

Seasonal differences in the transcriptome profile of the Zhedong white goose (*Anser cygnoides*) pituitary gland

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ABSTRACT In animals, the adaptation to breed at the time of greatest survival of the young is known as seasonal reproduction. This is mainly controlled by the photoperiod, which stimulates the hypothalamic-pituitary-gonadal axis and starts the breeding season. Herein, we have determined the seasonal changes in gene expression patterns of Zhedong white geese pituitary glands under a natural photoperiodism, conducted at autumn equinox (**AE**), winter solstice (**WS**), spring equinox (**SE**), and summer solstice (**SS**). Pair-wise comparisons of WS vs. AE, SE vs. WS, SS vs. SE, and AE vs. SS resulted in 1,139, 33, 704, and 3,503 differently expressed genes, respectively. When compared with SS, AE showed downregulation of genes, such as vasoactive intestinal peptide receptor, prolactin receptor, and thyroid hormone receptor beta,

whereas gonadotropin-releasing hormone II receptor was upregulated, indicating that these genes may be responsible for the transition from cessation to egg laying. In addition, the expression levels of 5 transcription factors (*POU1F1*, *Pitx2*, *NR5A1*, *NR4A2*, and *SREBF2*) and 6 circadian clock-associated genes (*Clock*, *Per2*, *ARNTL2*, *Eya3*, *Dio2*, and *NPAS2*) also changed seasonally. Gene Ontology term and Kyoto Encyclopedia of Genes and Genomes enrichment analysis revealed that “response to oxidative stress” and steroid biosynthesis pathway also participate in regulating the reproduction seasonality of geese. Overall, these results contribute to the identification of genes involved in seasonal reproduction, enabling a better understanding of the molecular mechanism underlying seasonal reproduction of geese.

Key words: Zhedong white goose, seasonal reproduction, pituitary gland, RNA-Seq analysis

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INTRODUCTION

Seasonal reproduction is an evolutionary adaptive strategy for animals living in areas with evident seasonal changes in environment, which in particular is important to ensure the survival of the offspring (Ikegami and Yoshimura, 2016; Nishiwaki-Ohkawa and Yoshimura, 2016). Although several factors change seasonally, for example, temperature, precipitation or humidity, the day length (photoperiod) is thought to be responsible for the detection of season variation by many organisms, such as plants (Konstantinova, 1966; Song et al., 2015),

insects (Tauber and Kyriacou, 2001; Saunders, 2013), and vertebrates (Hazlerigg and Wagner, 2006; Hazlerigg, 2012). Thus, this phenomenon is called photoperiodism. Based on the relative day length of their reproductive season, seasonal reproduction animals can be classified into long-day (**LD**) breeders, for example, hamsters and quail, and short-day (**SD**) breeders, for example, sheep and salmon (Nishiwaki-Ohkawa and Yoshimura, 2016). Long-day breeders develop gonads and display reproductive behaviors during spring when the days become longer and vice versa in SD breeders.

In vertebrates, reproductive process is primarily controlled by the hypothalamic-pituitary-gonadal (**HPG**) axis. Gonadotropin-releasing hormone (**GnRH**) produced by the hypothalamus stimulates the biosynthesis and release of luteinizing hormone (**LH**) and follicle-stimulating hormone (**FSH**) by the anterior pituitary gland, thus regulating gonadal activation. Prolactin (**PRL**) and its releasing hormone, vasoactive

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intestinal peptide (**VIP**) secreted by the hypothalamus, are also involved in the regulation of annual reproductive activities (Sharp and Blache, 2003; Sharp, 2005). For seasonal breeding animals, the HPG axis is activated at an appropriate time of the year, resulting in a dramatic change in gonadal size. This is particularly evident in birds (Dawson et al., 2001), in which gonadal weight increases more than a hundred-fold during the breeding season (Nicholls et al., 1983). After breeding, the HPG axis is automatically switched off, and the gonads start to regress, although photoperiod still increases. This phenomenon is known as photorefractoriness (Nicholls et al., 1988).

The underlying molecular mechanisms of avian seasonal reproduction have been studied extensively. In contrast to mammals, eyes and pineal gland of birds are not necessary for the regulation of seasonal reproduction because blind ducks and pinealectomized quail still show photostimulation-induced gonadal development (Siopes and Wilson, 1974). Subsequent reports have demonstrated that photoperiodic information is perceived by deep brain photoreceptors, such as OPN5-positive cerebrospinal fluid-contacting neurons, within the mediobasal hypothalamus (Sharp and Follett, 1969; Vigh and Vigh-Teichmann, 1998; Nakane et al., 2010; Yamashita et al., 2010). This information is transmitted to the par tuberalis in the pituitary gland, where the thyroid-stimulating hormone (**TSH**) is secreted. Long-day-induced TSH acts on the TSH receptor (**TSHR**) localized in ependymal cells, and upregulating the expression of type 2 deiodinase (**DIO2**) and downregulating type 3 deiodinase (**DIO3**) (Yoshimura et al., 2003; Nakao et al., 2008). *DIO2* encodes the thyroid hormone (**TH**)-activating enzyme that converts the prohormone thyroxine (**T₄**) into bioactive triiodothyronine (**T₃**), whereas *DIO3* turns both **T₃** and **T₄** into inactive metabolites (Yasuo et al., 2005). **T₃** has been reported to regulate morphological changes in GnRH nerve terminals and the glia, facilitating synthesis and release of GnRH and leading to gonadotropin secretion and gonadal development (Nakane and Yoshimura, 2014). In contrast to long days, the pattern of *DIO2* and *DIO3* expression during short days depletes **T₃**, and glial cells block the GnRH neuron connection with the basal lamina.

The circadian rhythm is a daily time-keeping mechanism fundamental to a wide range of species. It consists of the transcription-translation feedback of a highly conserved set of genes, collectively known as “clock genes.” It is now commonly accepted that the central core of this gene network consists of “positive” and “negative” elements. The “positive elements” are circadian locomotor output cycles kaput (*clock*) and the gene that encodes for brain-muscle Arnt-like protein 1 (*Arntl* or *Bmal1*). The “negative elements” are genes *period 1 to 3* (*Per 1-3*, but *Per1* has not yet been discovered in birds) and cryptochromes *cryptochrome 1-2* (*Cry1-2*). The products of these genes may dynamically interact to trigger rhythmic patterns of transcription, translation, biochemical and

physiological processes and ultimately organism behavior (Bell-Pedersen et al., 2005; Eckel-Mahan and Sassone-Corsi, 2013). The heterodimeric protein products of *Clock* and *Bmal1* bind to E-boxes in the promoter regions of *Pers* and *Crys* to facilitate their transcription. Following translation, proteins **PER** and **CRY** form heterocomplexes that enter the nucleus and inactivate **CLOCK** and **BMAL1** heterodimers, leading to negative feedback, inhibiting their own transcription and eventually shutting-off the loop (Reppert, 2000). Circadian rhythms exist in a wide range of physiological systems that include the reproductive axis. The circadian clock is closely related to the hypothalamus-pituitary regulation of multiple endocrine axes. It is well established that the circadian clock is involved in the photoperiodic response of various vertebrates, including fish (Underwood and Hyde, 1990), birds (Hamner, 1963; Meddle and Follett, 1997), and mammals (Elliott et al., 1972).

The Zhedong white goose (*Anser cygnoides*), a domestic goose breed in the east of Zhejiang province, is characterized by strict seasonality, high tendency of broodiness, and low egg-laying rate (Zhao et al., 2013). Its regular reproductive season spans from September to May of the following year, that is, from shortening days of early autumn to the longer days of early summer. This behavior results in poor laying performance. The *A. cygnoides* genome was finally sequenced in 2015, allowing us to perform a system-level analysis of photoperiodic time measurement in goose (Lu et al., 2015). In the present study, we have used high-throughput sequencing technology and bioinformatics to explore the endocrine mechanisms that regulate seasonal breeding in *A. cygnoides* and to identify genes that are differentially expressed in the pituitary gland at different seasons. Our results provide a better understanding of the molecular regulation that take place in seasonal reproduction.

MATERIALS AND METHODS

Ethics Statement

All animal experimentation was approved by the Institutional Animal Care and Use Committee (**IACUC**) of Zhejiang University. The methods and protocols were performed in accordance with the Measures for the Administration of Affairs Concerning Experimental Animal of Zhejiang Province, China (approved by the Zhejiang Provincial Government in 2009 and promulgated by Decree No. 263).

Goose Rearing and Sample Collection

Female Zhedong white geese (*A. cygnoides*) were reared in Xiangshan County, Zhejiang Province, China. They were raised according to common farm practice under the same environmental conditions with natural lighting and temperature. During the experiment, geese were fed ad libitum with rice grain,

supplemented with green grass or water plants whenever possible. The feed was given during daytime and geese had free access to drinking water. Twelve healthy Zhedong white geese hatched on the same day in January 2016 were selected for the experiments. They were tested at the autumn equinox (**AE**) and winter solstice (**WS**) in 2016 and at the spring equinox (**SE**) and summer solstice (**SS**) in 2017, representing different natural photoperiods. Each group consisted of 3 individuals, that is, biological replicates. The geese were anesthetized with sodium pentobarbital and euthanized by exsanguination. The pituitary gland, an oval body located in the sella turcica (hypophysial fossa), was carefully removed from each goose using fine pointed forceps. They were immediately wrapped in freezing tube, snap frozen in liquid nitrogen, and stored at -80°C until RNA extraction.

Total RNA Extraction, Library Preparation, and Illumina Sequencing

Total RNA was extracted from each pituitary gland using TRIzol Reagent (Invitrogen, Carlsbad, CA) followed by treatment with RNase-free DNase I (Thermo Scientific, Waltham, MA) to remove DNA contamination, according to the manufacturer's instructions. Purity, concentration, and integrity of total RNA were measured using a NanoPhotometer spectrophotometer (IMPLEN, Westlake Village, CA), Qubit RNA Assay Kit in Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA), and the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA), respectively. A total amount of 3 μg RNA per sample was used as input material for sequencing library construction. Sequencing libraries were prepared using the NEBNext Ultra RNA Library Prep Kit for Illumina (NEB, Ipswich, MA) following the instruction manual, and index codes were added to attribute sequences to each sample. Briefly, mRNAs were enriched from total RNA using poly-T oligo-attached magnetic beads, which were split into short fragments using divalent cations in NEB-Next First Strand Synthesis Reaction Buffer ($5\times$), then reversely transcribed into the first-strand cDNA with random hexamer primer and M-MuLV Reverse Transcriptase (RNase H-). The second-strand cDNA was synthesized using DNA Polymerase I and RNase H. Subsequently, the DNA fragments had their end repaired, with poly(A) added at the 3' ends. Ligation of NEBNext adaptors for hybridization was followed by purification with AMPure XP system (Beckman Coulter, Beverly, MA) to select cDNA fragments of 150–200 bp. Then PCR was carried out with Phusion High-Fidelity DNA polymerase (NEB, Ipswich, MA), universal PCR primers, and Index (X) primer. After purification of the PCR products, the quality of the cDNA libraries was assessed on an Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA), sequenced on the Illumina HiSeq 4000 platform where 150 bp paired-end reads were generated.

Quantity Control, Reads Mapping, and Gene Annotation

As a part of the quality-control procedure, the clean data were obtained by removing reads containing adapters, reads containing poly-N ($\text{N}\% > 10\%$), and low-quality reads from raw data. Probabilities Q20 and Q30 (probability of a miscalled base of 1 and 0.1%, respectively) and GC content of the clean data were calculated. The genome assembly and annotation of *A. cygnoides* were downloaded from GenBank database (accession code AOGC00000000). The filtered, high-quality sequencing reads subsequently were aligned to the assembled reference genome using TopHat (version 2.0.12) (Trapnell et al., 2009).

Gene Quantification and Differential Expression Analysis

HTSeq (version 0.6.1) was used to count the reads numbers mapped to each gene (Anders et al., 2015). Gene expression levels were estimated via FPKM (Fragments Per Kilobase of transcripts per Million fragments mapped) calculated based on the length of the gene and reads count mapped to a gene. Differential expression gene (DEG) analysis of the 4 groups was performed on the DESeq R package (version 1.18.0). To control false discovery rate, *P*-values were adjusted using the Benjamini and Hochberg's approach, and only genes with an adjust *P*-value < 0.05 were defined as DEG.

GO and KEGG Enrichment Analysis

Gene Ontology (GO) enrichment analysis of all DEG was implemented by the GOseq R package, in which gene length bias was corrected. GO terms with *P*-value less than 0.01 were considered significantly enriched by DEG.

Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used to find pathways in which the DEG were involved. Pathways with a *P*-value less than 0.05 were defined as significantly enriched pathways. KOBAS software was used to test the statistical enrichment of DEG in KEGG pathways.

Validation of RNA-seq–Based Quantitative RT-PCR

To confirm the DEGs detected by the RNA-seq approach, 15 DEG were selected randomly for qRT-PCR analysis. The qRT-PCR primers were designed using Primer Premier 5.0 software (www.PremierBiosoft.com) and synthesized by Generay biotechnology (Shanghai, China). The primer information is listed in Table 1. Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control. Each 20 μL reaction volume contained 10 μL $2\times$ ChamQ™ Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China), 0.5 μL 10 μM (each) forward and reverse primers, and 1.5 μL cDNA products. The final volume was adjusted using

Table 1. Primers used in the real-time quantitative PCR assay of genes.

Gene name	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	PCR product (bp)
<i>GAPDH</i>	CTGTCAAGGCTGAGAATG	CAAGAGGCATTGCTGACA	280
<i>POU1F1</i>	GAAGCGCAGAACCACCATAA	CCCCTCAGCCATCCTCATAA	106
<i>VIPR</i>	GGTGCTGGGAGGAAATAAT	GTCCAACGTCTGGGGAATG	141
<i>TRHR</i>	CTATTTCATTCTGTGGAT	TAACTGGCAGGTTTTTTTGTG	224
<i>PPARα</i>	ATGCCAAGGTCTGAGAAAGGC	GCCTGCAAGGATGACTCTG	168
<i>PGR</i>	TGAGGAAGTGCTGTCAAGCC	CTTCTGGCTCAATGCCTCGT	219
<i>Srebf2</i>	CCAGCAGCCCGTCATATAACC	CCAGAACCCTGCTGCTGGATG	119
<i>IRF2BP2</i>	GCATTGTCCGCCACAAAAT	CCTCGTCGGAAGGGATGAAG	157
<i>CLOCK</i>	TGGCTCCACCCATCATA	CTCGAACCTCCGCATAA	273
<i>PER2</i>	CAACCTGAATTAGAGGAAACCACA	AAGCAGGACACTGGACATGG	198
<i>CGA</i>	TTCTCATGCACGGTTGTCCA	CATTGGAGTGGGATAGGCC	119
<i>RELN</i>	CTCATCTGGCTCCTCACT	CCTTCTTCTCTGCTGTCTG	153
<i>PRL</i>	TGACCTCCTTGCTATCTG	TGTAATGAAACCCCGACC	164
<i>GNRHR</i>	CGCACAACTTACCCAG	AGCGTAGCAGCAGACCAT	128
<i>SDC1</i>	TTGAACCTAAAGCCCTGGC	CATTCTCCAGAGTCAGCC	243
<i>FSH</i>	CCAGCCTGTCCATTCTACT	ACCTGGGTAAACAAACGTCCTA	293

RNA-free water. The qRT-PCR was carried out on LightCycler 960 (Roche) as the following procedure: 1 cycle at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 s and annealing at 60°C for 60 s. All reactions were run in triplicate. Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method, and the WS group served as calibrator.

RESULTS

Overview of RNA-seq Analysis

To identify genes involved in the seasonal reproduction of goose *A. cygnoides*, we performed transcriptomic sequencing of the pituitary glands of 12 female geese at the 4 seasonal periods described (see [Materials and Methods](#)). A total of 704,035,936 raw reads with Q20 > 95% and Q30 > 89% were produced by Illumina HiSeq 4000 from 12 libraries, yielding 678,434,708 clean reads after quality control (see [Materials and Methods](#)). The clean reads were aligned to the reference genome of *A. cygnoides*, and 65.79 to 73.44% of the reads from each library were uniquely mapped, whereas 0.77 to 1.25% were multiple mapped, which was sufficient for subsequent analysis (see details in [Table 2](#)). All the sequence

data were submitted to the National Center for Biotechnology Information (<https://www.ncbi.nlm.nih.gov/sra>, NCBI) database (accession number SRP254115).

Identification of DEGs

For the 4 groups, the square correlation coefficient (R^2 values) between 3 individuals within each group were all above 0.928 ([Figure 1](#)), indicating that the three biological replicates were highly repetitive, and the experimental results were reliable and reasonable. Lowest values were found in AE and SS groups, which therefore showed the most variability in expression patterns.

The read count for each sample was standardized by DESeq and used for differential analysis. We identified 1,139, 33, 704, and 3,503 DEGs in the pairwise comparisons WS vs. AE, SE vs. WS, SS vs. SE, and AE vs. SS, respectively ([Supplementary Table 1](#)) distributed between upregulated and downregulated ([Figure 2A](#)). Only one DEG (ACY_000982, [*RELN*]) was present in all 4 comparisons ([Figure 2B](#)). Differences in gene expression level were visualized via a hierarchical clustering, which clearly displayed the variability in the overall patterns of DEG among the 4 seasons. A large

Table 2. Data quality and mapping data statistics of RNA-seq libraries.

Sample name	Raw reads	Clean reads	Q20 (%)	Q30 (%)	Total mapped	Multiple mapped	Uniquely mapped
AE1	51,733,302	50,103,882	96.07	91.02	33,466,593 (66.79%)	501,793 (1.00%)	32,964,800 (65.79%)
AE2	51,334,318	49,516,870	96.09	91.04	33,221,268 (67.09%)	468,132 (0.95%)	32,753,136 (66.15%)
AE3	55,244,430	53,636,366	96.21	91.26	37,383,365 (69.70%)	499,916 (0.93%)	36,883,449 (68.77%)
WS1	61,937,804	59,542,626	95.32	89.21	42,232,194 (70.93%)	745,463 (1.25%)	41,486,731 (69.68%)
WS2	65,701,866	63,219,712	95.78	90.19	46,179,485 (73.05%)	525,540 (0.83%)	45,653,945 (72.21%)
WS3	60,739,984	58,409,252	95.47	89.59	40,047,979 (68.56%)	491,402 (0.84%)	39,556,577 (67.72%)
SE1	59,318,364	57,141,918	96.58	91.86	42,517,522 (74.41%)	550,699 (0.96%)	41,966,823 (73.44%)
SE2	59,493,192	57,260,078	96.51	91.72	42,343,912 (73.95%)	599,498 (1.05%)	41,744,414 (72.90%)
SE3	60,894,962	58,719,002	96.66	92.28	42,800,229 (72.89%)	637,200 (1.09%)	42,163,029 (71.80%)
SS1	58,078,342	55,932,622	96.59	92.0	40,658,843 (72.69%)	461,450 (0.83%)	40,197,393 (71.87%)
SS2	59,175,570	57,173,964	96.72	92.26	40,898,203 (71.53%)	463,165 (0.81%)	40,435,038 (70.72%)
SS3	60,383,802	57,778,416	96.8	92.46	41,693,939 (72.16%)	445,167 (0.77%)	41,248,772 (71.39%)

AE, WS, SE, and SS represent the autumn equinox, winter solstice, spring equinox, and summer solstice, respectively; 1/2/3 represent 3 biological replicates of each group; Raw reads: all the original data produced by sequencing; Clean reads: the remaining reads after filtering of low quality reads from raw reads; Q20: the proportion of read base whose error rate is less than 1%; Q30: the proportion of read base whose error rate is less than 0.1%; Total mapped: the total reads amount mapped to the reference genome; Multiple mapped: the reads amount mapped to the reference genome more than one site; Uniquely mapped: the reads amount mapped to the reference genome only one site.

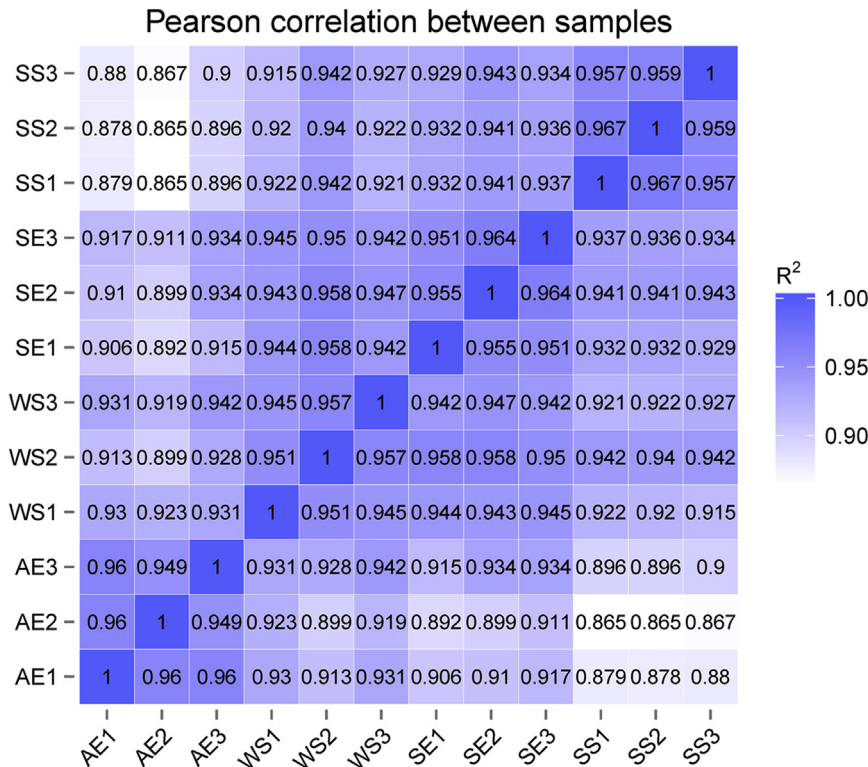


Figure 1. The Pearson correlation between individuals. Correlation coefficient with R^2 value. Abbreviations: AE, autumn equinox; SE, spring equinox; SS, summer solstice; WS, winter solstice. 1, 2, and 3 represent 3 biological replicates of each group.

number of genes show stage-specific expression with SS, WS, and SE, clustering away from AE (Figure 2C).

Hormone-Related Genes

Some DEG were related to three hormone-releasing and hormone receptors, as expected from the known role of the pituitary gland in regulating hormones and seasonal reproduction cycle. For these DEG, 3 different patterns were observed: (1) in AE vs. SS, vasoactive intestinal polypeptide receptor (*VIPR*) and thyrotropin-releasing hormone-degrading ectoenzyme were downregulated, whereas gonadotropin-releasing hormone II receptor was upregulated significantly; (2) in the comparisons WS vs. AE and AE vs. SS, prolactin receptor (*PRLR*), glucocorticoid receptor (*GR*), and thyroid hormone receptor beta were differentially expressed whereas AE had the lowest expression levels; finally (3) estrogen receptor 1 (*ER1*) was differentially expressed when comparing WS vs. AE, SS vs. SE, and AE vs. SS.

Pituitary Transcription Factors

Several pituitary transcription factors had lower expression levels in the pituitary glands of SS geese. These included pituitary homeobox 2 (*Pitx2*), Steroidogenic factor 1 (*SF-1*, also named nuclear receptor subfamily 5 group A member 1, *NR5A1*), nuclear receptor subfamily 4 group A member 2 (*NR4A2*), and sterol regulatory element-binding protein 2 (*SREBF2*). In contrast, level of pituitary-specific positive transcription

factor 1 (*POU1F1* or *Pit-1*) was higher, when compared with AE geese.

Clock Genes

We also observed seasonal expression differences of clock genes and proteins. For instance, circadian locomotor output cycles protein kaput (*Clock*), period circadian protein homolog 2 (*Per2*), aryl hydrocarbon receptor nuclear translocator-like protein 2 (*ARNTL2*, also called *BMAL2*), type II iodothyronine deiodinase (*DIO2*), aryl hydrocarbon receptor nuclear translocator 2 (*ARNT2*), CLOCK-interacting pacemaker (*CIPC*), casein kinase I isoform delta (*Csnk1d*), pericentrin (*PCNT*), and nuclear receptor subfamily 1 group D member 2 (*NR1D2*) were considered to be DEG when comparing AE group with at least one other group. Furthermore, the expression levels of these genes were the highest in the SS group and lowest in the AE group. Except for *Per2*, its lowest expression level appeared in the WS group, slightly lower than that in the AE group.

Functional Analysis of DEGs

To assign functional categories to the DEG obtained from pairwise comparisons, we performed GO enrichment analysis. GO terms were categorized into 3 main categories, including cellular component, biological process, and molecular function (Supplementary Table 2), and the top 30 highly enriched GO terms were presented in Figure 3. For WS vs. AE (Figure 3A), molecular function such as “binding,” “protein binding” and “ion

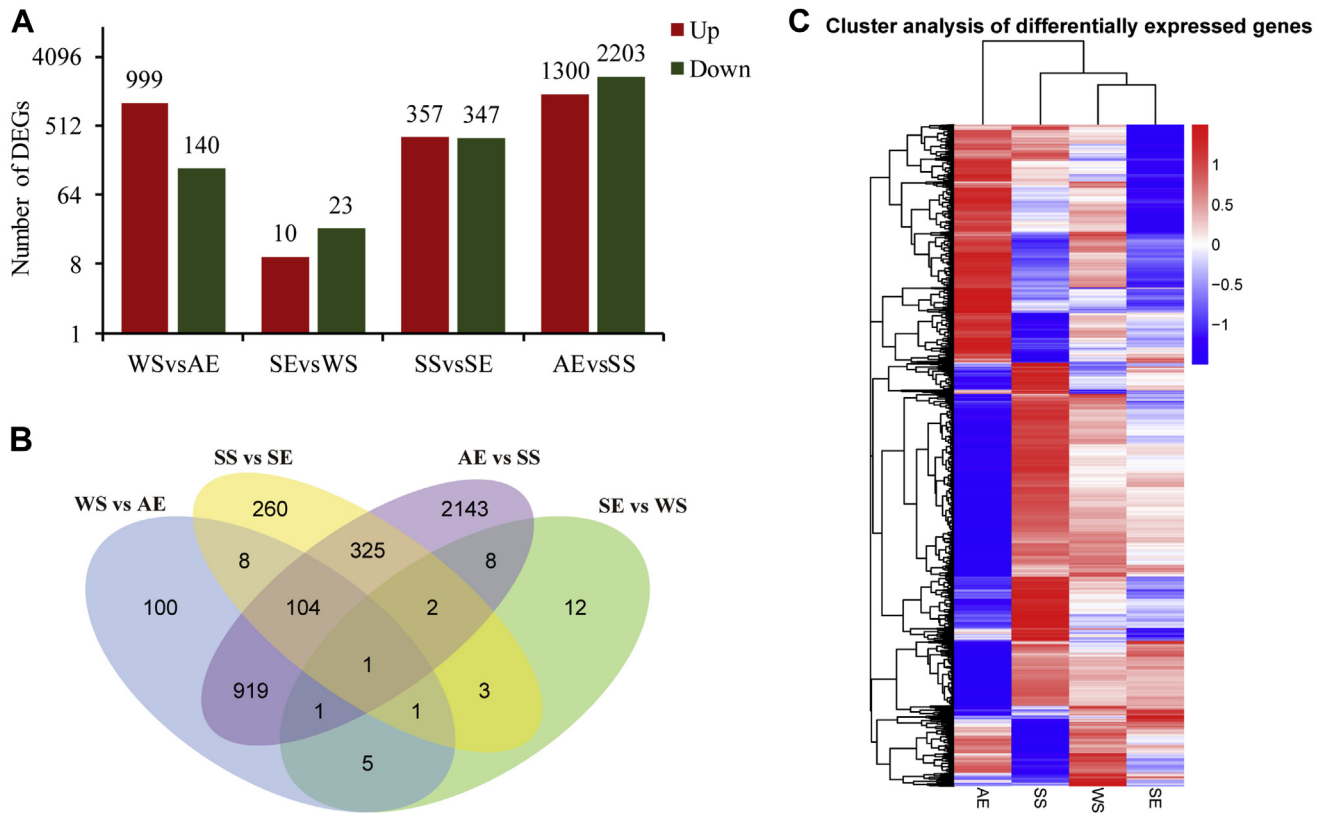


Figure 2. Statistics of differential expression gene (DEG). (A) Number of upregulated and downregulated DEG in each pairwise comparison. (B) Venn diagrams describing the numbers of unique and shared DEGs between samples. The sum of the numbers in each large circle represents the total number of DEG in the comparison combination, whereas overlapping portions of the circles represent the DEG shared between the combinations. (C) Heatmap comparing expression of the transcriptome at 4 time points. Intensity of color indicates expression levels. Red indicates upregulation and blue indicates downregulation. Similarity between individuals with hierarchical clustering is shown above the heatmap. AE samples were collected at the autumn equinox; WS samples were collected at the winter solstice; SE samples were collected at the spring equinox; and SS samples were collected at the summer solstice.

binding” accounted for the highest proportion of DEG. The significantly enriched cellular components included the “synapse part”, “presynaptic active zone” and “neuron part”. Genes also were involved in biological process, such as “cellular response to oxidative stress”, “response to oxidative stress” and “response to chemical”. Comparison SE vs. WS (Figure 3B) produced only 5 significantly enriched GO terms: “monooxygenase activity”, “MIS12 and MIND type complex”, “protein folding”, “tRNA wobble base modification” and “tRNA wobble uridine modification”. A total of 27 GO terms were significantly enriched of DEG in comparison SS vs. SE (Figure 3C). Among them, for example, “homeostatic process”, “biosynthetic process”, “oxidation-reduction process”, “response to oxidative stress,” and “TOR signaling” were the most important biological process terms. The molecular function category consisted of “protein transporter activity”, “antioxidant activity”, “oxidoreductase activity,” and “calcium channel activity”. For the DEG between AE vs. SS (Figure 3D), most were significantly associated with biological process, including “intracellular signal transduction”, “organic cyclic compound biosynthesis process”, “response to lipid”, “steroid hormone mediated signaling pathway”, “response to steroid hormone”, “transcription, DNA-templated,” and “cellular

response to oxidative stress”. The terms belonged to molecular functions, such as “binding”, “GTPase activator activity” and “hydrolase activity, acting on glycosyl bonds” were also significantly enriched. Finally, the term “response to oxidative stress” was enriched in 3 comparisons except SE vs. WS.

To understand the functional consequences of the expression variations in these 4 pairwise comparisons, the DEG found were mapped to 99, 18, 115, and 147 KEGG pathways, out of which 12, 4, 5, and 10 pathways reached significant levels, respectively. The top 20 pathways with high enrichment significance were displayed in Figure 4. In each comparison, the most significantly enriched pathways were the mTOR signaling pathway (Figure 4A), the extracellular matrix (ECM)-receptor interaction (Figure 4B), steroid biosynthesis (Figure 4C), and phosphatidylinositol signaling system (Figure 4D). Except in SE vs. WS, metabolic pathways accounted for the largest number of DEG (Supplementary Table 3). Eight KEGG pathways were enriched in two or more pairwise comparisons: “Steroid biosynthesis”, “GnRH signaling pathway”, “Lysine degradation”, “Inositol phosphate metabolism”, “Phosphatidylinositol signaling system”, “Focal adhesion”, “ECM-receptor interaction” and “Regulation of acting

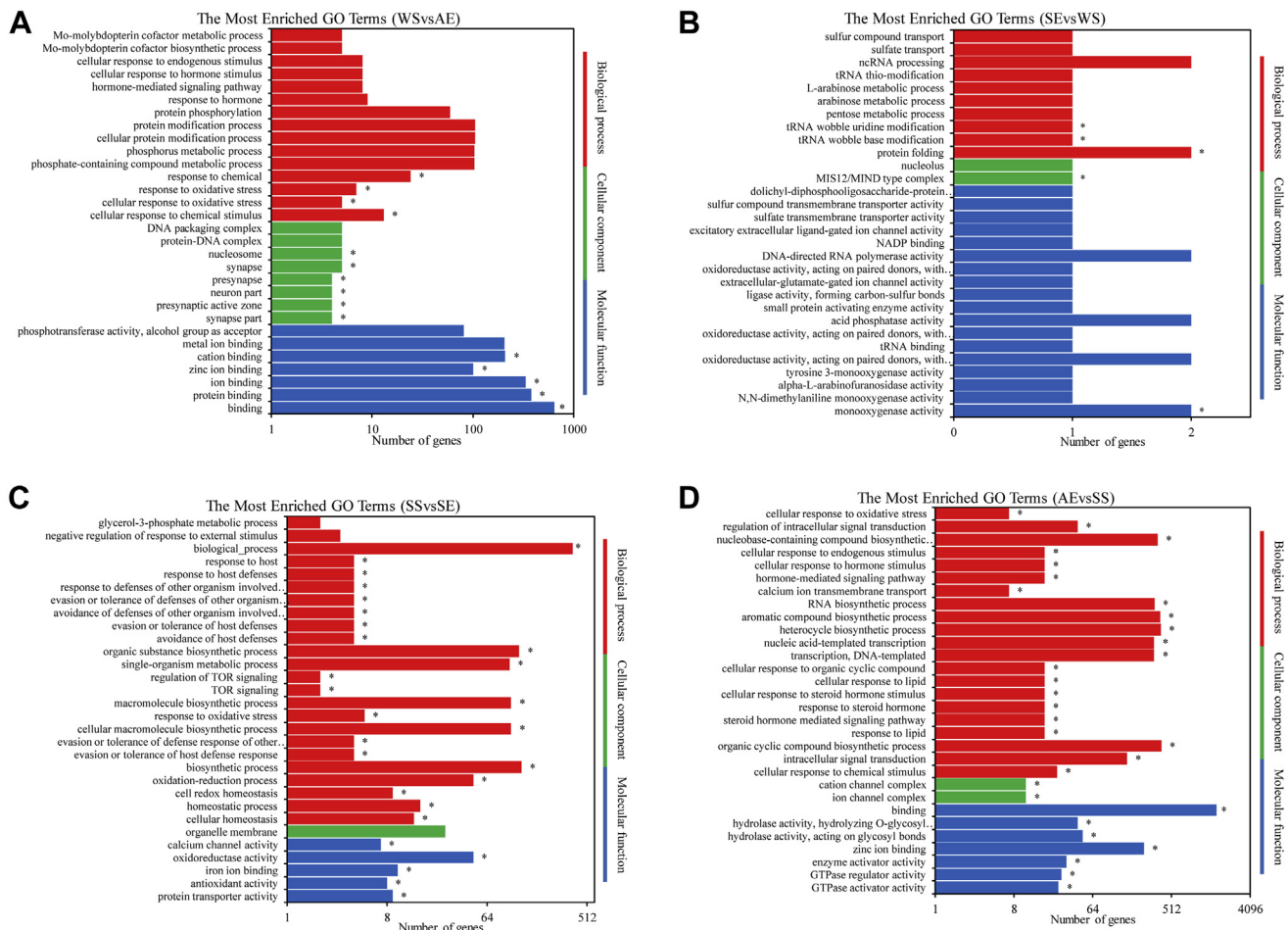


Figure 3. Gene Ontology (GO) functional enrichment of differential expression gene (DEG) between (A) WS vs. AE, (B) SE vs. WS, (C) SS vs. SE, (D) AE vs. SS. The ordinate coordinates represent 3 GO categories under the level of the GO term; the abscissa is the annotation of the term and the number of genes. Three different classifications of GO annotations of 3 basic categories are included (from top to bottom: biological processes, cellular component, and molecular function). The asterisk indicated significantly enriched ($P < 0.01$). Abbreviations: AE, autumn equinox; SE, spring equinox; SS, summer solstice; WS, winter solstice.

cytoskeleton". In the GnRH signaling pathway, expression of GnRH II receptor (gonadotropin-releasing hormone II receptor) increased, while epidermal growth factor receptor and adenylate cyclase type 1 (*ADCY1*) decreased significantly at AE. Steroid biosynthesis was the most highly represented functional group in SS vs. SE, and was also significantly enriched in AE vs. SS. Genes involved in the steroid biosynthesis pathway, for example, cholesterol side-chain cleavage enzyme (*CYP11A1*), farnesyl-diphosphate farnesyl-transferase 1 (*FDFT1*), delta (24)-sterol reductase (*DHCR24*) exhibited decreased expression levels from SE to SS, but were enhanced from SS to AE.

Validation of DEG by qPCR

To evaluate the RNA-seq data, expression levels of 15 randomly selected DEG were analyzed by RT-qPCR, and the \log_2 -fold change was calculated. The trend in expression variation of the 15 genes in intergroup comparisons was consistent with the transcriptome sequencing results (Figure 5). Overall, the RT-qPCR analysis indicated the reliability of the RNA-seq results, which can be further studied and analyzed.

DISCUSSION

To uncover the regulatory mechanisms that synchronize photoperiodic cues and reproduction of *A. cygnoides*, we have compared transcriptome levels in the pituitary gland, a crucial component of the HPG axis, between different reproductive states seasons. Some DEG found in pairwise comparisons were involved in signaling pathways related to reproductive performance, such as steroid biosynthesis, endocytosis, ECM-receptor interaction, and GnRH signaling pathway.

Genes Associated with Reproductive Hormones and Receptors

Genes known to be associated with reproductive hormones and receptors are candidate gene controlling the reproduction seasonality. In the hypothalamus, photoperiodic information regulates secretions of GnRH and VIP. In the pituitary, these stimulate secretions of LH and PRL, respectively. The latter control annual cycle of gonadal development and breeding activities (Sharp et al., 1998; Sharp and Blache, 2003). In female turkey, increased neuroendocrine activity of the VIP and PRL

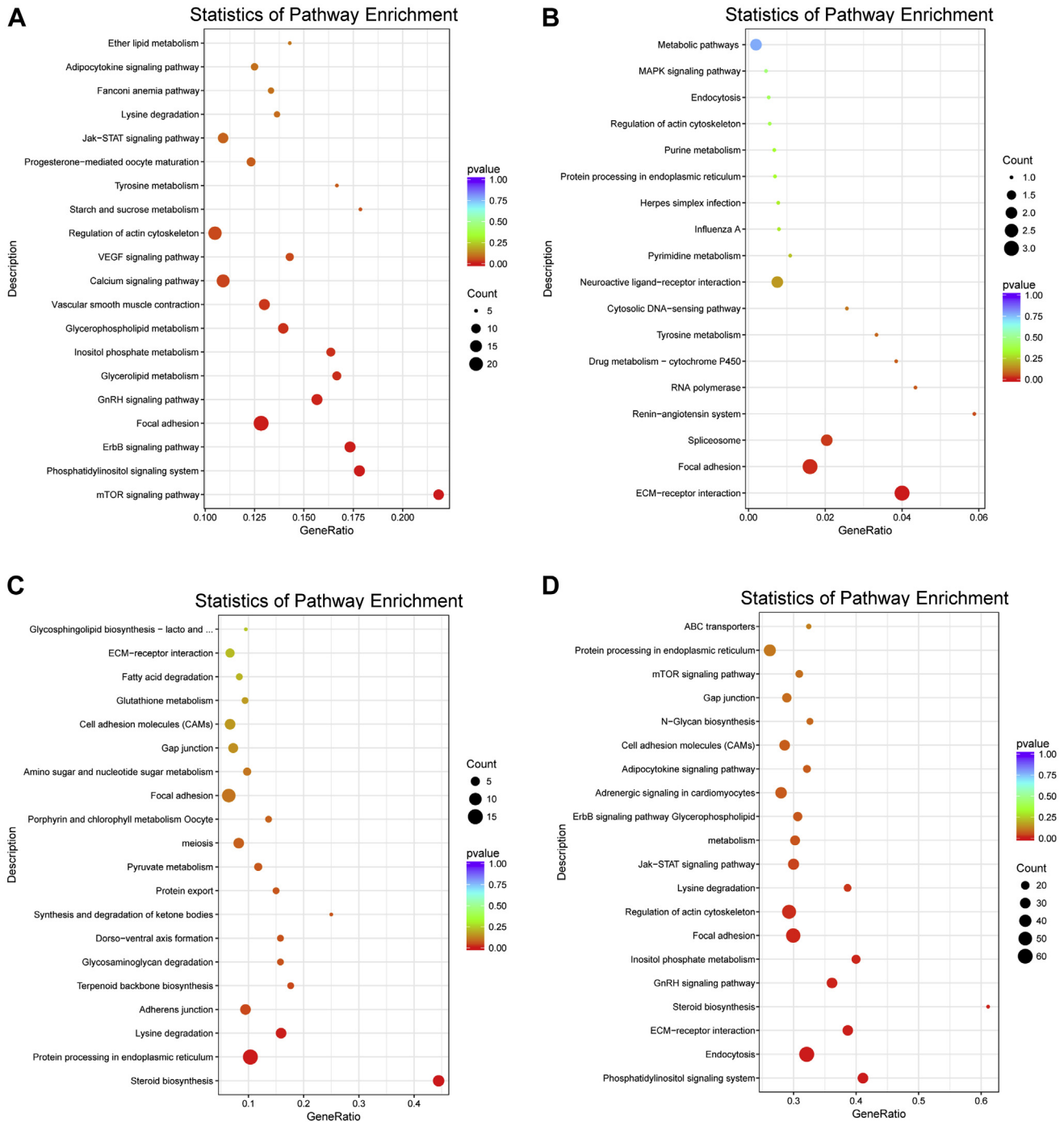


Figure 4. Pathways enrichment statistical scatter plot of differential expression genes (DEGs) between (A) WS vs. AE, (B) SE vs. WS, (C) SS vs. SE, (D) AE vs. SS. The vertical axis represents the name of pathway; the horizontal axis represents the pathways corresponding rich factor. The gene ratio refers to the ratio of the number of DEG enriched in the pathway and the number of all annotated genes in the pathway. Higher gene ratio indicates greater degrees of enrichment. The 20 pathways with the most significant enrichment were displayed in figure. Abbreviations: AE, autumn equinox; SE, spring equinox; SS, summer solstice; WS, winter solstice.

system suppressed the release of GnRH and LH, and lead to reduction of eggs production (Pitts et al., 1994). Our data show that GnRH receptor (*GnRHR*) expression level in the SS group was significantly lower than in the AE group, whereas for VIP receptor (*VIPR*), the results was opposite. GnRH receptor coupled to GnRH, plays an important role in activating the downstream pathway that regulate reproduction.

VIP is the avian PRL-releasing factor and its receptor *VIPR* was strongly suggested to play a prominent role in the regulation of PRL secretion at the pituitary level (Chaiseha et al., 2004). Decreased *GnRHR* receptor expression in SS geese reduces the sensitivity of the pituitary gland to stimulation by GnRH, and upregulated *VIPR* expression increased sensitivity to VIP and the release of PRL, leading to the cessation of egg

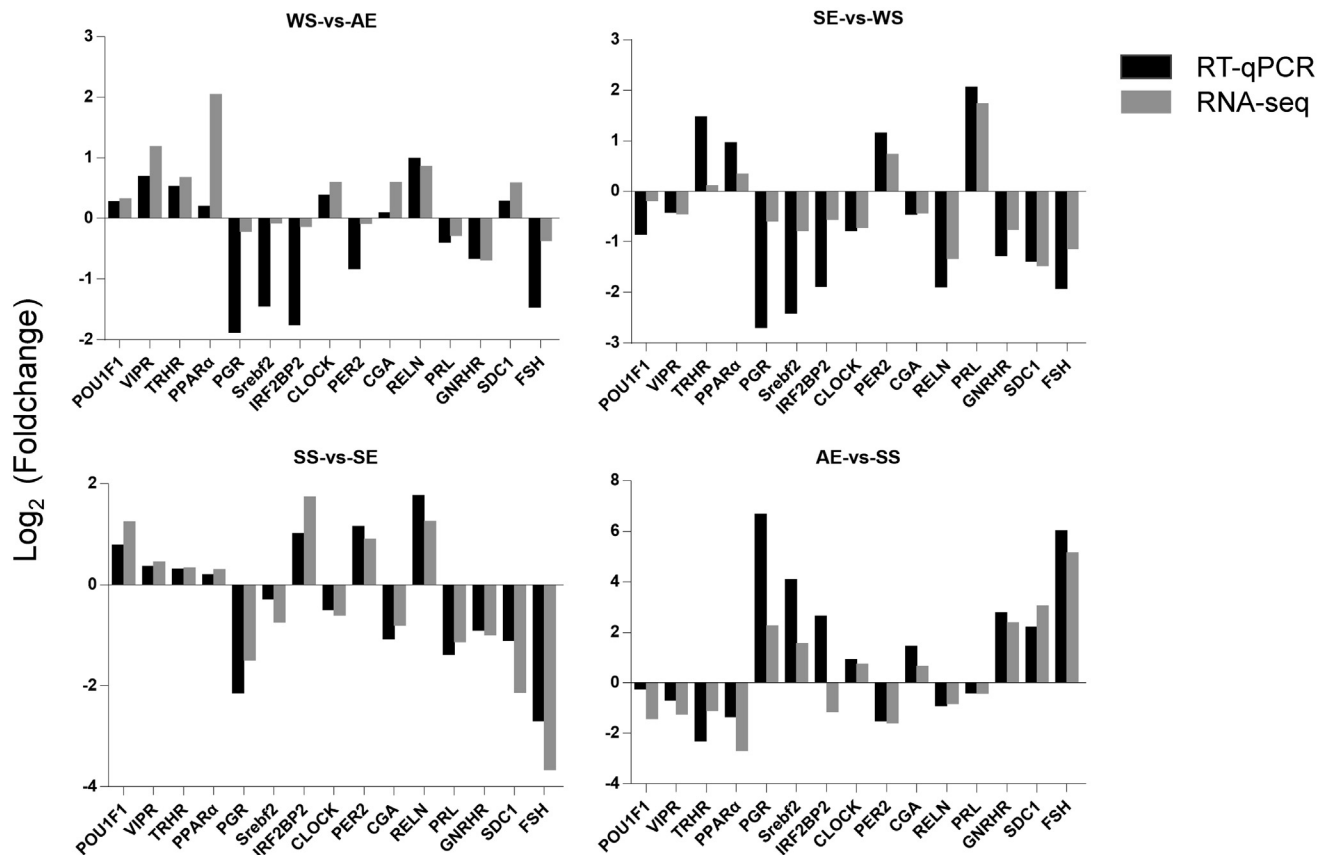


Figure 5. RT-qPCR validation of differential expression gene (DEG) obtained by RNA-seq. Total RNA extracted from the pituitary glands that were measured by qRT-PCR analysis; relative expression levels were calculated according to the $2^{-\Delta\Delta Ct}$ method using *GAPDH* as an internal reference gene.

production in the SS. We also found transcript expression changes of the PRL receptor (*PRLR*), which involves in the PRL signal transduction cascade and mediates various biological functions of PRL, including the reproductive processes of seasonally breeding species. *PRLR* mRNA may be regulated by prolactin in the pituitary (up) and hypothalamus (down) (Zhou et al., 1996). Our results indicate that these genes may participate in regulation of seasonal reproduction and in the reproductive stages transitions.

Circadian Clock-Associated Genes

The circadian rhythmicity of physiological systems provides an organism with the sense of time of day to ensure that physiological and behavioral events coincide (Kennaway, 2005). Circadian clock-associated genes changed significantly during season transitions. The clock protein (CLOCK) and aryl hydrocarbon receptor nuclear translocator-like (BMAL) protein, which can be substituted with Neuronal PAS domain protein 2 (NPAS2) and BMAL2, respectively, paired to form heterodimers CLOCK and BMAL1 or NPAS2 and BMAL2 that activate the transcription of their target genes, such as *Period* and *Cryptochrome* (Ikeda et al., 2000; Reick et al., 2001; Oishi et al., 2003; Sasaki et al.,

2009). We found no changes in *BMAL1* mRNA transcript expression in the pituitary across different seasons. *BMAL2*, in contrast, changed together with the *clock* gene, a DEG in comparisons WS vs. AE and AE vs. SS. This is consistent with the active role proposed for *BMAL2* in circadian translation (Sasaki et al., 2009).

The circadian clock gene *Per2* was highly expressed during long light, consistent with its reported light-inducible expression in different organs of other species (Yoshimura et al., 2000; Dardente et al., 2016). We found a significant enhancement of pituitary gland eyes absent 3 (*Eya3*) level in SS geese compared with SE and AE. In quail, induction of *Eya3* is an early-response gene in par tuberalis activated by long days, where expression patterns are closely matched to that of *TSH β* (Nakao et al., 2008). Three conserved E-box are found in *Eya3* promoter that suggest regulation by CLOCK and BMAL, which have an additive activation effect on *Eya3* promoter constructs (Dardente et al., 2010). Hypothalamic *Eya3* expression level increased in photorefractory buntings (Mishra et al., 2017). Overall, this supports that *Eya3* plays a role in maintenance of the photoperiodic-induced response (Majumdar et al., 2014; Majumdar et al., 2015) and an involvement of the circadian clock in the regulation of photoperiodism.

Thyroid-Stimulating Hormone

Thyroid-stimulating hormone from the pars tuberalis of the pituitary gland is induced by LD and acts on the ependymal cells to induce *DIO2*, responsible for converting thyroxine (T4) into triiodothyronine (T3). The enhanced transcription of *DIO2* we observed in SS geese may result from the increased day length, consistent with previous observation in Japanese quail (Nicholls et al., 1983) and chicken (Ono et al., 2009). Genes thyroid hormone receptor beta and thyrotropin-releasing hormone-degrading ectoenzyme were upregulated in SS geese, providing a rhythmic gating mechanism for thyroid hormone signaling. Glucocorticoids have been shown to control various physiological functions, notably those involved in development, metabolism, as well as induction of growth hormone expression during pituitary development (Ellestad et al., 2015), and exert most of their effects upon binding to the GR (encoded by *NR3A1* gene). In fact, GR is a central receptor and a transcription factor in regulating the HPA axis. These results highlight their potential role in regulating seasonal reproduction via photoperiodicity in goose.

Pituitary Transcription Factors

The present study also identified several pituitary transcription factors or regulators responsible for the reproduction transition between seasons: *POU1F1*, *NR4A2*, *SF-1*, *SREBF2*, and *Pitx2*.

POU1F1. *POU1F1* binds and transactivates the promoters of *PRL*, growth hormone (*GH*), and TSH beta subunit (*TSH β*) genes (Cohen et al., 1996; Miyai et al., 2005) and plays a role in regulating expression of genes for *TRH* receptor (Matre et al., 1999) and thyroid receptor (Wood et al., 1996). Estrogen responsiveness of the rat prolactin gene requires the presence of both estrogen receptor (*ER*) and *POU1F1*, suggesting a synergistic effect in inducing *PRL* gene transcription, synthesis, and secretion (Day et al., 1990; Nowakowski and Maurer, 1994; Ying and Lin, 2000). *POU1F1* also activated *PRL* gene expression in chicken (Ohkubo et al., 2000) and in domestic turkey (Weatherly et al., 2001).

NR4A2. *NR4A2* works in concert with *POU1F1* to enhance *PRL* expression in cell culture (Peel et al., 2020).

SF-1. *SF-1* regulates expression of the steroidogenic enzyme cytochrome P450 *CYP11 A* and *CYP11 B*. In *SF-1*-deleted mice, expression levels of both LH and FSH were markedly reduced and led to infertility and sexual immaturity (Ingraham et al., 1994; Zhao et al., 2001). *SF-1* was claimed to activate the transcription of gonadotrope-specific genes *LH β* , *GnRH* receptor, and neuronal nitric oxide synthase (Fowkes and Burrin, 2003), as well as targeting genes in the HPG axis that are critical for synthesis of progesterone, androgen, estrogens, and steroidogenesis (Jameson, 2004).

SREBF2. *SREBF2*, a key transcriptional regulator of cholesterol biosynthesis, was markedly induced by FSH

in the granulosa cells (Liu et al., 2009) and expression levels of *NR5A1* and *SREBF2* strongly associated with the reduced expression levels of steroidogenic genes (Shen et al., 2016).

Pitx2. *Pitx2* is important for pituitary early development, is an upstream regulator of *SF-1* gene (Suh et al., 2002) and is implicated in *SF-1* gene transcription (Shima et al., 2008). Reduced *Pitx2* caused decreased gonadotropes and thyrotropes, also involved in the transcription of *TSH β* , *GH* and *GHRHR* (Tremblay et al., 2000).

Therefore, we conclude that these 5 transcription factors may target hormone-release genes in goose pituitary gland and are important in the response to changes in photoperiod, triggering the hormonal responses.

Oxidative Stress

GO enrichment analysis of 3 pairwise comparisons show significant enrichment of “response to oxidative stress”, which may play a role in mediating seasonal reproduction transitions. Genes involved in include glutathione peroxidase 3 (*Gpx3*), sestrin-3 (*SESN3*), or bromodomain-containing protein 4 (*BRD4*). These protect against oxidative damage and genotoxic stresses (Budanov et al., 2004; Hussong et al., 2014). The link between reproduction and oxidative stress has been proposed (Metcalf and Monaghan, 2013) and oxidative stress likely underlie the trade-off between self-maintenance and investment in reproduction (Zimmer and Spencer, 2015). Reproduction is an energy-expensive process for females, with increased resource requirements and metabolism (Foucart et al., 2014). The energetic demands vary at different reproductive stages, and a concomitant change in oxidative stress may be also expected. For instance, song sparrows show increased oxidative stress during the laying period of reproduction as a result of nest predation (Travers et al., 2010). Therefore, our study supports a role for oxidative stress as a reproductive cost during reproductive stages changes.

Steroid Biosynthesis

KEGG enrichment analysis suggests that the involvement of genes enriched in steroid biosynthesis pathway, since expression was reduced sharply during the transition from SE to SS, but increased in AE vs. SS. Production of steroid hormones is associated with steroidogenic enzyme expression, whose changes during follicular development are initiated by gonadotropins (Nett et al., 2002). We observed reduction in mRNA expression levels of seven steroidogenic enzyme genes in the SS group (*CYP51A1*, *MSMO1*, *NSDHL*, *FDFT1*, *Lss*, *DHCR7*, and *DHCR24*). This might result in the decline of plasma level estrogen in egg-production ceased geese, consistent with results in broody chicken (Shen et al., 2016). Our data suggest that the interaction of steroid biosynthesis pathway and hormone genes, is in part, responsible for the seasonal reproduction in geese.

CONCLUSIONS

We have integrated high-throughput sequencing and bioinformatics to compare pituitary mRNA expression profiles among *A. cyanoides* at 4 season periods, identifying DEGs. Genes of *GnRHR*, *DIO2*, *POU1F1*, *Pitx2*, *NR3C1*, *Clock*, *BMAL2*, *Per2*, and *NPAS2* have been identified as candidate genes in molecular regulation of reproduction seasonality. GO and KEGG analyses suggest that response to oxidative stress, steroid hormone-mediated signaling pathway, steroid biosynthesis, and GnRH signaling pathway may also play a pivotal role in regulating seasonal reproduction.

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DISCLOSURES

The authors declared that they have no conflicts of interest to this work.

SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at <https://doi.org/10.1016/j.psj.2020.10.049>.

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