Effects of lighting regimes on performance, pineal melanopsin expression and melatonin content in native laying hens aged from 19 to 34 weeks

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ABSTRACT Melanopsin, a key light sensitive pigment, plays an important role in the regulation of biorhythm and photo-adaptation in poultry. This study aimed to investigate the effects of different lighting regimes on performance, pineal melanopsin expression and melatonin content in a native chicken, Beijing You Chicken (**BYC**) aged from 19 to 34 wk. A total of 900 nineteen-wk-old BYC female chicken having no significant body weight differences were randomly allocated to 3 groups with 3 replicates each, 100 birds each replicate, reared in individually lit floor pens with separate outdoor areas. Three different lighting regimes were used, including continuous 16 h (16L:8D, 6:00−22:00) for group 1, intermittent 16 h (12L:2D:4L:6D, 6:00 −18:00, 20:00−24:00) for group 2, and continuous 12 h (12L:12D, 6:00−18:00) for group 3, respectively. The performance was measured for 19 to 34 wk. Serum melatonin (**Mel**), prolactin (**Prl**), luteinizing hormone (**LH**), and 17-beta estradiol (**E2**) contents were measured at 24 wk, 29 wk, and 34 wk of age, the relative expression of pineal melanopsin gene (Opn4 mRNA) was measured on 1 d at 9:00, 13:00, 17:00, 21:00, 1:00, and 5:00 at 29 wk of age, and at the end of 29 wk and 34 wk. The results showed that the egg mass, egg-laying rate, and feed egg ratio of BYC were not affected by lighting regimes for 19 to 34 wk ($P > 0.05$), except for the average feed intake (**AFI**) ($P < 0.05$). The AFI in the 12L:12D group was significantly higher than that in the 16L:8D group ($P < 0.05$), but had no difference with that in the 12L:2D:4L:6D group. The pineal Opn4 mRNA level was significantly upregulated in the 12L:2D:4L:6D group and downregulated in the 12L:12D group when compared with 16L:8D group at 29 and 34 wks of age ($P < 0.05$). The Mel content in the 16L:8D group was lower than that in the other 2 groups at 29 wk of age ($P < 0.05$), there was no difference in Mel content between 16L:8D group and 12L:2D:4L:6D group at 34 wk of age ($P > 0.05$). The present study suggested that the pineal melanopsin expression of the birds in the intermittent 16 h lighting group was higher than in the continuous 16 h and 12 h lighting group, and a significant negative correlation was found between melanopsin expression and Mel content at 34 wk of age, which may interact to promote the photo-adaptation of the native chicken and affect the future laying performance.

Key words: lighting regime, performance, melanopsin, melatonin

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INTRODUCTION

It is important for life to adapt to the ambient light changes. It is said that there are three mechanisms for the photo-adaptation in the mammals: pupil constriction, light entrainment of circadian clock, and light modulation of neuroendocrine function (Nayak et al., 2007). These mechanisms were believed to work through cones and rods in the retina, but later it had been found that these mechanisms can rely on another regulatory pathway based on melanopsin (Hattar et al., 2002; Panda et al., 2002; Ruby et al., 2002).

Melanopsin, a key light sensitive pigment, is expressed in the retina, pineal gland and suprachiasmatic nucleus (**SCN**) in animals (Okabayashi et al., 2003; Nayak et al., 2007), especially more expressed in pineal gland of poultry (Holthues et al., 2005). The central oscillators that regulate bio-rhythm are explicitly located in SCN in mammals, whereas central oscillators are located in multiple tissues such as SCN, retina, and pineal gland in non-mammals (Gillette and Tischkau, 1999). The pineal gland of poultry can directly perceive light and participate in the regulation of biological clock and melatonin secretion through melanopsin (Natesan et al., 2002).

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Melatonin, secreted rhythmically by the pineal gland, is mainly involved in the process of light signal transmission and has a wide range of biological functions, such as maintaining circadian rhythm, improving the sleep, antistress, antioxidation, and enhancing the immunity (Reiter, 1991), the removal of pineal gland could lead to the disappearance of circadian synchronization (Gwinner et al., 1997).

Effects of lighting on birds mainly reflect in establishment of circadian rhythm (Dawson et al., 2001) and regulation of melatonin content (Appleby et al., 2004, Zeman et al., 2004). In view of the existence of light input pathway of melanopsin expression and output pathway of melatonin synthesis and secretion in the pineal gland, we hypothesized that the pineal melanopsin gene expression is affected by lighting regime and has a relationship with the melatonin content.

We previously used 6 lighting regimes, including 16L:8D (6:00−22:00); 12L:2D:4L:6D (6:00−18:00, 20:00−24:00); 14L:10D (6:00−20:00); 10L:2D:4L:8D (6:00−16:00,18:00−22:00); 12L:12D (6:00−18:00), and 8L:4D:4L:8D (6:00−14:00, 18:00−22:00), to study the effects on performance and egg quality in a native chicken −Beijing You Chicken (**BYC**) from 22 to 57 wk of age, and found that 12 h lighting is enough for meeting the requirement of the native chicken during the laying period (Geng et al., 2018), but in current commercial production, 16 h lighting were still popularly used during the egg-laying period. This present study used three commonly used lighting regimes from the above treatments, aims to study the effects of lighting regime on performance, melanopsin expression and melatonin content in the native laying hens, in order to provide the applicable reference for the role of melanopsin in poultry.

MATERIALS AND METHODS

Experimental Design and Birds

The experiment was conducted at the BYC Breeding Farm, Daxing district, Beijing. A total of 900 nineteen-wk-old commercial BYC laying hens with no significant body weight differences were moved from the rearing room (10 h lighting during 7−18 wk) and changed to the following 3 lighting regimes, including continuous 16 h (16L:8D, 6:00−22:00) for group 1, intermittent 16 h (12L:2D:4L:6D, 6:00−18:00, 20:00−24:00) for group 2, and continuous 12 h (12L:12D, 6:00−18:00) for group 3, respectively. The hens were randomly allocated to 3 groups with 3 replicates each, 100 birds each replicate, reared in individually lit floor pens with separate outdoor areas. The rearing and management were the same with the description by Geng et al. (2018).

In order to keep the same ranging time for the birds, the groups adopted the arrangement below: lights at 6:00 in the morning every day, birds fed 6:00 to 8:00, the birds range freely 8:00 to 14:00, and return to their pens at 14:00 when the second feeding begins. Special light-proof cloth and the light controller were used in each pen.

Table 1. Composition and nutrient levels of the basal diet.

Ingredients,%	19−21 wk	22−34 wk
Corn	65.5	64.0
Soybean meal	21.5	23.2
Wheat bran	5.0	3.8
Limestone	4	5
Layer premix[1]	4	4
Total	100	100
Nutrient level[2]		
ME/ (MJ/kg)	11.20	11.08
Crude protein/%	15.07	15.51
Calcium/%	2.03	2.75
Total phosphorus/%	0.51	0.51
Available phosphorus/%	0.29	0.29

[1]Layer premix provided per kilogram of diet: Vitamin A, 100−250 KIU; Vitamin D3, 60−80 KIU; Vitamin E, 0.5 KIU; Vitamin K3, 80 mg; Vitamin B1, 45 mg; Vitamin B2, 180 mg; Vitamin B6, 100 mg; Vitamin B12, 0.5 mg; D-Calcium-pantothenate, 220 mg; Nicotinamide, 720 mg; Folic acid, 20 mg; Biotin, 2 mg; Copper, 0.2−0.8 g, Ferrous iron, 1.5−5 g; Zinc, 0.8-2.4g; Manganese, 1.5−3 g; Iodine, 10−30 mg; Selenium, 2−6 mg

[2]Nutrient levels were calculated from the data provided by Feed Database in China (2013).

The incandescent lamps were used, and the bulbs were 2 m off the ground, and average light intensity was 10 lx. Appropriate temperature and moisture were manipulated. Under severe weather conditions such as thundering and raining days, the birds were confined inside the pen to reduce the stress. The birds were fed commercial corn-soybean-based diets, and the composition and nutrient levels were seen in Table 1.

The study was performed in accordance with local ethical guidelines and met the requirements of the Institutional Animal Care and Use Committee.

Measurement and Methods

Egg numbers and egg weight in each pen were recorded every day, feed intake was calculated each week, and weekly average feed intake (**AFI**), egg-laying rate, egg mass (**EM**), feed egg ratio (**FER**) for each replicate group were calculated for 19 to 34 wks.

Blood samples were taken at the end of 24 wk, 29 wk, and 34 wk, 10 birds each replicate were randomly selected. 4 ml blood was sampled each bird, centrifuged at 3,000 rpm for 10 min and the serum was stored at −20°C and melatonin (**Mel**), prolactin (**Prl**), luteinizing hormone (**LH**), and 17-beta-estradiol (**E2**) content were measured.

Serum Mel and E2 concentration were measured using the medical diagnosis radioimmunoassay (**RIA**) kits (Beijing North Institute of Biotechnology Co., Ltd., Beijing, China). The assay sensitivity was 0.2 pg/mL and intra-assay coefficient of variation was below 15%. Serum PRL concentration was measured by using a chicken Prl RIA according to Huang et al. (2008) with a little modification. Serum LH concentration was performed according to Krishnan et al. (1994). All the measurement were determined by using a

radioimmunoassay instrument (XH6080, Xi'an Nuclear Instrument Factory, Xi'an, China).

At 29 wk of age, one day was randomly selected and one bird from each replicate was randomly selected for blood sample at 9:00, 13:00, 17:00, 21:00, 1:00, and 5:00, respectively, then the bird was killed using cervical dislocation. The pineal gland were separated, cleaned with H_2O/DEPC, placed in RNAase-free storage tubes, then stored in −80°C for later use. The serum Mel content at different time points were determined.

At the end of 29 wk and 34 wk, after blood sampling, one bird randomly selected from each replicate group was euthanized and the pineal gland were separated, cleaned with H_2O/DEPC, placed in RNAase-free storage tubes and stored in −80°C for later use.

The relative expression level of pineal melanopsin gene (Opn4 mRNA) was determined by fluorescence quantitative PCR. Trizol reagent, inverse transcription regent (FastKing cDNA first chain synthesis kit), and quantitative PCR reagent (Talent Fluorescence Quantitative PCR kit, SYBR Green) were from Tiangen Biochemical Technology (Beijing, China) Co., Ltd., which was responsible for the design and synthesis of related primers. The fluorescence quantitative PCR were measured by using ABI 7500 Fast and Bio-Rad CFX96 (Bio-Rad, Hercules, CA). The Opn4 primer was designed and synthesized in reference to chicken melanopsin gene (Genebank Serial number: NM_001044653.1). The primer sequences utilized for qRT-PCR were the following: The forward primer: CACCATCAGTCCGTGCCTA; backward primer: TGTACAGACTTGTTGGCCTT. The ß-actin primer: forward primer: ATGTACCCTGGCATTGCTGAC; backward primer: CCAGACAGAGTACTTGCGCTCA.

The preparation and reaction procedures of the reaction system are carried out in accordance with the kit instructions.

The total pineal RNA was extracted using trizol reagent and 1.5% agarose gel electrophoresis was used to test the RNA integrity. The amplified fragments were consistent with the expected fragment size and had good specificity, which was used for subsequent analysis. RNA concentration (OD260) was determined by using Nano Drop D2000.

The reverse transcription system contained 1 μg of total RNA, 2 μL 5 × g DNA buffer, using RNase-free ddH_2O to dilute into 10 μL. Incubate 3 min at 42°C; synthesize the cDNA first chain. The reverse transcription: 2 μL 10 × King RT Buffer, 1 μL FastKing RT Enzyme Mix, 2 μL FQ-RT Primer Mix, 10 μL genome remover system, using RNase-Free ddH2O to reach 20 μL; 42°C, incubate 15 min; 95°C, incubate 3 min, put it on the ice. The reverse transcript product was diluted 10 times by using RNase-free ddH_2O and used as the template for quantitative PCR.

The qPCR mixture contained 10 μL 2 × Talent qPCR PreMix, 2 μL diluted template cDNA, 1 μL 5μM forward primer, 1μL 5μM backward primer, 0.4 μL 50 × ROX reference dye, use RNA-free-ddH_2O to dilute into 20 μL. The amplification profile: 1 × 95°C-3min; 1 × 95°C-20 s) , 40 × (60°C-30 s, 60°C-30 s), and then fluorescence signal was collected. The relative expression of gene used the -ΔΔCt method (Livak and Schmittgen, 2001).

Statistical Analyses

The data were expressed as mean ± SD, and analyzed statistically using the SPSS 25.0 Software for Windows (SPSS Inc. Chicago, IL). One-way analysis of variance (**ANOVA**) was used to analyze the effects of lighting regimes on performance, Opn4 mRNA level and Mel content. The percentage was arcsine transformed before analysis. The relationship between Opn4 mRNA level and the Mel content was assessed with Pearson's correlation coefficient. Correlation coefficients of r = 0.70 or higher was regarded as having a strong positive correlation, and when r = 0.30 to 0.70 the variable was regarded as having a moderate positive relationship, when r = −0.70 to −0.30 the variable was regarded as having a moderate negative relationship. Duncan's Test was used for multiple comparisons. $P < 0.05$ was regarded as statistically significant.

RESULTS

Performance

Table 2 showed that the EM, egg-laying rate and FER of BYC were not significantly affected by the lighting regimes for 19 to 34 wk ($P > 0.05$), except for the AFI ($P = 0.046$). The AFI in the 12L:12D group was significantly higher than that in the 16L:8D groups ($P < 0.05$), but had no difference with that in the 12L:2D:4L:6D group. There were no dead or culled chickens during the whole trial.

Table 2. Effects of lighting regime on performance of BYC aged 19 to 34 wk*.

Lighting regime	AFI/(kg)	EM/ (kg)	Egg-laying rate/(%)	FER/(kg:kg)
Continuous 16 h (16L:8D, 6:00-22:00)	0.62 ± 0.05[b]	0.15 ± 0.07	55.79 ± 7.15	4.13
Intermittent 16 h (12L:2D:4L:6D, 6:00-18:00. 20:00-24:00)	0.70 ± 0.06[ab]	0.17 ± 0.08	56.46 ± 8.67	4.12
Continuous 12 h (12L:12D, 6:00-18:00)	0.73 ± 0.07[a]	0.18 ± 0.10	57.15 ± 8.13	4.06
P value	0.046	0.074	0.109	0.089

Abbreviations: AFI, average feed intake; EM, egg mass; FER, feed egg ratio.
[ab]Values with different letter superscripts in the same column mean significant difference ($P < 0.05$).
*There were 100 randomly selected birds placed in each pen for the 3 replicate pens per treatment group, the same as the following tables.

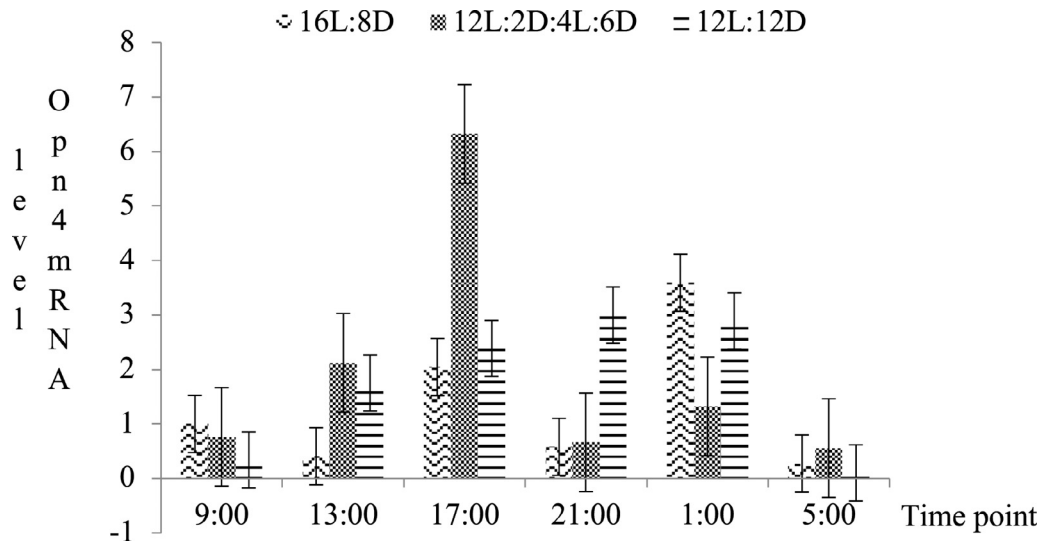

Figure 1. Effects of lighting regimes on the pineal Opn4 mRNA level at different time points*. *There had one chicken randomly sampled in each replicate at different time points.

Melanopsin Expression

Figure 1 indicated that pineal Opn4 mRNA level was significantly affected by the lighting regime at 13:00, 17:00, 21:00, and 1:00 ($P < 0.05$), but not affected at 9:00 and 5:00 at 29 of age ($P > 0.05$). The Opn4 mRNA level in the 12L:2D:4L:6D group were higher than those in the 16L:8D and 12L:12D groups at 17:00 ($P < 0.05$), but lower at 1:00; The Opn4 mRNA level in the 12L:12D group were higher than those in the 12L:2D:4L:6D and 16L:8D groups at 21:00 ($P < 0.05$). Different lighting regime affected the pineal Opn4 mRNA level, for example, at 17:00, the Opn4 mRNA level in 12L:2D:4L:6D group was significantly increased by nearly 3 times compared with those in the 16L:8D group and 12L:12D group ($P < 0.05$).

Figure 2 showed the Opn4 mRNA level at the end of 29 wk and 34 wk, and found that Opn4 mRNA level was different under 3 lighting regime groups, and it was significantly upregulated in the 12L:2D:4L:6D group and downregulated in the 12L:12D group when compared with 16L:8D group at the end of 29 wk and 34 wk ($P < 0.05$).

Mel Content

Figure 3 showed that there had no difference at 13:00, 17:00, 21:00, and 5:00 for the Mel content by different lighting regimes ($P > 0.05$), but the Mel content in the 16L:8D group was higher than those in the other 2 groups at 9:00 ($P < 0.05$), and Mel content in the 16L:8D group was higher than that in the 12L:2D:4L:6D group at 1:00 at 29 of age ($P < 0.05$) .

Table 3 showed that there had no difference in the Mel content at the end of 24 wk, but there had differences at the end of 29 wk and 34 wk by different lighting regimes. The Mel content in 16L:8D group was lower than that in the other 2 groups at the end of 29 wk ($P < 0.05$), there was no difference between 16L:8D group and 12L:2D:4L:6D group at the end of 34 wk ($P > 0.05$). At

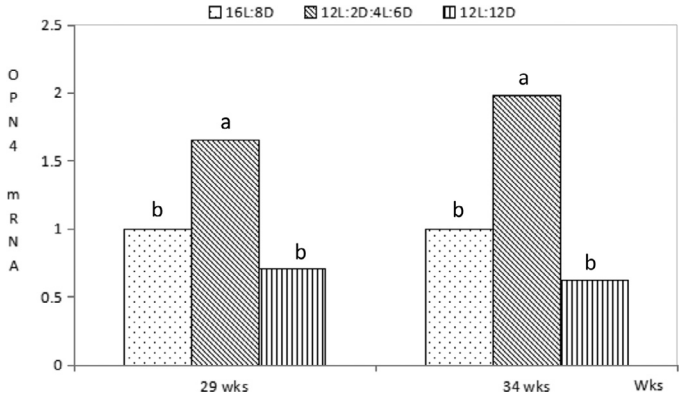

Figure 2. Effects of lighting regimes on pineal Opn4 mRNA level at 29 and 34 wk of age*. *There had 10 birds randomly sampled each replicate at the end of 29 wk and 34 wk.

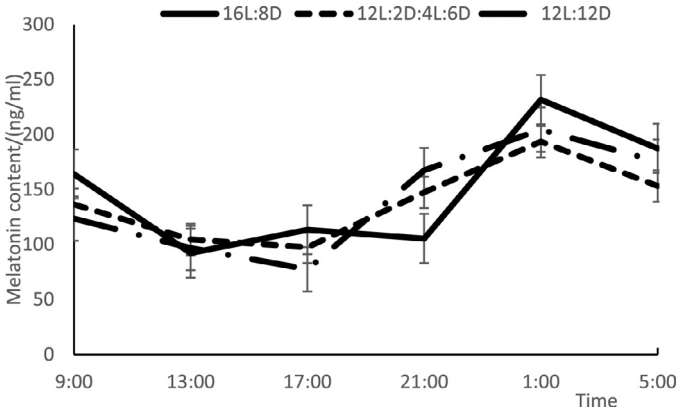

Figure 3. Effects of lighting regimes on the serum melatonin content*. *There had one chicken randomly sampled in each replicate at different time points.

Table 3. The melatonin and prolactin content under different lighting regime.

Lighting regime	Mel/(ng/mL)			Prl/(ng/mL)		
	24 wk	29 wk	34 wk	24 wk	29 wk	34 wk
Continuous 16 h (16L:8D, 6:00-22:00)	195.02 ± 20.31	150.43 ± 14.73[c]	149.62 ± 13.85[b]	29.78 ± 5.78[a]	30.14 ± 6.97	35.13 ± 10.60
Intermittent 16 h (12L:2D:4L:6D, 6:00-18:00. 20:00-24:00)	178.32 ± 15.69	219.47 ± 19.26[b]	175.81 ± 16.74[a]	20.28 ± 4.77[b]	28.56 ± 14.37	28.37 ± 11.56
Continuous 12 h (12L:12D, 6:00-18:00)	169.63 ± 14.58	251.06 ± 25.37[a]	126.92 ± 14.15[b]	33.46 ± 7.96[a]	36.39 ± 13.25	45.38 ± 12.37
P value	0.178	0.033	0.045	0.047	0.105	0.063

Abbreviations: Mel, Melatonin; Prl, Prolactin.
[abc]Values with different letter superscripts in the same column mean significant difference ($P < 0.05$).

the end of 24 wk, Prl content in groups 16L:8D and 12L:12D were greater than that in 12L:2D:4L:6D group ($P < 0.05$); At the end of 29 wk, there had no difference ($P > 0.05$), but close to a significance level at the end of 34 wk ($P = 0.063$). The LH and E2 content had no difference among the 3 groups ($P > 0.05$, not listed).

Table 4 showed that the pineal Opn4 mRNA level at different weeks of age is correlated with the Mel content to varying degrees, and the Opn4 mRNA level is moderately negatively correlated with the Mel content at the end of 34 wks ($R = -0.479$, $P < 0.05$).

DISCUSSION

There have been numerous studies about the effects of intermittent lighting on performance of hens (Rowland, 1985), but mostly concentrating on commercial high-producing layers, not on traditional or native chicken. Geweher and Freitas (2007) showed that intermittent lighting didn't affect the performance of the laying hens. A properly designed intermittent lighting (8L:6D:2L:8D) could reduce the mortality of Japanese quail (Zahoor et al., 2011). Our group adopted 6 kinds of lighting treatments to study the egg laying of BYC during 20 to 61wk, and found that the egg-laying rate was significantly higher in intermittent 16 h group than in continuous 16 h group (Geng et al, 2014). In this present study the intermittent 16 h lighting didn't affect the performance of BYC for 19 to 34 wk, the reason may be related to the short period of 19 to 34 wk, the egg production was in the peak rise period. But a higher AFI was found in the 12 h group than in the other two 16 h groups, which partly agreed with what Ma et al. (2011) indicated that the shorter the photoperiod, the longer the feeding duration and the greater the feed intake of the chicken.

Melanopsin plays an important role in behavioral adaptation to lighting in animal and poultry (Hatori and Panda, 2010; Geng, 2018). Melanopsin could mediate adaptive photoresponses, for example, the circadian activity rhythm of melanopsin-deficient mice exhibited a reduced sensitivity to light (Panda et al., 2002; Ruby et al., 2002). Melanoptin expression in the retina of rats showed circadian changes (Hannibal et al., 2007), obviously regulated by the external light and dark environment (Hannibal et al., 2005). Melanopsin was also rhythmically expressed in the pineal gland and retina of chickens (Bailey and Cassone, 2005; Holthues et al., 2005), the Opn4 mRNA level was the highest at night at Zeitgeber time 16 in chicken pineal gland (Chaurasia et al., 2005).

Jin et al. (2010) compared the effect of monochromatic light on transcription of melanopsin of Arbor Acre broiler, and showed that Opn4 mRNA expression of blue light group were higher than that of white light group either in the retinas or pineal glands. Haas et al. (2017) exposed Pekin drake to chronic red, long-day white, short-day white, or blue light, and found that there were no differences observed in relative Opn4 mRNA levels between any of the treatments. In this present study, we used white light sources, and found that the different lighting regime affected the daily pineal melanopsin expression.

Lima et al. (2006) found that melanopsin expression was 3-fold higher in retinas from the chicken kept under 6L:18D as compared with under 18L:6D. It seemed that the longer lighting had lower melanopsin expression. Wu et al. (2012) compared the changes of melanopsin expression in mouse retina exposed to 24 h of continuous light and 12 h of darkness, and found that the Opn4 mRNA level significantly decreased in 24 h group at 1 wk and 8 wk, indicating that continuous light may reduce the melanopsin expression. However, Kuenzel et al. (2015) transferred Cobb 500 broiler chicks in 8L:16D environment to stimulatory 16L:8D environment, and found that Opn4 expression was significantly increased. In this present study, the pineal Opn4 mRNA level in the intermittent 16 h lighting regime group (12L:2D:4L:6D) was significantly higher than continuous 16 h and 12 h lighting group at 29 wk and 34 wk of age, it appears that the 2 h darkness during continuous lighting can increase the adaptation of the birds, which partly support what Izzeldin and Kassim (2000) reported that the darkness could reduce hyperthermia and enhance acclimatization responses during acute heat stress. The improvements of adaptation during the dark period might be relevant to melatonin variation during the light and dark periods.

Table 4. Relationship between the Opn4 mRNA level and the Mel content.

Wk	Correlation coefficient (r)	*P* value
24	−0.246	0.063
29	−0.340	0.057
34	−0.479	0.048

The Mel is generally produced in dark period, while daytime light inhibits the Mel secretion from the pineal gland (Appleby et al., 2004). Özkan et al. (2006) reported that the Mel content reached its highest level after 4 h in dark under 12L:12D group, and showed obvious rhythmic pattern at 16L: 8D group compared with those under 24 L group for broilers (Özkan et al., 2012). This present study showed that there had no significant difference in the Mel content by the lighting regime at 24 wk of age, but there had significant differences at 29 wk and 34 wk of age by different lighting regimes. It seems that Mel content is affected by not only lighting regime but also the lighting duration.

The Mel is responsible for the activation of the inhibitory pathway of the reproductive axis (Ubuka et al., 2005), and regulate the synthesis and secretion of gonadal hormone by acting directly on target organs, such as hen's ovary (Zhang, 2019), while melanopsin could activate the neuroendocrine regulation of seasonal reproduction of birds, which could benefit for the reproductive performance of chicken (Kuenzel et al., 2015). We previously reported that the egg-laying rate of BYC was significantly higher in intermittent 16 h group than in continuous 16 h group for 20 to 61 wk (Geng et al, 2014), in this present study the pineal Opn4 mRNA level in the intermittent 16 h lighting regime group was significantly higher than continuous 16 h and 12 h lighting group, suggesting that the intermittent 16 h lighting may benefit the photo-adaptation of the native laying hens, the possibly interactive effects on laying performance by melanopsin and melatonin was the embodiment of the negative correlation between them.

CONCLUSIONS

The present study suggested that the pineal melanopsin expression of the birds in the intermittent 16 h lighting group was higher than in the continuous 16 h and 12 h lighting group, and a significant negative correlation was found between melanopsin expression and Mel content at 34 of age, which may interact to promote the photo-adaptation of the native chicken and affect the future laying performance.

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DISCLOSURES

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

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