Expression of GnIH and its effects on follicle development and steroidogenesis in quail ovaries under different photoperiods

Xiaoli Zhou,*; Danli Jiang,*; Zhuoshen Zhang,† Xu Shen,*; Jianqiu Pan,*; Danning Xu,*; Yunbo Tian,*; and Yunmao Huang,*;†

Zhongkai University of Agriculture and Engineering, Guangzhou 510225, China; and Guangdong Key Laboratory of Waterfowl Health Breeding, Guangzhou 510225, China

ABSTRACT Photoperiod is an important environmental factor that influence seasonal reproduction behavior in bird and GnIH can play a function in this process through the reproductive axis, and some studies suggest that GnIH may have a direct role at the gonadal level. To investigate the expression of GnIH and its effects on follicle development and steroidogenesis in quail ovaries under different photoperiods, 72 healthy laying quails of 8-wk-old were randomly divided into long day (LD) group [16 light (L): 8 dark (D)] (n = 36) and short day (SD) group (8L:16D) (n = 36). Samples were collected from each group on d1, d11, d22, and d36 of the experiment. The result showed that short day treatment upregulated the level of GnIH in the gonads (P < 0.05), decreased the expression level of CYP19A1,3β-HSD, StAR, LHR, and FSHR and increased the expression level of AMH, AMHR2, GDF9, and BMP15 to inhibit follicle development and ovulation, thus affecting the egg production performance of quails. In vitro culture of quail granulosa cells and treatment with different concentrations of GnIH (0, 1, 10, and 100 ng/mL) for 24 h. Result showed that GnIH inhibited the levels of FSHR, LHR, and steroid synthesis pathways in granulosa cells, upregulated the levels of AMHR2, GDF9, and BMP15. The results suggest that the inhibition of follicle development and reduced egg production in quail by short day treatment is due to GnIH acting at the gonadal level, and GnIH affected the steroid synthesis by inhibiting gonadotropin receptors.

Key words: GnIH, photoperiodism, follicle development, steroidogenesis, quail

INTRODUCTION

The photoperiod is an important regulator of seasonal reproduction in birds, and studies have shown that the photoperiod can act on the deep brain photoreceptor in hypothalamus to convert light signals into nerve impulses and then regulate reproductive activity through the hypothalamic-pituitary-gonadal reproductive axis (HPG) (Ikegami and Yoshimura, 2012). The HPG axis is a complete feedback system regulated by the central nervous system (Rose et al., 2022), mainly by gonadotropin-releasing hormone (GnRH) and gonadotropin-inhibiting hormone (GnIH) secreted by the hypothalamus to regulate the secretion of gonadotropins at the pituitary, which can influence the secretion of gonadotropins through the blood circulation, thereby promoting gonadal development and regulating animal reproduction (Tsutsui and Ubuka, 2020). Most studies have found that GnIH expression levels are negatively correlated with the reproductive status of animals under different photoperiods, with most of the alterations in photoperiod that result in the suppression or promotion of reproductive activity in birds, with GnIH levels increasing or decreasing (Ubuka et al., 2005; Bedecarrats et al., 2009; Dixit et al., 2017; Ouyang et al., 2021).

GnIH is a key inhibitory factor in the regulation of animal reproduction. In 2000, Tsutsui first isolated a novel neuropeptide with gonadotropin-releasing effects in the hypothalamus of Japanese quail (Tsutsui et al., 2000; Tsutsui, 2009). GnIH is a twelve amino acid residue short peptide neuropeptide hormone that acts by binding to its receptor (Yin et al., 2005; Tsutsui et al., 2018). Studies have confirmed that GnIH plays an important role in reproductive activity by inhibiting the synthesis and release of pituitary gonadotropins and suppressing gonadal development and activity in birds and mammals (Ubuka et al., 2006; Bentley et al., 2010; Tobari and Tsutsui, 2019). In addition to directly...
inhibiting pituitary gonadotropin synthesis and secretion, GnIH also acts directly on hypothalamic GnRH neurons through its receptors and inhibits GnRH synthesis and release (Bentley et al., 2006; Ubuka et al., 2008; Jadhao et al., 2017).

There are many studies on the mechanism of GnIH reproductive regulation, but almost of them focus on the hypothalamic-pituitary level, while there are very few studies related to the role of GnIH directly at the gonadal level (Bentley et al., 2008; Tsutsui et al., 2012; Tsutsui et al., 2015; Ullah et al., 2016). Many studies suggest that GnIH is also directly expressed in the gonads. The expression of GnIH and its receptors was detected in the testes of Eurasian marauders and was significantly lower in the breeding season than in the non-breeding season (Tsutsui et al., 2013); The expression of GnIH and its receptors in the chicken ovary decreases significantly before sexual maturity (Maddineni et al., 2008). Significant differences in the expression levels of GnIH receptors on follicular granulosa cells between pre-estrus and estrus in mice (Singh et al., 2011a). These studies suggest that GnIH/GnIHR can regulate follicle development or spermatogenesis directly at the gonadal level, but the molecular mechanisms involved are unclear and need to be further investigated.

So we investigated the expression of GnIH and its effect on follicle development and steroidogenesis in quail under different photoperiod, and the effect of GnIH on granulosa cell steroid synthesis and gonadotropin receptors was also investigated by culturing quail granulosa cells in vitro and adding exogenous GnIH peptide to demonstrate the mechanism of GnIH effect on follicular development.

**MATERIALS AND METHODS**

**Ethics Statement**

All operations in this study followed procedure no. 2021011118 approved by the Institutional Animal Care and Use Committee of Zhongkai University of Agriculture and Engineering.

**Animals and Light Schedules**

Seventy-two healthy laying female quails of 8-wk-old were selected and pre-fed for 15 d under 16 h light/8 h dark cycle (16L:8D), and randomly divided into 2 groups. The short day group (SD group, 8L:16D) were switched to the 8L:16D photoperiod for 5 wk while the long day group (LD group, 16L:8D) was maintained at 16L:8D. The experimental schedules are shown in Figure 1. During the experiment, quails were placed at a constant room temperature of 21°C ± 3°C, 50 to 60% relative humidity, fed with 30 g per day feed and water freely. Samples were taken on d1, d11, d22, and d36 of the experiment (n = 6), ovarian tissues (after removal of surface follicles >1 mm in diameter), yellow follicles (YF, ø > 3 mm), white follicles (WF, ø = 1 - 3mm) were collected and stored at −80°C. YF and WF were used for the quantitative real-time PCR (qRT-PCR), Ovary were used for the qRT-PCR and Western blot.

**Isolation and Culture of Granulosa Cells**

Yellow follicles (YFs, ø > 3 mm) were collected immediately from the ovaries of laying quails and placed in ice-cold phosphate-buffered saline (PBS, Gibco, Grand Island, NY) with 100 U/mL of penicillin or streptomycin (Gibco). After the yolk was drained out through an incision made in the follicular wall, the follicle was inverted and shaken in sterile PBS. Then the granulosa cells (GCs) were carefully separated from follicles, and treated with 0.2% collagenase II (Biosharp, China) at 37°C for 20 min, oscillated in a vortex pan every five minutes. An equal volume of M199 medium (M199, Gibco) containing 10% fetal bovine serum (FBS, Gibco, South America) was added to stop the digestion, and the cell suspension was filtered through a 70-μm cell strainer (JET BIOFIL). After centrifuging the filtrate at

Figure 1. Experimental design of this study. (A) All quails were pre-fed for 15 days under 16L:8D, and randomly divided into SD group and LD group. SD group were switched to the 8D:16L photoperiod for five weeks while LD group was maintained at 16L:8D. Samples were taken on d1, d11, d22, and d36 of the experiment. (B) granulosa cells were isolated from quails’ follicles and treated with different concentrations of GnIH (0, 1, 10, and 100 ng/mL) for 24 h.
1,000 rpm for 5 min at 4°C, it was resuspended in M199 medium containing 10% FBS and 100 U/mL of penicillin or streptomycin. Next, the cells were plated at a density of 5 x 10^5/mL cells in 6-well plates (Corning Costar) and cultured with 5% CO₂ at 39°C (Yu et al., 2017; Wu et al., 2019; Chen et al., 2021). When cells had reached confluence, the medium was replaced with M199 containing different concentrations of GnIH (0, 1, 10, and 100 ng/mL, GnIH: SIKPSAYLPLRF, Phoenix pharmaceuticals, inc., Beijing, China) for 24 h, each treatment was repeated three times. The granulosa cells were used for the qRT-PCR and Western blot.

**Real-Time Quantitative PCR**

Total RNA was extracted from the Ovaries, YF and WF with Trizol (Invitrogen, Foster City, CA), and the concentration was measured by absorbance at 260 nm using a spectrophotometer (260/280 ratio > 1.8). The PrimeScript™RT reagent kit with gDNA eraser (TAKARA, Japan) was used to synthesize complementary DNA (cDNA) for qRT-PCR. The primers for the qRT-PCR were designed according to the mRNA sequence of quail in GenBank (Table 1), and then synthesized by Sangon Biotech (Shanghai) Co., Ltd. qRT-PCR was performed in a 20 µL reaction volume using SYBR Green PCR Master Mix (Invitrogen) and 2.5 pmol primers using an ABI 7500 system (Applied Biosystems, Foster City, CA). The PCR procedure comprised pre-denaturation at 50°C for 2 min and then at 95°C for 10 min, all for one cycle; 95°C for 15 s and then the annealing temperature for 1 min, all for 40 cycles. Three replicates were undertaken for each sample. The results were analyzed using the 2^{-ΔΔCT} method. β-actin was used as an endogenous control, and sample of LD at d1 was used as calibrator.

**Western Blot Analysis**

Western blot was used to detect the protein levels. The protein from ovarian tissue and GCs were separated by electrophoresis. Transfer the protein to the PVDF membrane at 4°C. After blocking with 5% skimmed milk at room temperature for 2 h, the membranes were incubated with the primary antibody at 4°C overnight. The primary antibodies used were rabbit polyclonal StAR (Affinity Biosciences, China), CYP19A1 (HuAn Biotechnology Co., Ltd. China), 3β-HSD (Affinity Biosciences, China), GDF9 (Abcam, UK), BMP15 (Abcam), AMHR2 (R&D SYSTEMS, Shanghai, China), FSHR (Proteintech, China) and mouse monoclonal β-actin (Affinity Biosciences). After incubation with peroxidase-conjugated secondary antibodies (1:10,000) for 1 h at room temperature. The chemiluminescence images of the membranes were captured using a chemical luminescence immunity analyzer (Tanon, China) after incubation with an ECL kit (Biosharp, China). The western blot protein bands were analyzed in grayscale using the Image J software (https://imagej.en.softonic.com/).

**Statistical Analysis**

The vivo experimental data were analyzed by two-way ANOVA with time and photoperiod as variables, the vitro experimental data of qRT-PCR were analyzed

---

**Table 1. Primers for real-time quantitative PCR.**

| Gene   | Primer sequence (5’-3’)                              | Tm (°C) | PCR product(bp) |
|--------|------------------------------------------------------|---------|----------------|
| AMH    | F: ATCCAACACCACATCCCCACACT                          | 60      | 149            |
|        | R: AGAGTCTCATCATCTGGAAGGTCAA                        |         |                |
| AMHR2  | F: CTATTTGCCCCGGGATCC                               | 59      | 170            |
|        | R: CCTCATAGGCCAGACGGGAC                             |         |                |
| GDF9   | F: AGTACGCAAGGGTGACTGT                               | 59      | 184            |
|        | R: TTGTAAGTATGAGGCCCATC                             |         |                |
| BMP15  | F: CCGCTACAACCACGCATAT                              | 60      | 99             |
|        | R: GTGGTGGGCTAGGTTGAC                                |         |                |
| CYP19A1| F: ATGGAAGCATAACCTGGAAC                              | 60      | 118            |
|        | R: GGGGACTATGGCAACTC                                 |         |                |
| 3β-HSD | F: GTCAGCTCCTGTAACCC                                 | 60      | 128            |
|        | R: GGGGACTATGGCAACTC                                 |         |                |
| StAR   | F: AATGCTGATCGGGCCTACGC                              | 60      | 161            |
|        | R: TCCATTCTCTGTAAGGTTTCT                             |         |                |
| FSHR   | F: TGCTTTGCTCTGGAGGTTTCT                             | 59      | 119            |
|        | R: GTGAAAGCATACTGAGGTTTCT                            |         |                |
| LHR    | F: CCCAATGAAAGGCTCACA                                | 60      | 93             |
|        | R: CACTCTGCTGCTGGTTTCT                               |         |                |
| PRLR   | F: TGGTCTACAGGAAAGGACAGTTGAA                        | 60      | 110            |
|        | R: TGGTGGTCCATGGATTAGTGTTG                          |         |                |
| GnIH   | F: CCAGGAAATCTGGACAGGGAAT                           | 59      | 160            |
|        | R: TGACCAAGGCTTTCATCTGGTTGAA                        |         |                |
| GnIHR  | F: CTTGGACACTACCTGACGGAAG                           | 60      | 133            |
|        | R: GCAGATGACGGGAAAACCTCTC                           |         |                |
| β-actin| F: TGCGTGACATCAAGGAGAAC                             | 60      | 299            |
|        | R: TGCCAGGGTACATTTGAGGTA                            |         |                |
by one-way ANOVA and the western blot data were analyzed by t test using the Prism 7.0 software (Prism Software, Inc, San Diego, CA). All values were expressed as the mean ± SEM. Differences between means were considered significant at *P < 0.05* and for high significance at **P < 0.01**.

**RESULT**

**The Effect of Photoperiod on the Ovarian Development**

As showed in Figure 2, when quails were divided into different photoperiods, the egg laying rate was calculated, we found that the laying rate of LD group was maintained at 80% or even higher, but the laying rate of SD group gradually decreased, from the third week of the experiment, the laying rate of SD group was extremely significantly lower than that of LD group (**P < 0.0001**; Figure 2A). The egg production performance of birds depends on the development of their ovaries. During the experiment, we investigated the effect of different photoperiods on the development of follicles by dissecting and weighing the ovaries and counting the number of follicles at different stages of development. We found that the total ovarian weight (including ovarian tissues and follicles) of SD group was extremely significantly lower compared with LD group at d22 (**P < 0.001**) and d36 (**P < 0.01**; Figure 2B). By counting the follicles, it was found that after shortening the photoperiod, the number of YFs and WFs significantly decreased in the SD group, with a significant decrease in YFs d22 and d36 (**P < 0.001**; Figure 2C) and a significant decrease in WFs at d22 and d36 (**P < 0.0001**; Figure 2D).

**The Effects of Photoperiod on the Key Reproduction Regulatory Factors**

The expression levels of the key reproduction regulatory factors are illustrated in Figure 3. As observed, the relative expression of *GnIH* and *GnIHR* in YFs, WFs, and ovaries gradually increased as shortening the photoperiod, the expression levels of *GnIH* and *GnIHR* in YFs and ovarian in SD group were significantly higher than that of LD group at d22 (**P < 0.05**) and d36 (**P < 0.05 or 0.01**; Figures 3A, C, D, F), the difference of *GnIH* and *GnIHR* expression between LD and SD group were not significant in WFs (Figures 3B and 3E). The mRNA expression of *FSHR* in YFs in SD group were significantly or extremely significantly lower than that of LD group at d11 (**P < 0.01**) and d22 (**P < 0.05**; Figure 3G). While the relative expression of *FSHR* in ovaries gradually increased in SD group. The relative protein levels of FSHR in ovaries of SD group were significantly or extremely significantly higher than that of LD group at d22 (**P < 0.01**) and d36 (**P < 0.05**; Figure 6B). There was no significant change in the relative expression of *FSHR* of WFs between the 2 groups (Figure 3H). The expression levels of *LHR* in YFs in SD groups were extremely significantly declined at d22 (**P < 0.0001**) and...
The expression levels of LHR of WFs and ovaries and the expression levels of PRLR in YFs, WFs and ovaries were no difference between the LD group and SD group (Figures 3K−3O).

**Figure 3.** The effects of photoperiod on the key reproduction regulatory factors. Effect of photoperiod on the expression of GnIH, GnIHR, FSHR, LHR, and PRLR. Data are presented as $2^{-\Delta\Delta\text{CT}}$ method (normalized to housekeeping gene β-actin and sample of LD at d1 was used as calibrator.) Values are given as means ± s.e.m. a, b, and c indicates differences ($P < 0.05$) among the time. * indicates difference ($P < 0.05$) between the two groups, **, *** and **** indicate statistical significance based on $P < 0.05$, $P < 0.01$, and $P < 0.0001$, respectively.

**The Effects of Photoperiod on Steroid Biosynthesis Pathway**

The expression levels of the steroid biosynthesis pathway are presented in the Figure 4. As showed, compared with LD group, the relative expression of CYP19A1 in ovaries was significantly decreased in SD group at d22 and d36 ($P < 0.05$; Figure 3J). The expression levels of LHR of WFs and ovaries and the expression levels of PRLR in YFs, WFs and ovaries were no difference between the LD group and SD group (Figures 3K−3O). Also in YFs and WFs, the expression level of CYP19A1 significantly decreased in SD group at d36 ($P < 0.05$ or 0.0001). In SD group, the relative expression of 3β-HSD was significantly decreased in SYF at d22 ($P < 0.01$). The expression levels of StAR in YFs were significantly decreased at d22 ($P < 0.05$), but in WFs and ovaries, the expression levels of StAR were decreased in SD group when shorting the photoperiod, but there were no significant change between SD and LD group. In protein levels of ovaries, as showed in Figure 6, after shorting the photoperiod, the protein levels of CYP19A1 were significantly decreased in SD group at d11 ($P < 0.05$; Figure 6C), and the protein
levels of 3\(\beta\)-HSD were significantly decreased in SD group at d36 (\(P < 0.05\); Figure 6D). However, the relative protein levels of StAR in ovaries had no significant trend between the 2 groups (Figure 6E).

**The Effect of Photoperiod on TGF-B Family**

The expression levels of AMH, AMHR2, GDF9, and BMP15 were as showed in Figure 5. Relative expression levels of AMH in YFs were extremely significantly increased in SD group at d22 (\(P < 0.001\)) and d36 (\(P < 0.0001\)) (Figure 5A), and increased in ovaries in SD group at d22 and d36 although there was no significant difference between the 2 groups (Figure 5C). In WFs, the expression levels of AMH had no significant trend between the 2 groups. The relative expression of AMHR2 in YFs, WFs and ovaries had risen but there was no significant change between the 2 groups (Figures 5D–5F). In ovaries, the expression levels of GDF9 were significantly higher in SD group at d22 (\(P < 0.01\)) and d36 (\(P < 0.05\)) than that of LD group (Figure 5I). And in WFs, the expression levels of GDF9 were significantly increase in SD group at d22 (\(P < 0.05\); Figure 5H). However, the relative expression of BMP15 in YFs, WFs and ovaries had no significant trend between the 2 groups (Figures 5J–5L). The protein levels in ovaries were showed in Figure 6. While shorting the photoperiod, the protein levels of GDF9 had significant increase in SD group at d36 (\(P < 0.05\); Figure 6G).

**Effect of GnIH Treatment in Granulosa Cells**

In order to investigate whether the restricted follicular development of quail caused by altered photoperiod is associated with elevated levels of GnIH, we conducted in vitro experiments. Granulosa cells, the main functional cells of the follicle, were isolated and levels of reproduction-related factors and the TGF-\(\beta\) family were measured after the addition of different concentrations of GnIH.

As shown in Figure 7, GCs were treated with GnIH (0, 1, 10, 100 ng/mL) for 24 h and the gene expression was detected by qRT-PCR. The experimental results showed that compared with the control group, GnIH downregulated the relative expression of CYP19A1, 3\(\beta\)-HSD and StAR (Figure 7A) and upregulated the relative expression of AMHR2, GDF9, and BMP15 (Figure 7B). The relative expression of CYP19A1 showed a dose-dependent highly significant decrease (\(P < 0.01\) or \(P < 0.001\)), 10 and 100 ng/mL GnIH significantly downregulated the expression of 3\(\beta\)-HSD (\(P < 0.01\) or \(P < 0.05\)); 1 and 10 ng/mL GnIH significantly decreased the relative expression of LHR (\(P < 0.05\)); although the expression of FSHR decreased, there was no significant difference between the treatment group and the control group. There was no significant trend in the expression of AMH after GnIH treatment, but the relative expression of AMHR2 was significantly higher after GnIH.
treatment than in the control group ($P < 0.05$); GnIH increased the relative expression of $GDF9$ and $BMP15$, with 100 ng/mL GnIH significantly upregulated the relative expression of $GDF9$ ($P < 0.05$); 10 and 100 ng/mL GnIH significantly increased the relative expression of $BMP15$ ($P < 0.05$). In the protein levels, 10 ng/mL GnIH downregulated FSHR, CYP19A1 and 3β-HSD levels and upregulated AMHR2 and BMP15 levels (Figures 7C and 7D), with significant downregulation of CYP19A1 and 3β-HSD levels ($P < 0.01$ or $P < 0.05$), while 10ng/mL GnIH treatment had insignificant effects on StAR and GDF9.

**DISCUSSION**

Photoperiod is a key environmental factor affecting reproductive activity. In this study, the shortening of photoperiods in laying quails led to a reduction in egg production, a reduction in ovarian weight and a reduction in the number of follicles. The results are similar to previous studies where short day treatment of birds also led to a reduction in egg production and follicle numbers (Geng et al., 2018; Cui et al., 2021; Ouyang et al., 2021). The egg production performance of birds is mainly determined by the number of follicles that develop to ovulation. In this experiment, a shortened photoperiod led to a reduction in the number of follicles in quail, with WFs decreasing significantly by d11 and YFs only starting to decrease significantly at d22 and d36. Similar results were found in ducks, with the number of WFs varying most significantly between photoperiods (Cui et al., 2021), suggesting that photoperiodic effects on follicle development may take the lead in affecting the development of small follicles, resulting in small follicles failing to develop into large follicles and thus affecting the egg production performance of female birds.

Studies have shown that the relative expression of GnIH is photoperiodic (Dixit et al., 2020), the relative expression of GnIH and GnIHR in the follicles and ovaries of quail under different photoperiodic treatments
was measured by qRT-PCR, we found that the expression levels of GnIH/GnIHR in the gonads were also photoperiodic and showed an opposite trend to the development of the gonads. Studies have shown that changes in photoperiod affect changes in melatonin and that GnIH neurons express melatonin receptors, suggesting that photoperiod-induced changes in hypothalamic GnIH levels may be partially regulated by melatonin (Ubuka et al., 2005; Chowdhury et al., 2013; Soni et al., 2021). Also, melatonin can bind to its receptors at the gonadal level and regulate the expression and action of the GnIH system in the gonads (Mcguire et al., 2011). The short day photoperiods promotes melatonin secretion and melatonin receptors were found to be expressed in the gonads of birds (Sundaresan et al., 2009; Li et al., 2015). It was suggested that the increased levels of GnIH/GnIHR in the gonads could have been caused by a shortening of the photoperiod that resulted in elevated melatonin levels, but the mechanism of regulation needs to be further investigated.

Previous studies have found that when reproductive activity is inhibited in birds, it is mostly accompanied by elevated levels of GnIH/GnIHR in the hypothalamus-pituitary (Dixit et al., 2017; Zhu et al., 2017), in the present experiment, when the reproductive activity of quails were inhibited, the expression levels of GnIH/GnIHR were increased in follicles and ovaries, suggesting that GnIH/GnIHR in the gonads also played an inhibitory role follicular development. Follicle development requires gonadotropin stimulation, the pituitary gonadotropin secretion needs to bind to receptors on the gonads in order to regulate gonadal development. Follicle development requires gonadotropin stimulation, the pituitary gonadotropin secretion needs to bind to receptors on the gonads in order to regulate gonadal development (Simoni et al., 1997; Proszkowiec and Rzasa, 2001; Ghanem and Johnson, 2019). GnIH at the hypothalamic level plays an important inhibitory role, regulating ovarian function either directly by suppressing pituitary gonadotropin secretion or indirectly by suppressing pituitary gonadotropin secretion via hypothalamic GnRH. Thus, it is possible that gonadal secretion of GnIH may play an important regulatory role in the expression of gonadotropic receptors, thereby influencing the regulatory function of gonadotropins through binding to term receptors. In the present study, shortening the photoperiod resulted in an increase in the level of GnIH levels in

Figure 6. Effect of photoperiod on protein levels in ovary. Effect of photoperiod on protein levels of FSHR, CYP19A1, 3β-HSD, StAR, AMHR2, GDF9 and BMP15 in ovaries. Values are given as means ± s.e.m. * indicate statistical significance based on \( P < 0.05 \). a, b, and c indicate differences \( (P < 0.05) \) among the time. * indicates difference \( (P < 0.05) \) between the two groups.
the follicles and ovaries, with corresponding changes in FSHR and LHR levels found, and confirmed that GnIH can influence gonadotropin receptor expression at the gonadal level. Our study is similar to previous findings that high levels of FSHR and LHR accompany high levels of reproductive activity or egg production in birds (Liu and Zhang, 2008; Chen et al., 2020). FSHR and LHR regulate follicular growth and development and stimulate steroid hormone synthesis to induce ovulation and thus increase egg production. Therefore, inadequate expression of gonadotropic receptors at the gonadal level also severely diminishes the gonadotropin-promoting effect on gonadal function, affected the growth and development of follicles and ovulation, which ultimately led to lower egg production in the SD group. In this experiment, FSHR expression levels on WFs and YFs were significantly higher in the LD group than that in the SD group, the reason being that increased GnIH expression in follicles and ovaries under SD conditions suppressed FSHR expression, which interacted with reduced the FSH of pituitary to inhibit follicle development. Previous studies have found that only the binding of FSHR to FSH on GCs of follicles entering the hierarchical follicular sequence can give GCs the ability to synthesize the relevant hormones to further promote follicular growth and development (Kim and Johnson, 2018). In contrast, in pre-graded follicles, each follicle does not grow and develop at the same rate, the faster growing follicles are more sensitive to FSH, have a lower FSH threshold, and the FSHR on the faster growing follicles receives the signal from FSH and is more likely to respond to the signal, further promoting follicle development (Holesh et al., 2022). In our study, the ovarian tissue after removal of the developing follicle, short day treatment increased the FSHR levels, the reason for this may be that short day treatment slowed follicle development, allowing a large number of small and primordial follicles to accumulate in the ovarian stroma and stall development. These stalled follicles are therefore less sensitive to FSH and respond slowly to FSH. At the same time, SD treatment leads to an increase in AMH levels and it was found that AMH signaling leads to a basic absence of FSH signaling in small follicles, further affecting follicle growth and development (Huang et al., 2021). Also, these primary follicles also express FSHR to await the action of FSH to develop, with far more small follicles accumulating in the SD-treated ovaries than in the LD-treated ones, so that the total amount of FSHR detected in the SD-treated ones was greater than in the LD-treated ones. It was also found that even high expression of FSHR in small follicles failed to activate the cAMP signaling pathway to promote follicle development (Tilly et al., 1991; Johnson, 2015).

When the levels of FSHR and LHR at the gonadal level were reduced, we found that the efficiency of the
steroid production pathway was also affected. We examined key factors in the steroid synthesis pathway and found that shortening the photoperiod caused changes in the expression of these key factors. Shortening the photoperiod significantly downregulated CYP19A1 and 3β-HSD levels, and STAR levels were reduced but not significantly different between the two groups. In addition, in a study related to GnIH, it was found that GnIH can affect follicle development by inhibiting the synthesis of steroid hormones (Tsutsui and Ubuka, 2021), so we hypothesized that the shortening of photoperiod inhibited follicle development by upregulating the levels of GnIH, which inhibits the steroid synthesis pathway, mainly by downregulating the levels of CYP19A1 and 3β-HSD, leading to a blockage of the estrogen synthesis process. As STAR is also associated with cholesterol synthesis, it may be regulated by various factors, but the exact mechanism needs to be further investigated. To further validate the important role of GnIH at the gonadal level in the regulation of gonadotropin receptor expression, we conducted in vitro cellular experiments. Isolating quail granulosa cells in vitro and adding GnIH treatment, we found a significant decrease in key factors of the steroid synthesis pathway, and previous studies showed that GnIH treatment dose-dependently inhibited the levels of 3β-HSD, CYP19A1 and STAR in duck granulosa cells (Chen et al., 2021), similar results were found in mammalian studies, where GnIH treatment dose-dependently downregulated the expression levels of 3β-HSD and STAR to inhibit steroid hormone synthesis (Singh et al., 2011b). At the same time, GnIH treatment significantly down-regulated LHR expression levels, indicating a direct inhibitory effect of GnIH on follicle development and ovulation, further verifying that the effect of photoperiod alteration on follicle development and ovulation in vivo was caused by changes in GnIH expression levels.

The TGF-β family is also an important regulator in the process of follicular development, of which AMH is a regulator of follicular growth and development (Durlinger et al., 2002). AMH can exert a biological effect by binding to the specific receptor AMHR2 on granulosa cells, inhibiting the recruitment of primordial follicles and thus limiting follicular development (Johnson and Woods, 2009; Nilsson et al., 2011). High AMH levels can lead to restricted follicle development, preventing them from moving beyond the small precursor stage and ovulating regularly (Garg and Tal, 2016). The elevated AMH expression in ovaries and YFs after shortening the photoperiod in this experiment suggests that the photoperiod change mainly affects the levels of AMH in YFs and ovaries, leading to stalled follicle development. This result is consistent with the findings in Siberian rats and male quail, when shortened photoperiods elevated AMH levels in the ovaries of Siberian rats, stalling follicle development in the ovaries and limiting follicle development (Shahed and Young, 2013); short photoperiods elevated AMH and AMHR2 levels in the testes of male quail thereby inhibiting testicular development (Otake and Park, 2016). Studies in different species have shown that GDF9 and BMP15, which also belong to the TGF-β superfamily, also play an important regulatory role in follicle development and are important paracrine factors in the ovary. GDF9 inhibits progesterone synthesis (Hickey et al., 2005; Spicer et al., 2008) and chicken estradiol secretion, while BMP15 significantly inhibits gonadotropin-induced progesterone synthesis (Elis et al., 2007). Shortening the photoperiod in this study increased the protein levels of GDF9 and BMP15, inhibiting the secretion of progesterone and estradiol in the ovaries and follicles of quail, which in turn affected follicle development. It has been shown that GDF9 and BMP15 can also synergistically increase AMH levels (Pierre et al., 2016; Convisser et al., 2017), but the changes in AMH levels are photoperiodic in nature (Otake and Park, 2016). Therefore, it was not possible to confirm whether the elevated AMH levels in this experiment were caused by photoperiodic changes or by changes in the expression of GDF9 and BMP15 or GnIH, while the exact mechanism of action of which is still unclear and needs to be further investigated. However, in vitro cellular experiments, GnIH treatment had no significant effect on AMH expression levels, but upregulated the expression of its receptor AMHR2, we hypothesize that GnIH primarily affects hormone receptor expression levels at the gonadal level. GnIH also increased the expression levels of GDF9 and BMP15 in granulosa cells, suggesting that GnIH has an inhibitory effect on follicle growth. Thus, in the in vivo assay, the significant increase in AMH expression in the SD group was caused by photoperiodic changes and may not by GnIH, and the exact mechanism of action of photoperiodic effects on AMH levels still needs to be further investigated.

In summary, the altered photoperiod affected the expression level of GnIH in the gonads. Short day treatment upregulated the level of GnIH in follicles and ovaries, decreased the levels of FSHR and LHR and the expression level of key factors in the steroid synthesis pathway, increased the expression level of follicle growth inhibitory factors to inhibit follicle growth and ovulation, thus affecting the egg production performance of quail. At the same time, the direct inhibitory effect of GnIH on follicle development and ovulation was further verified by the vitro experiments, confirming that the impaired follicle development in quail was caused by elevated GnIH levels.

ACKNOWLEDGMENTS

Thanks to the site and equipment support of the Guangdong Province Key Laboratory of Waterfowl Healthy Breeding for their help.

Author contributions: YM.H, X.S, XL.Z, DL.J, JQ.P, DN.X and YB.T conceived the idea and designed the experiments, XL.Z, ZS.Z and YM.H wrote the manuscript, XL.Z, ZS.Z and DL.J performed experiments and data analysis, XL.Z, ZS.Z, DL.J and JQ.P help to
collection the samples. All authors read and approved the final manuscript.

This work was supported by National Natural Science Foundation of China (32072730), Guangdong Province Universities and Colleges Pearl River Scholar Funded Scheme (2018) and Major Fundamental Research Project of Educational Department of Guangdong Province (2018KZDXM039).

DISCLOSURES

The authors declare that they have no competing interests.

REFERENCES

Bedecarrats, G. Y., H. McFarlane, S. R. Maddineni, and R. Ramachandran. 2009. Gonadotropin-inhibitory hormone receptor signaling and its impact on reproduction in chickens. Gen. Comp. Endocrinol. 163:7–11.

Bentley, G. E., K. Tsutsui, and L. J. Kriegsfeld. 2010. Recent studies of gonadotropin-inhibitory hormone (GnIH) in the mammalian hypothalamus: pituitary and gonad. Brain Res. 1364:62–71.

Bentley, G. E., L. J. Kriegsfeld, T. Ougi, K. Ukena, S. O’Brien, N. Perfilieva, I. T. Moore, K. Tsutsui, and J. C. Wingfield. 2006. Interactions of gonadotropin-releasing hormone (GnRH) and gonadotropin-inhibitory hormone (GnIH) in birds and mammals. J. Exp. Zool. A Comp. Exp. Biol. 305:807–814.

Bentley, G. E., T. Ubuka, N. L. McGuire, V. S. Chowdhury, Y. Morita, T. Yano, I. Hasunuma, M. Binns, J. C. Wingfield, and K. Tsutsui. 2008. Gonadotropin-inhibitory hormone and its receptor in the avian reproductive system. Gen. Comp. Endocrinol. 156:34–43.

Chen, S., W. Liu, C. Yang, X. Li, X. Shen, D. Jiang, Y. Huang, and Y. Tian. 2021. Gonadotropin inhibitory hormone downregulates steroid hormone secretion and genes expressions in duck granulosa cells. Anim. Reprod. 18:e20210036.

Chen, W., W. G. Xia, D. Ruan, S. Wang, K. Abouelezz, S. L. Wang, Y. N. Zhang, and C. T. Zheng. 2020. Dietary calcium deficiency suppresses follicle selection in laying ducks through mechanism involving cyclic adenosine monophosphate-mediated signaling pathway. Animal 14:2100–2108.

Chowdhiury, V. S., T. Ubuka, and K. Tsutsui. 2013. Review: Melatonin stimulates the synthesis and release of gonadotropin-inhibitory hormone in birds. Gen. Comp. Endocrinol. 181:175–178.

Convissar, S., M. Armouti, M. A. Fierro, N. J. Winston, H. Scoccia, and J. A. Grootegoed, and A. P. Themmen. 2002. Anti-Mullerian hormone inhibits initiation of primordial follicle growth in the mouse ovary. J. Clin. Endocrinol. Metab. 101:2602–2611.

Dixit, A. S., N. S. Singh, and S. Byrsat. 2017. Role of GnIH in photoperiodic regulation of seasonal reproduction in the Eurasian tree sparrow. J. Exp. Biol. 220(Pt 20):3742–3750.

Dixit, A. S., S. Byrsat, and N. S. Singh. 2020. Circadian rhythm in photoperiodic expression of GnRH-I and GnIH regulating seasonal reproduction in the Eurasian tree sparrow, Passer montanus. J. Photochem. Photobiol. B 211:11993.

Durlinger, A. L., M. J. Grujters, P. Kramer, B. Karle, B. A. Ingraham, M. W. Nachtigal, J. T. Uilenbroek, J. A. Grooteveld, and A. P. Themmen. 2002. Anti-Mullerian hormone inhibits initiation of primordial follicle growth in the mouse ovary. Endocrinology 143:1076–1084.

Elis, S., J. Dupont, I. Couty, L. Persani, M. Govoroum, E. Blesbois, F. Batellic, and P. Monget. 2007. Expression and biological effects of bone morphogenetic protein-15 in the hen ovary. J. Endocrinol. 194:485–497.

Garg, D., and R. Tal. 2016. The role of AMH in the pathophysiology of polycystic ovarian syndrome. Reprod. Biomed. Online 33:15–28.

Geng, A. L., Y. Zhang, J. Zhang, H. H. Wang, Q. Chu, and H. G. Liu. 2018. Effects of lighting pattern and photoperiod on egg production and egg quality of a native chicken under free-range condition. Poult. Sci. 97:2378–2384.

Ghanem, K., and A. L. Johnson. 2019. Response of hen pre-recruitment ovarian follicles to follicle stimulating hormone, in vivo. Gen. Comp. Endocrinol. 270:41–47.

Hickey, T. E., D. L. Marraco, F. Amato, L. J. Ritter, R. J. Norman, T. B. Gillrist, and D. T. Armstrong. 2005. Androgens augment the mitogenic effects of oocyte-secreted factors and growth differentiation factor 9 on porcine granulosa cells. Biol. Reprod. 73:825–832.

Holesh, J. E., A. N. Bass, and M. Lord. 2022 May. Physiology, Ovulation, 8 (p. 28723025), Treasure Island, FL: StatPears, 28723025.

Huang, S. J., L. Purevsuren, F. Jin, Y. P. Zhang, C. Y. Liang, M. Q. Zhu, F. Wang, and C. L. Jia. 2021. Effect of anti-mullerian hormone on the development and selection of ovarian follicle in hens [e-pub ahead of print]. Poult. Sci. 100, doi: 10.1016/j.psj.2020.12.056, accessed October 24, 2022.

Iregami, K., and T. Yoshimura. 2012. Circadian clocks and the measurement of daylength in seasonal reproduction. Mol. Cell. Endocrinol. 349:76–81.

Jadhao, A. G., C. Pinelli, B. D’Aniello, and K. Tsutsui. 2017. Gonadotropin-inhibitory hormone (GnIH) in the amphibian brain and its relationship with the gonadotropin releasing hormone (GnRH) system: an overview. Gen. Comp. Endocrinol. 240:69–76.

Johnson, A. L. 2015. Ovarian follicle selection and granulosa cell differentiation. Poult. Sci. 94:781–785.

Johnson, A. L., and C. D. Woods. 2009. Dynamics of avian ovarian follicle development: cellular mechanisms of granulosa cell differentiation. Gen. Comp. Endocrinol. 163:12–17.

Kim, D., and A. L. Johnson. 2018. Differentiation of the granulosa layer from pre-hierarchical follicles associated with follicle-stimulating hormone receptor signaling. Mol. Reprod. Dev. 85:729–737.

Li, D. Y., N. Wu, J. B. Tu, Y. D. Hu, M. Y. Yang, H. D. Yin, B. L. Chen, H. L. Xu, Y. F. Yao, and Q. Zhu. 2015. Expression patterns of melatonin receptors in chicken ovarian follicles affected by monochromatic light. Genet. Mol. Res. 14:10072–10080.

Liu, H. Y., and C. Q. Zhang. 2008. Effects of daidzein on messenger ribonucleic Acid expression of gonadotropin receptors in chicken ovarian follicles. Poult. Sci. 87:541–545.

Maddineni, S. R., O. M. Ocon-Grove, S. M. Krzysik-Walker, G. R. Hindricks, and R. Ramachandran. 2008. Gonadotropin-inhibitory hormone (GnIH) receptor gene is expressed in the chicken ovary: potential role of GnIH in follicular maturation. Reproduction 135:267–274.

McGuire, N. L., K. Kangas, and G. E. Bentley. 2011. Effects of melatonin on peripheral reproductive function: regulation of testicular GnIH and testosterone. Endocrinology 152:3461–3470.

Ouyang, H., B. Yang, Y. Lao, J. Tang, Y. Tian, and Y. Huang. 2021. Photoperiod affects the laying performance of the mountain duck by regulating endocrine hormones and gene expression. Vet. Med. Sci. 7:1899–1906.

Pierre, A., A. Estienne, C. Racine, J. Y. Picard, R. Fanchin, B. Lahoz, J. L. Alabart, J. Folch, P. Jarrier, S. Fabre, D. Monmaux, and M. di Clemente. 2016. The bone morphogenetic protein 15 up-regulates the anti-Mullerian hormone receptor expression in granulosa cells. J. Clin. Endocrinol. Metab. 101:2602–2611.

Proszkiewicz, M., and J. Rzasa. 2001. Variation in the ovarian and plasma progesterone and estradiol levels of the domestic hen during a pause in laying. Folia. Biol. (Kraków) 49:285–289.

Rose, E. M., C. M. Haakenson, and G. F. Ball. 2022. Sex differences in seasonal brain plasticity and the neuroendocrine regulation of vocal behavior in songbirds. Horm. Behav. 142:105160.
Shahed, A., and K. A. Young. 2013. Anti-Mullerian hormone (AMH), inhibin-alpha, growth differentiation factor 9 (GDF9), and bone morphogenetic protein-15 (BMP15) mRNA and protein are influenced by photoperiod-induced ovarian regression and recrudescence in Siberian hamster ovaries. Mol. Reprod. Dev. 80:895–907.

Simoni, M., J. Gromoll, and E. Nieschlag. 1997. The follicle-stimulating hormone receptor: biochemistry, molecular biology, physiology, and pathophysiology. Endocr. Rev. 18:739–773.

Singh, P., A. Krishna, R. Sriskandam, and K. Tsutsui. 2011a. Immunohistochemical localization of GnIH and RFamide-related peptide-3 in the ovaries of mice during the estrous cycle. J. Mol. Histol. 42:371–381.

Singh, P., A. Krishna, and K. Tsutsui. 2011b. Effects of gonadotropin-inhibitory hormone on folliculogenesis and steroidogenesis of cyclic mice. Fertil. Steril. 95:1397–1404.

Soni, R., C. Haldar, and C. C. Mohini. 2021. Retinal and extra-retinal photoreceptor responses and reproductive performance of Japanese quail (Coturnix coturnix japonica) following exposure to different photoperiodic regime. Gen. Comp. Endocrinol. 302:113667.

Spicer, L. J., P. Y. Aad, D. T. Allen, S. Mazerbourg, A. H. Payne, and M. Shahab. 2016. Expression and actions of GnIH and its orthologs in vertebrates: current status and advanced knowledge. Neuropeptides 59:9–20.

Wu, Y., H. Xiao, J. Pi, H. Zhang, A. Pan, Y. Pu, Z. Liang, J. Shen, and J. Du. 2019. EGFR promotes the proliferation of quail follicular granulosa cells through the MAPK/extracellular signal-regulated kinase (ERK) signaling pathway. Cell Cycle 18:2742–2756.

Yu, G. M., N. Isobe, and T. Maeda. 2017. Protective effect of melatonin on LPS-stimulated granulosa cells in Japanese quail (Coturnix japonica): identification, expression and binding activity. J. Endocrinol. 184:257–266.

Zhu, H., X. Shao, Z. Chen, C. Wei, M. Lei, S. Ying, J. Yu, and Z. Shi. 2017. Induction of out-of-season egg laying by artificial photoperiod in Yangzhou geese and the associated endocrine and molecular regulation mechanisms. Anim. Reprod. Sci. 180:127–136.