGs were screened from the PPI network, as depicted in Fig. 7B, including the Wnt family member 3A (*WNT3A*), Wnt family member 8B (*WNT8B*), Wnt family member 4 (*WNT4*), Wnt family member 3 (*WNT3*), Wnt family member 6 (*WNT6*), Wnt family member 16 (*WNT16*), secreted frizzled related protein 1 (*SFRP1*), frizzled class receptor 9 (*FZD9*), sonic hedgehog signaling molecule (*SHH*), and paired box 6 (*PAX6*).

In addition, to identify hub genes responsible for differences

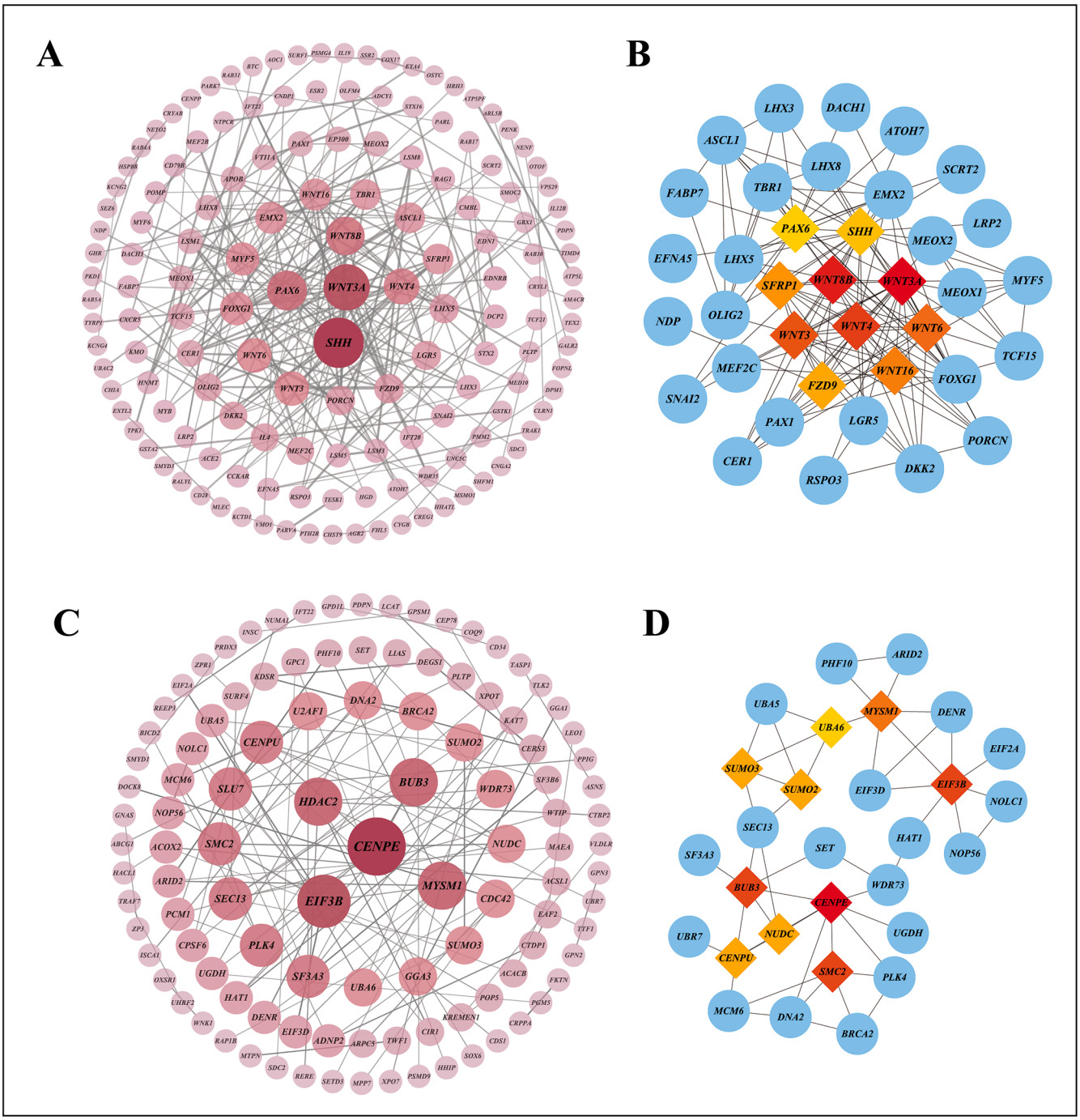

Fig. 7. Protein-protein interaction (PPI) network analysis of the identified testicular DEGs and module genes. (A) The PPI network constructed using the testicular DEGs enriched in either the top 10 GO terms or the top 10 KEGG pathways. The size (area) of each node was drawn proportional to its degree of interaction, and the color of each node indicated its betweenness centrality. (B) Visualization of the top 10 hub genes (locating in the inner circle) screened from the PPI network depicted in A using CytoHubba plugin of Cytoscape tool. (C) The PPI network constructed using the significantly enriched goose breed-related module genes under the cage-rearing system. (D) Visualization of the top 10 hub genes (locating in the inner circle) screened from the PPI network depicted in C.

observed in the goose breed-related testicular development and semen quality under CRS, another PPI network was constructed with the significantly enriched gene in the goose breed-related modules (MEgreen, MEtan, and MEblack) under CRS (Fig. 6C). As depicted in Fig. 7C, this network, which consisted of 109 nodes and 120 edges, displayed interactions among these selected module genes, and those genes within the nodes with darker color and bigger size might play more important roles. Furthermore, by using the CytoHubba plugin in Cytoscape, the top 10 hub genes were screened from the PPI network, as depicted in Fig. 7D, including the centromere protein E (*CENPE*), eukaryotic translation initiation factor 3 subunit B (*EIF3B*), structural maintenance of chromosomes 2 (*SMC2*), BUB3 mitotic checkpoint protein (*BUB3*), Myb like, SWIRM and MPN domains 1 (*MYSM1*), small ubiquitin like modifier 2 (*SUMO2*), small ubiquitin like modifier 3 (*SUMO3*), nuclear distribution C, dynein complex regulator (*NUDC*), centromere protein U (*CENPU*), and ubiquitin like modifier activating enzyme 6 (*UBA6*).

Validation of the expression profiles of several DEGs by RT-qPCR

To evaluate the accuracy of our transcriptomic sequencing data, a total of 8 DEGs, including *WNT3A, HIF1A, STMP1, MESD, CD59, KCTD1, MYCBP*, and *SEMA4D*, were randomly selected from our identified testicular DEGs for RT-qPCR validation. As shown in Fig. 8, the expression profiles of all selected DEGs determined by RT-qPCR showed similar trends to those observed by transcriptomic sequencing,

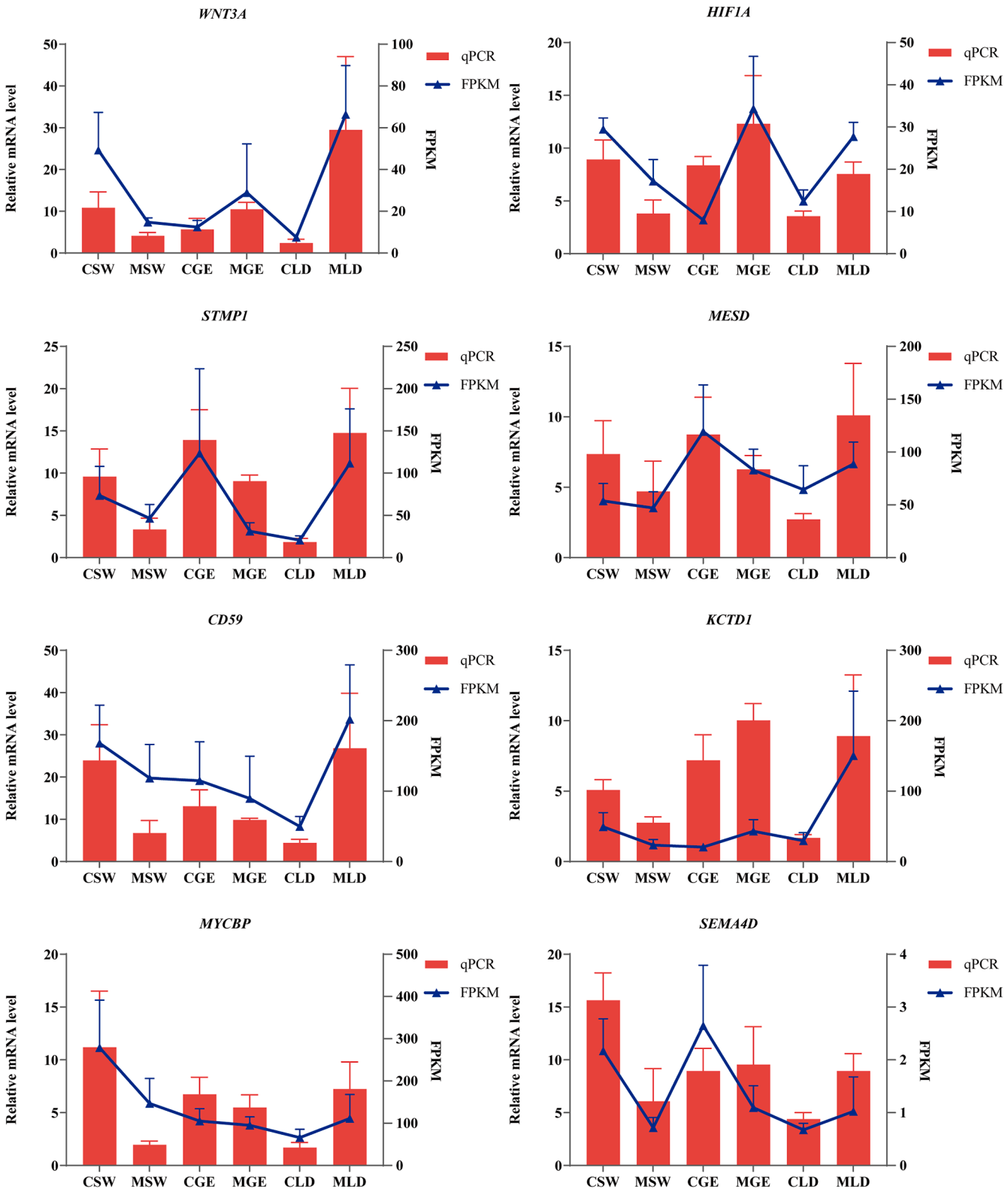

Fig. 8. The RT-qPCR validation of the expression patterns of 8 differentially expressed genes (DEGs) identified by RNA-Seq. The blue line represents the RNA-Seq results, while the red column represents the RT-qPCR results. CSW, Sichuan White goose (SW) under the cage-rearing system (CRS). MSW, SW under the net-floor mixed rearing system (MRS). CGE, Gang goose (GE) under CRS. MGE, GE under MRS. CLD, Landes goose (LD) under CRS. MLD, LD under MRS.

supporting the reliability of our RNA-seq results.

Discussion

As an important non-genetic factor, the rearing system has been widely reported to have significant effects on the health, welfare, and production efficiency of domestic birds such as chickens and ducks (Al-Ajeeli, et al., 2018; Wang, et al., 2015; Zhang, et al., 2018). With increasing demands for the transition of traditional extensive free-range rearing systems into modern intensive rearing ones in the Chinese goose industry due to environmental protection and efficient production, it is of great value to reveal the effects of dryland rearing systems on the goose testicular development and semen quality, which is crucial for increasing the economic gains from commercial goose breeder stocks. In the present study, the testicular morphological, histological, and transcriptomic responses of three goose breeds to the two dryland rearing systems (CRS *vs.* MRS) were systematically analyzed and compared. Our results showed that compared to SW, the rearing system showed more pronounced effects on the size, weight, and organ index of bilateral testes of GE and LD during the period from 10 weeks post-hatch to reach sexual maturation, but such effects seemed to be mitigated in LD and even reversed in GE when reaching body maturation. These observations were similar to those reported in male Rohman breeders, where their testicular weights differed among three different cage-rearing systems at either 105 or 210 days of age (Du, et al., 2021), demonstrating the age-dependent effects of rearing system on male poultry testicular morphology. Noticeably, the testicular size and weight of SW were significantly higher than those of GE and LD at 30 weeks of age under CRS, implying that there were breed-related differences in the testicular responses to the same rearing system.

In support of this, at the histological level, the rearing system showed differential effects on the testicular parenchymal-to-interstitial ratio, the number and density of seminiferous tubules, as well as the number of Leydig cells, Sertoli cells, spermatogonia, spermatids, and spermatozoa depending on the age and breed. Generally, in consistent with the above testicular morphological observations, most testicular histological parameters of all three goose breeds appeared to be higher under MRS than under CRS at 30 weeks of age, but the converse was seen in the testicular parenchymal-to-interstitial ratio, seminiferous epithelium thickness, seminiferous tubule diameter, and the number of spermatogonia and spermatozoa of either GE or LD at 43 weeks of age. These results suggested that the CRS may be more conducive than MRS to the goose testicular development during the period from 30 weeks of age (sexual maturation) to reach body maturation (43 weeks of age), although these effects differed among three goose breeds. Similarly, a previous study reported that the cage-reared broilers showed significantly improved fertility and hatchability compared to the floor-reared ones, demonstrating that the cage rearing system can enhance chicken reproductive performance (Andrews and Goodwin, 1973). Also, it has been shown that 10% of broiler deaths under intensive floor-rearing systems were cause by cannibalism, and this problem could be greatly mitigated by using the cage-rearing systems (El-Deek and El-Sabrout, 2019). Additionally, compared to CRS, the MRS can result in the uneven feeding and increased aggression among the poultry population, thereby raising levels of glucocorticoids and adrenaline, which consequently cause excessive energy expenditure and adversely affect the normal testicular development (Cerolini, et al., 2003). In this regard, it was speculated that the geese under CRS experienced less competition for food, limited activity, less bacterial contamination, and healthier environment. Moreover, the rearing system may show more pronounced effects during the critical period from week 30 to week 43 for the testicular development. Therefore, it was understandable that the geese testes under CRS generally developed better than those under MRS when reaching body maturation. In good accordance with the testicular development, the semen quality indicators (especially sperm density, sperm viability, morphological abnormal sperm, and semen quality factor) of all three goose breeds under CRS were generally better than those under MRS at 43 weeks of age, although the goose breed-related variations were seen in the ejaculate volume and sperm motility. These observations were well supported by our results of correlation analysis between the goose testicular histomorphological parameters and semen quality indicators, where the semen quality factor value showed a stronger association with the number of seminiferous tubules, Leydig cells, and Sertoli cells under CRS than under MRS. It has been widely accepted that both Leydig cells and Sertoli cells act as key drivers of testicular hormone production and sperm output (Dutta, et al., 2019; O'Donnell et al., 2022). Similarly, it was seen in roosters that the testicular development was closely related to semen quality, and both of them were influenced by the type of cage-rearing system (Du, et al., 2021). In Muscovy ducks, those reared under intensive rearing conditions generally showed higher semen volume and sperm concentration compared to semi-intensive rearing ones (Etuk, et al., 2006). Taken together, we concluded that compared to MRS, the CRS sustaining from week 10 to week 43 post-hatch could be more conducive to the goose testicular development and semen quality.

To unravel the underlying mechanisms responsible for differences in the testicular development and semen quality of three goose breeds between the two different rearing systems, we further analyzed and compared the testicular genome-wide transcriptomic changes. The largest number of DEGs identified in CRS *vs.* MRS was seen in the testes of LD, which implied that the testicular development and functions of LD may be more susceptible than either SW or GE to be influenced by the rearing system. By comparison, the fewest DEGs were seen in the testes of SW, and our PCA analysis showed that the testicular transcriptomic profiles were much more similar in SW between CRS and MRS. These results indicated that the testicular development and functions of SW were relatively less affected by the rearing system, which could be due to their stronger adaptability to environmental changes. The GO enrichment analysis showed that the rearing system-related DEGs identified in the testes of all three goose breeds were mostly enriched in the terms related to organ morphogenesis and development, such as neuron differentiation, somitogenesis, and positive regulation of myoblast differentiation, as well as cellular processes and functions, such as positive regulation of transcription by RNA polymerase II and regulation of cell-cell adhesion. Similarly, several studies have previously reported that organ morphogenesis and cellular processes play crucial roles during the testicular development (Aire, 2007; Estermann, et al., 2021a). Meanwhile, the KEGG enrichment analysis showed that these testicular DEGs were mainly enriched in the Wnt signaling, neuroactive ligand-receptor interaction, cytokine-cytokine receptor interaction, mTOR signaling, and metabolic pathways, which have been revealed to regulate the testicular growth and development (Hao, et al., 2024; Tang, et al., 2022).

Among them, the Wnt signaling pathway may play a more important role in the rearing system-mediated goose testicular development, because it was evidenced that the Wnt signaling participates in regulating multiple biological processes during testicular development (He, et al., 2018; Lee, et al., 2016). Additionally, both the neuroactive ligand-receptor interaction and mTOR signaling pathways could regulate the goose testicular development by affecting cellular functions, such as cell proliferation, survival, and metabolism (Chen, et al., 2021; Deng, et al., 2021; Guo, et al., 2023; Li, et al., 2022b). By constructing a PPI network using these testicular DEGs, we further identified 10 hub genes, including *WNT8B, FZD9, SHH, PAX6, WNT3A, SFRP1, WNT3, WNT6, WNT4*, and *WNT16*, most of which were involved in the Wnt signaling pathway. Among them, as a canonical Wnt ligand, *WNT3A* has been shown to be critical for regulating several cellular functions such as cell proliferation, differentiation, self-renewal, and motility (Kaur, et al., 2013) and to play a crucial role in the regulation of mouse and human spermatogenesis (Golestaneh, et al., 2009). As an activator of the Wnt signaling pathway, *WNT3* can initiate the canonical Wnt/β-catenin signal transduction by binding to its receptor (Basu, et al., 2018). Meantime, *WNT4* plays an important role in the testicular development

by regulating the functions of spermatogonial stem cells and supporting cells (Dong, et al., 2015), and *WNT6* uniquely expressed in Sertoli cells can mediate the proliferation of undifferentiated spermatogonia in the adult mouse testis (Takase and Nusse, 2016). Besides, as the Wnt signaling inhibitors, the *SFRP* family members including *SFRP1* play important roles in vertebrate embryonic and testicular development (Satoh, et al., 2006). Thus, these identified hub genes and their involved signaling pathways were considered as the promising targets for improving the testicular functions of male geese by developing new molecular breeding strategies.

Through the WGCNA analysis, we further identified key gene modules associated with the goose breed under CRS. By performing the KEGG enrichment analysis, it was shown that the SW-related module genes were mainly enriched in the metabolism-related pathways, including metabolic pathway, sphingolipid metabolism, and fatty acid metabolism. It has been reported that there was a close metabolic relationship between supporting cells and germ cells. Germ cells must obtain sufficient levels of energy substrates from supporting cells for survival and development, and some metabolic products, such as amino acids, carbohydrates, lipids, vitamins, and metal ions, are transferred between them (Boussouar and Benahmed, 2004; Meroni, et al., 2002; Mruk and Cheng, 2004). As for the GE-related module genes, most of them were significantly enriched in the cell cycle signaling pathway, which is well known for the regulatory roles in the division and differentiation of various somatic and germ cells. In the chicken testis, the development of germ cells depends on both the cell cycle control and meiosis *vs.* mitosis decision (Estermann, et al., 2021b). As for the LD-related module genes, they were most significantly enriched in the Wnt signaling pathway, further supporting the crucial role of this signaling in the testicular development. By constructing a PPI network using these goose breed-related module genes, we further identified several key genes that could be responsible for differences observed in the goose breed-related testicular development and semen quality under CRS, including *CENPE, SMC2, BUB3, NUDC, CENPU, SUMO2*, and *SUMO3*, most of which play critical roles during meiosis. As the two members of the *CENP* family, *CENPE* and *CENPU* are essential for cell division, DNA replication, and cell cycle regulation (Foltz, et al., 2006). *SMC2*, known as a member of the SMC protein family, plays a role in chromosome condensation, DNA recombination and repair, and gene regulation (Hirano, 2012). *BUB3* has also been reported to be important for meiosis (Cairo, et al., 2020). NUDC is a nuclear movement protein that binds to dynein, and has a role in control of mitosis and cytokinesis (Aumais, et al., 2003). As the two members of the *SUMO* family, *SUMO2* and *SUMO3* are involved in various regulatory events critical for cellular processes, including transcriptional regulation, cell cycle progression, and DNA repair (Shrivastava, et al., 2010). Also, SUMO proteins have been shown to be expressed in testicular cells and participate in regulating spermatogenesis (Vigodner, 2011). Thus, these screened signaling pathways, interaction networks, and hub genes were thought to play important roles in mediating the breed-dependent effects of CRS on the goose testicular development and semen quality.

Conclusion

In conclusion, the dryland rearing system exerted significant age-dependent effects on the testicular development and semen quality of all three goose breeds. Compared to MRS, the CRS sustaining from post-hatch week 10 until week 30 (reaching sexual maturation) did not significantly facilitate but even restricted the goose testicular development, especially in GE and LD; however, after a prolonged period from post-hatch week 30 to week 43 (reaching body maturation), the CRS were more conducive than MRS to both the testicular development and semen quality of all three goose breeds. Compared with GE and LD, there were relatively weaker impacts of rearing systems on the testicular histomorphological parameters, semen quality indicators, and transcriptomic profiles of SW, which could be due to their stronger adaptability to environmental changes. Through a systematic comparative transcriptomics analysis, several signaling pathways including the Wnt signaling as well as the involved hub genes (*WNT8B, FZD9, SHH, PAX6, WNT3A, SFRP1, WNT3, WNT6, WNT4*, and *WNT16*) were suggested to have important roles in mediating the effects of rearing systems on the goose testicular development and functions. Moreover, the metabolic-related, cell cycle, and Wnt signaling pathways seemed to be partially responsible for differences in the goose breed-related testicular development and semen quality under the same CRS, where a number of genes involved in meiosis, such as *CENPE, SMC2, BUB3, NUDC, CENPU, SUMO2*, and *SUMO3*, could have crucial roles.

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Declaration of competing interest

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

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2024.104589.

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