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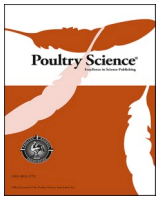

Full-Length Article

Long-24-h ahemeral light cycle improved eggshell quality of hens in late laying period

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ABSTRACT

Previous study indicated that long-24-h ahemeral light cycle could ameliorate the deterioration of eggshell quality, a significant issue in the later stages of egg production; nevertheless, the underlying process remains unclear. This study explored the mechanism of the long-24-h ahemeral light cycle improving eggshell quality of hens in late laying stage. Two sets of 260 Hy-Line brown laying hens, aged 74 weeks, were randomized, with 65 chickens in each group. One was treated to a standard light cycle (24 h, 16L:8D), whereas the other was subjected to a long-24 h ahemeral light cycle (28 h, 16L:12D). The experimental cycle was 12 weeks. The results indicated that the long-24 h ahemeral light cycle did not influence laying performance ($P > 0.05$); however, eggshell strength and thickness was dramatically enhanced ($P < 0.05$). The serum levels of $1,25(OH)_2D_3$ and ALP exhibited comparable changes, whereas PTH, PCT, and OT displayed contrasting variations between the two groups. Transcriptome sequencing revealed 889 common genes exhibiting rhythmic expression in both groups, with an average phase advance of 7.41 h. In comparison to the 16L:8D group, 97.41 % of genes exhibited phase advancement in the 16L:12D group. Based on the findings of the functional analysis, we identified four genes associated with bone metabolism and examined their expression patterns: *CTNNB1, BMPR2, BMP7, and PDIA3*. The expression trend of *BMPR2* gene was similar to that of serum $1,25(OH)_2D_3$, and the expression trend of *BMP7* gene was similar to that of serum OT. The mRNA expression patterns of *PDIA3* and *BMP7* were inversely related, whereas *CTNNB1* and *BMPR2* exhibited comparable trends. The mRNA expression of *CTNNB1, PDIA3*, and *BMP7* correlated with eggshell formation. The mRNA expression of *BMPR2* significantly elevated during eggshell formation in the 16L:8D group, whereas it remained low during the daylight in comparison to darkness in the 16L:12D group. In summary, the change of light cycle affects the biological rhythm of laying hens, and we found four genes related to bone metabolism, *CTNNB1, BMPR2, BMP7 and PDIA3*, which specifically responded to the long-24 h ahemeral light cycle and possibly participated in the improvement of eggshell quality.

Introduction

In recent years, the poultry industry has suggested extending the culling age of layers from the conventional 72 weeks to 80 weeks, or even 100 weeks (Bain et al., 2016; Fu et al., 2022). Nonetheless, the quality of eggshells necessarily diminishes progressively with the advancing age of laying hens on a weekly basis (Bell and Weaver, 2002). The ratio of broken eggs and eggshell strength during the late laying period increased by 20 % and fell by 8-25 %, respectively, compared to the peak time, significantly impacting economic advantages (Gu et al., 2021; Nys et al., 2004). Consequently, methods are required to be implemented to address the issue of eggshell quality in subsequent laying.

Light, a significant environmental component, has a vital role in numerous avian behaviors, including incubation, development, and rest (AI-Samrai et al., 2023; Geng et al., 2014; Kristensen et al., 2006; Yin

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Table 1
The distribution of light time of 16L:12D group in a week.

Item	Light time
Mon	7:00-23:00
Tue	11:00-3:00
Wen	15:00-7:00
Thr	19:00-11:00
Fri	23:00-15:00
Sat	
Sun	3:00-19:00

Table 2
The primer sequences.

Gene	Product size (bp)	Primer	Sequence (5′−3′)
PER2	115	F	CCAACTTTGTTGTGTGCTCCTTGC
		R	CTGGAACAAACAGGTTGGTGTGTG
CLOCK	121	F	GCTTCCAGGTAATGCTCGGAAG
		R	CCAGTCCTGTCGAATCTCACTGG
WDR72	171	F	TAGCGTAGTGTGCTGCGTGTG
		R	TCGTTCCAGTGTGCCTGTTTCA
GAPDH	153	F	AGGACCAGGTTGTCTCCTGT
		R	CCATCAAGTCCACAACACGG

Table 3
Impact of the long-24-h ahemeral light on egg production in laying hens aged 74 to 85 weeks.

Item/Group	16L:12D	16L:8D	SEM	*P*
BW, kg	1.83	1.87	0.02	0.36
Laying rate, %	66.76	69.69	1.47	0.19
Hen-day quality eggs, n	53.25	57.24	2.27	0.28
Hen-day eggs, n	56.52	58.54	0.98	0.16
FER[1]	3.05	3.15	0.11	0.65
Broken egg, %	4.40	5.15	0.94	0.67
Abnormal egg, %	1.04	0.57	0.35	0.47
Soft-shelled egg, %	0.33	0.19	0.08	0.32

[1]FER: Feed-to-egg ratios.

Table 4
Impact of the long-24-h ahemeral light cycle on the egg quality of laying hens at 80 weeks.

Item/Group	16L:12D	16L:8D	SEM	*P*
Shape index	1.33	1.33	0.01	0.54
Egg weight, g	61.69	58.65	1.31	0.06
Eggshell weight, g	5.76[a]	5.17[b]	0.25	0.02
Yolk weight, g	17.20[a]	16.00[b]	0.50	0.02
Albumen weight, g	38.73	37.48	0.65	0.21
Eggshell percentage, %	9.35	8.83	0.24	0.11
Yolk percentage, %	27.97	27.36	0.32	0.22
Albumen percentage, %	62.69	63.81	0.55	0.16
Eggshell thickness, mm	0.34[a]	0.32[b]	0.01	0.03
Eggshell strength, N	35.52[a]	30.56[b]	2.06	0.02

[ab] Indicates that values in the same row without a shared superscript differ significantly ($P < 0.05$).

et al., 2024). Furthermore, it can influence the production of circadian clock genes by acting as a regulator of biological rhythms, so impacting the biological functions of animals. The rhythmic fluctuations of *PER2* can expedite the ovulation process in laying hens (Zhang, 2016); the rhythmic variations of *PTGFR, AANAT*, and *SPP1* and *CA2* genes are associated with egg production process (Li, 2019), feeding process, and eggshell formation process (Zhang et al., 2022), respectively. The rhythm study is more appropriate for examining biological processes and may aid in understanding the mechanism of eggshell formation.

Research indicates that an ahemeral light cycle as a biological rhythm can enhance eggshell quality. Siopes and Neely (1997) observed that as the photoperiod extended from the normal duration to 28 h, eggshell thickness increased linearly; however, it drastically reduced beyond 28.5 h. Nordstrom and Ousterhout (1983) demonstrated that the quality of eggshells at 72 weeks might be enhanced by employing a 26-h ahemeral light cycle. Leeson et al. (1979) demonstrated that the eggshell was 6 to 10 % thicker when laid under a 27 to 28-h light cycle compared to the typical conditions. Our prior research indicated that a 28-h light cycle could enhance eggshell thickness, strength, and weight by 9.38 %, 13.90 %, and 13.28 %, respectively. Nevertheless, scant information has been disclosed regarding the chemical mechanism behind this behavior.

This study aims to investigate the effects of long-24-h ahemeral light cycle on eggshell quality, laying performance, hormone circadian expression related to bone and calcium metabolism, and gene rhythm expression in 74-85-week-old laying hens.

Materials and methods

Ethics statement

The utilization of research animals and the associated care methods adhered to national and institutional requirements, as approved by the Hebei Agriculture University Animal Care and Use Committee (Number: 2022118).

Experimental design and birds

This experiment utilized a single-factor design, comprising two treatments executed in distinct rooms (replicates), with complete independence among all rooms. Two groups of 260 Hy-Line brown laying hens, each including 65 hens, were randomly allocated to individual rooms at 73 weeks of age. The pre-feeding period is one week. One group was treated to a standard light cycle (24 h, 16L:8D), whereas the other was subjected to a long-24-h ahemeral light cycle (28 h, 16L:12D). The 16L:8D group turns on the light at 7:00 and turns off the light at 23:00 every day. The switching time in 16L: 12D group is not regular, and the details was clarified by Table 1. Rooms had independent temperature controls and were held at 21°C for the duration of the study. The instrument and the actual temperature were examined at regular intervals to make sure that the temperatures were in normal limits and there was no significant difference between replicates (rooms). Water was available for unrestricted use during the trial period. The avians were provided a commercial meal consisting of corn and soybeans, with calcium and CP contents of 4.20 % and 14.0 %, respectively.

Production performance

The fundamental metrics of production performance in two groups from weeks 74 to 85, encompassing egg weight, hen-day eggs and hen-day quality eggs, and feed consumption, were evaluated daily (six times per week for the 16L:12D group). After the experiment, the feed-to-egg ratios (FER) were computed. Defective eggs, including soft-shelled, broken, and abnormal eggs, were classified, and their percentages were computed relative to the total egg number.

Egg quality

At the 80th week, 50 eggs were collected from each replicate for the evaluation of egg quality. Previous studies have shown that the laying rhythm can be stabilized after 3 weeks of light stimulation (Liu et al., 2024). The dimensions of the egg, specifically length and width, were assessed using an egg shape determinator (FHK, Fujihira Industry Co., Ltd., Tokyo, Japan). The digital scale (Huachaoice Electrical Appliances, Shanghai, China) was utilized to measure the weights of the yolk, eggshell, egg, and albumen. The eggshell thickness was measured using a digital vernier calipers (MNT-LG11-121-1, Guangzhou Lige

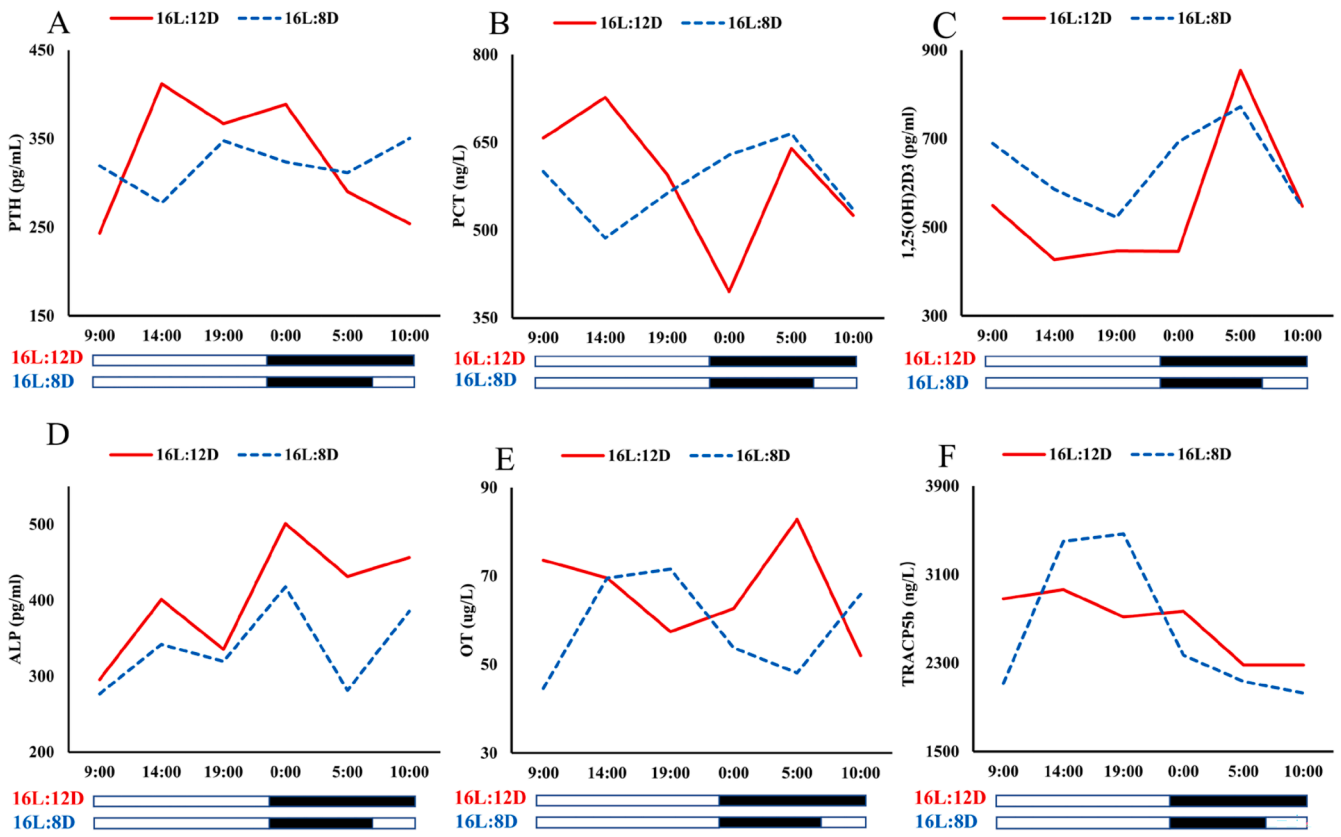

Fig. 1. Effect of the long-24-h ahemeral light cycle on serum bone and calcium metabolism markers at 85 weeks. The continuous red line denotes group 16L:12D, while the dashed blue line signifies group 16L:8D. The white boxes illustrated beneath the illustration signify periods of light illumination, whilst the black boxes denote periods of darkness.

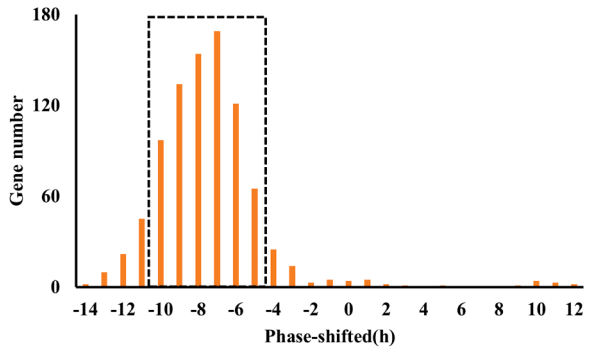

Fig. 2. Variation in peak phase among 889 prevalent circadian rhythm genes. The x-axis denotes the phase offset difference, while the y-axis indicates the quantity of genes.

Technology Co., Ltd., Guangzhou, China). The strength of eggshell was measured using the Egg Force Reader (ESTG-01, ORKA, Israel).

Hematological parameters

Zeitgeber time (ZT) denotes the standardized temporal reference in the light-dark cycle. When illuminated, it is documented as ZT0 (07:00 Beijing Time). On the basis of previous rhythm research mainly in 4-6 h (Du et al., 2024; Whittaker et al., 2023; Wolff et al., 2023; Zhang et al., 2023), this study selected 5 h. Meanwhile, because of the poor consistency of the chicken flock during the light on period, we selected ZT2 as the first sampling time point.

At 85 weeks, 12 hens were randomly selected from each group exhibiting a consistent oviposition pattern and collected blood samples every 5 h (ZT2, 7, 12, 17, 22, and 27). Commercial ELISA kits from Horabio Biotechnology Co., Ltd. in Shanghai, China, were utilized to quantify serum levels of parathyroid hormone (**PTH**), procalcitonin (**PCT**), 1,25-hydroxy vitamin D3 (**1,25(OH)$_2$D$_3$**), alkaline phosphatase (**ALP**), osteocalcin (**OT**), and tartrate-resistant acid phosphatase 5b (**TRACP5b**).

Transcript profiling

The Invitrogen Trizol Reagent (15596018, Ambion, Thermo Fisher, US) was utilized to extract total RNA from uterine tissues. The 2 % agarose gel electrophoresis and the A260/280 absorbance ratio were employed to assess the integrity of the 18S and 28S rRNA bands, as well as to evaluate the concentration and purity of RNA samples. The Illumina Hiseq 2500 system (Denovo Gene, Guangzhou, China) was employed for RNA sequencing. Fragments per kilobase of transcript per million mapped reads (**FPKM**) was employed to mitigate discrepancies across samples, hence facilitating the comparison of gene expression levels across various samples. ***Identification of Circadian mRNA and Enrichment Analysis***

At the 85th week, uterine tissues were harvested from 24 birds displaying analogous ovipositional patterns for transcriptome sequencing study, and 12 chickens for each group, which were consist with the samples used in the determination of blood indicators. The sample collection was conducted in six times. We utilized HTSeq to statistically compare the read count values of each gene, treating it as the baseline for gene expression. Genes were deemed expressed at a threshold of 0.1 FPKM in at least one time point, allowing for subsequent rhythmic gene analysis. The circacompare was employed to analyze the gene table across six time points for each group. A gene was identified as the

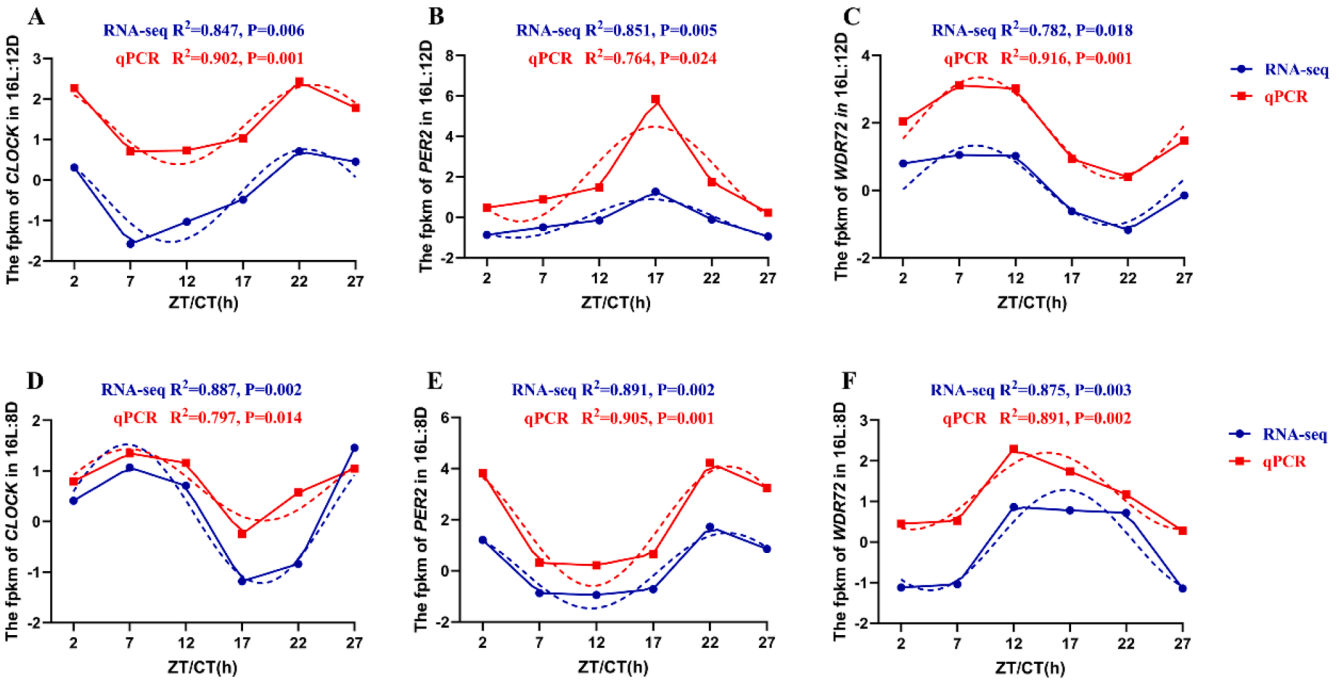

Fig. 3. qPCR confirmed the expression patterns of the selected genes. The solid line illustrates the gene expression trend for two groups, while the dashed line denotes the curve fitting. FPKM: fragments per kilobase of transcript per million mapped reads. Zeitgeber time (ZT) refers to the temporal framework inside the light-dark cycle.

circadian rhythmic gene with $P < 0.05$. We employed KOBAS 3.0 to conduct supplementary Gene Ontology (**GO**) and Kyoto Encyclopedia of Genes and Genomes (**KEGG**) enrichment analysis to determine the biological functions of the genes. Results with $P < 0.05$ were deemed significantly enhanced.

Real-time quantitative polymerase chain reaction (qPCR) analysis

Following the guidelines of the PrimeScript RT Reagent Kit (TaKaRa, Dalian, China), we reverse-transcribed the total RNA from the uterine tissues into cDNA. We conducted qPCR tests utilizing the KAPA SYBR Rapid universal qPCR kit (TaKaRa, Dalian, China) and the ABI Quant-Studio 7 Flex real-time detection system (Life Technologies, Carlsbad, CA). The expression levels of three genes were measured utilizing the $2^{-\Delta\Delta Ct}$ technique, with *GAPDH* as the normalization reference (Kenneth and Thomas, 2001). Table 1 shows the primer sequences.

Data analysis

Data are presented as the mean ± SEM. One-way ANOVA was employed to evaluate the data (SPSS 20.0, Inc., Chicago, IL). GraphPad Prism (Version 8.0.2) was used to evaluate the genetic rhythm. Results with $P < 0.05$ were deemed to reflect a statistically significant difference.

Results

Production performance

Table 2 illustrates the impact of the long-24-h ahemeral light on egg production in laying hens aged 74 to 85 weeks. No significant differences were observed in BW, laying rate, hen-day eggs and hen-day quality eggs, FER, and percentage of defective eggs between the 16L:12D group and the 16L:8D group ($P > 0.05$).

Egg quality

The effect of the long-24-h ahemeral light cycle on the egg quality of laying hens at 80 weeks were shown in Table 3. Results showed that the long-24-h ahemeral light cycle significantly enhanced the eggshell indicator including eggshell weight, eggshell thickness, and eggshell strength by 11.41 %, 6.25 %, and 16.23 %, respectively ($P < 0.05$). Meanwhile, the yolk weight was significantly increased in 16L:12D group ($P < 0.05$). And the long-24-h ahemeral light cycle had no significantly affect in other indicators ($P > 0.05$).

Table 4

Hematological parameters

Fig. 1 illustrates the impact of the long-24 h ahemeral light cycle on serum bone and calcium metabolism markers at 85 weeks. In the 16L:12D group, the serum levels of PTH, PCT, OT, and TRACP5b remained elevated during eggshell formation. In the 16L:8D group, serum levels of PTH and PCT remained elevated, while TRACP5b levels were maintained at a low level during eggshell formation (Fig. 1A-B, E-F). The serum content of $1,25(OH)_2D_3$ and ALP in darkness exceeded those observed during the daytime in both groups, however, there was no difference in its expression pattern (Fig. 1C-D).

Transcript profiling

The Illumina Hiseq2500 was utilized for sequencing cDNA library preparations. The supplementary document S1 indicates that the proportion of mapping reads varied between 92.58 % and 94.21 %. The mean values of Q20 and Q30 were found to be 97.83 % and 94.41 %, respectively.

Identification of circadian genes

The clean readings were aligned to the chicken genome (GRCg6a), which contains a reference annotation of 16,779 genes in the Ensembl

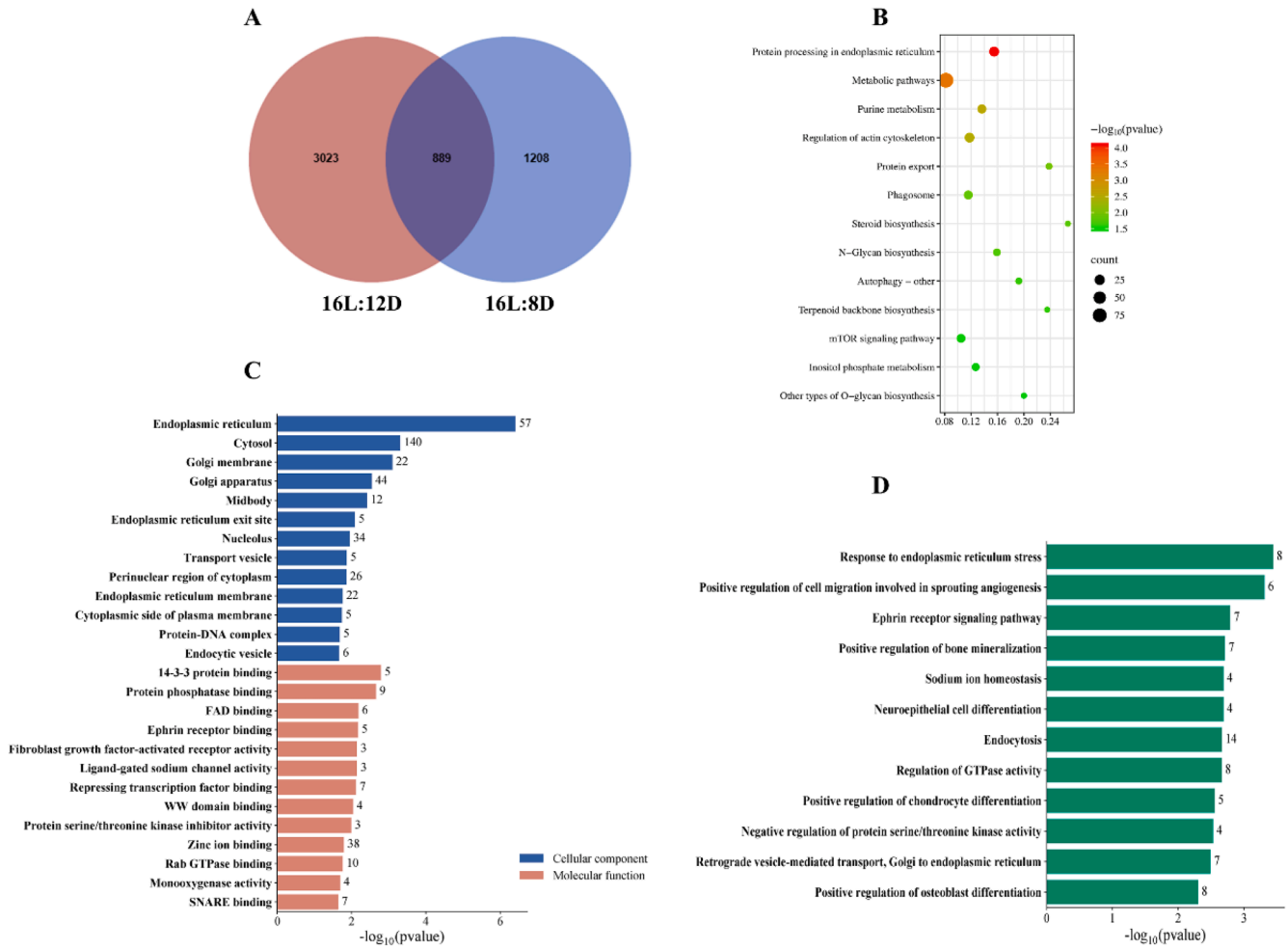

Fig. 4. Accumulative histogram of 889 common circadian rhythm genes (A), KEGG analysis (B), and GO analysis (C-D) of 889 common circadian rhythm genes. These 889 prevalent circadian rhythm genes result from the intersection of the 16L:12D and 16L:8D groups.

database. To filter the transcriptome data, a threshold of 0.1 FPKM was applied, resulting in the selection of 12,970 genes expressed at least one time point for further study. Gene expression data were evaluated for rhythmic oscillations utilizing the circacompare program. A gene was identified as the circadian rhythmic gene with $P < 0.05$. Our results indicate that there were 889 common genes exhibiting rhythmic oscillations in both groups (Fig. 4A). In comparison to the 16L:8D group, 97.41 % of genes exhibited a phase advancement in the 16L:12D group, with an average phase advance of 7.41 h relative to the 16L:8D group. The 889 common circadian rhythm genes exhibited a phase advance predominantly between 6 and 9 h (65.02 %) in contrast to the 16L:8D group (Fig. 2).

Enrichment analysis

GO and KEGG analysis revealed the biological functions of the 889 shared circadian rhythm genes (Fig. 4). The substantially enriched phrases predominantly (57.98 %) pertained to biological processes (**BP**) including positive regulation of bone mineralization, positive regulation of chondrocyte differentiation, positive regulation of osteoblast differentiation, and chondrocyte development (Fig. 4D). In the molecular function (**MF**) category, receptor activity, binding, and transporter activity were significantly enriched, whereas the cellular component (**CC**) included the endoplasmic reticulum, cytosol, and membrane (Fig. 4C). KEGG analysis revealed that 889 shared circadian rhythm genes were prominent in protein processing within the endoplasmic reticulum, metabolic pathways, purine metabolism, regulation of the actin cytoskeleton, and the mTOR signaling pathway (Fig. 4B). Based on the expression levels and the analytical outcomes of GO and KEGG function analysis, we identified four genes associated with bone metabolism: catenin beta 1 (***CTNNB1***), bone morphogenetic protein receptor 2 (***BMPR2***), bone morphogenetic protein 7 (***BMP7***), and protein disulphide isomerase (***PDIA3***).

Fig. 5A-B indicates that the alteration of the light cycle did not influence the mRNA expression patterns of *CTNNB1* and *BMPR2*, with mRNA expression occurring earlier in the 16L:12D group compared to the 16L:8D group. The mRNA expression patterns of *BMP7* and *PDIA3* were inversely correlated (Fig. 5C-D). Fig. 5 indicates that the expression levels of the *CTNNB1* and *PDIA3* genes remained elevated in both the 16L:12D and 16L:8D groups during the period of eggshell formation. During the period of eggshell formation, the mRNA expression of *BMPR2* was decreased in the 16L:12D group (Fig. 5B). The *PDIA3* levels in the 16L:12D group were elevated during the daytime compared to the darkness, whereas the *PDIA3* levels in the 16L:8D group were greater in the darkness than during the daytime (Fig. 5D).

Validation of RNA-Seq data via quantitative qPCR

To confirm the accuracy of the RNA sequencing expression results, three genes were randomly selected for validation by qPCR analysis: *CLOCK, PER2*, and *WDR72*. The qPCR results aligned with the transcriptome sequencing findings (Fig. 3), and three genes exhibited distinct circadian rhythm expression patterns ($P < 0.05$) in both groups, indicating that the circacompare program utilized for rhythm analysis is

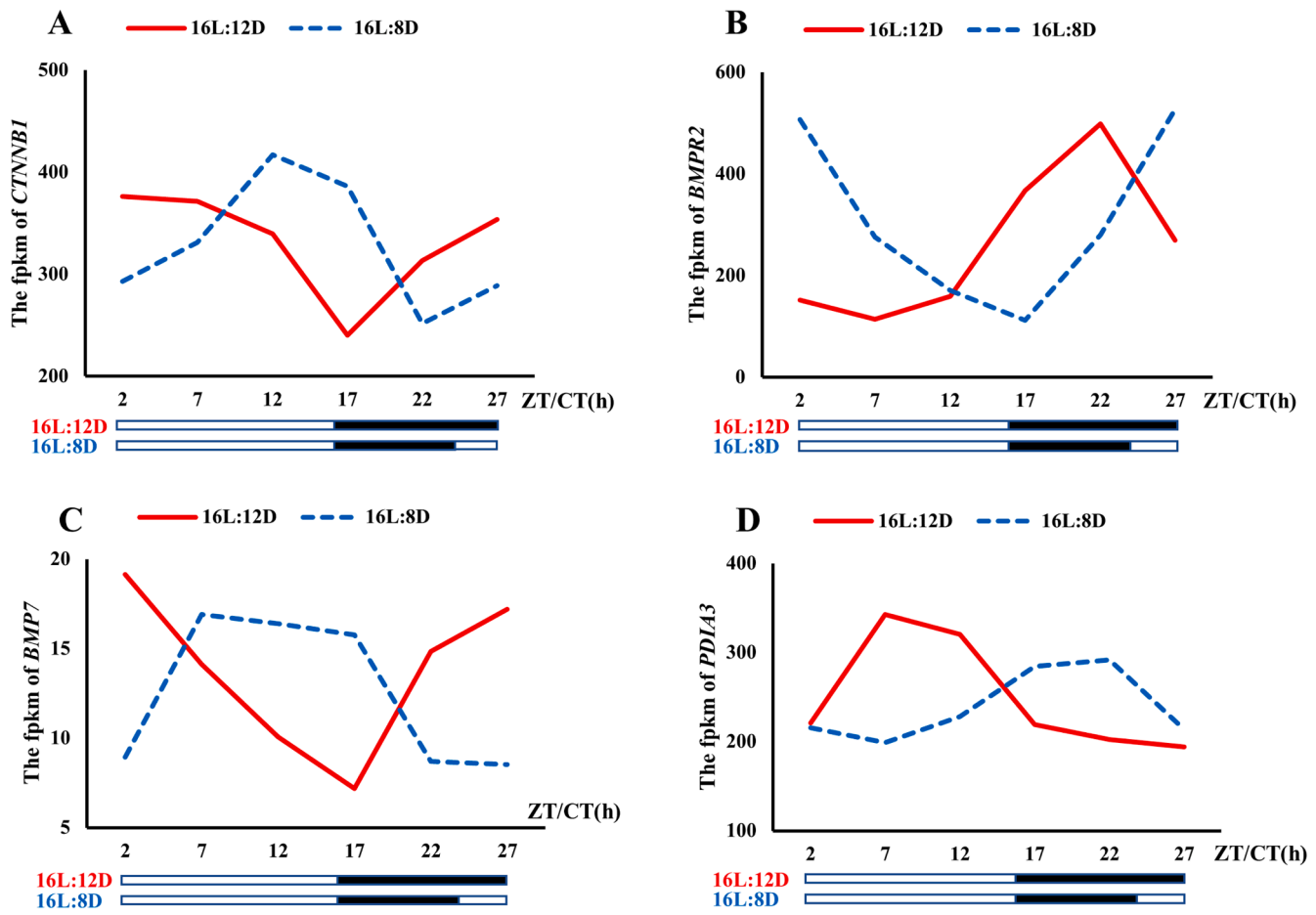

Fig. 5. Expression of four circadian genes related with bone metabolism in the uterine tissue of laying hens. Zeitgeber time (ZT) refers to the temporal framework inside the light-dark cycle.

reliable in this study.

Discussion

The deterioration of eggshell quality during the late laying stage, resulting in eggshell breakage, is the primary worry in the contemporary laying chicken industry. The ahemeral light cycle may enhance egg quality throughout both the peak laying time and the late laying stage, as corroborated by prior research. Prior research indicated that eggshells laid under extended light cycles were 6 to 10 % thicker (Leeson et al., 1979; Morris, 1973), corroborating our findings. In this study, the eggshell weight, thickness, and strength of the 16L:12D group were 11.40 %, 6.25 %, and 6.23 % more than those of the 16L:8D group, respectively. Consequently, prolonging the light cycle from 24 to 28 h in birds markedly enhanced eggshell quality.

RNA-seq results indicate that 889 genes are rhythmically expressed in both groups. In the 16L:12D group, the average phase advance of 889 genes was 7.41 h compared to the 16L:8D group. Our prior research indicated that the oviposition pattern advanced by 8 h when the light cycle increased from 24 h to 28 h (Liu et al., 2024), aligning with the observed 8-h advancement in rhythmic gene expression. The alteration of the light cycle, which induces a phase advance in rhythm genes, results in earlier laying behavior and helps sustain the 28-h oviposition pattern of hens during the late laying period. The 28-h oviposition pattern leads to extended eggshell formation durations, hence enhancing eggshell quality. Previous findings indicate that eggs produced under 27-h cycles remain in the shell gland for roughly one hour longer than those deposited under 24-h cycles, hence enhancing eggshell quality (Melek et al., 1973). Consequently, the extension of the eggshell formation period ultimately enhances eggshell quality. However, it is not known whether the improvement of eggshell quality is due to the increase of eggshell calcium content or the optimization of crystal structure, or the combination of the two. Further research is needed to determine.

The GO and KEGG analyses identified four genes associated with bone metabolism: *BMPR2, CTNNB1, BMP7*, and *PDIA3*. These genes participate in bone production, osteogenic differentiation, and the release of calcium from bone (Glass et al., 2005; Mount et al., 2006; Li et al., 2015, 2019; Qian et al., 2018, 2024; Zhang et al., 2019). The expression patterns of *CTNNB1, BMP7*, and *PDIA3* align with the process of eggshell formation. Comparable conclusions were found for blood PTH and PCT, indicating that the trends of PTH and PCT in the 16L:12D group exhibited opposing patterns relative to the normal group, with their content variations aligning with the eggshell formation process. The enhancement of eggshell quality is correlated with the prolongation of the eggshell formation period. Consequently, the enhancement of eggshell quality correlates with an extended duration of eggshell formation.

Approximately 60-70 % of the calcium necessary for eggshell formation is supplied by the diet, while the remainder is sourced from bone mobilization, which can account for up to 60 % during the late laying period (Bouvarel et al., 2011; Diana et al., 2021; Michael, 2001). In the 16L:12D group, oviposition shifted from daylight to nocturnal periods, resulting in a corresponding alteration in eggshell formation timing from night to day (Liu et al., 2024). Prior research (Olgun and Aygun, 2016) indicates that the calcium necessary for nocturnal eggshell formation predominantly derives from bone, with dietary sources also contributing, attributed to the altered oviposition pattern in the 16L:12D group,

which aligns with the heightened feeding frequency of chickens, potentially enhancing the efficacy of dietary calcium.

Prior research indicates that calcium ingested by laying hens during the day is deposited in the bones and subsequently mobilized at night for eggshell formation. When the light was turn off, the expression of *BMPR2* was significantly increased in the 16L:8D group. According to previous research (Chen et al., 2015; Li et al., 2015), the diminished expression of *BMPR2* may impede osteogenic differentiation, thereby hindering the incorporation of calcium into bone and elevating serum calcium levels. Therefore, high levels of *BMPR2* during eggshell formation time in 16L:8D group may result in the insufficient calcium supply, which may adversely affect eggshell quality. And the expression of *BMPR2* remained at a low level in the 16L:12D group during eggshell formation, indicating that the process of blood calcium entering the bone is prevented, which resulting in the increase of blood calcium. And the microbial abundance and diversity of the long-24-h ahemeral light cycle group were higher than those of the 24 h light cycle group during the light period (Unpublished Data). At the same time, previous studies have found that increased microbial abundance and diversity is conducive to the maintenance of biological functions (Liu et al., 2025; Lin et al., 2021). Therefore, we speculate that the increase of microbial abundance and diversity may promote the absorption and utilization of nutrients in the intestine.

At present, there are few studies on the molecular mechanism of long light cycle to improve the eggshell quality of laying hens in the late laying period. This experiment studied the effect of bone metabolism on the improvement of eggshell quality under the condition of long light cycle. However, the change of light cycle is essentially the change of circadian rhythm, and how clock genes regulate the expression of eggshell formation related genes and their interaction with each other is unclear.

Conclusion

This study investigated how the long-24-h ahemeral light cycle enhances eggshell quality of hens during the late laying period. In summary, the change of light cycle affects the biological rhythm of laying hens, and we found four genes related to bone metabolism, *CTNNB1, BMPR2, BMP7 and PDIA3*, which specifically responded to the long-24-h ahemeral light cycle and possibly participated in the improvement of eggshell quality.

Declaration of competing interest

No conflict of interest exits in the submission of this manuscript, and manuscript is approved by all authors for publication.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2025.104959.

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