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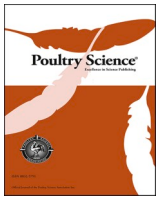

Full-Length Article

Molecular mechanisms of libido influencing semen quality in geese through the hypothalamic-pituitary-testicular-external genitalia axis

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A R T I C L E I N F O

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A B S T R A C T

Libido plays a crucial role in influencing semen quality, yet the underlying regulatory mechanisms remain unclear. As a central axis in male goose reproduction, the hypothalamic-pituitary-testicular-external genitalia (**HPTE**) axis may contribute to the regulation of this process. In this study, we established a rating scale for goose libido based on average number of massages to erection (**ANM**) and the erection type, and evaluated semen quality across the entire flock. Correlation analyses showed that ANM was negatively correlated with sperm concentration (**SC**), acrosome integrity (**AI**), and semen quality factor (**SQF**), while positively correlated with morphological abnormal sperm (**MAS**) ($P < 0.01$). A comparison of semen quality and testicular histology between high libido (**HG**) and low libido (**LG**) groups showed that SC and SQF were significantly higher and MAS was lower in HG ($P < 0.05$). The lumen diameter of seminiferous tubules (**LD**) ($P < 0.01$) and the number of Sertoli cells (**Sc**) ($P < 0.05$) were also significantly greater in HG. Further, the number of spermatogonia (**Sg**) was significantly ($P < 0.01$) lower, and spermatocyte (**Sp**) and elongated spermatid (**Se**) were significantly higher in HG ($P < 0.05$). Through transcriptome sequencing (**RNA-seq**), we identified 98, 163, 2,474 and 400 differentially expressed genes (**DEGs**) in the hypothalamus, pituitary, testis and external genitalia, respectively. Gene Ontology (**GO**) analysis indicated that the term "male gonad development" was significantly enriched in the hypothalamus. Here, the expression of *LHX9* was positively correlated with ANM, and negatively correlated with SC and SQF ($P < 0.05$). Additionally, *WNT4* was positively correlated with ANM and MAS ($P < 0.01$), and negatively correlated with SC ($P < 0.05$), suggesting that *LHX9* and *WNT4* might serve as key upstream regulatory genes. Further analysis through Weighted Gene Co-Expression Network Analysis (**WGCNA**) showed that the yellow module ($R = 0.89$, $P = 7e\text{-}09$) was strongly associated with testicular development, with genes predominantly involved in male reproductive process. Based on these findings, we screened genes significantly correlated with *LHX9* and *WNT4* from the yellow module ($|Cor| \geq 0.6$, $P < 0.05$). These genes were significantly enriched in 8 pathways, primarily associated with metabolic processes, including drug metabolism - other enzymes, metabolism of xenobiotics by cytochrome P450, metabolic pathways, pyrimidine metabolism, glycerolipid metabolism, and riboflavin metabolism. Using the Maximal Clique Centrality (**MCC**) algorithm in the CytoHubba plug-in, *SYCP3, DDX4, STRA8, AMH, MEIOB, CDT1, BCL2, PRIM1*, and *DLGAP5* were identified as hub genes. In conclusion, within the HPTE axis, libido might influence metabolism-related signaling pathways (mainly involving genes such as *SYCP3, DDX4, STRA8, AMH, MEIOB, CDT1, BCL2, PRIM1*, and *DLGAP5*) through *LHX9* and *WNT4* to regulate the development of the seminiferous tubules and germ cell number, ultimately affecting SC and MAS in geese. These findings offer practical insights into libido rating and shed light on the mechanisms by which libido regulates semen quality, potentially aiding in the improvement of goose breeding capacity.

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Introduction

Similar to other behaviors, libido is regulated by a combination of physiological mechanisms, learning experiences and motivation as a response to endogenous or exogenous stimuli (Bryant, 1989). Low libido could result in erection and ejaculation difficulties (Giatti et al., 2018), and it is an important parameter for assessing male reproductive potential (Ahmad, et al., 2005; Chenoweth, 1983).

In mammals, comprehensive criteria for assessing libido exist, allowing researchers to evaluate libido levels based on factors such as sexual interest, erection speed, whether or not ejaculation occurred and service traits (Frydrychová, et al., 2011; Islam, et al., 2018; Landaeta-Hernández, et al., 2001). However, in poultry, the relevant standards require further exploration and improvement. A study on *Rhynchotus rufescens* found that the average time from the onset of dorsal abdominal and cloacal massage to full ejaculation was approximately 2 min, with birds showing varying response times to stimulation—some requiring longer (Paranzini, et al., 2018). These findings suggested that the speed of response to massage seemed to partially reflect the libido level in birds. While our team previously developed a libido rating scale for ducks based on responses to massage and teasing training (Ouyang, et al., 2021), this scale does not apply to geese due to species differences. Therefore, there is an urgent need to develop a specific libido rating scale for geese to serve goose breeding.

Moreover, libido is a key factor influencing semen quality (Islam, et al., 2018). Studies on bulls have demonstrated that breeds with high libido also had superior semen quality (Rehman, et al., 2016), and libido was positively correlated with progressive motility, sperm concentration and normal sperm (Islam, et al., 2018). Similarly, ducks with higher libido showed better sperm viability, gonadal development (Hu, et al., 2023) and sperm count (Ouyang, et al., 2021). As an essential endocrine axis regulating reproduction in animals, the hypothalamic-pituitary-testicular axis likely plays an important role in this process (Dwyer and Quinton, 2019). Additionally, only about 3 % of male birds possess external genitalia for internal copulation, and waterfowl (e.g. ducks and geese) are representative of this group (Herrera, et al., 2013). The external genitalia is vital for normal erection and ejaculation in geese (Tang, et al., 2022). Ouyang et al. found that *TRAM2* expression and sequence differences were associated with duck libido (Ouyang, et al., 2021). However, the precise mechanism by which libido affects semen quality via the hypothalamic-pituitary-testicular-external genitalia (**HPTE**) axis remains unclear.

High libido and superior semen quality are desirable traits for a successful breeding program in geese. In this study, we implemented phenotypic selection by establishing a libido rating scale for geese. Additionally, we constructed mRNA expression profiles of hypothalamus, pituitary, testis and external genitalia with different libido using transcriptome sequencing (**RNA-seq**) to explore the complex molecular regulatory mechanisms behind them, providing a theoretical foundation for enhancing the breeding capacity of geese.

Materials and methods

Ethics statement

All animal handling procedures involved in this experiment were approved by the Institutional Animal Care and Use Committee (IACUC) of Sichuan Agricultural University (Chengdu Campus, Sichuan, China), License No. DKY20170913.

Goose massage training

Prior to the massage training, we examined the development of the external genitalia of each goose and eliminated geese with abnormal or incomplete external genitalia. Eventually, massage training was conducted on 150 male geese (Tianfu Meat Goose No.2), aged 247 days, from the Sichuan Agricultural University Waterfowls Breeding Farm (Ya'an, Sichuan, China) for 12 days. The training was divided into two stages: Stage I (day 1 to day 6) focused on establishing a sexual response, and Stage II (day 7 to day 12) aimed at stabilizing the sexual response to massage. The number of massages to cloacal erection and the level of cloacal erection were recorded daily throughout the training. The level of cloacal erection was categorized into 3 types: type A (base of the cloaca fully expanded, partial external genitalia palpable, very firm to the touch), type B (base the of cloaca partially expanded, soft to the touch), and type C (base of the cloaca not expanded). At the conclusion of the training, 6 libido grades were established based on the phenotypic data.

Semen quality assessment

After massage training, the semen quality of all geese was assessed in 3 separate trials, each conducted four days apart. The detailed procedures were as follows: (1) Semen was collected into a 150 mL beaker (Shubo, Chengdu, China) and the ejaculate volume (**EV**) of semen was measured using a 1.5 mL injector. (2) 10 μL of diluted semen (0.9 % saline) was pipetted to a disposable sperm counting plate, and at least 5 dynamic views were photographed at 100×. The Automated Sperm Analysis System (ML-810JZ) (Mailang, Nanning, China) was used to automatically calculate sperm viability (**SV**), sperm motility (**SM**), and sperm concentration (**SC**). Additionally, at least 5 static views were photographed at 100×, and normal and abnormal spermatozoa were manually labeled to record the morphological abnormal sperm (**MAS**). (3) Acrosome integrity (**AI**): 10 μL of diluted semen was evenly spread on a glass slide and air-dried. The slides were then fixed in phosphate-buffered saline solution containing 2 % glutaraldehyde (Sangon, Shanghai, China) for 30 min and subsequently stained with 5 % aniline blue (Sangon, Shanghai, China) mixed with 2 % acetic acid (pH =3.5) for 5 min. The acrosomes of 100 spermatozoa were observed at 1000× (Oil immersion), and the percentage of spermatozoa with intact acrosomes was calculated. (4) The semen quality factor (**SQF**) was calculated using the formula: SQF = EV (mL) × SC ($\times 10^6$mL) × live and morphologically normal sperm (%) (Liu, et al., 2008).

Sample collection

We randomly selected 3 geese from grades 5 and 6 to form the high libido group (**HG**) and 3 geese from grades 1 and 2 for the low libido group (**LG**). The experimental geese were euthanized through carbon dioxide inhalation followed by cervical dislocation after approximately 12 h fasting. The hypothalamus, pituitary, left testis and external genitalia were rapidly collected and frozen in liquid nitrogen, then stored at −80°C until sequencing. The right testis was preserved in 4 % paraformaldehyde fixative (Servicebio, Wuhan, China) for staining.

Testicular histological observation

The testis was removed from the fixative, trimmed, and placed in an embedding frame. It was then dehydrated using a gradient ethanol series before being immersed in paraffin. The paraffin-embedded testis was cut into 4 μm sections (Leica, Munich, Germany) and stained with hematoxylin-eosin. Imaging was performed using a Nikon DS-U3 mathematical pathology scanner (Nikon Corp, Tokyo, Japan). The structure of the testis was examined with Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, MD). For analysis, 5 random views from each section were selected, and the lumen diameter (**LD**) of intact seminiferous tubule was measured. Additionally, the numbers of Leydig cell (**Lc**), Sertoli cell (**Sc**), spermatogonia (**Sg**), spermatocyte (**Sp**), round spermatid (**Sr**) and elongated spermatid (**Se**) were counted in each view.

RNA extraction and sequencing

Total RNA was extracted from the hypothalamus, pituitary, testis, and external genitalia of each goose using the MJZol total RNA extraction kit (Majorbio, Shanghai, China) according to the manufacturer's operating manual. RNA integrity was assessed with an Agilent 5300 (Agilent Technologies, Santa Clara, CA, USA), and the RNA used for library preparation using Illumina® Stranded mRNA Prep, Ligation (Illumina, San Diego, CA) had an average RQN of 9.30 (range:8-10). 2 × 150 bp RNA-seq was performed using the Illumina NovaSeq X Plus sequencing platform. The RNA-seq data for this study could be obtained from the National Center for Biotechnology Information (**NCBI**) under BioProject ID PRJNA1154402.

Transcriptome alignment and assembly

Low quality reads were filtered using Fastp (version 0.23.4) (Chen, et al., 2018) to obtain clean reads. The clean reads were then aligned to the goose reference genome (BioProject ID PRJNA801885, data not released) using HISAT2 software (version 2.2.1) (Kim, et al., 2015). The resulting SAM (sequencing alignment/mapping) files were converted to BAM (binary alignment/mapping) format and sorted using SAMtools. Gene expression was then calculated using featureCounts (version 2.0.6) (Liao, et al., 2014) and normalized using the transcripts per million (**TPM**) method. Significant differential expression was identified based on the criteria $|\mathrm{Log_2(FC)}| \geq 1$ and $P < 0.05$.

Differentially expressed genes identification and functional analysis

DEseq2 (Love, et al., 2014) was used to identify intergroup differentially expressed genes (**DEGs**). Gene Ontology (**GO**) and Kyoto Encyclopedia of Genes and Genomes (**KEGG**) functional enrichment analysis were conducted using the online site KOBAS 3.0 (Bu, et al., 2021) (http://kobas.cbi.pku.edu.cn/kobas3/?$t = 1$) with *Gallus gallus* as the reference species. The Weighted Gene Co-Expression Network Analysis (**WGCNA**) was performed using the WGCNA package (version 1.72.5) (Langfelder and Horvath, 2008). DEGs interactions were referenced from the STRING 12 database (Szklarczyk, et al., 2019) (http://string-db.org/), Cytoscape (version 3.7.1) (Doncheva, et al., 2019) was employed for network visualization. GO enrichment results were further visualized using the ClueGO plug-in in Cytoscape (Bindea, et al., 2009). The CytoHubba plug-in was used to find hub genes using the Maximal Clique Centrality (**MCC**) algorithm (Chin, et al., 2014).

Validation of RNA-seq

qRT-PCR was performed on 6 randomly selected genes to validate the RNA-seq results. Total RNA extracted from the hypothalamus, pituitary, testis, and external genitalia was reverse transcribed into cDNA using the HiScript® III RT SuperMix for qPCR (+gDNA wiper) (Vazyme, Jiangsu, China). Primers were designed using the NCBI Primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi), and their specificity was confirmed through BLAST analysis (Table 1). qRT-PCR was performed using the Bio-Rad CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA, USA) with 3 replicates per sample. *β-actin* and *GAPDH* were used as housekeeping genes. Data were normalized using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001), followed by statistical analysis of the normalized results.

Statistical analysis

Statistical analysis was conducted using SPSS 27.0. Differences in semen quality between groups were evaluated using Student's *t* tests, and correlations were assessed using Spearman's correlation method. Statistical significance was set at $P < 0.05$.

Results and analysis

Massage training results and libido grading

As training progressed, both massage responsiveness and the level of cloacal erection were dramatically increased. Regarding massage responsiveness, the proportion of geese requiring more than 80 massages for cloacal erection decreased and remained low in stage II, while those needing fewer than 40 massages steadily increased (Fig. 1A). In terms of erection level, the percentage of individuals with type A cloacal erection rose from 73.86 % to 94.77 % during stage I and remained above 95 % in stage II (Fig. 1B). Based on these results, libido was graded according to the average number of massages to erection (**ANM**) and the number of type A erections achieved during the stabilization phase (stage II) (Table 2).

Correlation between libido and semen quality

Spearman's correlation analysis of training and semen quality assessment results revealed that the ANM was significantly and negatively correlated with SC, AI, and SQF, while showing a significant positive correlation with MAS ($P < 0.01$) (Table 3).

Comparison of semen quality and testicular histology between high and low libido groups

In terms of semen quality, SC and SQF were significantly higher in HG compared to LG, while MAS was significantly lower ($P < 0.05$) (Table 4). Additionally, we observed significant differences in testicular development between groups (Fig. 2A-B). The LD ($P < 0.01$) and the number of Sc ($P < 0.05$) in the seminiferous tubules were significantly higher in HG than in LG. No significant difference was noted in the number of Lc. Furthermore, the number of Sg ($P < 0.01$) was significantly lower in HG, whereas the numbers of Sp and Se ($P < 0.05$) was significantly higher. No significant difference was found in the number of Sr (Fig. 2C-D).

RNA-seq summary and identification of differentially expressed genes

An average of 27,205,489 raw reads were generated from each sample, yielding a total of 645,182,899 clean reads across 24 samples after thorough filtering. The maximum values for Q20 and Q30 were

Table 1
Primer sequences used in this study.

Symbol	Forward primer(5′−3′)	Reverse primer(5′−3′)	Product length (bp)
SYCP3	GCAATGGGATGTAGACGTGC	TGGTTTTCAGTCTCTGGCTTTG	122
LHX9	ATCCCAGAAGACAAAGCGCA	GCTTGAGGTCCTTGGCATCT	107
SYCE3	TAGTAATGCGCACCAACCCA	TCCCTTAGCACCTCTTGCCA	109
CDT1	AAGGTGCTGGCTGAGATGTT	GGTACACCGCCTTGATCTGG	160
PRIM1	CGGACTGGGAGATCATGCTG	GCAAATGGGTCGAACTGGTC	163
BCL2	GGATGCCTTCGTGGAGTTGT	CCGAGATAAGCGCCAAGAGT	126
β-actin	TGACAATGGCTCCGGTATGT	ACCATCACACCCTGATGTCTG	105
GAPDH	AGCAACATCAAGTGGGCAGA	CACCCATCACGAACATGGGA	157

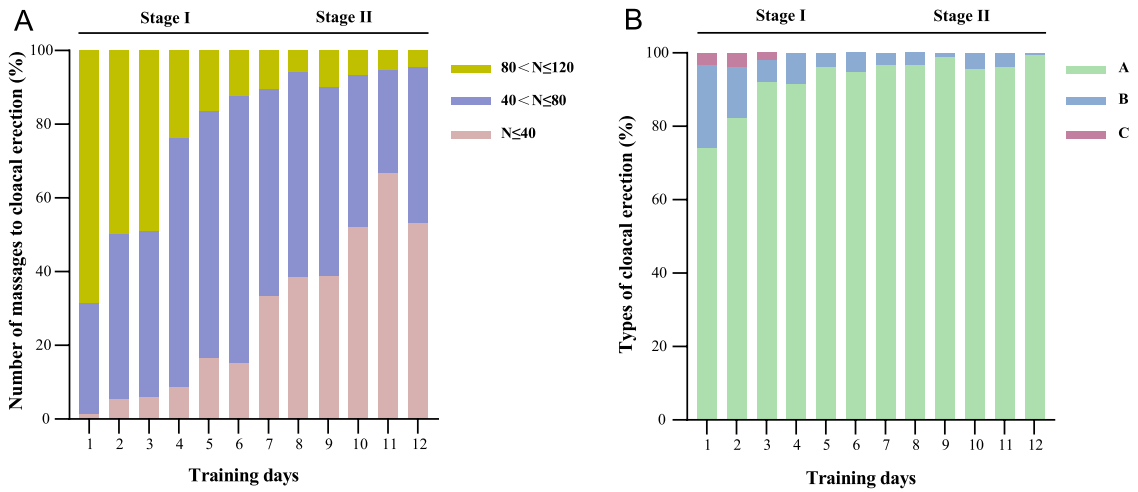

Fig. 1. ***Massage training results.*** (A) Trends in the number of massages required for cloacal erection over time. (B) Trends in the proportion of each cloacal erection type over time. Abbreviations: A: type A; B: type B; C: type C.

Table 2
Rating scale.

Grade	Training performance in stage II		Population	
	Average number of massages to erection	Number of type A erections achieved	Number	Ratio (%)
6	≤ 40	6	42	0.280
5	41 - 60	6	77	0.513
	≤ 40	5		
4	61 - 80	6	21	0.140
	41 - 60	5		
3	61 - 80	5	4	0.027
	≥ 81	6		
2	≤ 40	≤4	3	0.020
	41 - 60	≤4		
	≥ 81	5		
1	61 - 80	≤4	3	0.020
	≥ 81	≤4		

98.3 % and 95.42 %, and the minimum values were 97.02 % and 93.01 %, respectively. Mapping rates varied between 93.41 % and 95.72 %. These results indicate that the sequencing data quality was sufficient for subsequent analysis (Additional file: Table S1). We identified a total of 98, 163, 2,474 and 400 DEGs in the hypothalamus, pituitary, testis and external genitalia, respectively (Fig. 3A). In the hypothalamus, there were 59 up-regulated and 39 down-regulated genes. The pituitary contained 101 up-regulated and 62 down-regulated genes. In the testis, the majority of the genes were down-regulated, with 1,584 genes, and 890 genes were up-regulated. For the external genitalia, 142 genes were up-regulated, and 258 genes were up-regulated (Fig. 3B) (Additional file: Table S2).

Differently expressed genes functional analysis

The GO enrichment analysis revealed that 223, 204, 414 and 228 GO terms were significantly enriched in the hypothalamus, pituitary, testis and external genitalia, respectively ($P < 0.05$) (Additional file: Table S3). The top 30 GO terms were shown in Fig. 4A-D. Notably, the term "male gonad development" was significantly enriched in the hypothalamus, and the genes (*LHX9, SFRP1*, and *WNT4*) associated with it might be important upstream regulatory genes (Fig. 4A). Correlation analysis indicated that the expression level of *LHX9* was significantly positively correlated with ANM but negatively correlated with SC and SQF ($P < 0.05$). *WNT4* expression was significantly positively correlated with ANM and MAS ($P < 0.01$), and negatively correlated with SC ($P < 0.05$). While, *SFRP1* showed no significant correlation with these traits (Additional file Table S4). Therefore, *LHX9* and *WNT4* might be key upstream genes for libido influencing MAS, SC, and SQF in geese

Table 4
Comparison of semen quality between groups.

	EV (mL)	SV (%)	SM (%)	MAS (%)	SC (10^7/mL)	AI (%)	SQF
HG	0.18 ±0.02	73.77 ±3.52	62.14 ±4.06	6.95 ±1.75	45.81 ±18.51	93.95 ±2.69	57.41 ±28
LG	0.19 ±0.12	51.44 ±14.33	36.21 ±14.82	14.58 ±1.59	4.06 ±3.17	87.92 ±5.17	2.06 ±1.21
P	0.896	0.099	0.076	<0.05	<0.05	0.217	<0.05

Abbreviations: HG: high libido group; LG: low libido group; EV: ejaculate volume; SV: sperm viability; SM: sperm motility; MAS: morphological abnormal sperm; SC: sperm concentration; AI: acrosome integrity; SQF: semen quality factor.

Table 3
Results of correlation analysis between libido and semen quality.

	ANM	EV (mL)	SV (%)	SM (%)	MAS (%)	SC (10^7/mL)	AI (%)	SQF
ANM	1							
EV (mL)	−0.139	1						
SV (%)	−0.154	−0.05	1					
SM (%)	−0.128	−0.062	0.920**	1				
MAS (%)	0.255**	−0.027	−0.286**	−0.233**	1			
SC (10^7/mL)	−0.256**	0.014	0.195*	0.135	−0.323**	1		
AI (%)	−0.310**	0.054	0.175*	0.15	−0.260**	0.407**	1	
SQF	−0.334**	0.469**	0.332**	0.263**	−0.396**	0.833**	0.400**	1

Abbreviations: ANM: average number of massages to erection; EV: ejaculate volume; SV: sperm viability; SM: sperm motility; MAS: morphological abnormal sperm; SC: sperm concentration; AI: acrosome integrity; SQF: semen quality factor. ** $P<0.01$, * $P<0.05$.

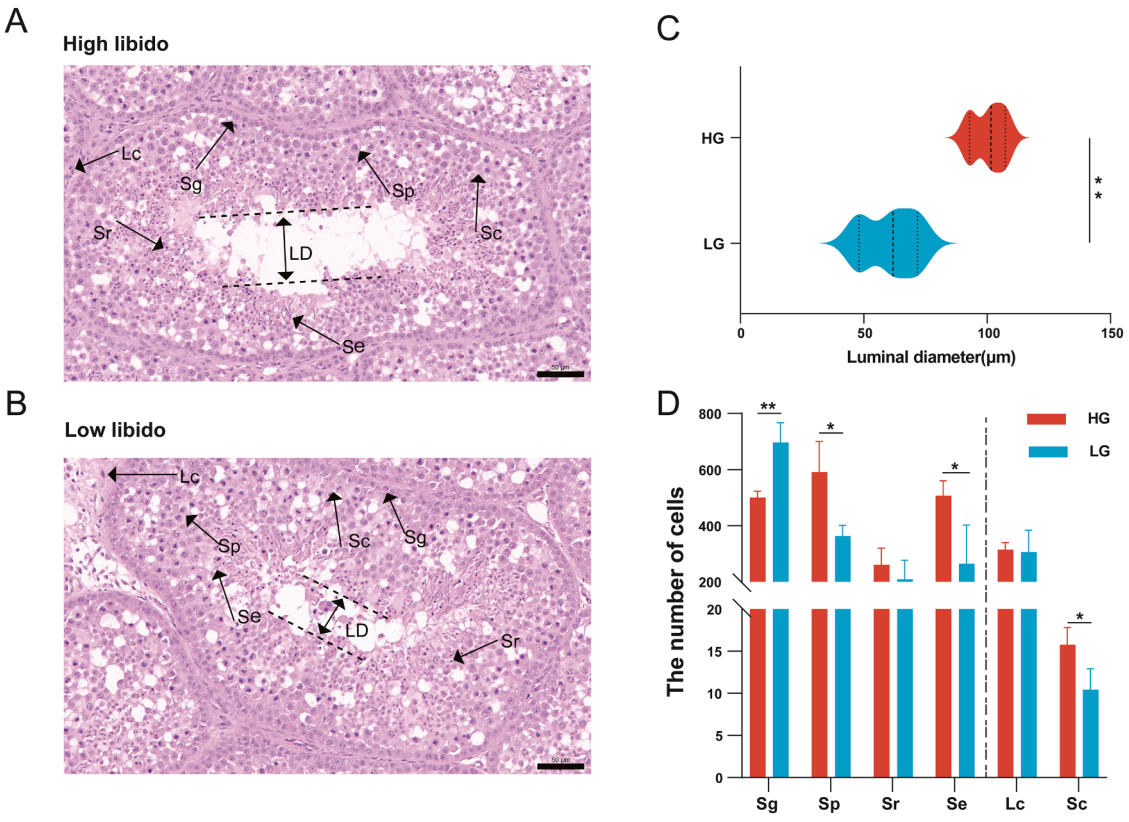

Fig. 2. ***Histologic observations of the testis.*** (A) Intra-testicular structure of HG. (B) Intra-testicular structure of LG. (C) Comparative results of the luminal diameter. (D) Comparison of germ cell numbers. Abbreviations: HG: high libido group; LG: low libido group; LD: lumen diameter; Lc: Leydig cell; Sc: Sertoli cell; Sg: spermatogonia; Sp: spermatocyte; Sr: round spermatid; Se: elongated spermatid.

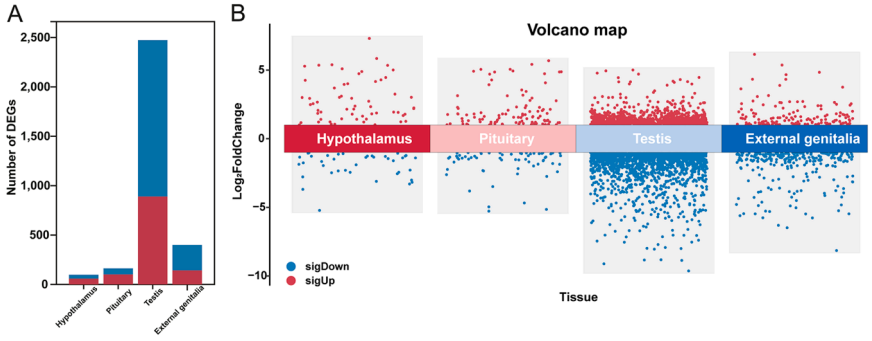

Fig. 3. Identification of DEGs in 4 tissues. (A) Number of DEGs. (B) Volcano map of DEGs.

through the HPTE axis.

Subsequent KEGG enrichment analysis revealed that 2, 9, 29, and 13 KEGG pathways were significantly enriched in the hypothalamus, pituitary, testis and external genitalia, respectively ($P < 0.05$) (Additional file: Table S5). The pathways identified in the hypothalamus were primarily related to signal transduction, including Wnt signaling pathway and MAPK signaling pathway (Fig. 5A). Meanwhile, metabolism-related pathways were enriched in the pituitary, including tryptophan metabolism, taurine and hypotaurine metabolism, and 2-oxocarboxylic acid metabolism (Fig. 5B). The testis had the highest number of significantly enriched pathways among all tissues, predominantly associated with metabolism and environmental information processing (Fig. 5C). In the external genitalia, the pathways were mainly associated with environmental information processing, including signal transduction (vascular smooth muscle contraction, calcium signaling pathway, apelin signaling pathway, MAPK signaling pathway, and Wnt signaling pathway) and signaling molecules and interaction (neuroactive ligand-receptor interaction, cell adhesion molecules, ECM-receptor interaction) (Fig. 5D).

Weighted gene co-expression network analysis and key module identification

To further investigate the key genes and their regulatory mechanisms by which libido affected the semen quality through the HPTE axis, we conducted WGCNA on DEGs identified from the 4 tissues. Genes exhibiting similar expression patterns were classified into the same module and a total of 11 modules were obtained (Fig. 6A). The turquoise ($R = 0.98$, $P = 3e\text{-}16$) and yellow ($R = 0.89$, $P = 7e\text{-}09$) modules were significantly correlated with testicular development (Fig. 6B). Enrichment analysis using ClueGO indicated that the genes within the turquoise module were primarily involved in inner dynein arm and protein retention in Golgi apparatus process. Meanwhile, the yellow module,

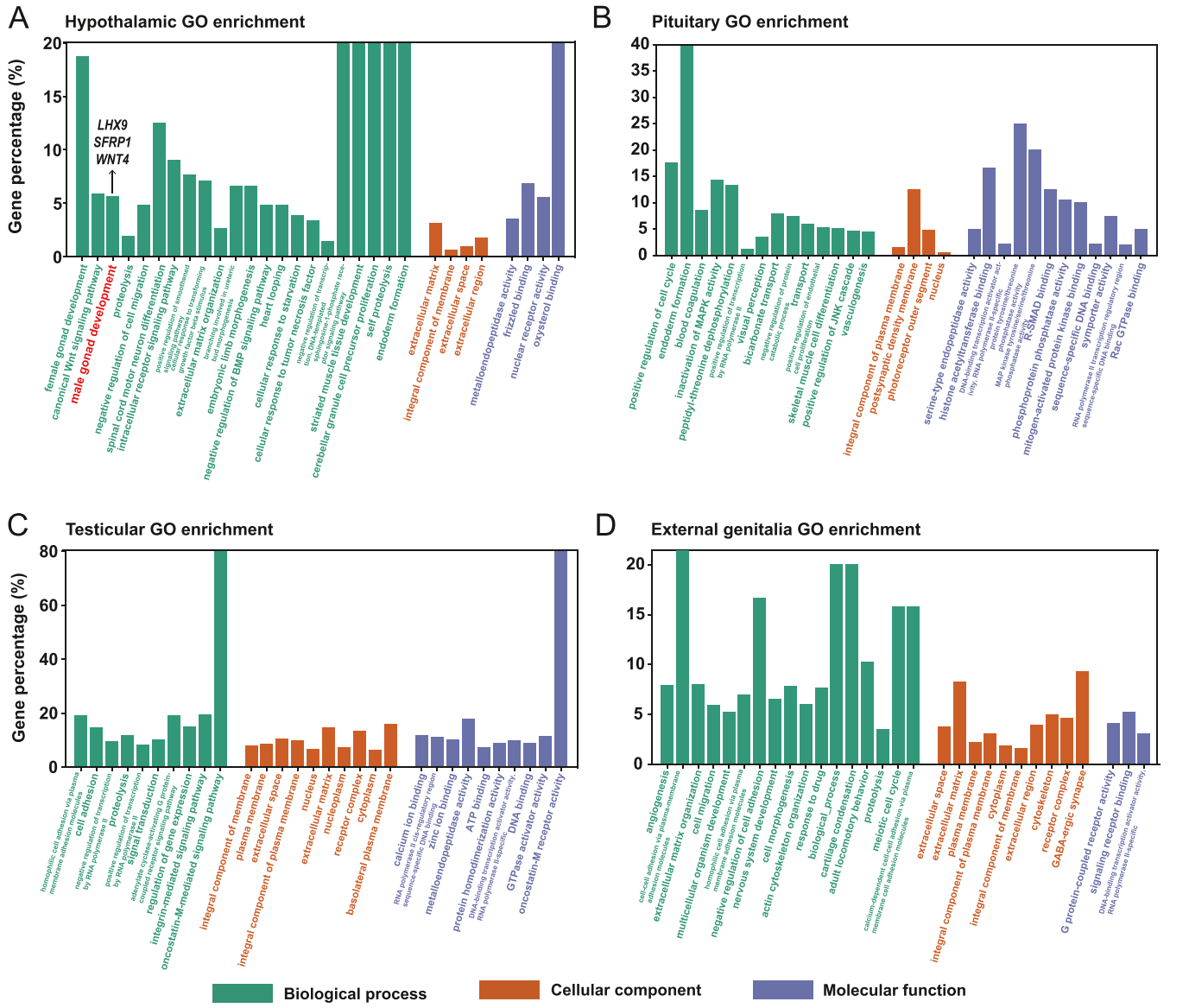

Fig. 4. Top 30 GO terms enriched by DEGs in hypothalamus (A), pituitary (B), testis (C) and external genitalia (D).

with genes mainly associated with the male reproductive process, appeared to be of greater importance (Fig. 6C).

Predictive regulating network construction and validation of RNA-seq

Based on these findings, we screened the genes significantly related (|Cor |≥0.6, $P<0.05$) to *LHX9* and *WNT4* from the yellow module, and constructed a correlation network (Fig. 7A). These genes were significantly enriched in 8 KEGG pathways, 6 of which were associated with metabolism (drug metabolism - other enzymes, metabolism of xenobiotics by cytochrome P450, metabolic pathways, pyrimidine metabolism, glycerolipid metabolism, and riboflavin metabolism). Subsequently, we developed a potential molecular regulatory network (Fig. 7B). Further, using the MCC algorithm in the CytoHubba plug-in, *SYCP3, DDX4, STRA8, AMH, MEIOB, CDT1, BCL2, PRIM1*, and *DLGAP5* were identified as hub genes. The qRT-PCR results of 6 randomly selected genes showed consistent trends between qRT-PCR and RNA-seq (Additional file: Fig. S1).

Discussion

With the widespread use of artificial insemination techniques in animal husbandry, there is a growing need to obtain as much high quality semen as possible to improve production efficiency (Henney, et al., 1990). Consequently, enhancing libido selection is crucial alongside focusing on semen quality. Dorsal abdominal and cloacal massage is a common method for collecting semen from poultry, leveraging sexual reflexes to elicit repetitive responses in a short period of time (Burrows and Quinn, 1935). During massage, we found significant individual differences in the number of massages required from the start of the massage to full ejaculation and the hardness of the cloacal erection. This finding was consistent with previous studies (Ouyang, et al., 2021; Paranzini, et al., 2018) but offered more nuanced insights. Thus, we developed a detailed training program and recorded phenotypes daily, and established a goose libido rating scale based on the number of massages to cloacal erection and the type of erection to aid in selection. Subsequent studies revealed a significant correlation between libido and semen quality, as well as a significant difference in semen quality between high and low libido geese, and these results were consistent with findings from other species (Hu, et al., 2023; Islam, et al.,

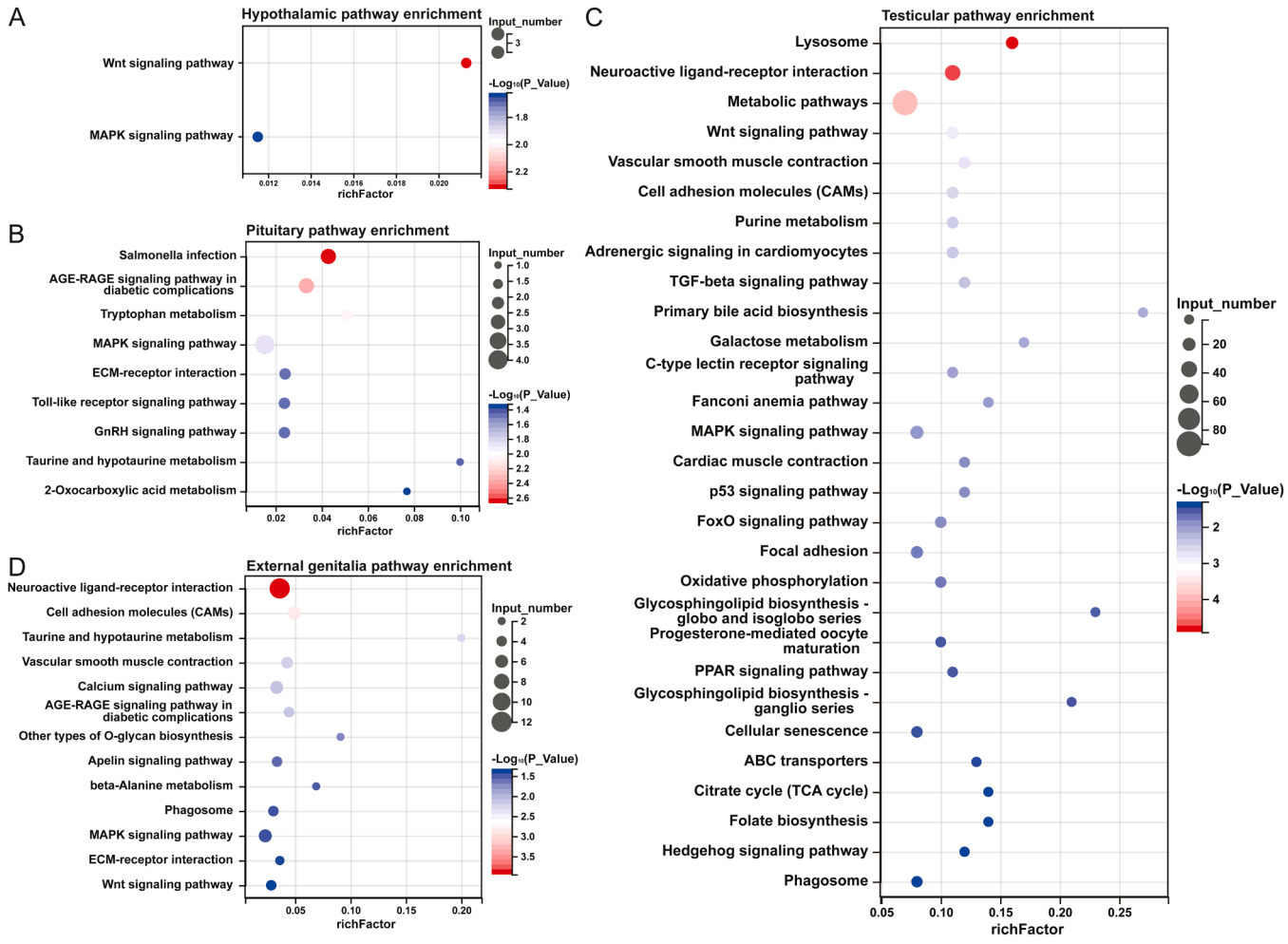

Fig. 5. Significantly enriched KEGG pathways in the hypothalamus (A), pituitary (B), testis (C), and external genitalia (D).

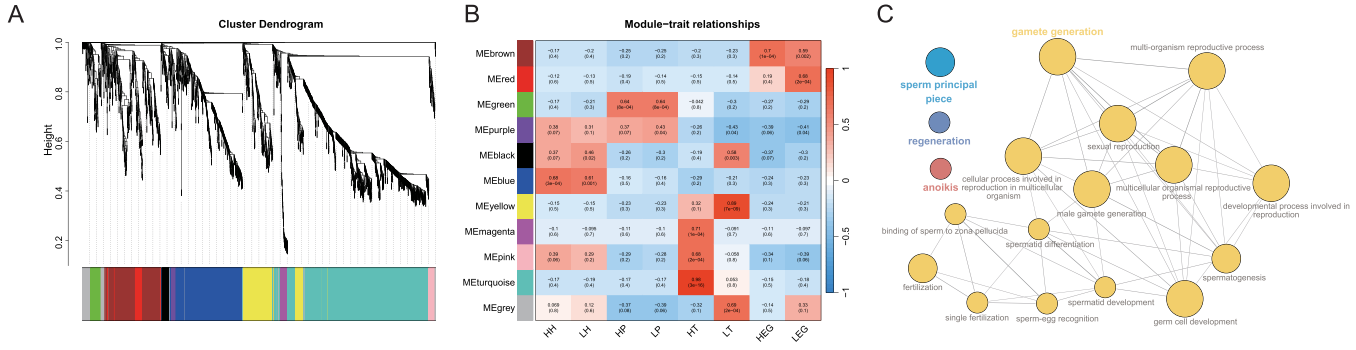

Fig. 6. ***Weighted gene co-expression network analysis results.*** (A) Hierarchical clustering tree. The same color represents the same clustering module. (B) Heatmap of correlations between modules and traits. The horizontal axis represents different traits and the vertical axis represents different gene modules. (C) Visualization of GO enrichment results of genes in the yellow module. Nodes of the same color represent the same functional group; the larger the node, the higher its significance.

2018).

Histological analysis revealed developmental abnormalities in the seminiferous tubules of LG testes compared to the HG, particularly characterized by lower LD and fewer Sc. Similar findings were noted in camel testes during the non-rutting season, suggesting that low libido geese might be in a sexually inactive state (Ali, et al., 2024). Additionally, a study indicated that chickens with low SM exhibited significantly lower LD compared to those with high SM (Sun, et al., 2019). In our study, lower LD was accompanied by lower SC and SQF and higher MAS. Furthermore, Sc serve as target cells for follicle-stimulating hormone (Means, et al., 1976; Wang et al., 2022a) and testosterone (Dimitriadis, et al., 2015), playing a crucial role in spermatogenesis and spermiogenesis by providing structural and nutritional support for germ cell development in the seminiferous tubules (Crisóstomo, et al., 2018; Griswold, 2018). Therefore, we speculated that less Sc might impair processes such as meiosis (O'Donnell, et al., 2022) and germ cell movement (Mruk and Cheng, 2004), ultimately leading to variations in germ cell numbers at different developmental stages.

To gain insights into the molecular regulatory mechanisms, we analyzed mRNA expression profiles in the hypothalamus, pituitary,

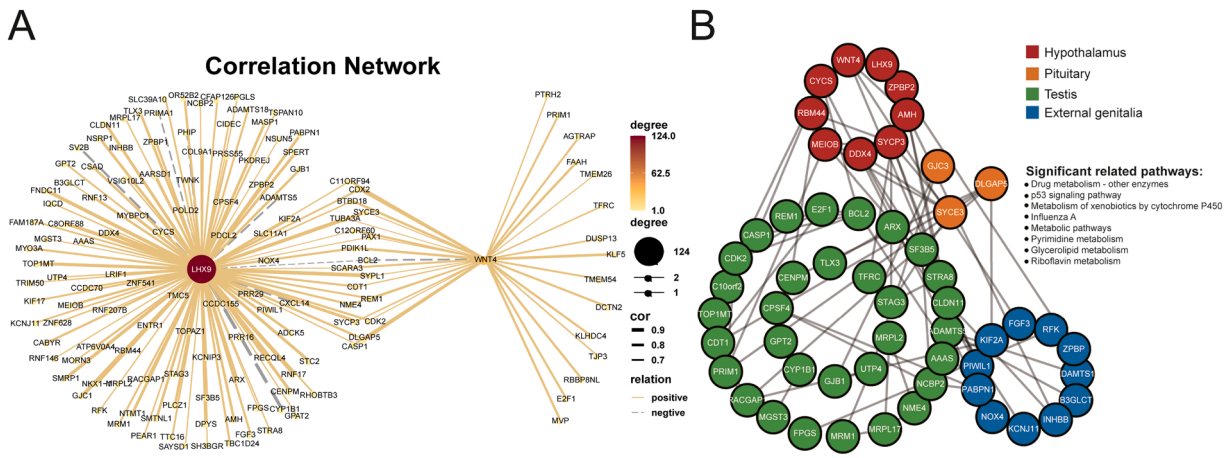

Fig. 7. ***Prediction of molecular mechanisms*** (A) Correlation network of *LHX9* and *WNT4* with yellow module genes. (B) Possible regulating networks in the HPTE axis.

testis and external genitalia. Although we identified the fewest DEGs in the hypothalamus, it was noteworthy that these DEGs were significantly enriched in the "male gonad development" GO term, including *LHX9, SFRP1*, and *WNT4*. LHX9 is a LIM-homeodomain (LIM-hd) transcription factor belonging to a growing family of developmental regulators (Failli, et al., 2000). They are particularly important in the development of the nervous system (Bertuzzi, et al., 1999; Liu, et al., 2015) and play key roles in cell determination (Mazaud, et al., 2002) and gonad formation (Birk, et al., 2000). *SFRP1* encodes a member of the SFRP family involved in Wnt signaling transduction (Wu, et al., 2017) and has been identified as a regulatory protein for spermatogenesis, tightly controlling sperm adhesion and sperm release in the adult rat testis (Wong, et al., 2013) and influencing testicular development (Zhao, et al., 2024). *WNT4* also belongs to the Wnt signaling pathway and encodes Wnt family member 4. In humans, mutations in *WNT4* were associated with low libido caused by disorders of sexual development (Ashfaq, et al., 2021). In addition this, it was reported to be engaged in regulating endothelial and steroidogenic cell migration in the developing mammalian gonad (Jeays-Ward, et al., 2003). As a critical pathway affecting male and female sexual development (Jeays-Ward, et al., 2004; Windley and Wilhelm, 2016), Wnt signaling transduction could affect the differentiation efficiency of GnRH neurons by influencing neurogenesis and altering the differentiation fate of neural progenitor cells (Wang et al., 2022b). And, previous studies have suggested that the Wnt signaling pathway might also regulate external genitalia development in geese (Tang, et al., 2022). Furthermore, studies in zebrafish demonstrated that *LHX9* could determine neuronal differentiation and partitioning in the caudal forebrain via regulating Wnt signaling (Peukert, et al., 2011). However, in our study, the regulatory mechanisms by which *LHX9* interacted with Wnt signaling in the hypothalamus, mediated by *SFRP1* and *WNT4*, required further investigation. Further analysis revealed that the expression of *LHX9* and *WNT4* were significantly correlated with libido and semen quality phenotypes, which were closely associated with metabolism-related pathways, including drug metabolism - other enzymes, metabolism of xenobiotics by cytochrome P450, metabolic pathways, pyrimidine metabolism, glycerolipid metabolism, and riboflavin metabolism (mainly involving genes such as *SYCP3, DDX4, STRA8, AMH, MEIOB, CDT1, BCL2, PRIM1*, and *DLGAP5*). The first two pathways were associated with xenobiotics biodegradation and metabolism. Xenobiotics are chemicals foreign to the organism (Iovdijová and Bencko, 2010) which could affect glucose metabolism and blood-testis barrier (Ghafouri-Fard, et al., 2021), potentially impairing spermatogenesis (Aly and Azhar, 2013; Kanter, et al., 2013; Rato, et al., 2012). Pyrimidines are essential for nucleic acid synthesis and energy transfer (Micheli, et al., 2011), and in bulls, pyrimidine metabolism in sperm has been shown to significantly affect fertility (Talluri, et al., 2022). Changes in pyrimidine metabolism in seminal plasma of infertile men have been noted, with certain types serving as potential infertility biomarkers (Lazzarino, et al., 2018). Additionally, lipids in boar seminal plasma extracellular vesicles might influence sperm motility through glycerolipid metabolism (Ding, et al., 2025). Further, riboflavin metabolism might affect spermatozoa in terms of energy metabolism, as riboflavin is a precursor of coenzymes for many mitochondrial metabolic enzymes (Amaral, 2022).

In conclusion, within the HPTE axis, libido might influence metabolism-related signaling pathways (mainly involving genes such as *SYCP3, DDX4, STRA8, AMH, MEIOB, CDT1, BCL2, PRIM1*, and *DLGAP5*) through *LHX9* and *WNT4* to regulate the development of the seminiferous tubules and germ cell number, ultimately affecting SC and MAS in geese.

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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.2024.104756.

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