Prolonged Dark Time Within A 24-h Light Regime Improves Eggshell Quality by Enhancing Calcium Deposition in Laying Hens

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Research

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Abstract

Background: The absorption and transportation of calcium and phosphorus is mainly relied on their corresponding transporters. Eggshell is mainly formed during dark time in one egg cycle. The aim of this study was to determine the effect of different light regime on eggshell quality and the expression of the relevant calcium and phosphorus transporters in laying hens. Seventy two 56-week-old laying hens were randomly divided into two groups and subjected to the following treatments: 16 h light: 8 h dark (control) and 9 h light: 15 h dark (LDP). The expression of phosphorus transporters type IIb Na/Pi co-transporter NaPi-IIa (Npt2a) and NaPi-IIb (Npt2b), calcium transporter calbindin-D28k (CaBP-D28k), and plasma membrane Ca ATPase 1b (PMCA1b) were measured in small intestine, kidney, and eggshell gland.

Results: The results showed that the feed intake ($P < 0.001$) and egg weight ($P = 0.05$) was decreased by LDP treatment, while laying rate, egg production, and feed efficiency were not significantly influenced ($P > 0.05$). Compared to control, eggshell hardness was increased ($P < 0.05$) by LDP treatment whereas eggshell thickness and eggshell percentage were not significant changed. Eggshell calcium (Ca) and phosphorus (P) contents were elevated in LDP-hens, compared to control birds. Compared to control birds, serum Ca ($P < 0.01$) and P levels ($P = 0.079$) at dark time were increased in LDP-hens while and alkaline phosphatase (ALP) activity was lowered ($P < 0.05$). The protein expression levels of CaBP-D28k and PMCA1b were not influenced in duodenum but were decreased at light time in jejunum of LDP hens. In kidney, the expression of *CaBP-D28k*, *PMCA1b* and *Npt2a* were not changed by LDP treatment. In eggshell gland, however, the expression of *CaBP-D28k* and *osteopontin (OPN)* were relative higher in LDP hens compared to control birds, whereas the PMCA1b expression was not altered.

Conclusions: The result indicates that the increased circulating Ca and P concentrations in dark time are favorable for the deposition of calcium and phosphorus in eggshell. The result offers an alternative strategy for the laying hen with a worse eggshell problem.

Introduction

Eggshell quality plays an important role in the production efficiency of commercial egg industry and the eggshell problems cause significant economic losses. The sustain of eggshell quality is essential for the persistency in lay of the long-life layer, which will be capable of producing 500 eggs in a laying cycle of 100 weeks [1]. The unsynchronous increase of calcium (Ca) deposition with eggshell weight is one of the underlying reason of poor shell quality as laying hen ages [2, 3].

It is crucial to provide calcium for the high metabolic calcium demand during eggshell mineralization. It is assumed that the continuous supply of calcium from digestive tract and bone mobilization could satisfy the requirement of eggshell formation. However, the limitation of hen access to feed during dark period [4] and bone loss with age [5, 6] make the effective calcium supply a problem in the laying period.

Photoperiod is generally considered as the primary cue to stimulate reproduction of laying hen [7]. The light source [8], spectrum [9, 10], intensity [11], and the uniformity of light distribution [12] all have an

influence on the laying performance. Altered photoperiod has an influence on eggshell quality. Eggshell quality is improved by increasing the photoperiod to 24 hours [13]. The midnight lighting programmes provide a means of supporting egg shell quality of older laying hens during the summer months without a significant reduction in egg production [14]. In contrast, an increase of 10% shell weight in hens under 27-h photoperiod (14 h light, 13 h dark) is ascribed to the increased time spent in the shell gland [15]. The underlying mechanism remains unclear.

In avian species, calbindin (CaBP-D28k), an intracellular Ca^{2+} -binding protein, transports Ca^{2+} from the apical to the basolateral membrane, is closely correlated with Ca^{2+} transport in the intestine, kidney, and the eggshell gland [16, 17, 18, 19, 20]. Calcium transporter CaBP-D28k is the primary Ca transcellular diffusion transporters in small intestine of birds [16].

Plasma membrane calcium ATPase (PMCA) participates in the translocation of calcium, mediates Ca^{2+} extrusion from cells [21, 22, 23]. The plasma membrane Ca ATPase 1b (PMCA1b) is the primary isomer expressed in small intestine, kidney, and uterus of birds [24, 25, 26, 27, 28]. The NaPi-IIb (NPT2b) cotransporter is the major phosphorus (P) transporter in small intestine that primary expressed at the brush-border membranes of epithelium [29]. Recently, our result indicates that duodenum has the highest expression level of NPT2b and is influenced by dietary phosphorus level [30]. Hence, we hypothesized that photoperiod take an effect on eggshell formation with the involvement of changed gene expression of the genes.

In this study, 56-weeks-old hens were employed to investigate the effect of prolonged dark period in 24-h cycle. The laying performance, eggshell quality, and the expression of P and Ca transporters in small intestine, kidney, and shell gland in laying hens were investigated.

Materials And Methods

All procedures used in this study were approved by the Animal Care Committee of Shandong Agricultural University and were carried out in accordance with the guidelines for experimental animals of the Ministry of Science and Technology (Beijing, China).

Animals

Seventy two 56-week-old ISA hens with similar body weight and laying rate were obtained. The hens were randomly divided into two groups of 36 hens and reared in two environmental controlled houses with the same rearing facilities. The laying hens were cage reared with two hens per cage (45 cm*35 cm*35 cm). Three cages were served as one replicate. The two groups of hens were reared in one of the two following light regimes: 16-h light and 8-h dark (16 L:8 D, control group) or 9-h light and 15-h dark (9 L:15 D, long-dark period treatment, LDP). The experiment lasted for 8 weeks. During the whole experimental period, the hens had free access to feed and water.

Feed intake, egg number, and egg weight were recorded daily, and laying rate and egg production were calculated. The egg quality was measured at 2, 4, 6, and 8 weeks after treatment. All the eggs were collected for three consecutive days every two weeks and the eggshell hardness, percentage of eggshell, yolk weight, albumen height, Haugh unit, eggshell thickness (mean from the equator and two ends), shape index, and egg yolk color were measured.

At the end of the experiment, sixteen hens were randomly selected from each treatment and sampled at the midpoint of light and dark periods respectively, 13:00 pm and 01:00 am in control group and 9:30 am and 21:30 pm in 9 L:15 D group (Fig. 1). After a blood sample was obtained from a wing vein, the hens were sacrificed by exsanguination after cervical dislocation [31,32]. The duodenum, jejunum, ileum, kidney, and shell gland mucosa were sampled. Tissue samples were immediately snap-frozen in liquid nitrogen and stored at -80°C for further analysis. Serum was separated by centrifugation at 1,500 *g* for 15 min and stored at -20 °C until analysis.

Egg quality measurement

Shell thickness was determined at the sharp, blunt ends, and equator by an eggshell Thickness Meter (EFG-0503, ROBOTMATION, Japan), and took the average of the three data. Egg shape index was calculated by long diameter/short diameter. Eggshell breaking strength was determined based upon the vertical axis measured by an eggshell strength tester (EFG-0503, ROBOTMATION, Japan). After breakout, yolk was separated and weighed. Relative weight of yolk was calculated against egg weight. The albumen height, yolk color, and Haugh unit were detected by the multifunctional egg detector (EMT-5200, JapanRobotmation). Eggshells were weighed after dried. Relative weight of eggshells was calculated against egg weight.

Determination of calcium and phosphorus in eggshell

Determination of calcium content in feed and eggshell with potassium permanganate titration[33,34]. Phosphorus concentration was measured at 420 nm using a spectrophotometer (UV-2450; Shimadzu Corp., Kyoto, Japan).

Serum calcium, phosphorus, and alkaline phosphatase analysis

Serum Ca and P levels and alkaline phosphatase (ALP) activity were measured with commercial kits (Sichuan Mike Biotechnology Co., Ltd., China) by automatic biochemistry analyzer (7020 Clinical Analyzer: Hitachi High-Tech GLOBAL) [35].

Total RNA extraction and Real-Time PCR analyses

The mRNA expression levels of *NaPi-IIa* (*Npt2a*), *Npt2b*, *CaBP-D28k*, *PMCA1b* were measured by real-time PCR. Total RNA was extracted from the duodenum, jejunum, ileum, kidney, and eggshell gland using TRIzol reagent (TransGen Biotech, China). The primers used in this study were designed using Primer 5.0 software and synthesized by Sangon Biotech (Shanghai, China, Table 1). Total RNA concentration was

then quantified by measuring the absorbance at 260 nm in a photometer (Eppendorf Germany). Ratios of absorbance (260/280 nm) were between 1.75 and 2.01 for all preparations. Next, reverse transcription was performed using total RNA (1 µg) for first-strand cDNA synthesis with the Transcriptor First Strand cDNA Synthesis Kit (Roche, Germany). The cDNA was amplified in a 20 µL PCR reaction system containing 0.2 µM of each specific primer (Sangon, China) and of the SYBR Green master mix (Roche, Germany) according to the manufacturer's instructions. Real-time PCR was performed at the ABI QuantStudio 7500 PCR machine (Applied Biosystems; Thermo, United States) [34,36]. The mRNA values were normalized with the expression of chicken b-actin mRNA. The analyses of the relative gene expression data were performed by the $2^{-\Delta\Delta CT}$ method [37, 38, 39]. All of the samples were run in duplicate, and the primers were designed to span an intron to avoid genomic DNA contamination. Therefore, all gene transcription results are reported as the n-fold difference relative to the calibrator. The specificity of the amplification product was verified by the standard curve and dissolution curve.

Western blot analysis

The tissue samples were homogenized in 1 mL of lysis buffer (Beyotime, China). After centrifuged at 12000 *g* for 10 min at 4 °C to remove cellular debris, the supernatant was collected and then quantified for protein by the method of BCA protein assay kit (Beyotime, China) according to the manufacturer's protocol. An equal amount of proteins were separated by 10% SDS polyacrylamide gels (Bio-Rad, Richmond, 246 CA) and the proteins were transferred onto polyvinylidene fluoride membrane (Millipore, United States) at 200 mA for 2 h in a Tris-glycine buffer with 20% anhydrous ethanol at 4 °C. Then membranes were blocked with western blocking buffer (Beyotime, China) for 1 h at room temperature. The membranes were incubated with specific primary antibodies at 4 °C with gentle shaking overnight. The primary antibodies used were anti-Calbindin-d28k (Sigma, United States), anti-PMCA (Thermo, United States), anti-β-actin and anti-β-tubulin (Beyotime, China). The membrane was washed with Tris-buffered saline/Tween buffer for 10 min three times, and then the membranes were incubated with secondary antibodies (HRP-conjugated anti-rabbit or anti-mouse IgG, 1:1000; Beyotime) for 4 h at 4 °C. After being washed as before, membranes were then visualized by exposure to Hyperfilm ECL (Beyotime, China). Western blots were developed and quantified using BioSpectrum 810 with VisionWorks LS 7.1 software (UVP LLC) [36, 38]. The band intensity was normalized to the β-actin or β-tubulin band in the same sample.

Statistical analyses

Prior to analysis, all data were examined for the homogeneity and normal distribution plots of variances among the treatments by using UNIVARIATE procedure. The main effect of photoperiod treatment was evaluated by a one-way ANOVA and a two-way ANOVA with the Statistical Analysis Systems statistical software package (Version 8e, SAS Institute, Cary, NC, USA). $P < 0.05$ was considered statistically significant.

Results

Laying performance

Compared with control, feed intake ($P < 0.001$) and egg weight ($P < 0.05$) were decreased by LDP treatment, while the laying rate and feed to egg ratio were not significantly influenced by treatment ($P > 0.05$; Table 2, Fig. 2).

Egg quality

Compared with the control group, eggshell hardness was significantly increased by LDP treatment ($P < 0.05$; Table 3, Fig. 3a). In contrast, eggshell thickness, egg shape index, albumen height, yolk color, Haugh units, percentage of yolk and eggshell were not changed by treatment ($P > 0.05$; Table 3).

Ca and P contents in eggshell

Compared with the control group, eggshell Ca ($P < 0.05$) and P ($P < 0.01$) contents were increased by LDP treatment (Fig. 3d, e). The LDP-hens consumed less Ca and P compared to control ($P < 0.01$; Fig. 3f, g). In contrast, the Ca excretion via eggshell of was not changed ($P > 0.05$) while P excretion was increased significantly ($P < 0.01$; Fig. 3i).

Serum parameters

In control hens, the serum Ca and P concentrations were higher in light time ($P < 0.05$) compared to that in dark time, whereas there was no difference ($P > 0.05$) between light and dark time in LDP treatment (Fig. 4a, b). Moreover, serum Ca level in dark time was higher in LDP treatment ($P < 0.05$) compared with control (Fig. 4a). The ALP activity had no detectable difference ($P > 0.05$) in light and dark time in both control and LDP treatments. Compared to control, the hens in LDP treatment had lower ALP activity during dark time ($P < 0.05$; Fig. 4c).

The expression of calcium transporters

In light time, the mRNA expression level of *CaBP-D28k* in the eggshell gland was lower ($P < 0.05$) in both treatments, compared to that in the dark time (Fig. 5a). In contrast, there was no difference in the protein level of CaBP-D28k ($P < 0.05$) between light and dark time (Fig. 5b). In light time, however, the protein level of CaBP-D28k was higher in control than that in LDP treatment ($P < 0.05$). Neither the mRNA nor the protein level of *PMCA1b* was altered by photoperiod treatment ($P > 0.05$; Fig. 5d-f).

In the control group, the mRNA expression level of *CaBP-D28k* was higher in dark period compared to the light period in duodenum and jejunum ($P < 0.05$) but not in ileum ($P > 0.05$; Fig. 6, 7, 8). In LDP treatment, however, there was no difference in the mRNA expression level of *CaBP-D28k* between light and dark period ($P > 0.05$). The mRNA level of *PMCA1b* was increased during dark period in the duodenum of control hens compared with the light period ($P < 0.05$), whereas no detectable influence ($P > 0.05$) was observed in jejunum and ileum of control hens. In LDP treatment, the expression of *PMCA1b* had no difference between light and dark time ($P > 0.05$). In duodenum and jejunum, the expression of *NPT2b*

was not changed by light and dark cycle or photoperiod treatment ($P > 0.05$). In ileum, however, the *PMCA1b* expression was decreased ($P < 0.05$) during dark time in LDP treatment compared to that in the light or dark time of control (Fig. 8).

In kidney, the light and dark period had no significant ($P > 0.05$) influence on the mRNA level of *CaBP-D28k* and *PMCA1b* and the protein level of CaBP-D28k and PMCA1b (Fig. 9).

The expression of phosphorus transporters

In control group, there was no difference ($P > 0.05$) in the mRNA level of *Npt2a* in the kidney between light and dark time (Fig. 10a). In contrast, the *Npt2a* mRNA level was higher ($P < 0.05$) in light time than that in dark time. The mRNA expression level of *Npt2b* in the duodenum and jejunum was not affected by light treatment ($P > 0.05$; Fig. 10b, c). However, in the LDP group the mRNA expression level of *Npt2b* in ileum was significantly upregulated in light time, compared with dark time ($P < 0.05$; Fig. 10d). The mRNA expression level of *Npt2b* in control group was significant higher than LDP group at dark time in ileum ($P < 0.05$).

Discussion

In the present study, the result indicated that prolonged dark time in a 24 h light-dark cycle decreases feed intake, hen-day egg production, and egg weight but has no unfavorable influence on feed efficiency. Longer dark time increases Ca and P contents of eggshell and improves eggshell strength. The result suggests that the elevated serum Ca and P concentrations at dark time are involved in the improved eggshell quality. The result highlights an effective method to ameliorate eggshell quality at late-phase of laying period.

Prolonged dark time has a disadvantage effect on laying performance

Chicken is sensitive to the change of photoperiod. Many studies have been conducted to investigate the effect of changed light-dark period, light source, light intensity, and intermittent lighting [11, 40, 41]. In the present study, the prolonged dark period in 24-h cycle had a negative influence on the laying rate, egg weight, and egg production. However, the decreased laying performance seemed to be a result of reduced feed intake in LDP-hens, as the egg to feed ratio was not significantly influenced by photoperiod treatment. In broiler under 16 L:8 D cycle, the feed intake in the dark is almost negligible and take account of less than 3% of total feed consumption [42]. In laying hen subjected to an asymmetric pattern of 0.25 L:0.75 D for 16 h followed by 8 D, the reduction in feed intake is ascribed to reduced total activity time [43]. Hence, the result suggests that the reduced feed intake in LDP hens is a result of shorter light time and in turn shorter forage time.

Prolonged dark period improves eggshell quality by elevating Ca level during dark period

During the eight weeks experiment period, the egg quality was evaluated every two weeks. The elevated eggshell strength in LDP-hens was in accordance with the egg broken rate, which had a 36.5% decrease

in LDP treatment (Control, 2.60% vs. LDP, 1.65%). Hence, the result indicated that LDP could improve eggshell quality. Eggshell strength and thickness were improved in high Ca consumption group [44]. The inconsistent response of eggshell thickness and eggshell strength is in line with the previous works [45, 46]. The breaking strength and shell thickness are respectively negatively and positively influenced by increasing dietary phosphorus and calcium contents [47]. Except of the shell thickness, the calcium content in eggshell also contributes to eggshell hardness. Although the eggshell thickness is not necessary to correlate with its calcium content [48], the calcium concentration in eggshell decreases with age in laying hens [49]. In the present study, the calcium and phosphorus concentrations were all increased in the eggs from LDP-hens, suggesting that the improved eggshell strength is associated with the elevated Ca and P contents in the eggshell.

The elevated plasma Ca and Pi levels are associated with the improved eggshell strength [46]. The serum Ca exhibits a circadian rhythm which varied with the changed light: dark cycle in laying hens [50]. In the present study, the blood samples were obtained at the intermittent points of light- and dark-period, respectively. The relative higher serum Ca and P levels in light period compared to the dark period indicated that there is a circadian rhythm in the blood Ca and P levels. This result was in line with the previous results [51, 52]. Parsons and Combs (1981) [51] reported that blood Ca^{2+} concentration varies with the egg position in the oviduct, reaches the maximum level following oviposition and drops until an uncalcified egg entered the shell gland. The daily change of blood P, however, has different reports. The blood P follows a pattern opposite that of Ca [51] or shows a similar trend as blood Ca [52]. Except of the daily egg cycle, blood Ca^{2+} and P levels are related to the light-dark cycle or feed intake. In 4-month-old pullets, serum Ca is lower during daylight hours while is higher during the dark period [51], indicating daylight exposure and/or feed intake take an influence on blood Ca. In LDP-hens of the present study, however, the circadian rhythm in the blood Ca and P levels disappeared and the serum Ca and P were kept high levels in both light- and dark-period, implying that a more sufficient supply of blood Ca and P during the dark hours. It is speculated that the increase of blood Ca^{2+} is a result of additional release of bone Ca during the night hours [53]. In contrary, the relative lower activity of ALP at dark time in LDP hens indicated the reduced Ca mobilization from bone during dark time. Similarly, the Ca level in pullets, without the interruption of eggshell formation, is significantly elevated during the dark period, dropping to low levels during daylight hours when feeding [51]. The underlying mechanism needs to be investigated further. Collectively, the result suggests that the prolonged dark time is beneficial for the eggshell formation and calcium deposition during night and the increased circulating Ca and P levels during dark time should be at least partially responsible for the elevated eggshell Ca and P contents in LDP treatment.

OPN, a component of the organic matrix of eggshell, play an important role in egg shell calcification. OPN is mainly secreted by the epithelial cells of the eggshell gland lumen [54,55]. In the present study, the expression level of *OPN* was higher at dark time compared to light time, in line with the work by Pines and Knopov (1995) [56], who reported that the expression of osteopontin in eggshell gland showed circadian rhythm and the peak value of osteopontin expression appeared in dark period. When the egg enters the uterus, the pressure on the uterine wall is the key factor causing the upregulation of OPN expression [57].

The relative higher expression levels of OPN at both light (4.87 folds) and dark (1.28 folds) periods compared to control suggests that upregulated OPN expression is involved in the enhanced eggshell strength.

Calcium transport is not altered by lighting program

In laying hens, CaBP-D28k localizes at the intestinal enterocyte cytoplasm, and highly expresses in duodenum and followed by jejunum and ileum [30, 58, 59]. The ingested calcium is absorbed into the vascular system in duodenum and upper jejunum with the involvement of CaBP-D28k [60]. PMCA mediates Ca^{2+} extrusion from cells [21, 22, 23]. Therefore, we determined the expression of the *CaBP-D28k* and *PMCA1b* in the intestinal tract, kidney, and eggshell gland. The relative higher expression level of *CaBP-D28k* in duodenum and jejunum and *PMCA1b* in duodenum at dark time was observed in control hens but not in LDP hens. The protein level of CaBP-D28k and PMCA1b, however, had no detectable difference between control and LDP-hens at either day or dark time. Hence, the result suggests that Ca absorption in duodenum is not changed by light regime. In jejunum, the protein levels of CaBP-D28k and PMCA1b were higher in control hen at day time, compared to LDP-hens. The chyme remained in digestive tract at measuring time may be responsible at least for the relative lower expression level of CaBP-D28k and PMCA1b in LDP hens. In the study, the hens were sampled at the intermediate point of dark period, 4- and 7.5-h after light out in control and LDP groups respectively. Moreover, the LDP hens consumed less feed (89.9% of control) than control ones.

The protein levels of CaBP-D28k and PMCA1b in the kidney were not changed by photoperiod treatment and light-dark cycle, suggesting that calcium reabsorption in the kidney plays a less important role in the elevated circulating calcium concentration during dark period in LDP hens. In shell gland, however, the unchanged CaBP-D28k and PMCA1b levels in dark time between control and LDP treatments, suggesting that Ca secretion by shell gland is not changed by light regime treatment. Collectively, the result implies that higher circulating Ca concentration at dark time in LDP hens should be responsible for the increased Ca deposition in eggshell and in turn, the improved eggshell quality.

Phosphorus transport is not altered by photoperiod

There are two Na/Pi cotransporter families, Na/Pi-II and NaPi-III. NaPi-II has three kinds of homologs, NaPi-IIa (NPt2a), NaPi-IIb (NPt2b), and NaPi-IIc (NPt2c). In small intestine, P absorption is primary adjusted by NPt2b [61, 62, 63, 64, 65] and the expression of gene *NPt2b* in intestinal tract mainly locates at the duodenum [30, 63]. In the present study, the expression of *NPt2b* was not influenced by photoperiod in duodenum and jejunum, whereas the *NPt2b* level was lower in the dark period in ileum, indicating that light regime treatment has no effect on P absorption. As there is no available antibody on NPt2a, the protein level of NPt2a was not determined in the present study. The elevated circulating P ($P = 0.079$) in LDP hens at dark time remains to be elucidated.

In kidney, *NPt2a* is the main type of P transporter. The adaptive capacity of kidney in P transport takes the most important role in the maintenance of P homeostasis in laying hens [66]. The relative lower

expression level of *NPT2a* in LDP-hens was in line with the high serum P level. This result was in accordance with the previous work by Li et al. (2018) [30] who reported that laying hens fed a diet with higher available phosphorus had relative lower *NPT2a* mRNA level.

Conclusions

In conclusion, the laying performance of hens under 9 L:15 D light regime was lowered compared to the hens at 16 L:8 D light regime. Prolonged dark time from 8-h to 15-h improves eggshell strength and eggshell calcium and phosphorus contents. The result indicates that the increased circulating Ca and P concentrations in dark time are favorable for the deposition of calcium and phosphorus in eggshell. The result offers an alternative strategy for the laying hen with a worse eggshell problem.

Declarations

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Competing interests

The authors declare that they have no conflicts of interest. We greatly thank the reviewers for their valuable comments and suggestions on the paper.

Ethics approval and consent to participate

All procedures used in this study were approved by the Animal Care Committee of Shandong Agricultural University and were carried out in accordance with the guidelines for experimental animals of the Ministry of Science and Technology (Beijing, China).

Consent for publication

Not applicable.

Availability of data and material

All data is available upon request.

Authors' contributions

All authors contributed to the study conception and design. Qian Xin, Xiaojuan Wang, and Hai Lin conceived and designed the experiments, wrote manuscript; Qian Xin performed the experiments and analyzed the data; Jingpeng Zhao designed the experimental diet and gave suggestions to data analysis; Haifang Li and Hongchao Jiao provided essential reagents; and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1
Gene-specific primer of related genes

Gene	Sequences (5'→3')	Accession NO.	Product size (bp)
CaBP-D28	F: TGTTATGGAGTGCAGGATGG R: TAGAGCGAACAAGCAGGTGA	NM_205513	131
PMCA1b	F: TTCAGGTACTCATGTGATGGAAGG R: CAGCCCCAAGCAAGGTAAAG	XM_015277056	98
NPt2a	F: CCAAAGTGCACGGCTTCT R: TGGGAGGTCAGT GTTGATGA	XM_015293846	249
NPt2b	F: ACTGGCTTGCTGTGTTTGC R: AGGGGCATCTTCACCACTTT	NM_204474	113
β-actin	F: CTGGCACCTAGCACAATGAA R: CTGCTTGCTGATCCACATCT	NM_205518	123

Table 2
Effects of different light regime on laying performance of hens

Items	16 L:8 D	9 L:15 D	<i>P</i>
Laying rate, %	87.9 ± 2.44	80.1 ± 4.2	0.142
Egg weight, g	67.6 ± 1.02 ^a	64.9 ± 0.67 ^b	0.050
Feed intake, g/hen per d	140.9 ± 1.25 ^a	126.7 ± 2.32 ^b	< 0.001
Hen-day egg production, g/d	59.4 ± 1.98	52.1 ± 2.9	0.063
Feed efficiency, g/g	2.38 ± 0.07	2.5 ± 0.13	0.466
Egg broken rate, %	2.60 ± 0.78	1.65 ± 0.81	0.395
Data were presented as mean ± SEM (<i>n</i> = 6). ^{a-b} Means with different superscripts differ significantly (<i>P</i> < 0.05)			

Table 3
Effects of different light regime on egg quality of laying hens

Items	16 L:8 D	9 L:15 D	<i>P</i>
Shell thickness, mm	0.346 ± 0.003	0.352 ± 0.004	0.277
Egg shape index	1.29 ± 0.01	1.3 ± 0.01	0.508
Eggshell hardness, kg. f	3.81 ± 0.05 ^b	4.14 ± 0.1 ^a	0.011
Albumen height, mm	4.4 ± 0.13	4.57 ± 0.14	0.377
Yolk color	7.67 ± 0.06	7.61 ± 0.1	0.617
Haugh units	60.0 ± 0.8	62.3 ± 1.5	0.214
Percentage of yolk, %	28.5 ± 0.5	28.8 ± 0.6	0.698
Percentage of eggshell, %	10.9 ± 0.1	11.2 ± 0.2	0.141
Data were presented as mean ± SEM (<i>n</i> = 6). ^{a-b} Means with different superscripts differ significantly (<i>P</i> < 0.05)			

Figures

Fig. 1

Figure 1

The schematic graph of experimental protocol. (a) 16 L:8 D, control, (b) 9 L:15 D, experimental group

Fig. 2

Figure 2

Effects of different light regimes on laying performance of hens. Egg production (a), egg weight (b), feed intake (c), feed efficiency (d), and hen-day egg production (e). Data were presented as means $\pm$ SEM (n = 6); *P < 0.05

Fig. 3

Figure 3

Effects of different light regimes on eggshell quality and calcium and phosphorus metabolism of laying hens. Eggshell hardness (a), percentage of eggshell (b), eggshell thickness (c), calcium content in eggshell (d), phosphorus content in eggshell (e), dietary calcium intake (f), dietary phosphorus intake (g), calcium excreted from eggshell (h), and phosphorus excreted from eggshell. Data were presented as means $\pm$ SEM (n = 6); *P < 0.05, **P < 0.01

Fig. 4

Figure 4

Effects of different light regimes on serum calcium (a), phosphorus (b), and alkaline phosphatase (c). Data were presented as means $\pm$ SD (n = 8); *P < 0.05, **P < 0.01

Fig. 5

Figure 5

Effect of different light regime on the mRNA expression level and protein expression level of calbindin-D28k (CaBP-D28k) and plasma membrane calcium ATPase 1b (PMCA1b) in eggshell gland of laying hens. The mRNA and protein levels of CaBP-D28k (a, e); the mRNA and protein expression levels of PMCA1b (b, f); the mRNA and protein expression levels of OPN (c). Western blot assay showing the

protein expression of CaBP-D28k and PMCA1b (d). β -actin were used as the loading control. The values are presented as the means $\pm$ SD (n = 8); *P < 0.05, ***P < 0.0001

Fig. 6

Figure 6

Effect of different light regime on the mRNA expression level and protein expression level of calbindin-D28k (CaBP-D28k) and plasma membrane calcium ATPase 1b (PMCA1b) in duodenum of laying hens. The mRNA and protein levels of CaBP-D28k (a, d); the mRNA and protein expression levels of PMCA1b (b,

e). Western blot assay showing the protein expression of CaBP-D28k and PMCA1b (c). β -tubulin were used as the loading control. The values are presented as the means $\pm$ SD ($n = 8$); * $P < 0.05$, ** $P < 0.01$

Fig. 7

Figure 7

Effect of different light regime on the mRNA expression level and protein expression level of calbindin-D28k (CaBP-D28k) and plasma membrane calcium ATPase 1b (PMCA1b) in jejunum of laying hens. The mRNA and protein levels of CaBP-D28k (a, d); the mRNA and protein expression levels of PMCA1b (b, e).

Western blot assay showing the protein expression of CaBP-D28k and PMCA1b (c). β -tubulin were used as the loading control. The values are presented as the means $\pm$ SD ($n = 8$); * $P < 0.05$, ** $P < 0.01$

Fig. 8

Figure 8

Effect of different light regime on the mRNA expression level and protein expression level of calbindin-D28k (CaBP-D28k) and plasma membrane calcium ATPase 1b (PMCA1b) in ileum of laying hens. The mRNA and protein levels of CaBP-D28k (a, d); the mRNA and protein expression levels of PMCA1b (b, e).

Western blot assay showing the protein expression of CaBP-D28k and PMCA1b (c). β -tubulin were used as the loading control. The values are presented as the means $\pm$ SD (n = 8); *P < 0.05

Fig. 9

Figure 9

Effect of different light regime on the mRNA expression level and protein expression level of calbindin-D28k (CaBP-D28k) and plasma membrane calcium ATPase 1b (PMCA1b) in kidney of laying hens. The mRNA and protein levels of CaBP-D28k (a, d); the mRNA and protein expression levels of PMCA1b (b, e).

Western blot assay showing the protein expression of CaBP-D28k and PMCA1b (c). β -actin were used as the loading control. The values are presented as the means $\pm$ SD (n = 8); *P < 0.05

Fig. 10

Figure 10

The mRNA expression level of type II-A Na/Pi co-transporter (NPT2a) in kidney and the mRNA expression level of type II-B Na/Pi co-transporter (NPT2b) in small intestine of laying hens. Kidney (a); duodenum (b); jejunum (c); ileum (d). Data were presented as means $\pm$ SD (n = 8); *P < 0.05