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Changes in goose hypothalamus under different photocycles: RNA-Seq reveals new pathways and molecules

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ABSTRACT

Reproduction in avian species is regulated by light, and the hypothalamus is the most important regulator of the response to light signals. The objective of this study was to identify the key molecules and signaling pathways in the hypothalamus that regulate reproduction in birds based on the photoperiod. Yangzhou geese kept in a closed goose barn were selected for transcriptome sequencing. The light program during rearing was as follows: a short photoperiod (light[L]: dark[D]=6.5 h:17.5 h) for 60 days, followed by a prolonged photoperiod (L:*D* = 11.5 h:12.5 h). Samples were collected on the 38th day of the short photoperiod (SP), the 20th (LPS), 73rd (LPP), and 135th (LPF) days of the long photoperiod. Hypothalamus were collected to screen for key avian reproduction-related genes and signaling pathways affected by the photoperiod using RNA sequencing (RNA-seq) analysis. In the SP group, the testis gradually enlarged and mature sperm were observed in the curved seminiferous tubules after a prolonged photoperiod. Meanwhile, the concentrations of testosterone (T), follicle-stimulating hormone (FSH) and luteinizing hormone (LH) increased in the blood. The RNA-seq results revealed that compared with the SP group, the LPS group showed 24 significantly upregulated genes, and 9 significantly downregulated genes. Compared with the LPS, the LPP group showed 7 significantly upregulated genes, and 27 significantly downregulated genes. Compared with the LPP group, the LPF group showed 7 significantly upregulated genes, and 5 significantly downregulated genes. Differentially expressed genes were mainly enriched in neuroactive ligand-receptor interaction, ovarian steroidogenesis, thyroid hormone synthesis, GnRH secretion, and GnRH signaling pathway, etc. Key genes, including *GAL, GnRH, UCN3, CGA* and *CYP19A1*, were identified. This study extends the theory of the central regulation of avian reproductive activity by light.

Introduction

Goose farming is an efficient and low-cost poultry industry that has shown steady growth globally. However, the seasonal reproduction of geese has resulted in low reproductive performance, the scarcity of goslings, and soaring prices, hindering the rapid development of the goose industry. Mature artificial light technology has been developed to regulate the reproductive activities of breeding geese; however, the intrinsic molecular mechanism of photoperiodic regulation in breeding geese requires further elucidation.

Avian species are typically light-sensitive seasonal breeds. Unlike in mammals, light signals can directly penetrate the avian cranium to act on deep-brain photoreceptors (DBPs) (Pérez, 2022), such as melanopsin (Opn4), neuropsin (Opn5) and vertebrate ancient opsin (VAO), which perceive light signals and are involved in avian reproductive activity (Bailey, 2005; Chaurasia et al., 2005; Halford, 2009; Nakane and Yoshimura, 2010; Davies, 2012). These photoreceptors further stimulate the production of thyroid hormones (TSH) by influencing the pituitary tubercle. TSH secreted by the pituitary gland prompts hypothalamic ventricular cells to express deiodinase iodothyronine type II (DIO2). DIO2 converts thyroxine (T4) into the biologically active triiodothyronine (T3), which regulates the secretion of hypothalamic

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gonadotropin-releasing hormone (GnRH) (Nakane and Yoshimura, 2010; Yoshimura T, 2013). GnRH enters the anterior pituitary gland through the pituitary portal system and stimulates the pituitary gland to secrete follicle stimulating hormone (FSH) and luteinizing hormone (LH), which travel from the bloodstream to the gonads, thereby promoting gonadal development and the secretion of related hormones, such as testosterone (T) and estradiol (E2) (ONAGBESAN, 2006, SHARP 2005). Therefore, light regulates avian reproduction by modulating the activity of the hypothalamic-pituitary-gonadal (HPG) axis, which affects sex hormone secretion (Bentley, 1996; Ikegami, 2013; Yoshimura, 2013; Kuenzel., 2014). However, this theory still needs to be refined (Dardente, 2022). More research is needed to determine whether other ways of regulating reproduction by light signals exist.

Yangzhou geese were used as the experimental model in this study. Samples were collected from geese exposed to different photoperiods, and hypothalamic tissues were analyzed using RNA sequencing (RNA-seq) technology. We explored the key molecules modulating the influence of light on avian reproduction, with the intention of improving our knowledge of the mechanism whereby light regulates the reproductive behavior of breeding geese. The findings of this study provide reference and research information for poultry breeding and promote the development of the animal husbandry industry.

Materials and methods

Animal experiment design and sample collection

The experiments were carried out in Guiliu Animal Husbandry Co., LTD., and the test subjects were Yangzhou geese (male to female ratio was 1:5), which were raised in a fully enclosed shed with artificial control of light and temperature. as follows: firstly, Yangzhou geese were treated with ultra-long photoperiod (L:*D* = 20 h: 4 h) for 1 month to quickly end the egg laying activity. A short photoperiod (L:*D* = 6.5 h: 17.5 h) was treated for 2 months; then the photoperiod was extended to 11.5 h (L:*D* = 11.5 h: 12.5 h). Sampling was carried out according to the laying time nodes of the geese (the short photoperiod, the long photoperiod-start, the long photoperiod-peak, the long photoperiod-finish). Sampling was performed on the 38th day of the short photoperiod (SP) and the 20th (LPS), 73rd (LPP), and 135th days (LPF) of the long photoperiod, and five Yangzhou geese (male) were selected from each group.

Testis morphometry and histology

After the testicular tissue was isolated, about 0.5 cm * 0.5cm* 1 cm testicular tissue was quickly cut and soaked with 4 % paraformaldehyde. The fixed samples were dehydrated, embedded in paraffin wax, and cut into continuous sections (4 μm). The sections were dewaxed, then rinsed with alcohol and distilled water, stained with hematoxylin, rinsed with tap water and 1 % hydrochloric ethanol, and stained with 5 % eosin. Testicular tissue sections were evaluated using a microscope and microcomp integrated digital imaging analysis system (Olympus SZX10, Tokyo, Japan). The test was entrusted to Wuhan servicebio technology Co.

Total RNA extraction

Grind the hypothalamic tissue in liquid nitrogen, remove a portion of the tissue and place it in a 2 mL enzyme free centrifuge tube. Add 1 mL of trizol, mix thoroughly with a vortex mixer, and let it stand at room temperature for 30 s. Subsequently, add 200 μL chloroform, shake for 15 s, and then let it stand at room temperature for 5 min. Next, centrifuge at 12000 rpm and 4 °C for 15 min. After centrifugation, absorb approximately 500 μL of the upper aqueous phase and transfer it to a 1.5 mL enzyme free centrifuge tube. An equal volume of pre-cooled isopropyl alcohol was added, mixed, and then placed at −20 °C for 1 h. The sample tube was removed and centrifuged at 12000 rpm and 4 °C for 15 min. After centrifugation, the liquid was poured off, 1 mL 75 % ethanol was added, the precipitate was suspended by gently swirling the bottom of the tube, left to stand at room temperature for 3 min, then centrifuged at 12000 rpm and 4 °C for 5 min and washed twice. Finally, after a rapid centrifugation, the ethanol was cleaned using a pipette gun, the tube cover was opened, and it was placed in a super clean bench to dry for 5 min. According to the amount of RNA precipitation, an appropriate amount of DEPC water was added and left for 5 min in a 55 °C-water bath, then the RNA was centrifuged, mixed and re-centrifuged. Eventually, the concentration of total RNA in the samples was determined using Nanodrop 2000 instrument.

RNA-seq experimental procedure

The hypothalamus tissues of Yangzhou geese were collected for RNA-seq analysis. After the extraction of hypothalamic RNA, eukaryotic mRNA with polyA tail was enriched using Oligo (dT) magnetic beads, and the mRNA was broken by ultrasound. Using fragmented mRNA as template, the first strand of cDNA was synthesized using random oligonucleotides, then the RNA strand was degraded by RNaseH, and the second strand of cDNA was synthesized using DNA polymerase I. The purified double-stranded cDNA was end-repaired, A-tail was added, and sequencing joints were connected. cDNA of about 200 bp was screened by AMPure XP beads for PCR amplification, and then PCR products were purified by AMPure XP beads to finally obtain A library. The resulting cDNA libraries were sequenced using Illumina Novaseq 6000. The sequencing of this experiment was entrusted to Gene Denovo Biotechnology Co., Ltd (Guangzhou, China).

Gene expression assay

Total RNA is extracted from the tissue, and real-time fluorescence quantitative PCR was performed to detect the expression of mRNA of reproduction-related genes. The primers used for qPCR are listed in Table 1. The Ct value was used to calculate the number of corresponding gene copies according to the standard curves. The concentrations of each DNA sample were adjusted to 10 ng/uL. The qPCR mixtures consisted of 0.2 μM concentration of each primer, 10 μL of Genious 2X SYBR Green SYBR qPCR mix (Vazyme, China), 1 μL of template DNA, and the final volume was adjusted to 20 μL by adding ddH_2O. Mx3000 P (Stratagene, San Diego, CA, USA) was employed for amplification and quantification. The protocol was conducted as follows: 30 s at 95°C, then 40 cycles of 5 s at 95°C, 30 s at the annealing temperature, extension for another 30 s at 72°C and a simultaneous fluorescence signal scanning at 72°C, and then a melt curve stage with temperature ramping from 65 to 95°C. The relative expression level was calculated using the $2^{-\Delta\Delta Ct}$ method, and the internal reference gene was GAPDH. The primers were designed using Primer 5.0 software and synthesized by Prime Biology Co.

Johnson score

The Johnson score is a quantitative scoring system used to assess the degree of spermiogenesis and spermiogenesis disorders in seminiferous

Table 1
PCR primers sequence.

Genes	Gene bank ID	Primer sequence(5′to 3′)
GAPDH	NM_204305.1	F:GCCATCACAGCCACACAGA
		R:TTTCCCCACAGCCTTAGCA
GnRH	EF495207.1	F:CTGGGACCCTTGCTGTTTTG
		R:AGGGGACTTCCAACCATCAC
GAL	XM_013195301.2	F:TACCTACTTGGGCCACATGC
		R:TTGTCAAGTGCTCCCACCTC
SPX	XM_013184566.2	F:AGTGCTCCTCAGGCTCATTT
		R:TCTGAGATGAATCGCCGTCC

tubules in males and is mainly used for the analysis of testicular biopsy specimens. It is divided into 10 levels, with higher scores indicating better spermiogenesis and lower scores indicating more severe spermiogenesis disorders.

Statistical analysis

Data such as gene expressions, were analyzed by one-way ANOVA using SPSS software (version 20.0, SPSS Inc., Chicago, IL, USA). The Duncan multiple range test was used to compare the significant differences between the means. Repetition ($n = 3$) was used as the experimental unit. The results are expressed as mean ± standard error. $p < 0.05$ was considered statistically significant. The GraphPad (GraphPad Software, San Diego, USA) was utilized for presentation of data (Fig. 1).

Result

Influence of photoperiod on testicles of Yangzhou goose

As can be seen from Table 2, compared with the three groups after prolonged photoperiod, the relative weight of the left and right testis in the SP group was the smallest. After prolonging the photoperiod, the relative weight of testes gradually increased greatly. The relative weight of left and right testes was the largest in the LPP group, and compared with SP group, there was no significant difference in relative weight between the LPS group and the LPF group ($p > 0.05$). HE staining showed that there were no mature spermatozoa in the SP group, whereas there were many mature spermatozoa in the LPP group (Fig. 2). Johnson scores showed that the mean scores of the four groups were 2.6, 6.4, 9.2, and 5.2. The SP group was significantly lower than the other three groups, while the LPP group score was significantly higher than the other three groups, and the LPS group was significantly higher than the LPF group ($p < 0.05$).

Detection of hormone in serum of Yangzhou goose

As can be seen from Table 3, the concentrations of T, FSH and LH in the blood of Yangzhou geese in the LPP group were significantly higher than the SP and the LPS groups ($p < 0.05$). The concentration of FSH in blood of Yangzhou geese in LPF group was significantly higher than the SP and the LPS groups ($p < 0.05$).

Genome comparison result

The Clean Reads of each sample were sequentially compared with the reference genome (GCF_000971095-1) using HISAT2.

Typically, when a suitable reference genome is chosen and there is no contamination in the relevant experiments, the mapping rate of sequencing sequences generated by the experiments is usually higher than 65 %. In this experiment, while ensuring the quality of the reference genome, the ratio of RNA-seq data and reference genome sequence aligned to unique sites on the reference genome exceeded 70 %, resulting in an overall alignment rate greater than 72 %. These findings indicate a strong agreement between theoretical expectations and measured values, validating their utility for subsequent analysis (Table 4).

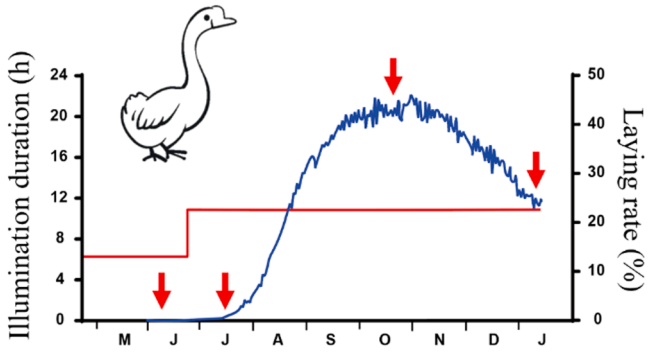

Fig. 1. Schematic diagram of sampling time points. Ultra-long photoperiod (L:*D* = 20 h: 4 h) for one month to rapidly end egg-laying activity. Short photoperiod (L:*D* = 6.5 h: 17.5 h) was treated for 2 months, then the photoperiod was extended to 11.5 h (L:*D* = 11.5 h: 12.5 h). Samples were conducted on the 38th day of short photoperiod (SP), the 20th (LPS), 73rd (LPP), and 135th (LPF) day of long photoperiod.

Table 2
Effect of photoperiod on appearance and organizational structure of testis of Yangzhou geese.

Sample	SP	LPS	LPP	LPF
Left testis/body weight (10^{-3})	0.26 ± 0.06^{c}	0.66 ± 0.01^{b}	1.96 ± 0.13^{a}	0.74 ± 0.22^{b}
Right testis/ body weight (10^{-3})	0.19 ± 0.04^{b}	0.49 ± 0.01^{b}	1.41 ± 0.17^{a}	0.56 ± 0.19^{b}
Johnson score	2.6 ± 0.24^{d}	6.4 ± 0.20^{b}	9.2 ± 0.20^{a}	5.2 ± 0.24^{c}

Differential gene analysis

Based on gene expression information, principal component analysis was performed. In the analysis results, the more similar the samples are, the closer the samples on the PCA plot are, while the samples from different effective treatments are usually farther apart. Discard SP-3 and LPS-1 due to outliers. As shown in Fig. 3A, the SP group is clearly distinguished from the LPS, the LPP, the LPF groups. Genes with FDR < 0.05 and $|\log2FC| > 1$ were screened as differentially expressed genes (DEGs), with twenty-four genes and nine genes downregulated in expression in the LPS group compared to the SP group; seven genes upregulated and twenty-seven genes downregulated in the LPP group compared to the LPS group; and seven genes upregulated and five genes downregulated in the LPF group compared to the LPP group (Fig. 3B,C). Fig. 3D shows the TOP 20 genes with the greatest overall difference between the two groups of samples according to maximum log2 (FC). Among them, the DEGs of SP vs LPS include *GnRH, CTXND1, CNP3, ARMC3, ankrd34b, UCN3, Mycl, capn5, Greb1, GAL, CGA, CYP19A1*, etc. DEGs of LPS vs LPP include *CTXND1, GnRH, CGA, GAL*, etc. DEGs of LPP vs LPF include *CTXND1, GnIH*, et al.

Functional enrichment analysis of differentially expressed genes

GO is a database established by the Gene Ontology Consortium, and this database can categorize the genes in the selected gene set: biological processes involved, components that make up the cell, molecular functions achieved, etc. As shown in Fig. 4A, it can be observed that compared with the SP group, the DEGs in the LPS group are mainly enriched in hormone activity, axon terminus, neuron projection terminus, neuropeptide hormone activity, receptor agonist activity, receptor activator activity, receptor regulator activity, etc.; the LPP group compared to the LPS group, the DEGs mainly focus on response to gonadotropin, system process, endocrine hormone secretion, cellular response to follicle-stimulating hormone, cellular response to gonadotropin stimulus, regulation of steroid hormone secretion, endocrine process, steroid hormone secretion, hormone activity, etc.; the LPF group compared to the LPP group, the DEGs mainly focused on chorismate mutase activity, N(6)-L-threonylcarbamoyladenine synthase, biliverdin reductase activity, acylphosphatase activity, mitochondrial tRNA threonylcarbamoyladenine modification, establishment of glial cell membrane secretion, and hormone activity modification, establishment of glial blood-brain barrier, etc.

KEGG is a knowledgebase used for systematic analysis of gene

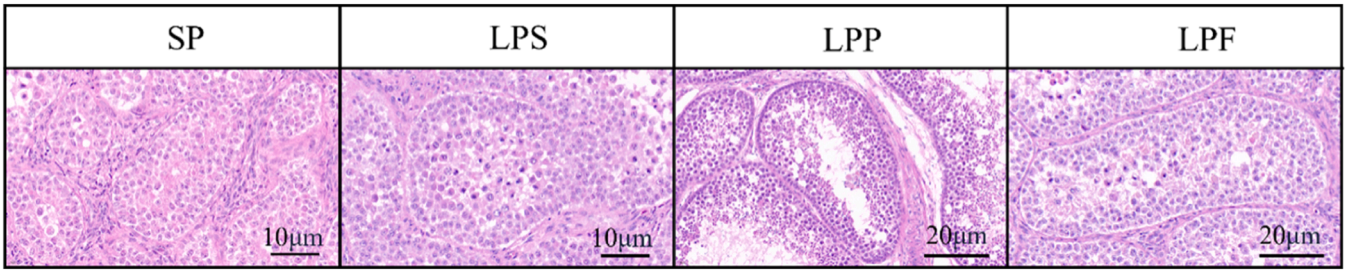

Fig. 2. Effect of photoperiod on appearance and organizational structure of testis of Yangzhou goose. HE of testis of Yangzhou goose under different magnification microscope. The data were analyzed as Mean ± standard error (Mean ± SE), with different lowercase letters indicating significant difference ($p < 0.05$) and no letters indicating no significant difference ($p > 0.05$), $n = 5$.

Table 3
Effect of photoperiod on hormone levels in blood of Yangzhou geese.

Sample	SP	LPS	LPP	LPF
T	178.48 ± 8.05 ^{b}	187.89 ± 7.32 ^{b}	267.02 ± 13.54 ^{a}	264.33 ± 13.34 ^{ab}
FSH	2.43 ± 0.25 ^{c}	2.58 ± 0.28 ^{c}	5.65 ± 0.37 ^{a}	4.69 ± 0.27 ^{b}
LH	93.54 ± 6.23 ^{b}	99.233 ± 8.84 ^{b}	136.533 ± 9.28 ^{a}	123.868 ± 6.28 ^{ab}

Note: The data were analyzed as Mean ± standard error (Mean ± SE), with different lowercase letters indicating significant difference ($p < 0.05$) and no letters indicating no significant difference ($p > 0.05$), $n = 5$.

Table 4
Comparison of reference genomes.

Sample	Total	Unmapped (%)	Unique Mapped (%)	Multiple Mapped (%)	Total Mapped (%)
SP-1	37063746	5691610 (15.36 %)	30994914 (83.63 %)	377222 (1.02 %)	31372136 (84.64 %)
SP −2	42449632	11304787 (26.63 %)	30731799 (72.40 %)	413046 (0.97 %)	31144845 (73.37 %)
SP −3	47300870	7118503 (15.05 %)	39672570 (83.87 %)	509797 (1.08 %)	40182367 (84.95 %)
LPS-1	35081252	5820466 (16.59 %)	28848346 (82.23 %)	412440 (1.18 %)	29260786 (83.41 %)
LPS −2	43330506	6546893 (15.11 %)	36298027 (83.77 %)	485586 (1.12 %)	36783613 (84.89 %)
LPS −3	44399498	6697568 (15.08 %)	37256169 (83.91 %)	445761 (1.00 %)	37701930 (84.92 %)
LPP-1	42864384	6625309 (15.46 %)	35781252 (83.48 %)	457823 (1.07 %)	36239075 (84.54 %)
LPP −2	38340158	5942390 (15.50 %)	31993660 (83.45 %)	404108 (1.05 %)	32397768 (84.50 %)
LPP −3	39204688	6069905 (15.48 %)	32738679 (83.51 %)	396104 (1.01 %)	33134783 (84.52 %)
LPF-1	36451236	5347796 (14.67 %)	30719385 (84.28 %)	384055 (1.05 %)	31103440 (85.33 %)
LPF-2	44244888	6688970 (15.12 %)	37088109 (83.82 %)	467809 (1.06 %)	37555918 (84.88 %)
LPF-3	49197906	8092803 (16.45 %)	40581516 (82.49 %)	523587 (1.06 %)	41105103 (83.55 %)

Note: Sample: sample name; Total: Number of reads after ribosome filtration; Unmapped (%): Number of reads that were not mapped to the reference genome and proportion of valid reads; Unique Mapped (%): The number of reads in the reference genome and the proportion of effective reads; Multiple Mapped (%): The number of reads in the reference genome and the proportion of effective reads in multiple comparisons; Total Mapped (%): The number of all reads that can be mapped to the genome and the proportion of valid reads, $n = 3$.

functions and linking genomic and functional information. Using the KEGG database, gene sets can be categorized based on the metabolic pathways in which genes participate or the functions they perform. The LPS group compared to the SP group, DEGs were mainly enriched in neuroactive ligand-receptor interaction, ovarian steroidogenesis, GnRH secretion, GnRH signaling pathway, etc.; the LPP group compared to the LPS group, DEGs was mainly enriched in neuroactive ligand-receptor interaction, ovarian steroidogenesis, thyroid hormone synthesis, GnRH secretion, GnRH signaling pathway, etc. (Fig. 4B). DEGs in the neuroactive ligand-receptor interaction pathway were *NTS, GAL, FSHB, LHB, TSHB, GnRH, UCN3, CGA*; DEGs in the ovarian steroidogenesis pathway include *LHB, FSHB, CYP19A1, CGA*; GnRH secretion pathway include *GnRH, LHB, CGA*; GnRH signaling pathway include *GnRH, CGA*; DEGs in the thyroid hormone synthesis pathway were *TSHB, CGA, SLC26A4* (Table 5). Based on Fig. 3D and Table 5, we screened the top 20 DEGs of SP vs LPS or LPS vs LPP or LPP vs LPF, and the genes that were simultaneously changed in the significance pathways, including *GAL, GnRH, UCN3, CGA, CYP19A1* mRNA. Correlation analysis showed that *UCN3* mRNA was positively correlated with testis/body weight and length diameter of both sides of testis (Fig. 5).

qRT-PCR Validation of differentially expressed genes

In order to verify the accuracy of the results of RNA-seq, 3 DEGs were selected and verified by qRT-PCR. The results showed that the results of RNA-seq were highly reliable and repeatable (Fig. 6).

Discussion

Animals develop survival strategies to reproduce in the most suitable environment for their offspring. Therefore, they breed only during certain seasons (Leska, 2007). Long-day animals breed in spring and summer, in response to increases in sunlight duration and temperature, whereas short-day animals breed, in the fall and winter, in response to decreases in sunlight duration and temperature. Geese are typical seasonal breeding animals, and changing the light parameters is an effective way to regulate their reproductive performance. For example, in an experiment on Japanese quails, testis growth was promoted when the light duration was greater than 11.5 h, while it was not promoted when the light duration was less than 11.5 h (Ikegami, 2013). In the present study, the testis:body weight ratio of Yangzhou geese increased significantly after a prolonged photoperiod, which is consistent with the findings of the abovementioned studies, and confirms that light affects testicular development.

Previous studies have shown that spermatozoa are produced by the fine tubule at sexual maturity, and testicles may experience a period of slight enlargement during periods of frequent sexual activity (Dardente, 2022). Testosterone (T) is an important sex hormone in male birds including geese. T can stimulate the development of male reproductive

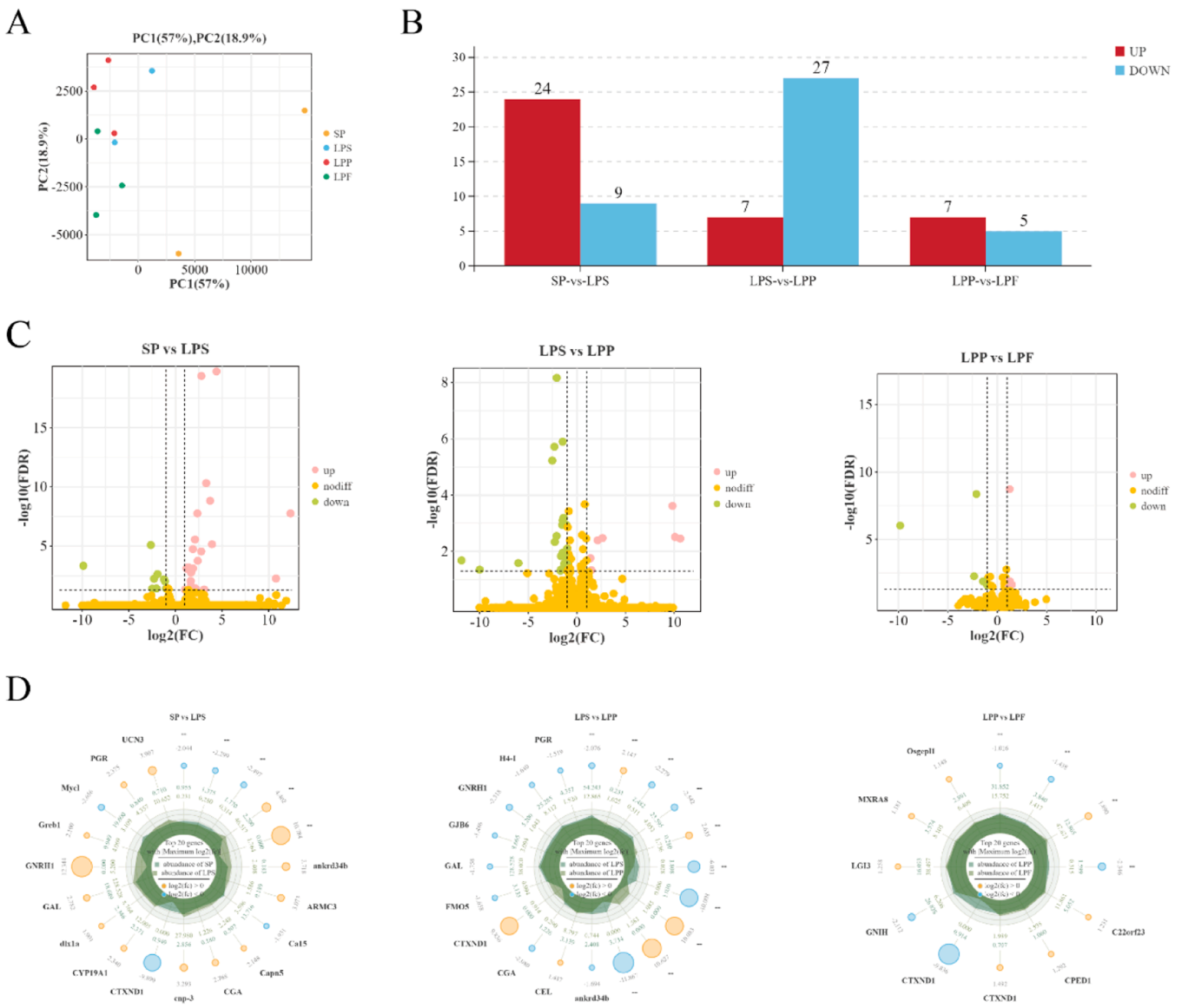

Fig. 3. Basic analysis of differentially expressed genes. A. Plot of principal component analysis of samples; B. Statistical plots of SP vs LPS, LPS vs LPP, and LPP vs LPF DEGs; C. Volcanic maps of SP vs LPS, LPS vs LPP, and LPP vs LPF DEGs; D. Radar map of SP vs LPS, LPS vs LPP, and LPP vs LPF DEGs (top20), $n = 3$.

organs and maintain their sexual function. T can also maintain the secondary sexual characteristics of males and promote sperm production, development, and maturation (Leska, 2007). T levels remain high during the reproductive season, which is extremely important to nourish the development of germ cells in the spermatogenic tubules. In the present study, the T concentration in the blood of Yangzhou geese significantly increased after a prolonged photoperiod, and mature sperm were observed, as indicated by hematoxylin and eosin (HE) staining. Some studies have found that, with the prolongation of the photoperiod, the spermatogenic tubule area and sperm count increase significantly, which is consistent with the effect of the prolongation of the photoperiod on the promotion of gonad development (Pérez, 2022). Therefore, the results of this study showed that the appropriate light conditions can cause Yangzhou geese to enter the breeding period and change the appearance and internal structure of the testis of the geese. The Johnson scores of the SP, LPS, LPP and LPF groups were 2.6, 6.4, 9.2, and 5.2 points, respectively, and these Johnson scores were significantly higher in the groups exposed to a prolonged photoperiod than in the SP group. The Johnson score indicated that the seminiferous tubules in the testis of geese in the SP group only contained supporting cells, with few spermatogonia, and the geese had not begun to breed. After prolonged light exposure, the number and types of reproductive cells in the testis gradually increased. In the LPS group, early sperm cells appeared in the seminiferous tubules. By the peak of egg-laying (the LPP group), the testis has strong spermatogenic function, and all types of reproductive cells (including spermatogonia, spermatocytes, spermatids, and mature sperm cells) were present in a normal state and were abundant. Compared with the LPS and LPP groups, the LPF group showed a decrease in the Johnson score, indicating that the geese in this group gradually stopped their breeding activities. Both this study and previous studies have shown that the photoperiod affects testicular development (Yoshimura, 2013; Zhang, 2019), but little is known about the regulatory mechanism.

The hypothalamus regulates reproductive behavior in animals. However, the classical theory of light-signal regulation of reproduction states that birds, unlike mammals, perceive light through DBPs (Pérez, 2022), resulting in local thyroid hormone activation in the medial basal region of the hypothalamus. Local bioactive TSH control seasonal GnRH and subsequent gonadotropin secretion (Yoshimura, 2013). Therefore, the hypothalamus is the most important target tissue for investigating

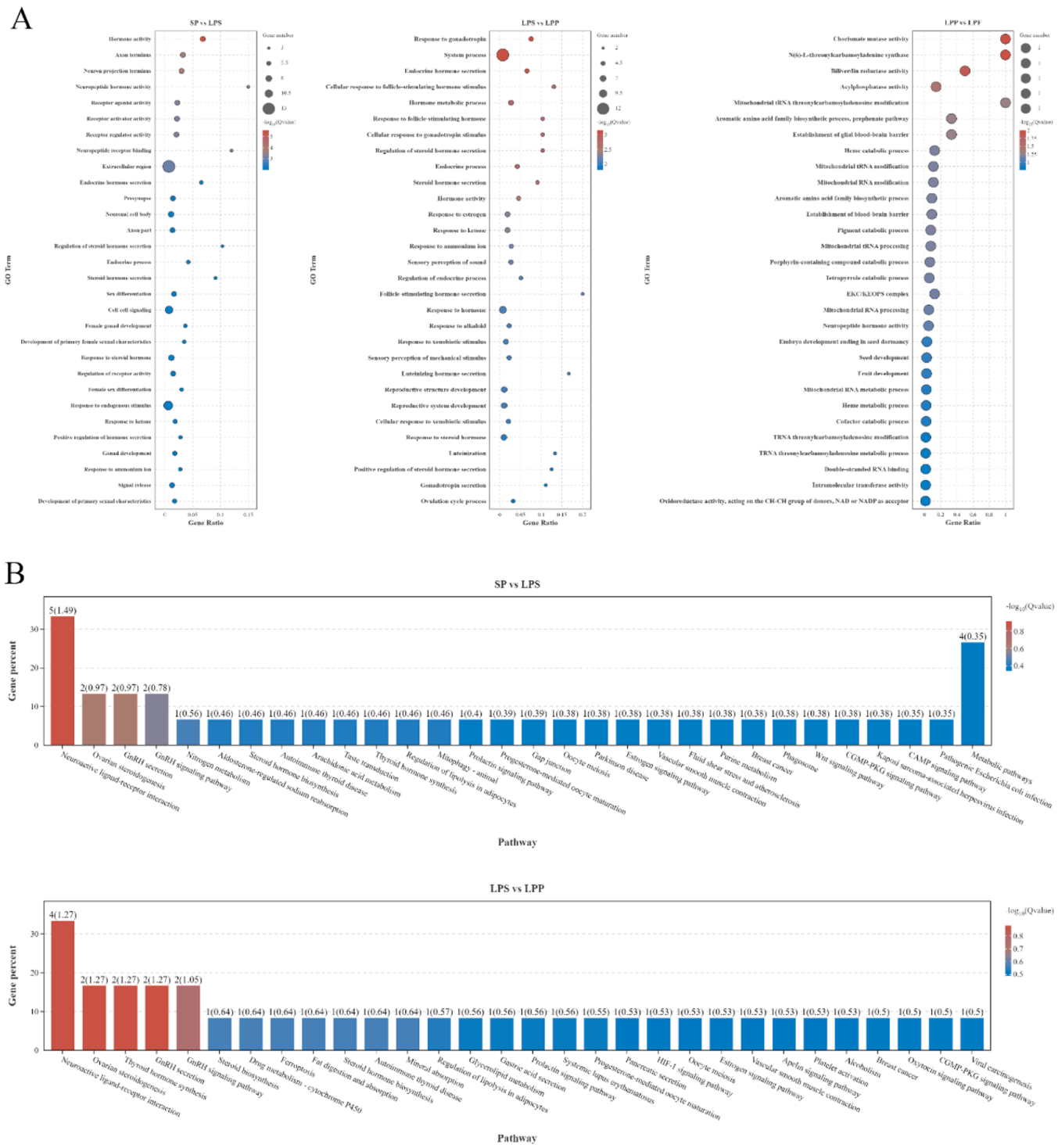

Fig. 4. Functional enrichment analysis of differentially expressed genes. A. GO enrichment analysis of DEGs in SP vs LPS, LPS vs LPP, and LPP vs LPF; B. KEGG enrichment analysis of DEGs in SP vs LPS, LPS vs LPP, and LPP vs LPF, $n = 3$.

the light-mediated regulation of avian reproduction. In this study, we collected hypothalamic tissues from geese under different photoperiods and analyzed transcriptional changes using RNA-seq. Principal component analysis (PCA) showed that the transcriptome of the SP group was significantly different from that of the LPS, LPP, and LPF groups. Twenty-four genes showed upregulated expression and nine genes with downregulated expression in the LPS group compared to the SP group, seven genes with upregulated expression and twenty-seven genes showed downregulated expression in the LPP group compared to the LPS group, and seven genes showed upregulated expression, and five genes showed downregulated expression in the LPF group compared to the LPP group. In this experimental model, the geese were in the non-breeding period under short-light conditions, and in the breeding period under long-light conditions. Therefore, a significant difference was observed between the short-photoperiod and long-photoperiod groups, which confirms the important role of the hypothalamus in light-mediated reproductive regulation.

The DEGs were significantly enriched in neuroactive ligand-receptor

Table 5
Differentially expressed genes in KEGG.

Pathway	Gene
SP vs LPS	
Neuroactive ligand-receptor interaction	*NTS, GAL, FSHB, LHB, TSHB, GnRH, UCN3, CGA*
Ovarian steroidogenesis	*LHB, FSHB, CYP19A1, CGA*
GnRH secretion	*GnRH, LHB, CGA*
GnRH signaling pathway	*GnRH, CGA*
LPS vs LPP	
Neuroactive ligand-receptor interaction	*NTS, GAL, FSHB, LHB, TSHB, GnRH, UCN3, CGA*
Ovarian steroidogenesis	*LHB, FSHB, CYP19A1, CGA*
Thyroid hormone synthesis	*TSHB, CGA, SLC26A4*
GnRH secretion	*GnRH, LHB, CGA*
GnRH signaling pathway	*GnRH, CGA*

interaction, ovarian steroidogenesis, thyroid hormone synthesis, GnRH secretion, and GnRH signaling pathways. Genes in the neuroactive ligand-receptor interaction pathway with differential expression levels between the LPS and LPP groups were *NTS, GAL, FSHB, LHB, TSHB, GnRH, UCN3*, and *CGA*. The neuroactive ligand-receptor interaction pathway can regulate avian laying performance (Zhang at al., 2019). Furthermore, studies in mammals and fish have also shown that this signaling pathway is actively involved in regulating reproduction (Xu et al., 2015). The abovementioned studies have shown that neuroactive ligand-receptor interactions play an important role in regulating avian reproductive activities. The genes in the GnRH secretion pathway that were differentially expressed between the LPS and LPP groups included *GnRH, LHB*, and *CGA*; while those in the thyroid hormone synthesis pathway that were differentially expressed in the LPP group included *TSHB, CGA*, and *SLC26A4*. Bentley (1996) found that GnRH can induce the rhythmic secretion of LH and FSH by binding to gonadotropin-secreting cell-specific receptors in the pituitary system and activating the adenylate cyclase cAMP protein kinase pathway. This induces the secretion of LH and FSH, thereby inducing avian entry into the reproductive phase. In young mid-pubertal males, the level of *GnRH* mRNA expression in the hypothalamus is elevated after a prolonged photoperiod, along with a significant increase in blood LH levels (Kiss and Peczely., 1987). In addition, Dunn et al. (Dunn et al., 2003) found that estrogen mediates the elevation of gonadotropin (FSH and LH) levels in the blood of hens after long-light treatment. The RNA-seq results of the present study showed that *GnRH, FSHB*, and *LHB* mRNA expression levels were significantly upregulated in the LPS group.

We identified the top 20 DEGs between the SP and LPS, LPS and LPP, LPP and LPF groups. Genes with concurrent changes in relevant pathways, including *CGA, UCN3, CYP19A1*, and *GAL* mRNA, and *GnRH, FSHB, LHB*, and *TSHB* mRNA based on previous reports, were further analyzed to understand the important candidate genes associated with poultry reproductive traits. Chromogranin (CGA) is a 49 kDa protein localized to the secretory granules of neuroendocrine cells (O'Connor et al., 1984). Evidence suggests that CGA functions as a prohormone for biologically active peptides. CGA and its fragment are postulated to function as general modulators of hormonal release (Cohn et al., 1990). CGA promotes the storage and secretion of FSH and LH in rodents (Crawford et al., 2002). In the present study, serum FSH and LH concentrations were significantly increased after prolonged illumination, suggesting that CGA might be related to these effects. UCNs belong to the corticotropin-releasing hormone (CRH) family and include UCN1, UCN2, and UCN3 (Hauger et al., 2003). This family is a key regulator of the HPA axis, which promotes the release of adrenocorticotropic hormones and corticosteroids. UCNs are found in the central nervous system (CNS) and peripheral tissues, including those of the digestive, cardiovascular, immune, reproductive, and endocrine systems (Squillacioti et al., 2011, Squillacioti et al., 2014). Squuollacioti has been suggested (Squillacioti C, 2016) that UCNs may play roles in regulating mitotic and apoptotic events that occur during spermatogenesis, and steroidogenesis through autocrine/paracrine mechanisms. Previous studies of UCNs have focused on domestic animals (Deussing et al., 2010; Shemesh et al., 2016), and studies on UCNs in poultry are limited. Correlation analysis showed that *UCN3* mRNA levels were positively correlated with testis/body weight and the Johnson score, but the specific mechanism needs to be further studied. Previous studies have suggested that

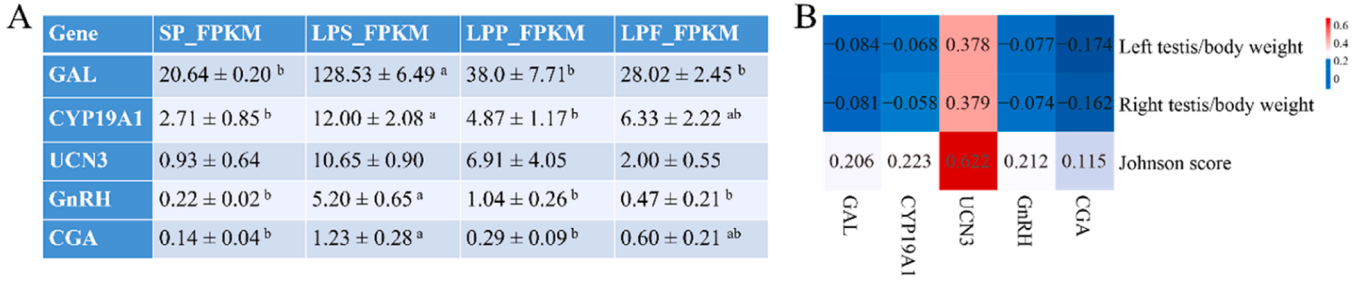

Fig. 5. Expression levels of differentially expressed genes in KEGG and correlation analysis. A: expression of differentially expressed genes in KEGG. B: Correlation analysis of DEGs with testicular relative weight and Johnson score. SP, LPS: $n = 2$, LPP, LPF: $n = 3$. Data were analyzed as Mean ± SE, different lowercase letters indicated significant difference ($p < 0.05$), and no letters indicated no significant difference ($p > 0.05$).

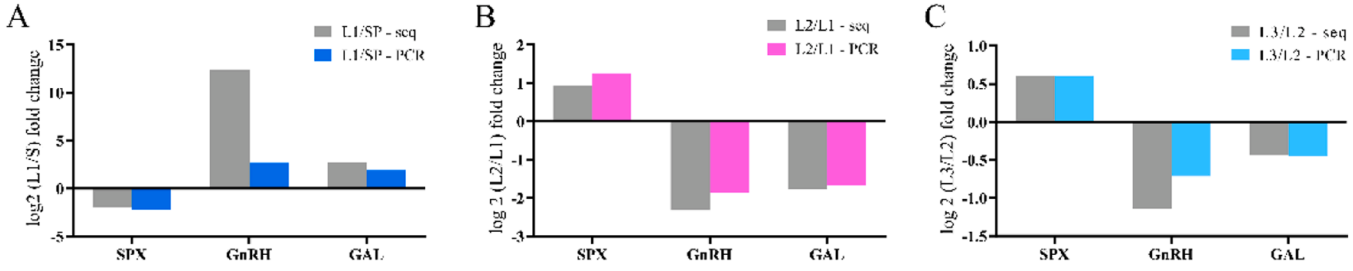

Fig. 6. qRT-PCR verification of differentially expressed genes. $2^{-\Delta\Delta Ct}$ was used to calculate the average of relative gene expression levels obtained by real-time PCR method ($n = 3$), and then the log2 (A: gene expression level LPS/ gene expression level SP; B: gene expression level LPP/ gene expression level LPS; C: gene expression level LPF/ gene expression level LPP) in real-time PCR and RNA-seq results was calculated separately.

CYP19A1 may regulate seasonal reproduction through the cAMP, ovarian steroidogenic, calcium, GnRH, and steroid biosynthesis signaling pathways (Zhu et al., 2023). Glycopeptide, a 29 amino acid peptide, is a cellular messenger (neurotransmitter/neuromodulator) within the central and peripheral nervous systems that regulates a wide range of physiological functions, including injury perception; arousal/sleep regulation; cognition; and many facets of neuroendocrine activity related to feeding, energy metabolism, thermoregulation, osmotic and water homeostasis, and reproduction. Glycopeptides are involved in LHRH production and subsequently in estrogen-triggered LH surges during the pre-estrus period in rats through a positive feedback mechanism (Pau et al., 1997). Glycopeptides reduce the inhibitory tone of this neurotransmitter by inhibiting dopamine release and stimulating prolactin secretion from the pituitary gland (Merchenthaler et al., 2010).

Conclusions

Reproductive performance is one of the most important economic traits in poultry farming, and seasonal reproduction is the main bottleneck limiting the high fecundity of geese. Therefore, identifying the molecules that affect avian reproduction is crucial. The findings of this study indicated that the photoperiod affects avian reproductive performance through neuroactive ligand-receptor interaction, ovarian steroidogenesis, thyroid hormone synthesis, GnRH secretion, and GnRH signaling pathways, and other pathways that regulate avian reproductive performance. Additionally, genes such as *GnRH, CGA, UCN3, CYP19A1*, and *GAL* play key roles. Our results provide valuable information for identifying genes associated with avian reproductive traits.

Declaration

Ethics approval and consent to participate

The Animal Management and Ethics Committee of Nanjing Agricultural University (IACUC) approved all animal procedures. Sampling and slaughter procedures were in accordance with “Guidelines on Ethical Treatment of Experimental Animals” (2006) No. 398 set by the Ministry of Science and Technology, China and “Regulation Regarding the Management and Treatment of Experimental Animals” (2008) No. 45 formulated by the Jiangsu Provincial People’s Government.

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Authors’ contributions

YF performed experiments, analyzed the data and drafted the manuscript. JL and NY conceived the idea, designed the experiment, and finalized the manuscript. All authors read and approved the final manuscript.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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References

Bailey, M.J., Cassone, V.M., 2005. Melanopsin expression in the chick retina and pineal gland. Brain Res. Mol. Brain Res. 134, 345–348.
Bentley, G.E., 1996. Thyroxine and photorefractoriness in the European starling. Diss Univ. Bristol 1.
Chaurasia, S.S., Rollag, M.D., Jiang, G., Hayes, W.P., Haque, R., Natesan, A., Zatz, M., Tosini, G., Liu, C., Korf, H.W., Iuvone, P.M., Provencio, I., 2005. Molecular cloning, localization and circadian expression of chicken melanopsin (Opn4): differential regulation of expression in pineal and retinal cell types. J. Neurochem. Jan, 92, 158–170.
Cohn, D.V., Fasciotto, B.H., Gorr, S.U., Parkins, F., Shioi, J., Levine, M.A., Greeley Jr., G. H., 1990. The putative role of secretoryprotein-l/chromogranin A as a precursor for regulatory hormones. Prog. Clin. Biol. Res. 332, 51–66.
Crawford, J.L., McNeilly, A.S., 2002. Co-localisation of gonadotrophins and granins in gonadotrophs at different stages of the oestrous cycle in sheep. J Endocrinol Aug 174, 179–194.
Dardente, H., Simonneaux, V., 2022. GnRH and the photoperiodic control of seasonal reproduction: delegating the task to kisspeptin and RFRP-3. J. Neuroendocrinol. 34, e13124.
Davies, W.I., Turton, M., Peirson, S.N., Follett, B.K., Halford, S., Garcia-Fernandez, J.M., Sharp, P.J., Hankins, M.W., Foster, R.G., 2012. Vertebrate ancient opsin photopigment spectra and the avian photoperiodic response. Biol. Lett. 8, 291–294, 23.
Deussing, J.M., Breu, J., Kühne, C., Kallnik, M., Bunck, M., Glasl, L., Yen, Y.C., Schmidt, M.V., Zurmühlen, R., Vogl, A.M., Gailus-Durner, V., Fuchs, H., Hölter, S.M., Wotjak, C.T., Landgraf, R., de Angelis, M.H., Holsboer, F., Wurst, W., 2010. Urocortin 3 modulates social discrimination abilities via corticotropin-releasing hormone receptor type 2. J. Neurosci. 30, 9103–9116, 7.
Dunn, I.C., Lewis, P.D., Wilson, P.W., Sharp, P.J., 2003. Acceleration of maturation of FSH and LH responses to photostimulation in prepubertal domestic hens by oestrogen. Reproduction. 126, 217–225.
Halford, S., Pires, S.S., Turton, M., Zheng, L., González-Menéndez, I., Davies, W.L., Peirson, S.N., García-Fernández, J.M., Hankins, M.W., Foster, R.G., 2009. VA opsin-based photoreceptors in the hypothalamus of birds. Curr Biol. 19, 1396–1402, 25.
Hauger, R.L., Grigoriadis, D.E., Dallman, M.F., Plotsky, P.M., Vale, W.W., Dautzenberg, F.M., 2003. International Union of Pharmacology. XXXVI. Current status of the nomenclature for receptors for corticotropin-releasing factor and their ligands. Pharmacol. Rev. 55, 21–26.
Ikegami, K., Yoshimura, T., 2013. Seasonal time measurement during reproduction. J. Reprod. Dev. 59, 327–333.
Kuenzel, W.J., 2014. Research advances made in the avian brain and their relevance to poultry scientists. Poult. Sci. 93, 2945–2952.
Kiss, J.Z., Péczely, P., 1987. Distribution of tyrosine-hydroxylase (TH)-immunoreactive neurons in the diencephalon of the pigeon (Columba livia domestica). J. Comp. Neurol. 257, 333–346. Mar, 15.
Leska, A., Dusza, L., 2007. Seasonal changes in the hypothalamo-pituitary-gonadal axis in birds. Reprod. Biol. 7, 99–126.
Merchenthaler, I., 2010. Galanin and the neuroendocrine axes. Exp. Suppl. 102, 71–85.
Nakane, Y., Ikegami, K., Ono, H., Yamamoto, N., Yoshida, S., Hirunagi, K., Ebihara, S., Kubo, Y., Yoshimura, T., 2010. A mammalian neural tissue opsin (Opsin 5) is a deep brain photoreceptor in birds. Proc. Natl. Acad. Sci. U S. A 107, 15264–15268. Aug, 24.
Nakane, Y., Yoshimura, T., 2010. Deep brain photoreceptors and a seasonal signal transduction cascade in birds. Cell Tissue Res. 342, 341–344.
O’Connor, D.T., Frigon, R.P., Sokoloff, R.L., 1984. Human chromogranin A. Purification and characterization from catecholamine storage vesicles of human pheochromocytoma. Hypertension 6, 2–12. Jan-Feb.
Onagbesan, O.M., Metayer, S., Tona, K., Williams, J., Decuypere, E., Bruggeman, V., 2006. Effects of genotype and feed allowance on plasma luteinizing hormones, follicle-stimulating hormones, progesterone, estradiol levels, follicle differentiation, and egg production rates of broiler breeder hens. Poult Sci. 85, 1245–1258.
Pau, K.Y., Spies, H.G., 1997. Neuroendocrine signals in the regulation of gonadotropin-releasing hormone secretion. Chin. J. Physiol. 40, 181–196, 1997 Dec 31.
Pérez, J.H., 2022. Light receptors in the avian brain and seasonal reproduction. J. Exp. Zool. a Ecol. Integr. Physiol. 337, 985–993. Dec.
Sharp, P.J., 2005. Photoperiodic regulation of seasonal breeding in birds. Ann N Y Acad Sci. 1040, 189–199.
Shemesh, Y., Forkosh, O., Mahn, M., Anpilov, S., Sztainberg, Y., Manashirov, S., Shlapobersky, T., Elliott, E., Tabouy, L., Ezra, G., Adler, E.S., Ben-Efraim, Y.J., Gil, S., Kuperman, Y., Haramati, S., Dine, J., Eder, M., Deussing, J.M., Schneidman, E., Yizhar, O., Chen, A., 2016. Ucn3 and CRF-R2 in the medial amygdala regulate complex social dynamics. Nat. Neurosci. 19, 1489–1496. Nov.
Squillacioti, C., De Luca, A., Alì, S., Ciarcia, R., Germano, G., Vittoria, A., Mirabella, N., 2014. Presence and distribution of urocortin and corticotrophin-releasing hormone receptors in the bovine thyroid gland. Anat Histol Embryol. 43, 429–434.
Squillacioti, C., De Luca, A., Liguori, G., Alì, S., Germano, G., Vassalotti, G., Navas, L., Mirabella, N., 2016. Urocortinergic system in the testes of normal and cryptorchid dogs. Ann. Anat. 207, 91–96. Sep.
Squillacioti, C., De Luca, A., Liguori, G., Paino, S., Mirabella, N., 2011. Expression of urocortin and corticotropin-releasing hormone receptors in the bovine adrenal gland. Gen. Comp. Endocrinol. 172, 416–422. Jul, 1.
Xu, S., Wang, D., Zhou, D., Lin, Y., Che, L., Fang, Z., Wu, D., 2015. Reproductive hormone and transcriptomic responses of pituitary tissue in anestrus gilts induced by nutrient restriction. PLoS. One 10, e0143219. Nov, 18.

Yoshimura, T., 2013. Thyroid hormone and seasonal regulation of reproduction. Front. Neuroendocrinol. 34, 157–166. Aug.
Zhang, T., Chen, L., Han, K., Zhang, X., Zhang, G., Dai, G., Wang, J., Xie, K., 2019. Transcriptome analysis of ovary in relatively greater and lesser egg producing Jinghai Yellow Chicken. Anim. Reprod. Sci. 208, 106114. Sep.
Zhu, M., Yang, Y., Yang, H., Zhao, Z., Zhang, H., Blair, H.T., Zheng, W., Wang, M., Fang, C., Yu, Q., Zhou, H., Qi, H., 2023. Whole-genome resequencing of the native sheep provides insights into the microevolution and identifies genes associated with reproduction traits. BMC. Genomics. 24, 392. Jul, 11.