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Melatonin Secretion in Regulating the Circadian Rhythms of Reproduction in Goose (*Anser cygnoides*)

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Abstract: Circadian rhythms affect the physiology and behavior of most organisms. The ovulation–laying cycle of poultry exhibits evident rhythmic patterns. However, the underlying biological mechanism has remained unclear. Herein, Yangzhou goose (*Anser cygnoides*) were selected at 6:00, 12:00, 18:00, and 24:00 (n = 6/timepoint) to investigate the regulation of circadian egg-laying through the light-driven melatonin secretion. Our study revealed that the laying rates displayed diurnal fluctuations, with a peak of 40% of eggs being laid between 4:00 and 7:00. The cosine analysis revealed that the expression of clock genes exhibited rhythmicities ($p < 0.05$). Relevantly, melatonin secretion also displayed circadian rhythmicity and sharply decreases with increasing amount of light ($p < 0.001$). The immunohistochemical analysis found that the melatonin receptor is highly expressed during the night period. Notably, tissue distribution analysis further revealed that the melatonin receptor genes showed a decreasing trend in the pineal gland and hypothalamic–pituitary–gonad (HPG) axis throughout the day. Concomitantly, the expression of reproduction-related genes at 12:00 was significantly higher than that at 24:00 ($p < 0.01$). Taken together, these data suggested cyclical secretion of melatonin in response to photoperiod, which acts as a neuroendocrine transducer of circadian rhythm and the time preference of reproduction in domestic geese.

Keywords: circadian rhythms; goose; hypothalamic–pituitary–gonadal (HPG) axis; melatonin; reproduction

Citation: Bao, Q.; Liu, D.; Guo, Y.; Gu, W.; Cao, Z.; Zhang, Y.; Zhang, Y.; Xu, Q.; Chen, G. Melatonin Secretion in Regulating the Circadian Rhythms of Reproduction in Goose (*Anser cygnoides*). *Agriculture* **2023**, *13*, 1620. https://doi.org/10.3390/agriculture13081620

Received: 12 July 2023
Revised: 3 August 2023
Accepted: 14 August 2023
Published: 17 August 2023

1. Introduction

Circadian rhythms are endogenous adaptation mechanisms employed by living organisms to better adapt to the environment, and they play a fundamental characteristic in regulating various life activities. In order to survive and reproduce, the reproductive system of animals must be synchronized with favorable external stimuli and internal factors that enable or even optimize reproduction [1,2]. According to previous studies, the ovulation–laying cycle of female poultry exhibited a clear rhythm and environmental adaptability, especially regarding the time preference of laying eggs [3]. High-yielding hens have strict laying time preferences, mainly concentrated in the morning under natural photoperiod conditions [4]. It is still noteworthy that the cluster trait of hens was interrupted when the egg-laying time was delayed until the afternoon [5,6]. Moreover, a great number of studies have proved that poultry typically present with spontaneous ovulation, and the follicles need to go through a long process of growth and development to reach the mature state before ovulation occurs [7,8]. Follicles transform into hierarchical follicles and acquire the ovulatory potential under the synergistic action of reproductive endocrine hormones

and various growth factors [9–11]. Subsequently, the gonadal axis induces ovulation of the mature follicle where the process of egg-laying is strictly dependent on ovulation. Among them, gonadotropin-releasing hormone (GnRH) and gonadotropins (Gths) play an important role in the process of follicular development and differentiation [12]. The Gths are classified as follicle-stimulating hormone (FSH) and luteinizing hormone (LH) [13]. Meanwhile, ovulation is triggered by a rapid increase in luteinizing hormone (LH) from the pituitary gland [14]. Studies have found that GnRH, secreted by the hypothalamus, enters the blood and stimulates the release of FSH, LH, and other glucocorticoids from the anterior pituitary through the hypophyseal portal system [15]. These hormones, via the bloodstream, act on the ovary to stimulate a series of functions related to ovulation by promoting the production of Estrogen (E2) and Progesterone (P4) [16]. Although the period of egg formation is closely related to reproductive endocrine activity, activation of the hypothalamic–pituitary–gonadal (HPG) axis is also controlled by the intrinsic time-keeping mechanism.

Light is a crucial exogenous environmental factor for controlling many physiological and behavioral processes in animals [17]. Unlike mammals, birds possess a singular endocrine gland, the pineal gland, which can detect and respond to external light stimuli [18]. In recent years, it has been confirmed in several studies that the pineal gland is responsible for converting light signals into hormone signals and plays a vital role in the regulation of reproductive processes [19]. Although many secretions of the pineal gland have been discovered in recent years, melatonin is considered the chief product. The regulation of melatonin release is governed by sympathetic afferentation to the pineal gland, mediating the suppressive impact of light on the secretion [19]. However, the hormone melatonin, which is synthesized by the pineal gland, plays a crucial role in the regulation of the circadian rhythm [20]. At present, multiple core clock genes have been confirmed in the pineal gland of birds, including *Clock*, *Bmal* (*Bmal1* and *Bmal2*), *Per* (*Per2* and *Per3*), and *Cry* (*Cry1* and *Cry2*) genes [21,22]. The synthesis of melatonin is governed by the endogenous circadian clock of the pineal gland, resulting in rhythm oscillations on a near 24 h cycle [23]. Consequently, melatonin secretion exhibits a diurnal variation, with low levels during the day and high levels at night [24]. Therefore, the pineal gland of birds is characterized by the presence of both light input and melatonin output channels, indicating its dual functionality in terms of sympathetic innervation and circadian rhythm [25–27]. The pineal gland is also considered a dominant circadian oscillator, playing a crucial role in regulating the organism's internal clock [20]. Notably, the melatonin secreted by the pineal gland also influences follicle development and ovulation via the hypothalamic–pituitary–gonad (HPG) axis. In addition, melatonin could also directly regulate the activity of the ovary through its interaction with specific receptors, thereby influencing the reproductive endocrine system of the organism [28–30]. There are three melatonin receptor subtypes, melatonin receptor 1A (MT1, Mel-1a), melatonin receptor 1B (MT2, Mel-1b), melatonin receptor 1C (MT3, Mel-1c), have been identified [31]. The presence of melatonin receptors 1A in the ovaries of African ostrich chicks suggested that melatonin serves as a significant physiological regulator in the maturation of ovarian granulosa cells [32]. For most species, it has been demonstrated that the light input and melatonin output pathways are closely related to internal circadian oscillators, however, the molecular mechanisms governing the regulation of the egg-laying circadian rhythm remain inadequately understood in geese [26,27].

Numerous studies have reported that geese exhibit a characteristic egg-laying time preference, but the precise involvement of the pineal gland in the modulation of reproduction rhythms remains ambiguous. In this study, Yangzhou geese (*Anser cygnoides*) during the egg-laying peak period were selected. On the basis of elucidating the egg-laying preference and the rhythmic expression of clock genes in the pineal gland, the circadian regulation of melatonin secretion was further explored. As a result, these data deepen the understanding of the physiological mechanism of circadian behavioral rhythms and provide a theoretical basis for improving egg production in domestic geese.

2. Materials and Methods

2.1. Ethics Statement

This study has been approved by Yangzhou University's Ethics Committee on Animal Experiments for all animal experiments and protocols (Approval Code: 154-2020, Government of Jiangsu Province, China). Any experimentation involving geese was conducted in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals, as approved by the State Council of the People's Republic of China.

2.2. Experimental Design and Sample Collection

This study included female Yangzhou geese raised in the conventional method of stocking in the Yangzhou Tiange Goose Industry Development Co., Ltd., Jiangsu Province of China (32°64′ N, 119°34′ E). This experiment was performed at 28 °C/14 °C (day/night), on a 13L: 11D natural lighting photoperiod. For recording reproductive performance under natural lighting photoperiod, lights were not turned on at the end of the light phase. The geese' house was disinfected and segregated before the start of the experiment and breeding geese by means of flat farming. The general ventilation system was natural through two large doors and ventilation openings (2.0 m high) along the walls and along the roof. All animals were fed the same diet during the experiment (crude protein: 16%, metabolizable energy: 11.29 MJ/kg, Table 1). Feed and water were provided ad libitum. In the peak laying period, a total of 48 healthy Yangzhou geese 1R6 (68 weeks old) were selected for the experiments. They were tested at 6:00, 12:00, 18:00, and 24:00 for successive 2 days, representing different natural photoperiods, respectively. Each group was comprised of 6 individuals, specifically referred to as biological replicates. During each sampling period, the experimental subjects were female geese whose weights varied by no more than 3% of the average. Then serum was obtained through centrifugation at 3000× *g* for 15 min, the collected serum was stored at −80 °C. In this study, these geese were euthanized under sodium pentobarbital anesthesia and dissected to obtain the pineal gland, hypothalamus, pituitary, and ovary (six samples per composite were used for each test). These fresh tissues were immediately frozen in liquid nitrogen and stocked at −80 °C until RNA isolation. Finally, the collected pineal glands were subjected to further immunohistochemistry experiments. A summary of the sample collection and analysis process is presented in Figure 1.

Table 1. Ingredient and nutrient levels of the experimental diet in geese (air-dry basis).

Item	Content	Item	Content
Metabolic energy (MJ/kg)	11.29	Zinc (mg/kg)	33
Crude protein (%)	16	Calcium (g/kg)	45
Corn powder (g/kg)	403	Manganese (mg/kg)	55
Crushed wheat (g/kg)	250	Available phosphorus (g/kg)	35
Bean cake powder (g/kg)	135	Vitamin A (IU/kg)	4409
Green hay powder (g/kg)	127	Vitamin D3 (IU/kg)	661
Salt (g/kg)	5		

2.3. Reproductive Performance and Immunohistochemical Observations

The global horizontal irradiations (GHI) and egg-laying rates were also continuously monitored during the two weeks prior to sampling. On a daily basis, egg-laying rates were calculated according to the formula: egg-laying rate (%) = (number of eggs divided by total number of geese) × 100%.

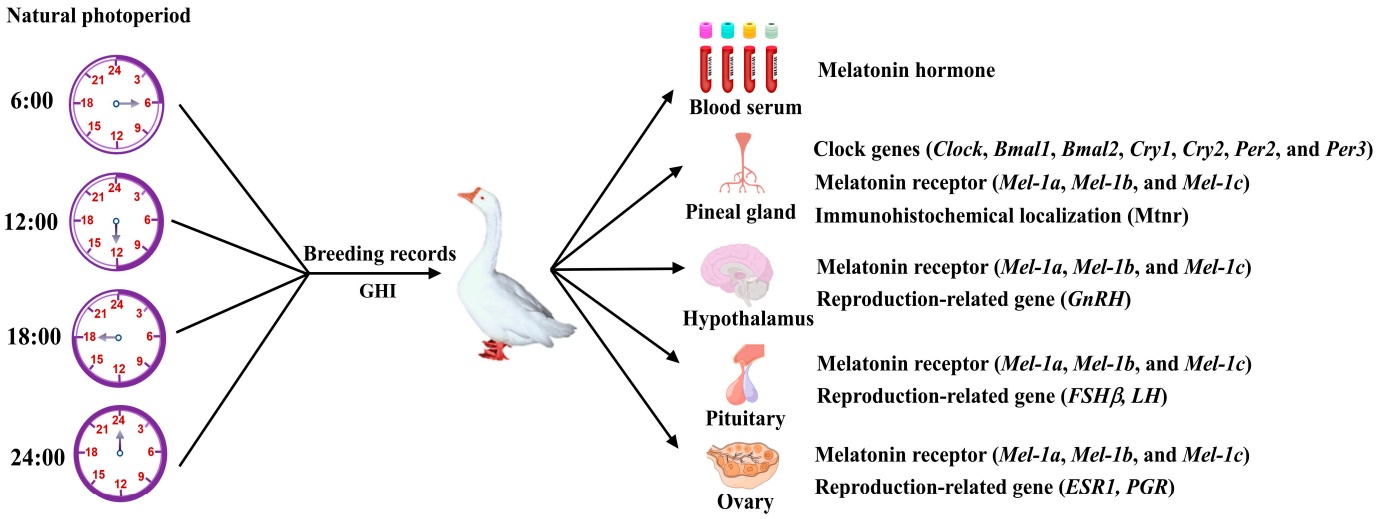

Figure 1. A summary of the sample collection and analysis process.

The immunohistochemistry procedure involved dewaxing the slides thrice in xylene for 15 min each and placing them in an EDTA buffer for microwave repair. Endogenous peroxidase activity was quenched with 3% H_2O_2 for 10 min. Then, the slides were incubated with primary antibodies overnight (Mtnr, 1:1000, ab87639; Abcam, Cambridge, UK). The WB experiments were performed to verify the specificity of the Mtnr antibody. After washing, the second antibody, rabbit anti-mouse IgG (ab6728; Abcam, Cambridge, UK), was added dropwise and incubated for 30 min. After washing again, freshly prepared 3,3′-diaminobenzidine (DAB) chromogenic reagent was added dropwise to the slides. After 10 min of chromogenic reaction, the reaction was terminated, followed by hematoxylin staining, alcohol dehydration (70%, 90%, and 100%), xylene clarification, and neutral resin mounting. The imaging system (Thermo Fisher Scientific, Waltham, MA, USA) was employed to capture both bright-field and fluorescence images (using a GFP filter) of the stained sections. The mean integrated optical density (IOD) of the immunohistochemical results was evaluated using Image-Pro Plus software (Image-Pro Plus, v 6.0).

2.4. RNA Extraction and Quantitative RT-PCR

Approximately 30 mg of tissue was used for RNA extraction. Total RNA was extracted with Trizol (Invitrogen, San Diego, CA, USA) in accordance with the manufacturer's recommendations. The primers (Table 2) were synthesized by TSINGKE biological technology (Nanjing, China). Furthermore, the stably expressed reference gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was employed to standardize target gene expression. Subsequently, RT-PCR was performed with a Quant Studio 5 (Applied Biosystems, Thermo Fisher Scientific) using the SYBR Green Master Mix (ABclonal, Wuhan, China). This study utilized an RT-PCR thermal cycling program consisting of an initial denaturation step at 95 °C for 5 min, followed by 41 cycles of amplification at 95 °C for 20 s and 60 °C for 30 s. The relative amount of each mRNA was calculated by the DCt method. Each reaction was performed in triplicate, and the data represent the mean of three independent experiments.

Table 2. The primers used in a quantitative RT-PCR assay for gene expression.

Gene Name	Accession Number	Primer Sequence (5′-3′)	PCR Product (bp)
Mel-1a	XM_048078352.1	F: TCATGCACGTTTGCACAGTC R: GTGGTCTCAGTCTGGGGTTG	169
Mel-1b	XM_013178069.2	F: CACGGTGGTGGACATCT R: CAGTGGGTATGGATACAAGG	139
Mel-1c	XM_048075512.1	F: CAGATAAGTGGGTTCCTGATGGG R: ACCGAAGGCTGTGGCAGATGTAG	103
Clock	XM_048049947.1	F: CAGAAAGACGGCGAGAAC R: GGAGGAAGCAGATGGTGT	155
Bmal1	XM_013189028.2	F: CCAGGTGGAACAAGAGC R: GATGTATCAGGCGGTGG	146
Bmal2	XM_048050182.1	F: GTTTTACGGATGGCTGTG R: CTGCTGCCCTAAGGATTA	127
Cry1	XM_048057002.1	F: TGGCTGTTTGTCTTGTCG R: CCGTGGATTGTTAGTCGC	145
Cry2	XM_048065130.1	F: GGCCATCATTAGCCGTAT R: CACATCATCGTGGTTCTCC	106
Per2	XM_048062937.1	F: GGCTTCTCCACCTTTATTCC R: TTGCTGCCTATGGCTCCT	141
Per3	XM_048067384.1	F: CGTCCTGTGAATGCTCTG R: CTTGTCCACGTAGTTCTGCT	114
GnRH [a]	DQ023158	F: GAAGATCTTGGTCGGTGTCCTCCTGT R: AATCTCCTTTCTTCTGGCTTCTCCTTC	262
FSHβ	XM_013177587.2	F: CACCAGTATCATCCGTTCAGC R: CAGTGCTATCAGTGTCACAGGTC	153
LH [b]	DQ023159	F: GGTGTATCGCAGCCCTTTG R: TATCAGAGCCACGGGGAGG	133
ESR1	XM_013178332.2	F: GCAAAGAGAGTTCCAGGATTTGT R: AGCGCCAGACTAAGCCAATC	103
PGR	XM_013178063.2	F: GCGTCCTCTACAAGACCGAA R: GGATTCTGTATCTGGCCGCA	227
GAPDH	XM_013199522.2	F: GTGGTGCAAGAGGCATTGCTGAC R: GCTGATGCTCCCATGTTCGTGAT	86

[a] Primers from Huang, Y M., et al. (2008) [33]. [b] Primers from Zhang, X., et al. (2013) [34].

2.5. Measurement of Serum Melatonin Hormone Concentrations

To estimate hormone levels, blood samples were subjected to centrifugation, and serum melatonin hormone (MT) levels were measured by ELISA kits (Qisong Biological, Beijing, China). The manufacturers' protocols were adhered to during the execution of operations. ELISA tests were repeated three times. The ELISA assay exhibited a sensitivity of 1.55 pg/mL and did not exhibit any cross-reactivity with other structural analogues. Additionally, intra-assay and inter-assay variation coefficients were less than 15%. Finally, the absorbance of each well was measured at 450 nm. The ELISA data were calculated using a model as below.

$$y = A2 + (A1 - A2)/(1 + (x/x0) \hat{} p)$$

R^2 values for all standard curves were higher than 0.98.

2.6. Statistical Analysis

All results were expressed as Mean ± standard error mean (SEM). These data underwent Kolmogorov–Smirnov (KS) analysis for normality of distribution, and nonnormal values were log-transformed for regression analysis. One-way *ANOVA* was performed, and multiple comparisons were made using the Duncan test before conducting the analysis. Significance was set at the 0.05 level ($p < 0.05$). In addition, non-linear regression was used

to analyze the rhythmic expression of core clock genes, with the model equation formula as follows:

$$Y(t) = M + A \times \cos(\omega \times t - C)$$

Statistical analyses were conducted using the SPSS 20.0 software package (IBM Corp, Armonk, NY, USA). Spearman correlation tests are conducted utilizing the R programming language (version 4.2.0) to establish correlations.

3. Results

3.1. The Temporal Dynamics of Egg-Laying Preference in Goose

The characteristics of laying time in Yangzhou geese during the 14-day observation period were shown in Figure 2. Our study revealed that Yangzhou geese exhibited an average oviposition interval of approximately 47.74 h (Figure 2A). Among the individuals observed, approximately 43% oviposition interval within the time frame of 46–48 h, while around 76% oviposition interval within the range of 43–51 h. Moreover, the GHI was recorded throughout the day (Figure 2B). This result showed that the GHI appeared at 5:00 in the morning, then reached a peak value greater than 900 W/m^2 at 11:00, and disappeared at 19:00. The time preferences of laying were also investigated (Figure 2C). Approximately 40% of the eggs were laid between 4:00 and 7:00. In contrast, only 7.9% of eggs were laid between 12:00 and 24:00.

3.2. Circadian Expression of Clock Genes in Pineal Glands

To investigate the circadian rhythm of clock genes in the pineal gland, we examined their expression trends over a 48 h period. The results revealed that the pineal gland of Yangzhou geese exhibited expression of all clock genes, namely *Clock*, *Bmal1*, *Bmal2*, *Cry1*, *Cry2*, *Per2*, and *Per3* (Figure 3, Table 3). Among them, the three positive regulatory genes *Clock, Bmal1*, and *Bmal2* all showed an oscillation trend that increased during the day and decreased at night (Figure 3A–C). The cosine period regression analysis revealed the presence of a 24 h circadian rhythm in the cosine curve of *Clock* ($R^2 = 0.925$, $p = 0.001$) and *Bmal2* ($R^2 = 0.851$, $p = 0.0011$), with discernible differences in amplitude. Notably, the amplitude of oscillation in *Bmal2* expression was relatively smaller compared to that of *Bmal1*. Furthermore, the negative regulatory genes, *Cry1*, *Cry2*, *Per2*, and *Per3,* exhibited contrasting rhythmic expression patterns to that of positive regulatory genes, demonstrating a consistent decrease in expression during the day and elevating at night (Figure 3D–G). It is worth noting that these clock genes exhibit significant circadian oscillations ($p < 0.05$). Meanwhile, the cosine fitting of *Cry2*, *Per2*, and *Per3* gene expression falls within the range of 0.7–0.8 ($p = 0.003$, $p = 0.003$, and $p = 0.009$, respectively). In addition, the peak times of expression for the clock genes were different. The negatively regulated genes reached their peak expression levels between 22:00 and 2:00, which was in contrast to the positively regulated genes.

Table 3. Cosine characteristics of clock gene expression in pineal gland.

Gene	Mesor ± SEM	Amplitude	Acrophase (hh:mm)	Cosine *p*-Value
Clock	1.10 ± 0.13	0.313	12:22	0.001 *
Bmal1	1.37 ± 0.08	0.418	13:02	n.s.
Bmal2	1.19 ± 0.06	0.278	17:48	0.001 *
Cry1	1.59 ± 0.03	0.754	22:06	0.046 *
Cry2	0.98 ± 0.05	0.446	02:32	0.003 *
Per2	1.14 ± 0.07	0.413	00:19	0.003 *
Per3	1.08 ± 0.05	0.385	01:05	0.009 *

Amplitude and Acrophase are calculated by nonlinear regression fit of a cosine function. * Cosinor analyses validated a statistically significant circadian rhythm ($p < 0.05$). n.s. indicates no significant level in cosine test ($p > 0.05$).

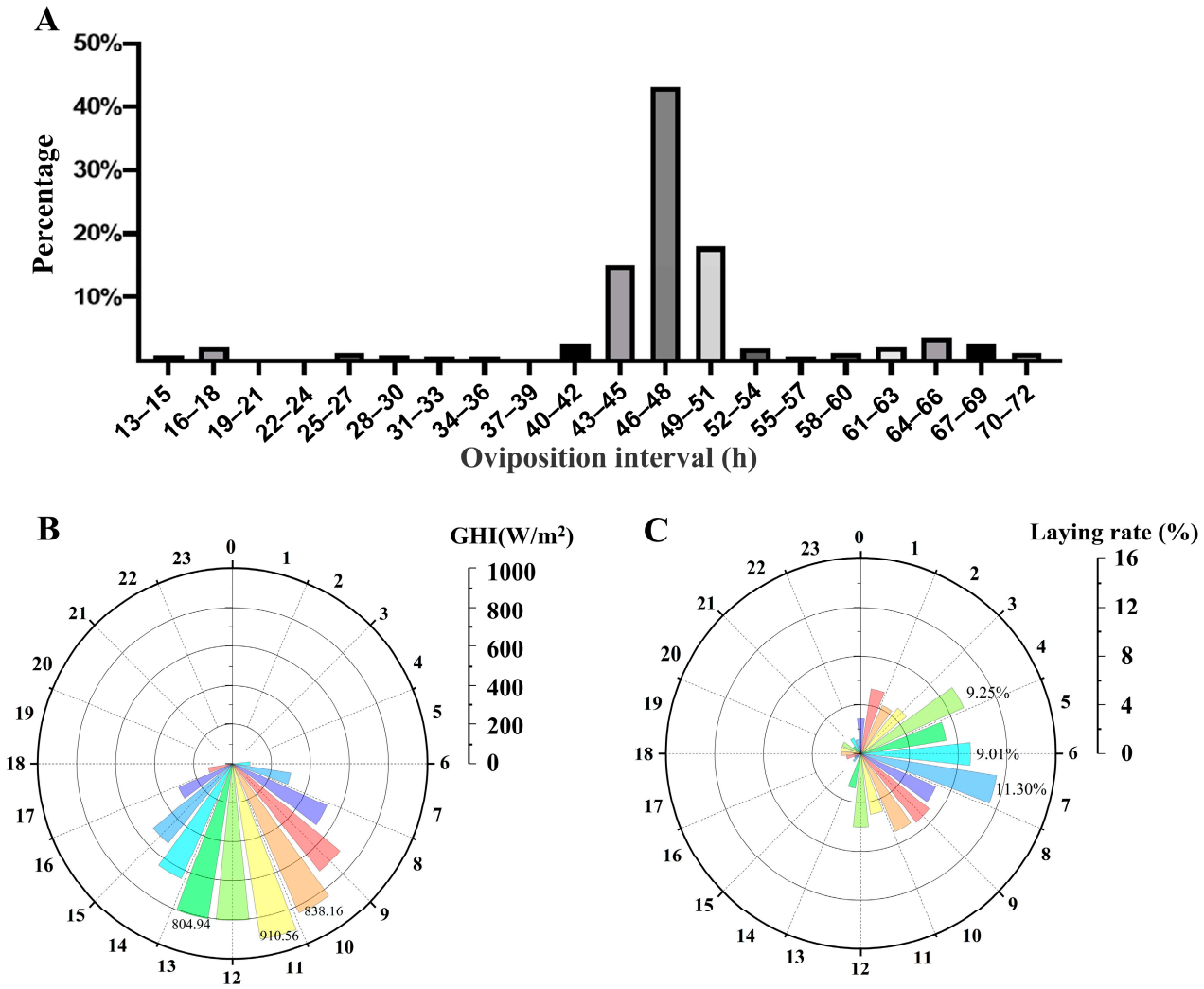

Figure 2. Characteristics of egg-laying preference in Yangzhou goose under natural photoperiod. (**A**) Oviposition interval. Recorded the duration between two consecutive ovipositions of geese during the 14-day observation period, and present these data as a percentage. (**B**) Global horizontal irradiations (GHI). The data were obtained from the European Centre for Medium-Range Weather Forecasts (ECMWF). (**C**) The observations of the egg-laying time. Each outer circle represents 24 h, and the vertical coordinates represent the laying rate. The columns of different colors correspond to the different time points.

3.3. The Dynamics of Melatonin Secretion and Immunohistochemical Localization

The dynamic change of the melatonin hormone is shown in Table 4. Melatonin secretion exhibited a typical diurnal trend. The results showed that melatonin content was the highest at 24:00 ($p < 0.001$). Subsequently, as the duration of exposure to light increased, the concentration of melatonin decreased rapidly, resulting in a significantly lower concentration of melatonin at 12:00 compared to 6:00 ($p < 0.001$).

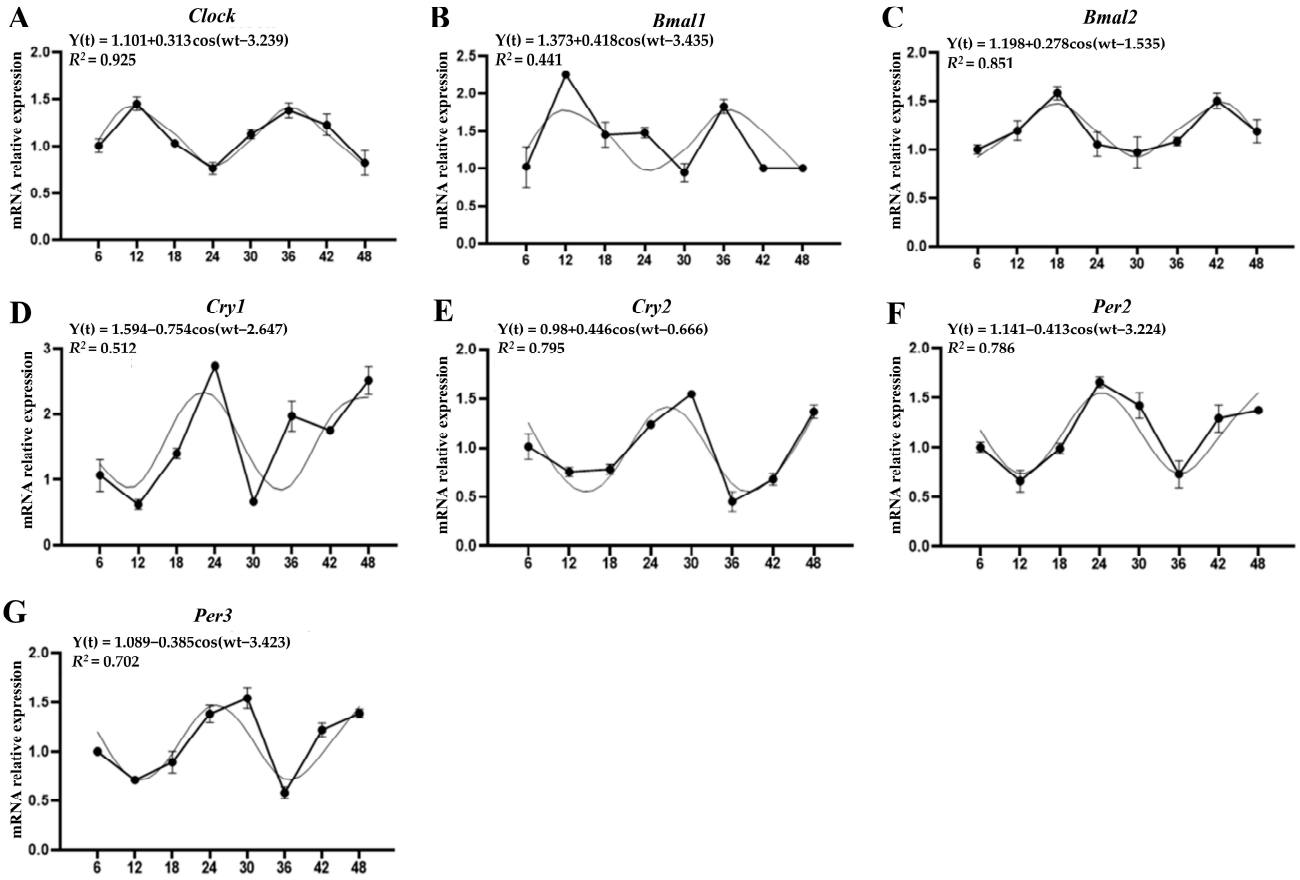

Figure 3. The expression of clock gene in the pineal gland according to the continuous experiment time (6 h, 12 h, 18 h, 24 h, 30 h, 36 h, 42 h, and 48 h). The smooth curves are describing function fits. The R^2 value was used to determine the degree of fit. The closer the R^2 value is to 1, the better the fitting degree is, and vice versa.

Table 4. The characteristics of melatonin hormone secretion.

Parameters	6:00	12:00	18:00	24:00
MT (pg/mL)	5.093 ± 0.295 ^{b}	4.103 ± 0.125 ^{c}	3.997 ± 0.094 ^{c}	6.146 ± 0.243 ^{a}

Note: The bloods of geese were collected every 6 h, and the content of melatonin was determined by ELISA. Mean ± SEM was used to represent all data, and different letters showed significant differences ($p < 0.05$).

To further explore the expression of the Mtnr, immunohistochemical analysis was conducted on both the pineal gland and peripheral tissues at distinct times of light (12:00) and dark (24:00), respectively (Figure 4). Mtnr were widely expressed in different issues. In the pineal gland, the expression of Mtnr was observed to be widespread in the pineal organ, with the greatest level found in close proximity to the connective tissue capsule (Figure 4A,E). Furthermore, the expression level of Mtnr was significantly higher during the dark periods compared to periods of light ($p < 0.001$). Different from the pineal gland, while the expression of Mtnr was elevated during the dark period in the hypothalamus, no statistically significant differences in circadian were observed ($p > 0.05$, Figure 4B). The Mtnr exhibited abundant expression at the edge of the pituitary, with a significant increase in expression levels during the night as light exposure decreased ($p < 0.001$, Figure 4C). Similarly, the ovary, as evidenced by immunohistochemical analysis, indicated that the theca layer of ovarian follicles exhibited a light yellow or even brownish-yellow hue, signifying a significant expression of Mtnr (Figure 4D). Concurrently, the expression of

Mntr during the dark period exhibited a significant increase compared to the light period ($p < 0.001$).

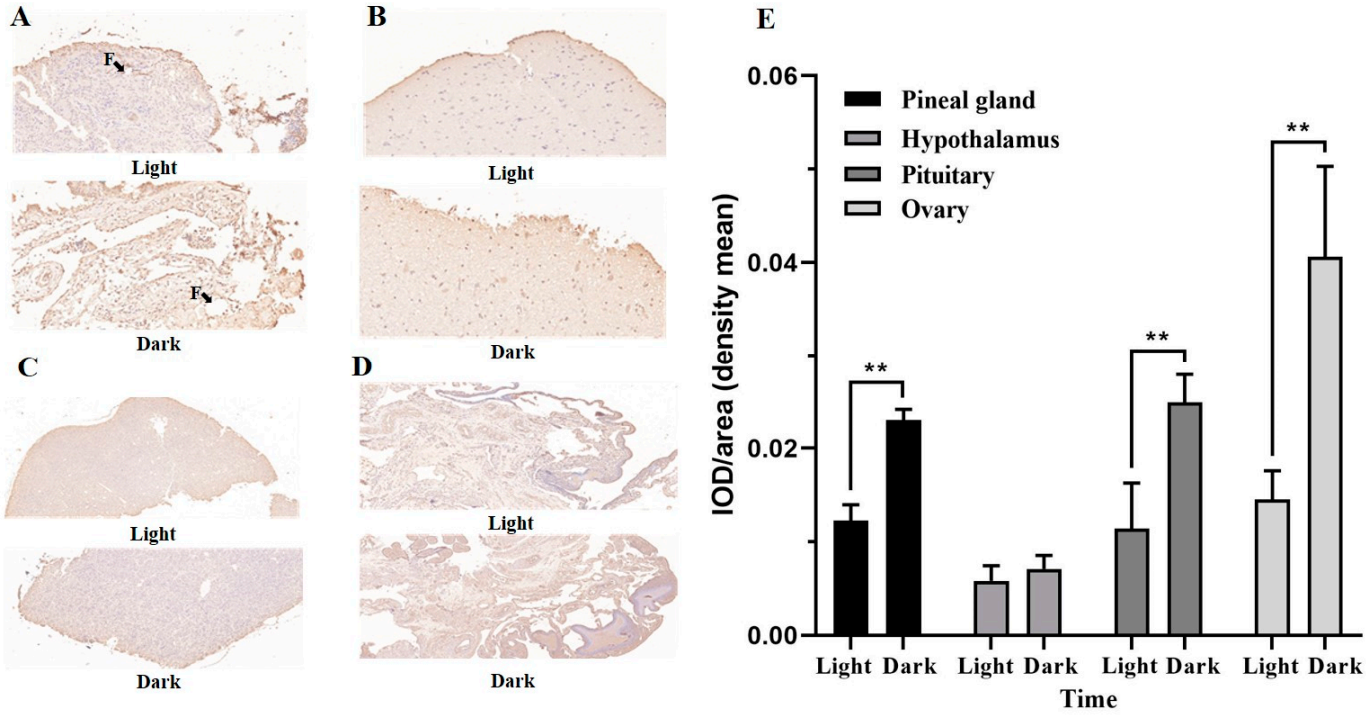

Figure 4. Various tissue sections were subjected to immunohistochemical staining. (**A**–**D**), the result of immunohistochemical staining; (**E**), average optical density; (**A**), pineal gland; (**B**), hypothalamus; (**C**), pituitary; (**D**), ovary; Light = 12:00, Dark = 24:00. Arrow indicates follicle-like structure (F). Asterisk (*) denotes significant differences, multiple asterisks indicate level of significance (** $p < 0.01$).

3.4. Tissue Distribution and Circadian Expression of Mtnrs Genes

Many physiological functions of melatonin are achieved by binding to receptors, so we explored the circadian regulation of *Mtnrs* genes in the pineal gland and peripheral tissue. The *Mtnrs* genes exhibited extensive distribution across various tissues, including the pineal gland, pituitary, hypophysis, and ovary (Figure 5). Real-time PCR analysis demonstrated that there is a significant circadian oscillation in the expression of *Mel-1a* and *Mel-1c* within the pineal gland (Figure 5A). Specifically, the expression levels at 24:00 exhibited a significant increase compared to the other three time points ($p < 0.001$). On the other hand, the expression level of *Mel-1b* in the pineal gland remained relatively constant and was notably lower than that of *Mel-1a* and *Mel-1c* ($p < 0.001$). In addition, in comparison to other tissues, the expression of *Mel-1b* was notably elevated in the hypothalamus and pituitary ($p > 0.05$, Figure 5B,C), with the most prominent expression observed at 24:00. However, the expression levels of *Mel-1a* and *Mel-1c* in the hypothalamus were relatively stable throughout the day (Figure 5B). In the interim, the *Mel-1a* expression within the pituitary persisted at a low level throughout the day and exhibited significantly lower in comparison to the other subtypes ($p < 0.001$, Figure 5C). At 24:00, the expression level of *Mel-1b* in the pituitary gland exhibited a minimum 5-fold increase in comparison to that at 12:00 ($p < 0.001$). Finally, the results indicate that the ovary exhibited the highest expression levels of the three melatonin receptor subtypes at 24:00, with *Mel-1c* demonstrating the largest circadian fluctuation (Figure 5D). Furthermore, the expression levels of *Mel-1b* and *Mel-1c* at 24:00 were significantly higher than those observed at 12:00 ($p < 0.001$).

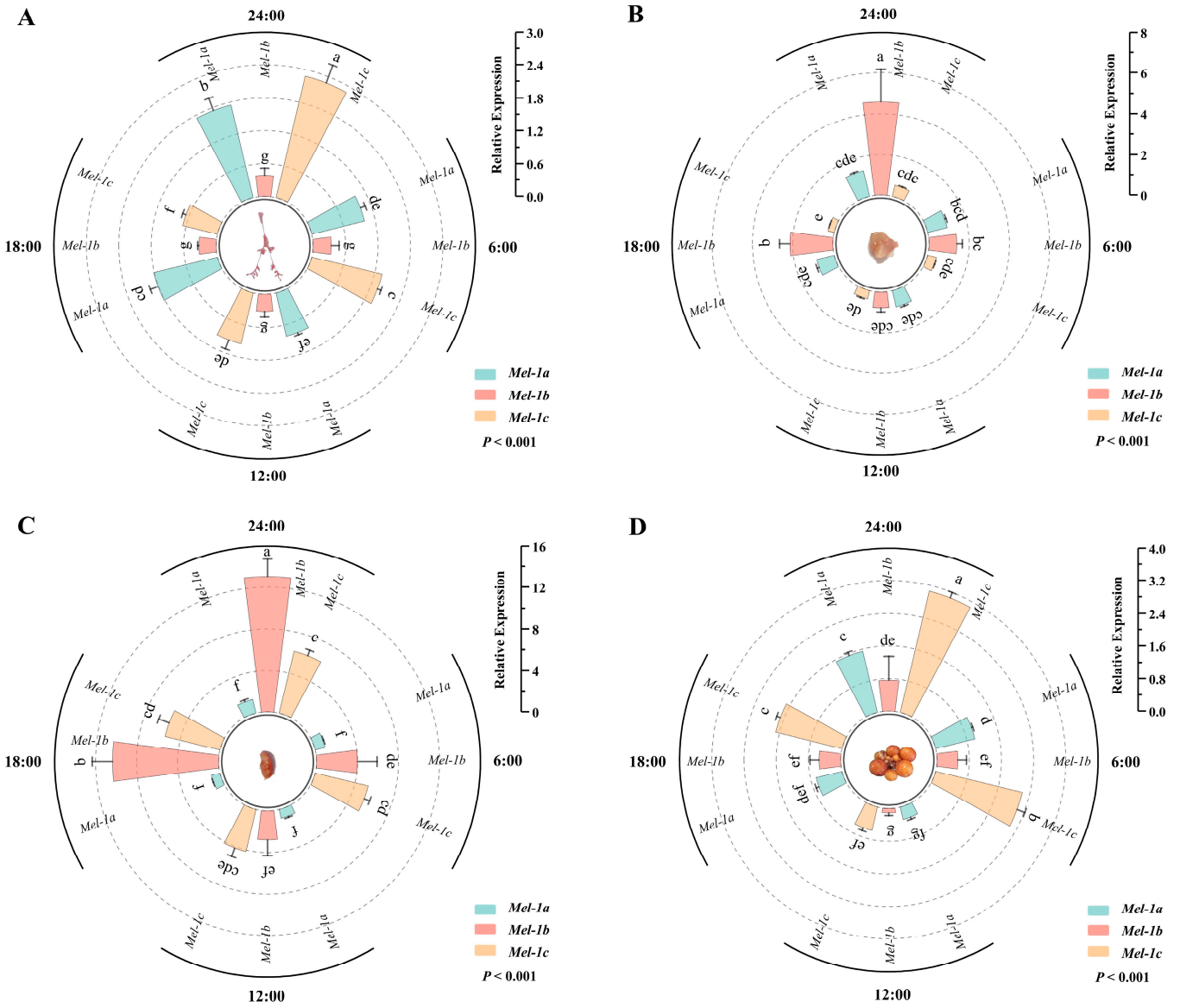

Figure 5. The tissue distribution and circadian expression of *Mtnr* genes in the pineal gland (**A**), hypothalamus (**B**), pituitary (**C**), and ovary (**D**). The vertical coordinates represent mRNA relative expression, and the data are shown with the mean ± SEM. Statistical analysis is conducted using two-way *ANOVA*, with different letters indicating statistically significant differences among groups ($p < 0.05$).

3.5. Circadian Oscillation of the Reproduction-Related Genes in HPG Axis

A diagram showing the changes in reproduction-related genes with respect to circadian cycles can be found in Figure 6. The expression level of *GnRH* exhibited a rapid increase from 6:00 to 12:00 ($p < 0.001$), followed by a decline as the duration of light decreased, reaching its lowest point at 24:00 (Figure 6A). Correspondingly, the expression levels of *FSHβ* and *LH* showed similar circadian fluctuations, with a statistically significant increase observed at 12:00 in comparison to other times of the day ($p < 0.001$, Figure 6B,C). Notably, the expression level of *LH* exhibited large-amplitude fluctuations, whereby the expression level at 12:00 was approximately 30-fold higher than that at 24:00 ($p < 0.001$). In the ovary, the actions of *P4* and *E2* are mainly mediated through their respective nuclear receptors, namely the progesterone receptor (*PGR*) and estrogen receptor (*ESR1*) (Figure 6D,E). The study revealed that the levels of *ESR1* expression did not exhibit a significant difference between 12:00 and 18:00 ($p > 0.05$), but were significantly elevated compared to other times

of the day ($p < 0.001$). Furthermore, while *PGR* exhibited high levels at 12:00 ($p < 0.001$), its expression remained relatively stable across other time points.

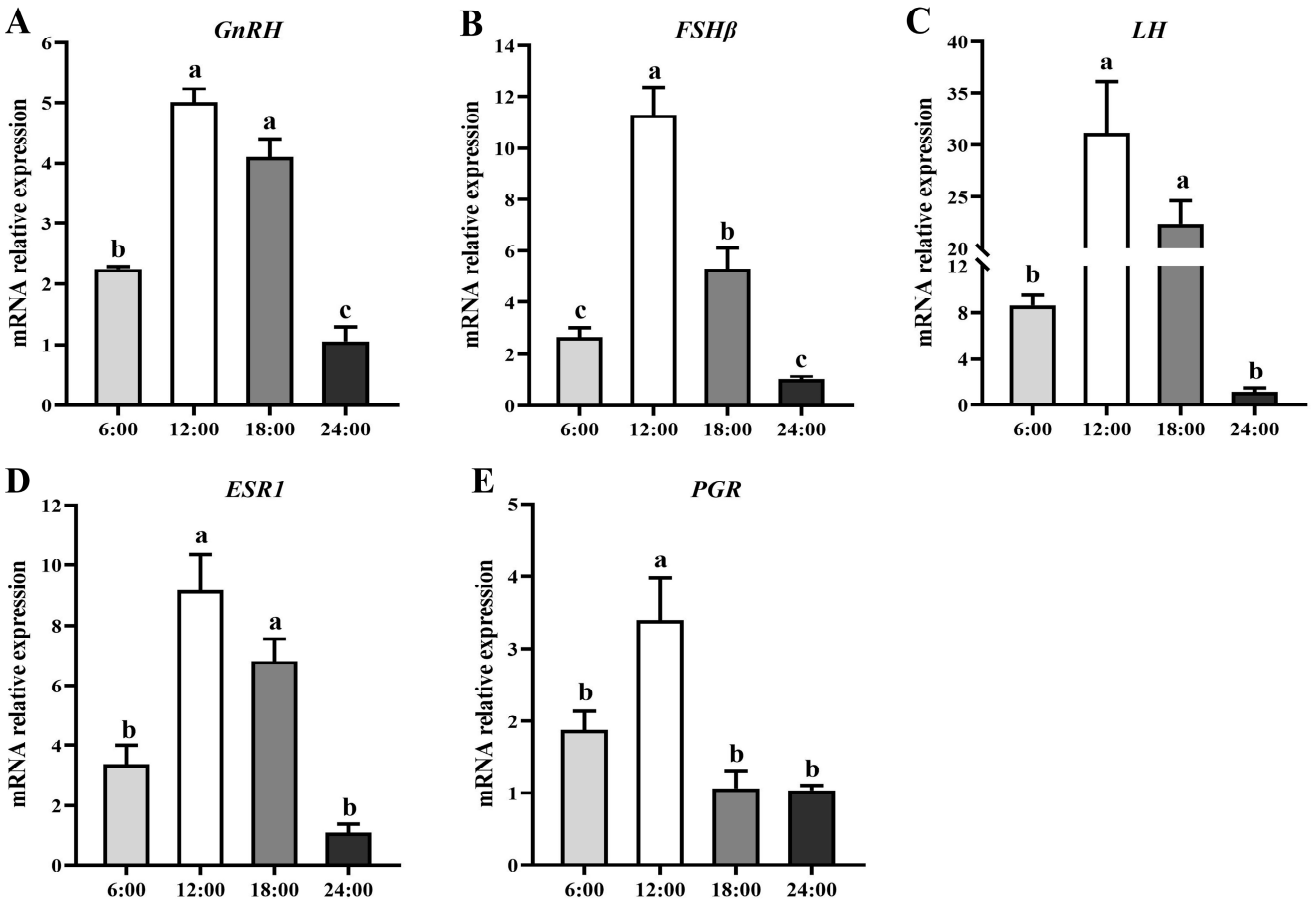

Figure 6. Relative mRNA expression of the reproduction-related genes. (**A**) *GnRH* mRNA expression in hypothalamus. (**B**,**C**) *FSH* and *LH* mRNA expression in the pituitary. (**D**,**E**) *ESR1* and *PGR* expression mRNAs in the ovary. RNA levels are normalized by *GAPDH*, with mean + SEM. Statistically significant differences were indicated with different letters ($p < 0.05$ by one-way *ANOVA*).

3.6. Correlation Analysis of Melatonin Hormone and Reproductive-Related Genes

The correlation analysis between the melatonin hormone and reproductive-related genes was presented as a chordal graph (Figure 7A) and a correlation heatmap (Figure 7B). The present investigation revealed a negative association between melatonin hormone and reproductive-related genes, with a significant correlation observed between melatonin hormone and *GnRH* ($R^2 = -0.8$, $p = 0.044$, Table S1). In addition, there were also some degree relationships between different reproductive-related genes. The results indicate that *GnRH* exhibited a positive association with *FSH*, *LH*, *ESR1*, and *PGR1* (Figure 7A), with a statistically significant correlation observed between *LH* ($p = 0.001$) and *ESR1* ($p < 0.001$). Similarly, *FSH* was significantly positively correlated with *LH* ($p = 0.047$). Furthermore, in comparison to other genes involved in reproduction, the positive associations observed between *PGR* and other reproductive-related genes did not reach statistical significance ($p > 0.05$, Figure 7B).

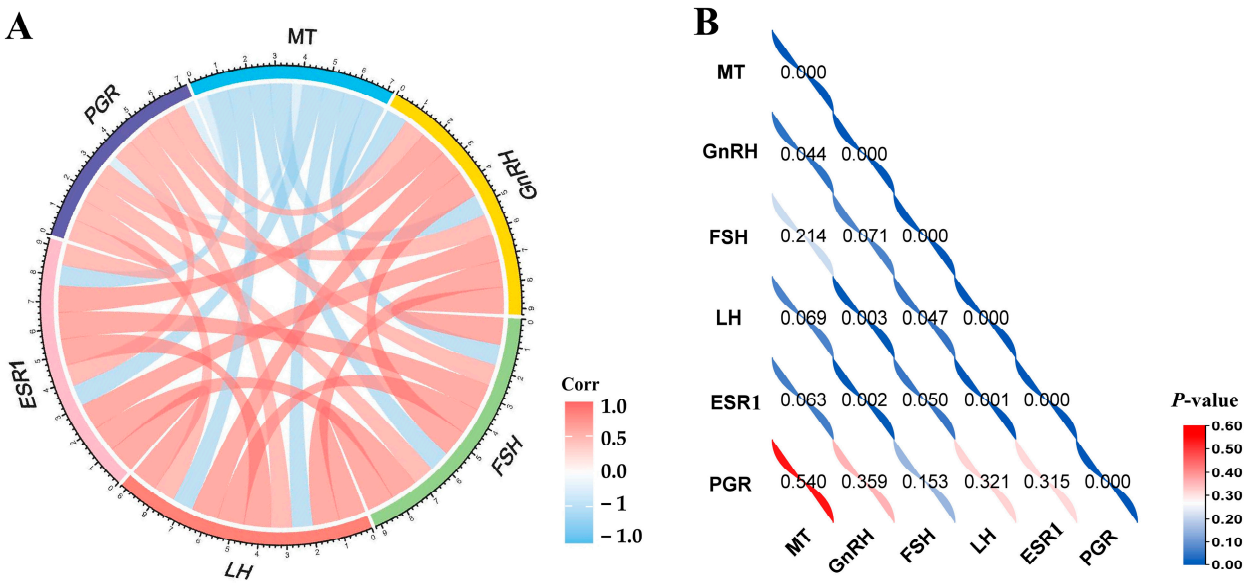

Figure 7. Correlation Analysis of melatonin hormone and reproductive-related genes. (**A**) Chord diagram showing the correlation between the melatonin hormone and reproductive-related genes. (**B**) Heatmap showed the correlation. The value *p* is the *p*-value of observing the Spearman correlation. Significant correlations were filtered by *p*-value (*p*-value < 0.05).

4. Discussion

To further reveal the regulatory mechanisms of circadian reproduction, we explored the expression of core clock genes and the secretory activity characterizations of the pineal gland under the normal day and night light cycles, with a particular emphasis on the regulation of the HPG axis. As shown in this study, the pineal gland in response to photoperiod regularly triggers ovulation and maintains the clear time preference of egg-laying in geese. In this way, the cyclical secretion of melatonin by the pineal gland provides the neuroendocrine control of reproduction with an internal molecular representation of changes in the photoperiod (Figure 8).

The circadian rhythm is a vital biological process that enables animals to acclimate to their external environment [35]. Studies have confirmed that the ovulation–laying cycle of poultry exhibited obvious rhythmicity and adaptability [36]. The egg production of poultry determines reproductive performance, which is an important economic characteristic. In this study, these geese were bred to get fertile eggs, and the probing of reproductive performance was of high significance. In this research, we observed Yangzhou geese during their peak laying period and discovered that they exhibited a distinct preference for laying duration. Unlike mammals, poultry differed in both anatomy and physiology, as their ovaries contain a great number of hierarchical follicles [7]. Among them, the process of egg formation in poultry involves multiple stages, including the development of the ovarian follicle, ovulation of the largest follicle, formation of the egg white and the eggshell membrane, calcification of the eggshell, and oviposition [10,11]. Animals synchronize their physiological state with external stimuli to accurately time their reproductive activities. Furthermore, previous studies have shown that high-yielding laying hens exhibit an egg-laying interval of 25–27 h under natural photoperiod conditions [37,38]. However, geese, unlike chickens, exhibited an egg-laying interval of approximately 46–48 h, potentially attributable to the longer duration of egg retention within the oviduct [39]. Meanwhile, our study also revealed that most female Yangzhou geese exhibited egg-laying preference in the morning. A previous observations found that nearly 10% of Sichuan white geese and Zhedong white geese laid eggs at 6:00 [40]. Similarly, in our study, we observed that the laying of eggs by Yangzhou geese predominantly occurred during the hours of 4:00 to 7:00. As such, it is recommended that breeders engage in a comprehensive cleaning

of the goose house during the afternoon to mitigate any potential adverse effects on the egg-laying process of geese.

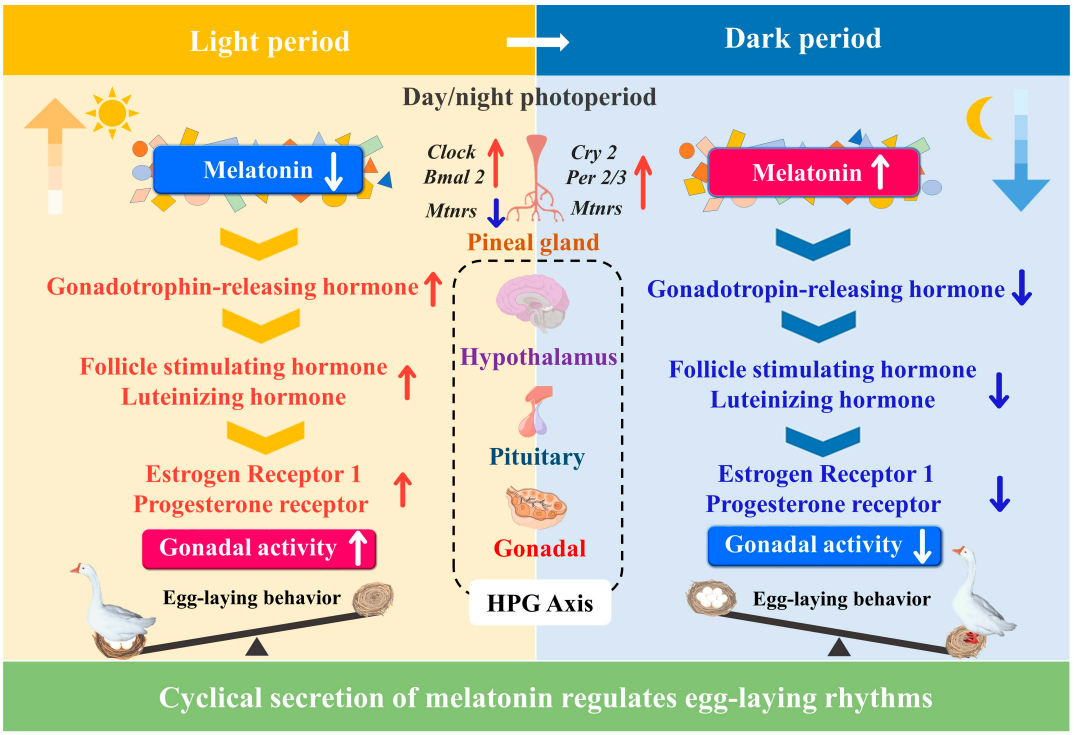

Figure 8. Schematic summarizing the interaction mechanism between the cyclical secretion of melatonin and egg-laying behavior.

Furthermore, the circadian clock plays a crucial role in regulating behavioral regularities and physiological processes, including but not limited to endocrine hormone production, lipid metabolism, and maintenance of body temperature [36,41]. Studies have proved that light signals can influence the activity of the hypothalamus–pituitary–gonad axis (HPG axis) through the biological clock, thereby impacting the reproductive activities of animals [25,38]. Hence, investigating the rhythmic oscillation of circadian clocks is essential for comprehending the rhythmic patterns of egg production. Clock genes are crucial regulators of the biological rhythm, potentially linked to external environmental factors, including light, temperature, and other variables [42]. During the diurnal cycle, the pineal gland of White Leghorn chicken (*Gallus gallus*) exhibits higher expression levels of clock genes during the light compared to the night [43]. Moreover, the Rhythmic oscillation of the core circadian has also been shown in sparrows [44]. The present investigation revealed that the *Clock* and *Bmal* genes exhibited their highest expression levels at noon in the pineal gland, whereas the *Cry* and *Per* genes demonstrated their expression peaks in the late night and early hours of the morning. Some previous studies found that the transcription factors *Bmal* and *Clock*, belonging to the bHLH PAS family, activate the transcription of *Per* and *Cry* genes in vertebrates by binding to E-box promoter elements located in their respective promoters [44]. Meanwhile, the formation of a heterodimer between *Per* and *Cry* results in the repression of *Per* and *Cry* transcription by acting on the *Clock-Bmal* heterodimer [45]. The negative feedback loop comprised of these genes serves as the fundamental mechanism of the circadian clock in animals [46]. Our findings align with previous research conducted on mammals. Specifically, in rats exposed to a 12 h light and 12 h dark cycle, the expression of *Clock* was observed to be greater during the light than during the dark, suggesting that light cues may influence the expression of the core circadian clock [47].

The pineal gland, as a crucial core biological clock in poultry, plays an important role in endocrine regulation by sensing photoperiod information [19]. It was reported that a light

regime could alter the morphology of the pineal gland [18]. Numerous studies have found that the synthesis and secretion of melatonin are closely related to light [19,48]. A study has confirmed that the pineal gland of reptiles, fish, and birds has the ability to respond to light stimulation, thereby regulating the release of hormones [49]. Moreover, the pineal gland further regulates growth and sexual maturation through the secretion of melatonin [50]. Pineal melatonin mostly acts as an endocrine agent, whereas melatonin performs not only autocrine functions but also functions in a paracrine manner [51,52]. Melatonin secretion follows a pattern of low levels during the day and high levels during the night in response to alterations in light duration [53]. The same observation was found in this research. Furthermore, as a short-day breeding species, most birds experience a suppression of gonadal activity due to the presence of melatonin [54]. Typically, melatonin exerts effects on the reproductive endocrine system by binding to the receptors [28]. The results of the immunohistochemical analysis in our study indicate that the expression of melatonin receptors was notably elevated during the dark phase in nearly all examined tissues, in contrast to the daytime. However, part of the staining is close to the pineal gland surface. It is likely that there is an edge effect for the immunohistochemical staining. Similarly, we examined the tissue distribution of the melatonin receptor gene. Three melatonin receptor subtypes are widely distributed in the pineal and HPG axis. This result was consistent with a study by Valent et al., (1999), which also demonstrated that multiple melatonin receptor subtypes expressed in most tissues and control gonadal development and sexual maturation [55].

5. Conclusions

In this study, our data demonstrate that the female Yangzhou geese have a distinct circadian preference for egg-laying, and the change of photoperiod signal was a significant regulator. As the central regulator of the biological clock, the pineal gland regulated the rhythmic release of melatonin in response to photic cues and effected on the hypothalamus. Afterward, the hypothalamus periodically releases GnRH to act on the pituitary gland and ovary, prompting follicle maturation and regular ovulation in poultry. In summary, our study provided basic information on circadian preference in reproduction. It also provided the inspiration for the relationship between melatonin and reproductive endocrine.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture13081620/s1, Table S1: Spearman coefficient of determination (R^2).

Author Contributions: Conceptualization, Q.B., D.L. and Q.X.; methodology, Q.B. and G.C.; formal analysis, W.G. and Q.X.; data curation, Y.Z. (Yang Zhang) and Y.Z. (Yu Zhang); writing—original draft preparation, Q.B. and Z.C.; writing—review and editing, Q.B. and Y.G.; funding acquisition, G.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Science and Technology Project of Jiangsu Province (BE2022350).

Institutional Review Board Statement: All animal experimentations and protocols were approved by the Ethics Committee on Animal Experiments of Yangzhou University (Approval Code: 154-2020, Government of Jiangsu Province, China). Any experimentation involving geese was conducted in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals, as approved by the State Council of the People's Republic of China.

Informed Consent Statement: Not applicable.

Acknowledgments: We thank Home for Researchers (www.home-for-researchers.com, accessed on 3 May 2023) for their help with language and figures.

Conflicts of Interest: The authors declare no conflict of interest.

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