

Circadian zinc feeding regime in laying hens related to laying performance, oxidation status, and interaction of zinc and calcium

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ABSTRACT This study investigated that circadian zinc (Zn) feeding regime affected laying performance, Zn and calcium (Ca) status, antioxidant capacity and gene expression of circadian clock, and Ca and Zn transporter in laying hens. In total, 162 of 21-wk Hyline Sophie laying hens were assigned randomly into 3 groups including CON group (Control Zn, basal diets supplemented 60 mg/kg Zn), HL group (high-low Zn, basal diets supplemented 120 mg/kg Zn—basal diets), and LH group (low-high Zn, basal diets—basal diets supplemented 120 mg/kg Zn), which were fed at 0,530 h and 1,530 h, respectively. Blood, tibia, duodenum, and eggshell gland samples were collected at 8 h intervals with starting at 0,000 h in 1 d after 10 wk of experiment. Compared with CON group: 1) Feed conversion ratio (FCR) of LH and HL group decreased significantly ($P < 0.05$); 2) in serum, total antioxidant capacity and CuZn-superoxide dismutase (SOD) at 0,000 h increased significantly, as well as Ca and Zn concentration of tibia at 0,800 h in LH

group ($P < 0.05$); 3) in duodenum, mRNA expression of calbindin-d28k (CaBP) and NCX1 at 1,600 h in HL group upregulated significantly, as well as Per2 and Per3 at 0,000 h, CLOCK, Cry2, Per2, and Per3 at 1,600 h ($P < 0.05$). But, Zn5 at 0,800 h in HL group downregulated significantly ($P < 0.05$). 4) In eggshell gland, the mRNA expression of CaBP at 0,000 h and Zn5 at 1,600 h in HL group downregulated significantly ($P < 0.05$). However, SOD at 1,600 h in HL group upregulated significantly, as well as Cry1 and Per3 at 0,800 h in HL group upregulated significantly ($P < 0.05$). In conclusion, circadian Zn feeding diet regime was beneficial to improvement of FCR. The regulation of laying hens' circadian rhythms affected Zn and Ca transporter and interrelationship between Ca and Zn metabolism, also altered antioxidant capacity in present study. Therefore, circadian Zn feeding regime can be considered as a new method to improve laying performance in laying hens.

Key words: zinc, circadian feeding regime, calcium, circadian rhythms, laying hens

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INTRODUCTION

Trace minerals are essential nutrients in wide physiological processes, and deficiency may result in poor health status and production (Lopez-Alonso, 2012). They are necessary to maintain body function to optimize growth and reproduction and to stimulate immune response and as defense against oxidative stress and cell

damage (Liu et al., 2014). Their presence in feed with adequate quantities is taken for granted (Lopez-Alonso, 2012). Zinc (Zn), as a cofactor in over 300 Zn metalloenzymes, is one of the most abundant trace minerals in cells. It is essential for growth and development of nearly all organisms (Reed et al., 2014). Zinc is also one of main trace minerals involved in eggshell formation process and plays a major role in antioxidation, growth and development, immune system, and stress in poultry production systems (Dikmen et al., 2015; Naz et al., 2016; Sacan et al., 2016). One mineral intake at high level has interaction effects on other minerals (Sirirat et al., 2012). Previous study demonstrates that calcium (Ca) addition may cause poor utilization of Zn (Forbes et al., 1979), and excessive Ca decreases Zn absorption

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because of competition for mineral binding ligands (Ao and Pierce, 2013).

Laying hens utilize Ca more efficiently than most animals, but unbalanced Ca utilization will manifest poor quality eggshell (Taylor et al., 2013). Serum Ca exhibits a circadian rhythm which varies with changing light: dark cycle (Pablos et al., 1995). Circadian rhythm regulates wide metabolic and physiologic activities about 24 h circle (Li and Zhang, 2015; Tahara and Shibata, 2016). Our previous studies report that dynamic supplementation with uridine or methionine affects circadian variations of lipid metabolism in liver both in mice or laying hens (Liu et al., 2019a,b). The rhythms are regulated by circadian oscillators in poultry pineal gland (Pablos et al., 1995), and circadian pacemaking system in bird requires interaction that pineal gland mediates various physiological function (Woller and Gonze, 2013). Circadian timekeeping system synchronizes slaves clock in peripheral organ to adapt to daily alternating period during feed and forage or sleep and fast and to regulate metabolic homeostasis corresponding to environmental change, most light/dark cycle (Saini et al., 2011; Chen et al., 2013). Sooncharernying et al. review that total Ca concentration in plasma has a cyclic fluctuation during egg laying cycle (Sooncharernying and Edwards, 1989), and pattern of serum Ca is altered by diets restriction in laying hens (Roland et al., 1972). Optimal nutrition, with adequate trace mineral levels, guarantees proper functions of the organism, including structural, physiological, catalytic, and regulatory (Suttle, 2009). In our previous studies, dynamic feeding low and high methionine or Ca diet schedule alters circadian variation of serum Ca (Liu et al., 2017; Lin et al., 2018b). Specially, dynamic feeding low and high Ca diets regime alters serum Zn levels (Lin et al. 2018a).

It was proposed that feeding time of Zn coupling with circadian rhythm may affect serum and tibia minerals concentration in laying hens. No attempt was made to determine relationship between circadian rhythm and Zn in laying hens. Therefore, the objective of present study was conducted to investigate laying hens' circadian rhythms which affect laying performance and egg quality through investigating the relationship of Zn and Ca in egg yolk, serum and tibia, as well as gene expression of circadian clock, Ca and Zn transporter in laying when related to dynamic feeding schedule with low and high Zn diets.

MATERIALS AND METHODS

All experimental and sample collection procedures were carried out according to the Chinese guidelines for animal welfare and approved by the Institutional Animal Care and Use Committee of the Hunan Agricultural University.

Diets

Diets were as the same as Table 1, were corn-soybean-based including 28.22 mg/kg Zn, and fed as a granule

Table 1. Composition of the basal diet for the laying hens.¹

Item	Ingredients (%)
Corn	60.00
Soybean meal (43%)	22.00
Wheat bran	6.00
Limestone	7.98
Dicalcium phosphate	1.31
NaCl	0.30
Zeolite powder	2.09
DL-methionine	0.06
Premix ²	0.26
Total	100.00
Nutrient and energy content (%) ³	
ME (kcal/kg)	2,614.16
Crude protein	16.95
Calcium	3.40
Total phosphorus	0.49
Available phosphorus	0.35
Methionine	0.30
Methionine + Cystine	0.57
Lysine	0.76

¹Analyzed Zn concentration (mg/kg) of basal dietary was 28.22.

²Supplied per kilogram of diet: 12,000 IU of retinyl acetate; 3,000 IU of 1,25-hydroxy-cholecalciferol; 30 mg of DL- α -tocopheryl acetate; 6 mg of menadione; 3 mg of thiamine propyl disulfide; 9 mg of riboflavin tetrabutylate; 6 mg of pyridoxine; 0.03 mg of cobalamin; 0.15 mg of D-biotin; 18 mg of D-Pantothenic acid; 1.5 mg of Folic acid; 6 mg of nicotinamide; 18.15 mg of ethoxyquin; 50 mg of choline chloride; 10 mg of phytase; 0.004 mg of ubiquitin calcium; 5.12 mg of copper (Cu), copper glycine form; 72 mg of iron (Fe), iron glycine form; 60 mg of zinc (Zn), zinc methionine form; 84.8 mg of manganese (Mn), manganese glycine form; 0.64 mg of iodine (I); 0.32 mg of cobalt (Co). 0.27 mg Cystine, 0.76 mg Lysine, 0.58 mg Threonine, 0.18 mg Tryptophan. (Iron glycine form, zinc methionine form, manganese glycine form and copper glycine form were provided by Tanke Bio-tech Co., Ltd., 510890, Guangzhou).

³Calculated values.

form. All nutrients reached or exceeded the nutrients recommendation (NRC, 1994) for laying hen, except concentrations of Zn. Three diets were formulated only with different Zn concentrations. They were basal diet which served as low Zn diet, control Zn diet (basal diet + 60 mg/kg Zn), and high Zn diet (basal diet + 120 mg/kg Zn). They served for 3 treatments, which were fed at 0,530 h and 1,530 h daily: CON group (Control Zn diet, which fed at 0,530 h and 1,530 h, respectively), HL group (High Zn diet fed at 0,530 h, and low Zn diet fed at 1,530 h, respectively), and LH group (Low Zn diet fed at 0,530 h, and high Zn diet fed at 1,530 h, respectively). The ingredients compositions and nutrients contents of diets were shown in Table 1.

Birds and Management

A total of 162 Hyline Sophie laying hens included in Hyline strain were selected from a commercial flock (Huangpu District, Guangzhou City, China) and randomly divided into 3 equal groups with 6 replicates of 9 hens each. The trial lasted for 70 d, from 21st to 30th wk (June–August) age of hens. Three birds were housed in a (38 × 35 × 35 cm) wire cage with 3 ladders, and then 3 wire cages formed an experimental unit which was randomly distributed in the shed. The lighting regimen used was a 16 h light and 8 h darkness cycle with light beginning at 0,500 h local time. The birds

were fed twice daily (0,530 h and 1,530 h) and allowed ad libitum access to water and treatment diets during the experiment period. Before providing new diets, the residual feeds were cleaned away, and the weights of new and residual diets were recorded to calculate feed intake (FI) of birds daily. Dietary treatments were as following: CON (received control diet at both 0,530 h and 1,530 h); LH (received low Zn diet at 0,530 h and high Zn diet at 1,530 h); HL (received high Zn diet at 0,530 h and low Zn diet at 1,530 h).

Sample Collection

Since the last day of the trial to the following 2 d, total 108 birds, 12 birds per treatment (2 per replicate) were killed at 8-h intervals, (3 times per day) in a daily cycle, starting at 0,000 h, blood, eggshell gland, duodenum, and left tibia samples were collected at the same time. Blood was centrifuged for 15 min at $3,000 \times g$ under normal temperature to obtain serum and then stored at -20°C for subsequent blood mineral analysis. The eggshell gland, duodenum, and left tibia were dissected and immediately frozen in liquid nitrogen and stored at -80°C until further analysis (Xie et al., 2016).

Performance

During 10-wk experimental period, egg production (EP) and egg weight (EW) were obtained daily by replicate, and FI was also obtained daily by replicate to calculate EP, EW, and feed conversion ratio (FCR). The incidence of soft-shelled and cracked eggs was also recorded.

Egg Quality Traits

Total 72 normal eggs were randomly and equally selected on equal basis ($n = 24$ eggs/group) from each treatment to evaluate the egg quality traits at the end of the experiment. Egg quality traits including EW, albumen height, haugh unit, eggshell strength, and shell thickness were evaluated. Egg weight, albumen height, and haugh unit were determined by egg quality analyzer (EA-01, Orka Food Technology Ltd., Ramat HaSharon, Israel). Eggshell strength was determined by an egg force reader (Orka Food Technology Ltd.). Shell thickness was determined by shell thickness gauge (Orka Food Technology Ltd.) at 3 different locations (bottom, middle, and top of the egg) (Lin et al., 2020).

Mineral Analysis

Egg yolk, serum, and tibia sample dilution and preparation of standards were prepared with ultrapure water and ultrapure acids (HNO_3 : $\text{H}_4\text{Cl} = 4:1$). Samples were analyzed with preliminary treatment via electric oven digestion. Tibia samples were ground firstly, then dried in oven at 105°C for 24 h, and then 0.2 g of bone powder were dissolved in ultrapure acids for 24 h to determine

minerals concentration in ICP-OES (iCAP 6000, Shanghai, China) (Ni et al., 2002).

Serum Biochemical Parameters Analysis

Serum total antioxidant capacity (T-AOC) and CuZn-superoxide dismutase (CuZn-SOD), carbonic anhydrase (CA) of HL group, as well as serum alkaline phosphatase (ALP) activity, and malondialdehyde (MDA) were evaluated according our previous study (Liu et al., 2019a; Lin et al., 2020).

RNA Extraction and Quantitative Real-Time PCR Analysis

Approximately 100 mg of duodenum or eggshell gland tissue were pulverized in liquid nitrogen. Total RNA was isolated from homogenate with the TRIzol reagent and then treated using DNase I guided by the manufacturer's instructions. First-strand cDNA was synthesized using Oligo (dT) 20 and Superscript II reverse transcriptase. TRIzol, DNase I, and reverse transcriptase were provided by Takara Bio Inc. (Kusatsu, Shiga, Japan). Primer was designed in NCBI according to the gene sequence of chick (<http://www.ncbi.nlm.nih.gov/>) to receive an amplification product (Table 2).

Real-time PCR were performed as previous study (Xie et al., 2016). In brief, 1.5 μL cDNA templates were added to total volume of 10 μL including 5 μL SYBR premix and ROX mixture, 2.9 μL dH₂O, and 0.3 $\mu\text{mol/L}$ of forward and reverse primer each. After a predenaturation program (10 s at 95°C), 40 cycle of amplification was performed that each cycle consists 95°C for 5 s and 60°C for 20 s followed by melting curve program (60°C – 99°C with a heating rate of 0.1°C/s and fluorescence measurement). The relative levels of mRNA expression were calculated with $2^{-\Delta\Delta\text{Ct}}$ method after normalization using β -actin as housekeeping gene to be internal control.

Statistical Analysis

This experiment was performed with completely randomized design. All data in this experiment were shown as mean \pm SEM. Significant difference among treatments means was determined by one-way analysis of variance followed by Duncan's multiple-ranges test (SPSS, 17.0). The difference was considered significant when $P < 0.05$.

RESULTS

Effects of Circadian Zinc Feeding Regime on Laying Performance and Egg Quality Traits

Laying performance of hens fed with dynamic Zn diets was summarized in Table 3. No significant effects were found on egg production, EW, soft shelled or cracked egg rate, and morning and afternoon laying ratio

Table 2. Sequence of primers for real-time PCR.

Name of target gene	Accession number	Nucleotide sequence of primers(5'-3')
CLOCK	AF246959.1	F: TTCTGTCTTCTCATCTGCTGGA R: GGTGCTCTTTGGGCTATTG
BMAL1	AF193070.1	F: CCATCCTACTGTCCCTGGAA R: TGGCGATGATCCTCTTATCC
CRY1	NM_204245.1	F: AGACCACGAATGAACGCCAA R: CAGGACAAACAGCCAAAGCG
CRY2	NM_204244.1	F: AGTCCCTACCTGCGTTTGG R: CTTCGGGTGTTTGTGGCTG
PER2	NM_204262.1	F: ACCATCCCAGTTTCTGCCC R: GTTGTGGGAAAGGGCTAGGG
PER3	NM_001289779.2	F: TTCCCTTTTCCTTTGGCTTT R: GGAAACAACCTCCCAGTGAA
CaBP-d28k	NM_205513.1	F:GACTTCATGCAGACATGGAGA R:CTTGATCGTACATCTCAAAGGC
NCX1	DQ987923.1	F:TGCCCTGACCATCATTCGTC R:CGGGCTTGAAGACCACTGTC
CA	Z14957.1	F: CCTCCGACAAGTCAAGT CTGCAA R: TCAVGGCCATCCACAG TGTGCTC
CuZn-SOD	NM_205064.1	F: GAAGGCCGTGTGCGTGATGA R: CACGGAAGAGCAAGTACAGC
ALP	NM_205360.1	F: TACCTCTGCGGCGTCAAA GCCAA R: AATGCCACCCGCTTGCCTTCAT
Zn1	AJ619980	F: TCGGAGTGCCCTTCTTCT R: AAGGAGCTGTCAGGTCTGTAAT
Zn5	NM-001031419	F: ATGCTGTTGTGGGATGTA R: TTGCTTTGGCTGGTCTC
β -actin	L08165.1	F: TTAATCGCCTCTGTGAAGGC R: TCCTAGACTGTGGGGACTG

($P > 0.05$) in 3 treatments. However, FCR of LH and HL group decreased significantly ($P < 0.05$) and the soft shelled or cracked eggs rate reduced 0.42 and 0.74%, respectively, without significant difference ($P > 0.05$), when compared with CON group. In addition, egg quality traits of hens fed with dynamic Zn diet was summarized in Table 4. Dynamic Zn feeding regime had no effects on egg quality traits in 3 treatments ($P > 0.05$).

Effects of Circadian Zinc Feeding Regime on Feed Intake and Zinc Intake

Feed intake and Zn intake of hens fed with dynamic Zn diet were showed in Table 5. Results showed that morning Zn intake and afternoon Zn intake were significant ($P < 0.05$) influenced by circadian Zn feeding regime. Daily total FI and Zn intake of 3 treatments were similar ($P > 0.05$).

Effects of Circadian Zinc Feeding Regime on Egg Yolk Minerals Deposition

Effects of circadian Zn feeding regimen on egg yolk minerals deposition were shown in Table 6. Compared with CON group, circadian Zn feeding regime decreased significantly Ca concentration of egg yolk in LH or HL group ($P < 0.05$). Specially, the Ca: P ratio and Ca: Zn ratio of egg yolk decreased significantly in both LH and HL group ($P < 0.05$).

Effects of Circadian Zinc Feeding Regime on Serum Biochemical Parameters

Serum biochemical parameters results of 3 treatments were shown in Figure 1. Compared with CON group, at 0,000 h, circadian Zn feeding regime increased significantly serum T-AOC and CuZn-SOD in both LH and

Table 3. Effects of circadian zinc feeding regime on laying performance during 10 wk.¹

Laying performance	Dietary treatment			SEM	P-value
	CON group	LH group	HL group		
Egg production (%)	83.99 ± 1.72	85.85 ± 2.11	87.70 ± 1.10	1.64	0.331
Feed conversion ratio (g of feed/g of egg)	2.04 ± 0.03 ^a	1.93 ± 0.04 ^b	1.92 ± 0.03 ^b	0.03	0.041
Morning egg production (%)	84.04 ± 2.82	81.85 ± 2.44	83.12 ± 1.20	2.15	0.793
Afternoon egg production (%)	15.96 ± 2.82	18.15 ± 2.44	16.88 ± 1.20	2.15	0.793
Egg weight (g of egg)	55.23 ± 0.39	54.56 ± 0.99	54.31 ± 0.63	0.67	0.654
Soft shelled or cracked eggs rate (%)	1.17 ± 0.61	0.75 ± 0.36	0.43 ± 0.31	0.43	0.520

Abbreviations: CON, control; HL, Zinc: high-low; LH, Zinc: low-high.

¹Data are means of 6 replicates per dietary treatment. Values in the same row with different superscript are significantly different ($P < 0.05$) by one-way ANOVA.

Table 4. Effects of circadian zinc feeding regime on 70th-d egg quality traits.¹

Egg quality	Dietary treatment			SEM	P-value
	CON group	LH group	HL group		
Egg weight(g)	55.45 ± 0.65	57.73 ± 1.25	55.37 ± 1.81	1.24	0.381
Egg shape	1.30 ± 0.01	1.30 ± 0.01	1.31 ± 0.01	0.01	0.842
Albumen height(mm)	7.05 ± 0.25	6.75 ± 0.66	6.75 ± 0.38	0.43	0.871
Yolk color	4.35 ± 0.43	4.28 ± 0.35	4.98 ± 0.45	0.41	0.433
Haugh unit (U)	85.28 ± 1.41	81.82 ± 4.42	83.45 ± 1.99	2.60	0.707
Eggshell strength (kg)	3.99 ± 0.24	3.63 ± 0.19	4.05 ± 0.16	0.20	0.302
Eggshell thickness (mm)	0.36 ± 0.00	0.35 ± 0.00	0.36 ± 0.00	0.00	0.425

Abbreviations: CON, control; HL, Zinc: high-low; LH, Zinc: low-high.

¹Data are means of 6 replicates per dietary treatment. Values in the same row with different superscript are significantly different ($P < 0.05$) by one-way ANOVA.

HL group ($P < 0.05$). At 1,600 h, it increased significantly serum CA of HL group, as well as serum ALP activity in LH group ($P < 0.05$). However, it decreased significantly serum MDA at 0,800 h in HL group and serum CA and ALP at 1,600 h in LH group ($P < 0.05$).

Effects of Circadian Zinc Feeding Regime on Minerals Concentration in Serum and Tibia

Serum minerals concentration results of 3 treatments were shown in Figure 2. Compared with CON group, at 0,000 h, circadian Zn feeding regime increased significantly serum P level but decreased significantly Ca: P ratio in HL group ($P < 0.05$). It decreased significantly serum Ca level at 0,800 h in LH group ($P < 0.05$). At 1,600 h, it increased significantly serum Zn level but decreased significantly Ca: Zn ratio in HL group ($P < 0.05$). In addition, compared with CON group, the tibia Ca and Zn concentrations of LH group increased significantly at 0,800 h under the circadian Zn feeding regime ($P < 0.05$, Figure 3).

Effects of Circadian Zinc Feeding Regime on mRNA Expression Level of Biological Enzyme Gene in Duodenum and Eggshell Gland

Effects of circadian Zn feeding regime on mRNA expression level of biological enzyme gene in duodenum of 3 treatments were presented in Figure 4. Compared

with CON group, at 0,000 h, circadian Zn feeding regime upregulated significantly mRNA expression level of CA in duodenum of LH group, as well as ALP at 1,600 h in duodenum of HL group ($P < 0.05$).

Effects of circadian Zn feeding regime on mRNA expression level of biological enzyme genes in eggshell gland of 3 treatments were presented in Figure 5. Compared with CON group, circadian Zn feeding regime downregulated significantly mRNA expression level of CA at 0,000 h and at 0,800 h in eggshell gland of LH group, as well as CA at 0,800 h of HL group ($P < 0.05$). However, it upregulated significantly mRNA expression levels of ALP and SOD at 1,600 h in eggshell gland of HL group ($P < 0.05$).

Effects of Circadian Zinc Feeding Regime on the mRNA Expression Level of Zinc and Calcium Transporter in Duodenum and Eggshell Gland

Effects of circadian Zn feeding regime on mRNA expression level of Zn and Ca transporter in duodenum of 3 treatments were presented in Figure 6. Compared with CON group, at 0,000 h, circadian Zn feeding regime downregulated significantly mRNA expression level of NCX1 in duodenum of LH group ($P < 0.05$) but upregulated significantly Zn1 ($P < 0.05$). At 0,800 h, it downregulated significantly mRNA expression level of Zn5 in duodenum of HL group. However, it upregulated significantly mRNA expression level of calbindin-d28k (CaBP) and NCX1 at 1,600 h in HL group ($P < 0.05$).

Table 5. Effects of circadian zinc feeding regime on feed intake and calculated zinc intake of laying hens during 10 wk.¹

Item	Dietary treatment			SEM	P-value
	CON group	LH group	HL group		
Morning feed intake (g/bird/day)	42.88 ± 0.80	41.05 ± 0.85	42.51 ± 1.06	0.91	0.350
Afternoon feed intake (g/bird/day)	49.68 ± 0.75	47.62 ± 1.73	47.39 ± 0.37	0.95	0.302
Total feed intake (g/bird/day)	92.56 ± 1.49	88.67 ± 2.38	89.90 ± 1.31	1.73	0.317
Morning zinc intake (mg/bird/day)	3.97 ± 0.06 ^b	1.19 ± 0.02 ^c	6.63 ± 0.21 ^a	0.10	<0.001
Afternoon zinc intake (mg/bird/day)	4.16 ± 0.07 ^b	6.89 ± 0.26 ^a	1.22 ± 0.03 ^c	0.12	<0.001
Total zinc intake (mg/bird/day)	8.14 ± 0.09	8.08 ± 0.26	7.84 ± 0.23	0.19	0.590

Abbreviations: CON, control; HL, Zinc: high-low; LH, Zinc: low-high.

¹Data are means of 6 replicates per dietary treatment. Values in the same row with different superscript are significantly different ($P < 0.05$) by one-way ANOVA.

Table 6. Effects of circadian zinc feeding regime on 70th-d egg yolk mineral deposition.¹

Item	Dietary treatment			SEM	P-value
	CON group	LH group	HL group		
Zn(mg/kg)	35.83 ± 2.06	35.11 ± 1.03	32.69 ± 1.52	1.53	0.369
Ca(g/kg)	2.26 ± 0.09 ^a	1.85 ± 0.12 ^b	1.36 ± 0.04 ^c	0.08	<0.001
P(g/kg)	5.54 ± 0.09	5.35 ± 0.07	5.05 ± 0.19	0.12	0.052
Ca/P-ratio	0.41 ± 0.01 ^a	0.34 ± 0.02 ^b	0.27 ± 0.01 ^c	0.01	<0.001
Ca/Zn-ratio	63.54 ± 1.74 ^a	52.76 ± 3.28 ^b	41.86 ± 1.30 ^c	2.11	<0.001

Abbreviations: CON, control; HL, Zinc: high-low; LH, Zinc: low-high.

¹Data are means of 6 replicates per dietary treatment. Values in the same row with different superscript are significantly different ($P < 0.05$) by one-way ANOVA.

Effects of circadian Zn feeding regime on mRNA expression level of Zn and Ca transporter in eggshell gland of 3 treatments were shown in Figure 7. Compared with CON group, circadian Zn feeding regime downregulated significantly mRNA expression level of CaBP, Zn1, and Zn5 at 0,000 h in eggshell gland of LH and HL group, as well as Zn5 at 1,600 h of HL group ($P < 0.05$).

Effects of Circadian Zinc Feeding Regime on mRNA Expression Level of Circadian Gene in Duodenum and Eggshell Gland

Effects of circadian Zn feeding regime on mRNA expression level of circadian gene in duodenum of 3 treatments were presented in Figure 8. Compared with CON

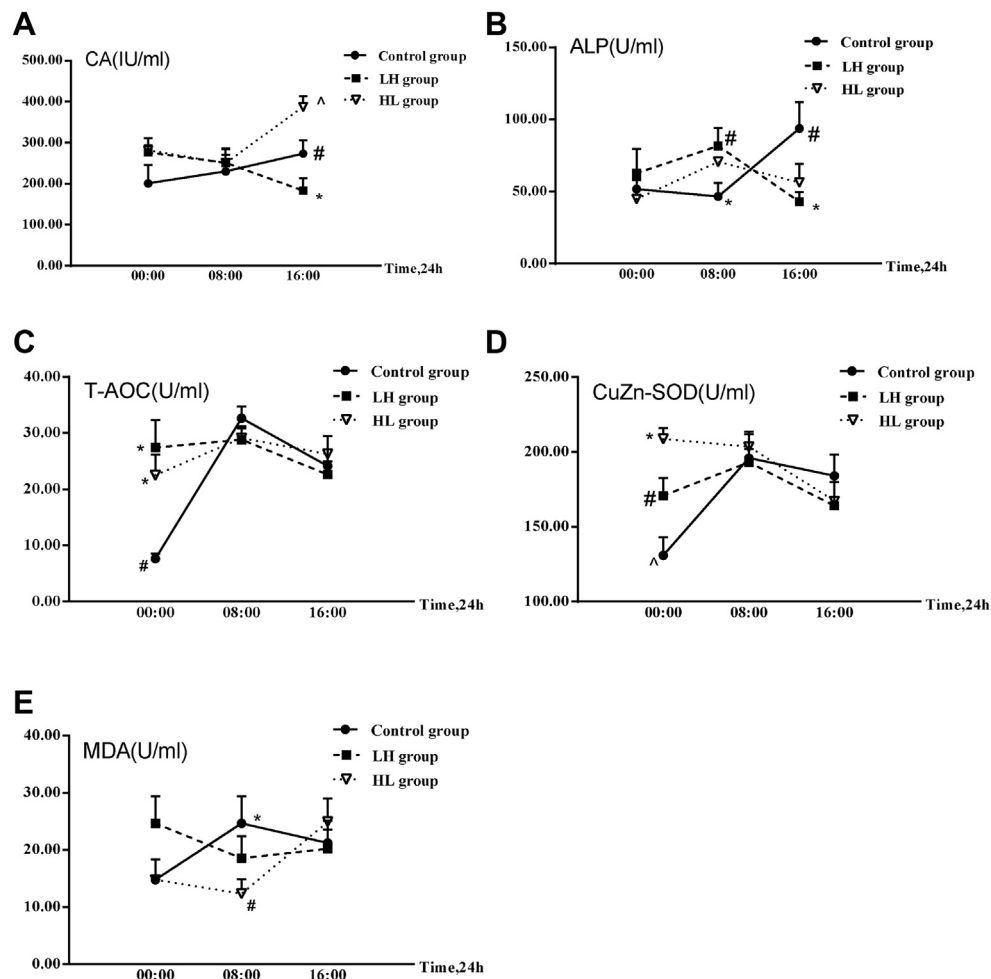


Figure 1. Effects of circadian zinc feeding regime on serum biochemical parameters after 10 wk. Carbonic anhydrase (CA), alkaline phosphatase (ALP), total antioxidant capacity (T-AOC) and CuZn-superoxide dismutase (CuZn-SOD), and malondialdehyde (MDA) is, respectively, shown in panels A, B, C, D and E. Data are means of 6 replicates per dietary treatment per time point, * and # denote significant ($P < 0.05$) differences for either: a) the LH group from the CON group or b) the HL group from the CON group. Abbreviations: CON, control; HL, Zinc: high-low; LH, Zinc: low-high.

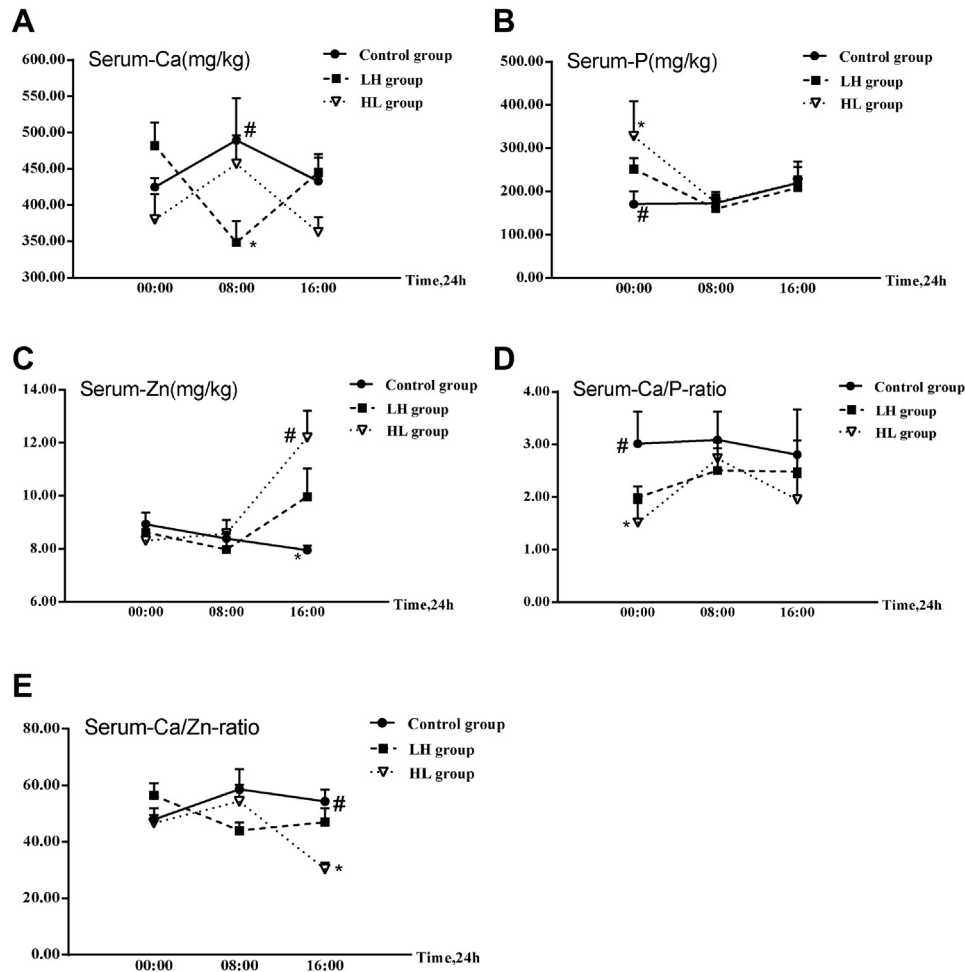


Figure 2. Effects of circadian zinc feeding regime on serum mineral level after 10 wk. Calcium (Ca), phosphorus (P), zinc (Zn), Ca: P ratio (Ca/P-ratio), and Ca: Zn ratio (Ca/Zn-ratio) is, respectively, shown in panels A, B, C, D and E. Data are means of 6 replicates per dietary treatment per time point, * and # denote significant ($P < 0.05$) differences for either a) the LH group from the CON group or b) the HL group from the CON group. Abbreviations: CON, control; HL, Zinc: high-low; LH, Zinc: low-high.

group, at 0,000 h, circadian Zn feeding regime upregulated mRNA expression level of Per2 and Per3 in duodenum of HL group ($P < 0.05$), as well as CLOCK, Cry2, Per2, and Per3 at 1,600 h in duodenum of HL group ($P < 0.05$). However, it decreased significantly BMAL1 in duodenum of HL group ($P < 0.05$).

Effects of circadian Zn feeding regime on mRNA expression level of circadian gene in eggshell gland of 3 treatments were presented in Figure 9. Compared with CON group, at 0,800 h, circadian Zn feeding regime upregulated mRNA expression level of Cry1 and Per3 in eggshell gland of HL group ($P < 0.05$), as well as Cry2 at 1,600 h in eggshell gland of LH group ($P < 0.05$).

DISCUSSION

Antioxidant system comprising metal enzyme CuZn-SOD has a cellular protective action against oxidative stress and dietary trace elements could help to maintain appropriate antioxidant balances (Putker and O'Neill, 2016). Zinc is involved in metabolism of oxygen and biochemistry of redox reactions included in CuZn-SOD, which catalyzes the dismutation of superoxide

(Pappas et al., 2011). In present study, significant correlation between good FCR and the highest or lowest morning Zn intake value in HL or LH group compared with CON group during 10 wk of trial period. Furthermore, serum T-AOC and CuZn-SOD at 0,000 h in LH or HL group increased. MDA is product of lipid peroxidation, and increased MDA levels are indexes of enhanced lipid peroxidation (Liu et al., 2015; Li et al., 2018). In this study, serum MDA at 0,800 h in HL group decreased. These indicated that dynamic Zn supplement schedule improved the antioxidant capacity of laying hens, which was contributed to the improvement of FCR of LH or HL group. During egg shell forming, there are increases in activities of ALP in blood of laying hens because of calcification process (Khawaja et al., 2013). In present study, the serum ALP at 0,800 h increased in LH group, and at 1,600 h, the mRNA expression levels of duodenum ALP, eggshell gland CuZn-SOD and ALP in HL group were upregulated. All above results indicated dynamic Zn supplement schedule improved FCR possibly through enhancing laying hens' antioxidant capacity. Furthermore, serum CA activity of HL group at 1,600 h increased accordingly. Thus, it was speculated

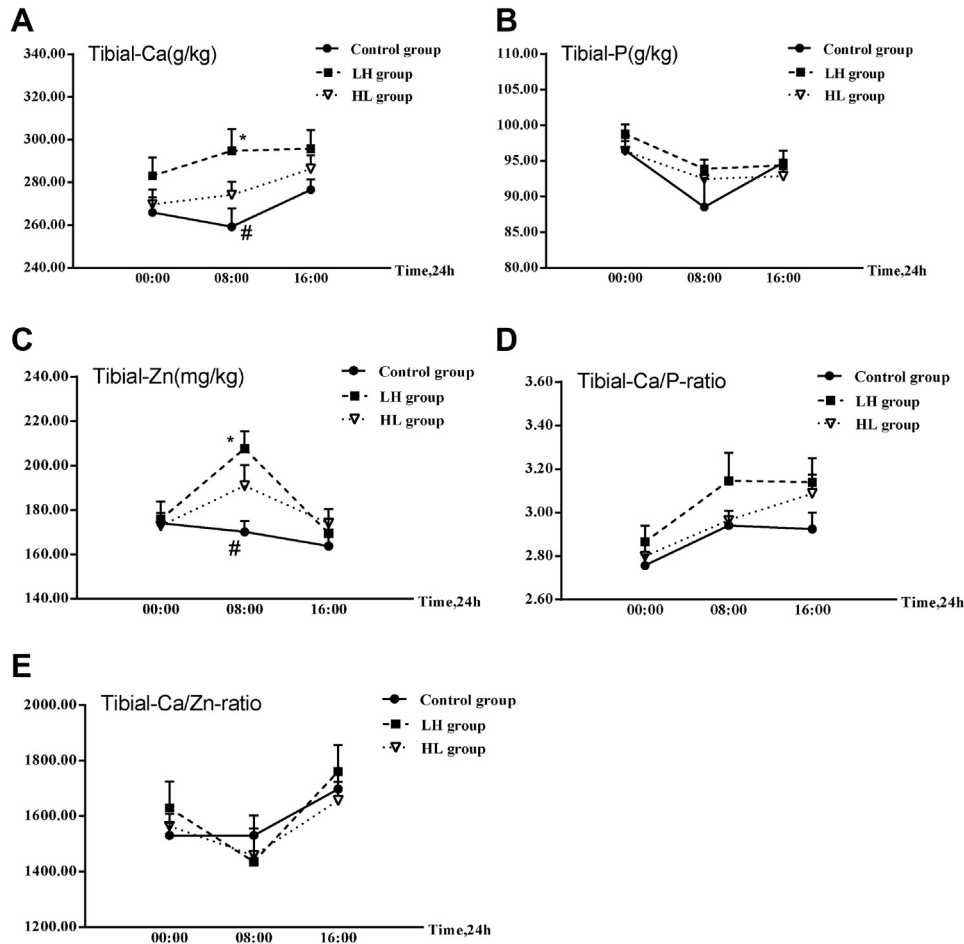


Figure 3. Effects of circadian zinc feeding regime on tibia mineral deposition after 10 wk. Calcium (Ca), phosphorus (P), zinc (Zn), Ca: P ratio (Ca/P-ratio), and Ca: Zn ratio (Ca/Zn-ratio) is, respectively, shown in panels A, B, C, D and E. Data are means of 6 replicates per dietary treatment per time point, * and # denote significant ($P < 0.05$) differences for either a) the LH group from the CON group or b) the HL group from the CON group. Abbreviations: CON, control; LH, Zinc: low-high; HL, Zinc: high-low.

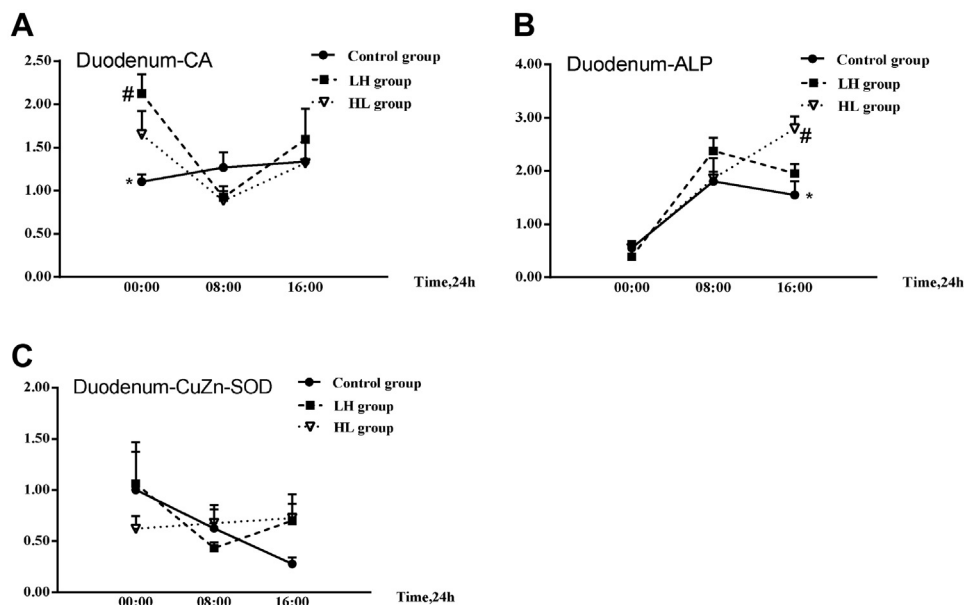


Figure 4. Effects of circadian zinc feeding regime on duodenum biological enzyme after 10 wk. The mRNA relative expression level of CA, ALP and CuZn-SOD is, respectively, shown in panels A, B and C. Data are means of 6 replicates per dietary treatment per time point, * and # denote significant ($P < 0.05$) differences for either a) the LH group from the CON group or b) the HL group from the CON group. Abbreviations: CON, control; LH, Zinc: low-high; HL, Zinc: high-low.

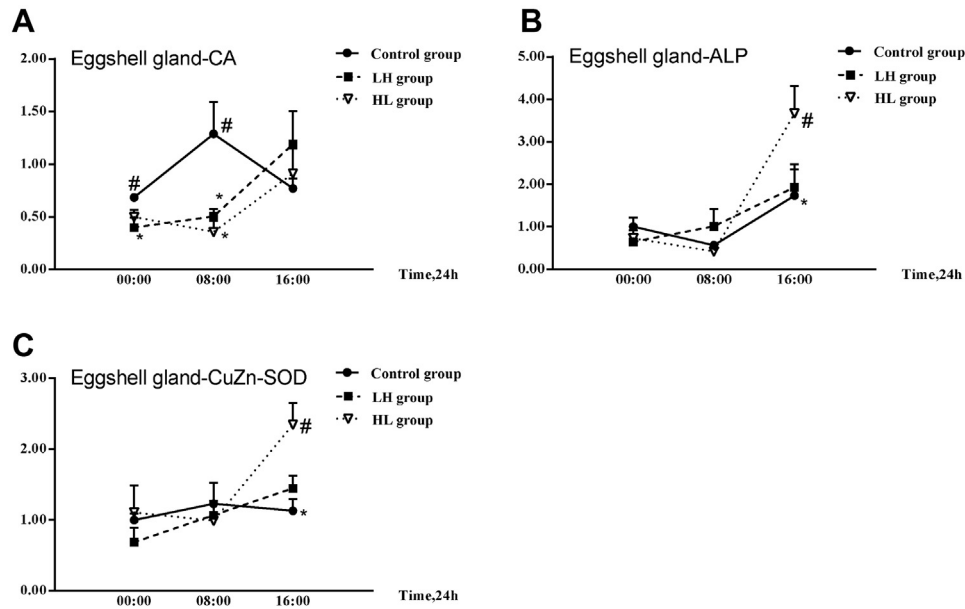


Figure 5. Effects of circadian zinc feeding regime on eggshell gland biological enzyme after 10 wk. The mRNA relative expression level of CA, ALP and CuZn-SOD is, respectively, shown in panels A, B and C. Data are means of 6 replicates per dietary treatment per time point, * and # denote significant ($P < 0.05$) differences for either a) the LH group from the CON group or b) the HL group from the CON group. Abbreviations: CON, control; LH, Zinc: low-high; HL, Zinc: high-low.

that increase of CA may be caused by the role of Zn as a component of CA, which is crucial for supplying carbonate ions during eggshell formation (TuMová et al., 2014). In present study, the mRNA expression level of duodenum CA at 0,000 h in LH group was upregulated. It was speculated that circadian Zn feeding regime affected FCR possibly through regulation of activities of Zn linking antioxidation and key biological enzymes including ALP and CA in eggshell forming.

Serum minerals levels are results of homeostatic regulation of minerals, also are the most important indicators

of birds' minerals nutritional status (Zhou et al., 2008). In present study, serum Zn value of HL group increased at 1,600 h, the possible reason is that Zn may regulate activity of key enzymes involved in the process of membrane in forming eggshell (Utlu et al., 2007), and eggshell forming may be started from the afternoon under the circadian feeding regime with high Zn diets in morning and low Zn diets in afternoon. Egg is an important international food, and hens may deposit minerals into the egg (Hargitai et al., 2016; Jia et al., 2016). In present study, circadian Zn feeding regime affected Ca retention

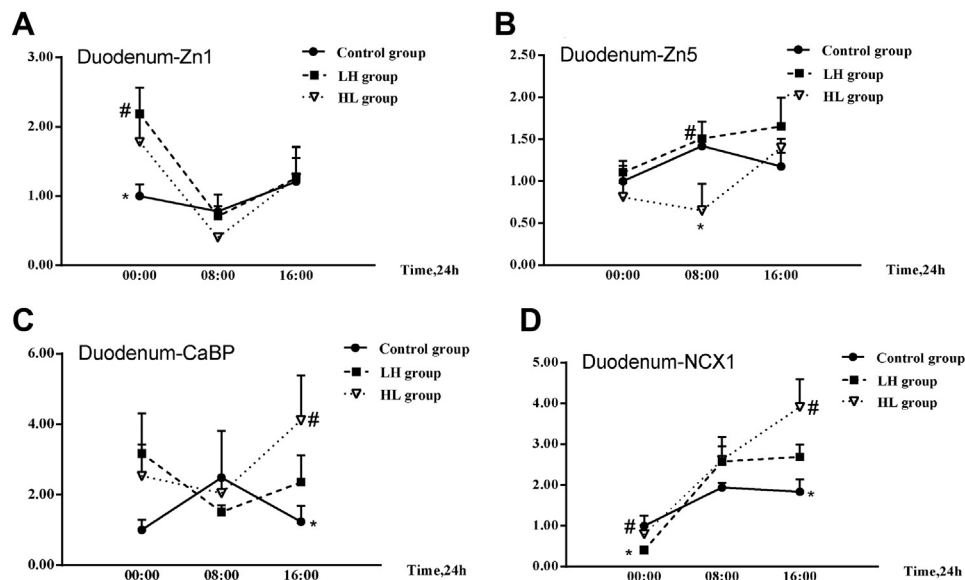


Figure 6. Effects of circadian zinc feeding regime on duodenum zinc and calcium transporter gene after 10 wk. The mRNA relative expression level of Zn1, Zn5, CaBP and NCX1 is, respectively, shown in panels A, B, C and D. Data are means of 6 replicates per dietary treatment per time point, * and # denote significant ($P < 0.05$) differences for either a) the LH group from the CON group or b) the HL group from the CON group. Abbreviations: CON, control; LH, Zinc: low-high; HL, Zinc: high-low.

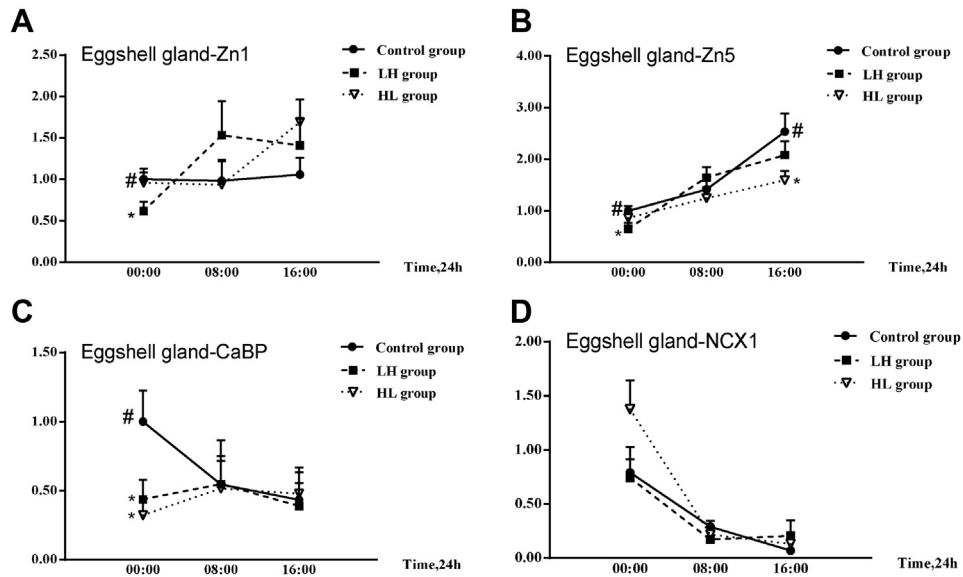


Figure 7. Effects of circadian zinc feeding regime on eggshell gland zinc and calcium transporter gene after 10 wk. The mRNA relative expression level of Zn1, Zn5, CaBP and NCX1 is, respectively, shown in panels A, B, C and D. Data are means of 6 replicates per dietary treatment per time point, * and # denote significant ($P < 0.05$) differences for either a) the LH group from the CON group or b) the HL group from the CON group. Abbreviations: CON, control; LH, Zinc: low-high; HL, Zinc: high-low.

in egg yolk, especially, both highest and lowest morning Zn supplement decreased Ca concentration, Ca: P and Ca: Zn ratio in egg yolk. These may be connected to

serum Zn increase and Ca: Zn ratio decrease at 1,600 h in HL group, and tibia Ca and Zn level at 0,800 h in LH group increased respectively in present study. Tissue

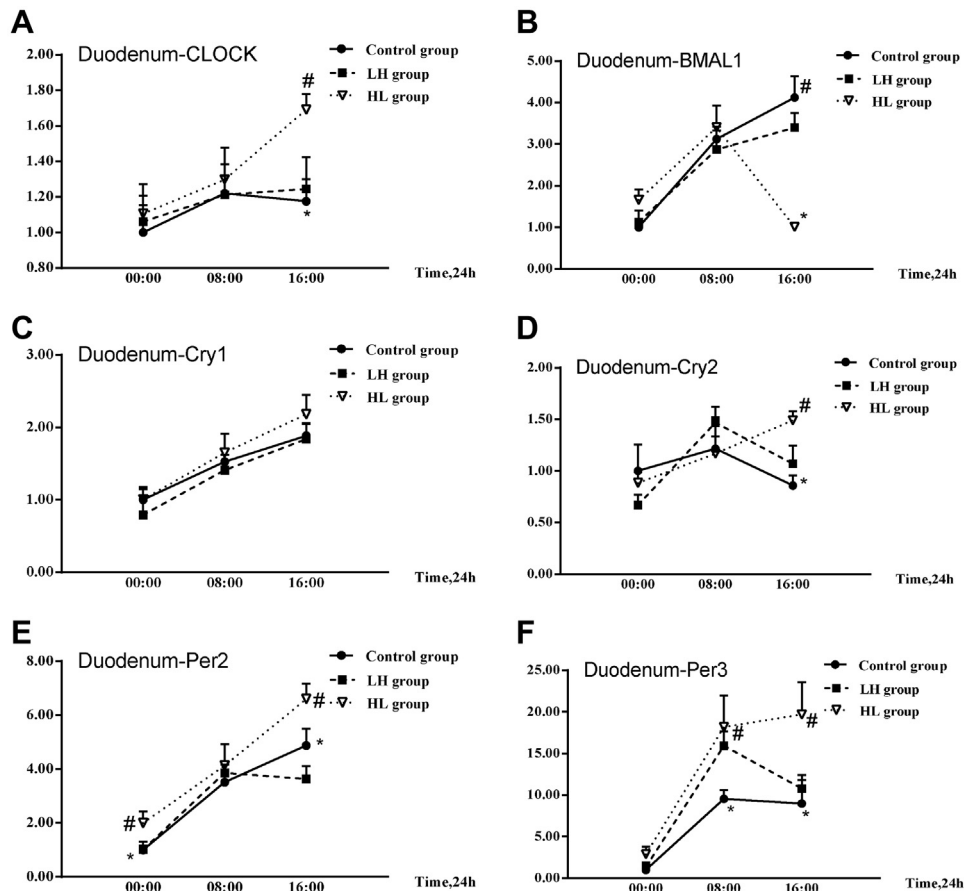


Figure 8. Effects of circadian zinc feeding regime on duodenum circadian gene after 10 wk. The mRNA relative expression level of CLOCK, BMAL1, Cry1, Cry2, Per2 and Per3 is, respectively, shown in panels A, B, C, D, E and F. Data are means of 6 replicates per dietary treatment per time point, * and # denote significant ($P < 0.05$) differences for either a) the LH group from the CON group or b) the HL group from the CON group. Abbreviations: CON, control; LH, Zinc: low-high; HL, Zinc: high-low.

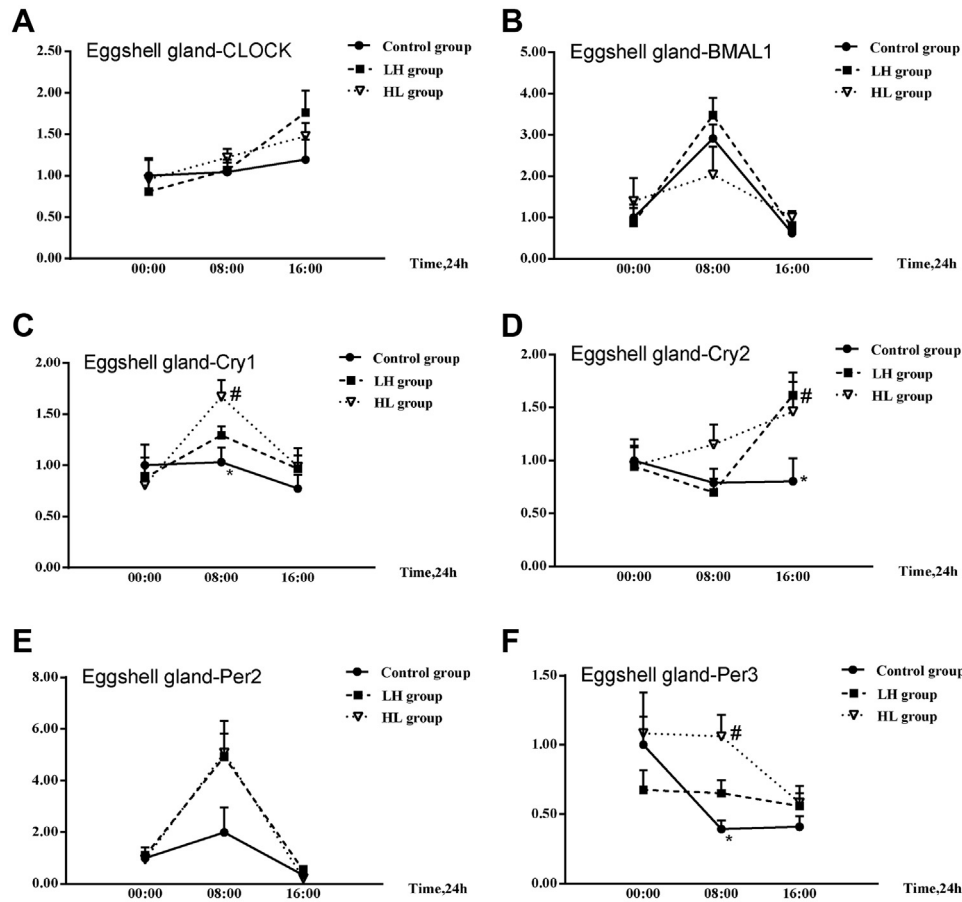


Figure 9. Effects of circadian zinc feeding regime on eggshell gland circadian gene after 10 wk. The mRNA relative expression level of CLOCK, BMAL1, Cry1, Cry2, Per2 and Per3 is, respectively, shown in panels A, B, C, D, E and F. Data are means of 6 replicates per dietary treatment per time point, * and # denote significant ($P < 0.05$) differences for either a) the LH group from the CON group or b) the HL group from the CON group. Abbreviations: CON, control; LH, Zinc: low-high; HL, Zinc: high-low.

mineral concentration level is usually used to evaluate mineral status in animals (Feng et al., 2009). In this study, the Zn and Ca changing status of serum and tibia suggested that Ca may be interacted with Zn at level of absorption, distribution, retention (Pappas et al., 2011), and time. It demonstrated that dynamic changes of Zn intake in the morning and afternoon affected serum, tibia, and egg yolk Zn and Ca deposition in laying hens. This was related to Zn and Ca metabolism in physiological processes.

Calcium is a critical nutrient to ensure the production of egg with good quality eggshell and must have adequate level and be well balanced in ration (Rodrigues et al., 2013), and in present study, serum Ca value at 0,800 h in LH group decreased significantly, it was speculated that serum Ca can be influenced by the dietary Zn and time of intake, and low Zn feed intake may affect the absorption of Ca. The principal site of mineral absorption in animal is the small intestine (Veum et al., 2009). On one hand, in present study, dynamic Zn supplement schedule affected Zn transporter protein genes. In LH group, mRNA expression level of duodenum Zn1 at 0,000 h increased, but eggshell gland Zn5 at 0,000 h and 0,800 h decreased, as well as duodenum Zn5 at 0,800 h and eggshell gland Zn5 at 1,600 h in HL group.

And these changes may influence Ca metabolism. On the other hand, CaBP and $\text{Na}^+/\text{Ca}^{2+}$ exchangers (NCX) were considered to be involved in Ca transporters (Bar, 2009). Protein NCX is primarily as plasma membrane Ca^{2+} efflux mechanism, NCX1 occurs at almost all cells (Hudecova et al., 2011). The present study showed that dynamic Zn supplement schedule leads to Ca metabolism changes in laying hens, which provided molecular support that Ca transporter like NCX1 and CaBP at 1,600 h of duodenum in HL group increased significantly, but CaBP at 0,000 h of eggshell gland both in LH and HL group decreased significantly. Obviously, these observations demonstrated that there were interaction effects between Ca and Zn metabolism. However, interactions of minerals are complex, and study indicates that increase of Ca intake may reduce bioavailability of Zn in animal, and this effect may be dose-dependent (Ao and Pierce, 2013). It was speculated that circadian Zn supplement regime affected Ca deposition in egg via interaction between Ca and Zn metabolism in laying hens. Present observations suggested that this Ca and Zn metabolism regulation was under control of circadian time-keeping system.

Circadian rhythms play a major role in regulating the digestive system (Froy and Nava, 2007), resulting that a

connection between Zn intake and other minerals metabolism in body. Calcium was connected, and the present study showed that Zn intake changes in the morning and afternoon led to egg yolk, serum, and tibia Ca deposition changes in laying hens. Circadian clock mechanisms in the brain and peripheral tissue are consist of CLOCK, BMAL1, core clock proteins period (**PER**), and cryptochrome (**CRY**) gene (St John et al., 2014; Coogan et al., 2016). Various peripheral tissue shows circadian rhythm (Ando et al., 2005), and peripheral tissue also contains circadian clock regulating physiological function (Tahara and Shibata, 2016), such as metabolism in intestine (Froy, 2010). In present study, Per2 and Per3 at 0,000 h, CLOCK, Cry2, Per2, and Per3 at 1,600 h were significantly upregulating in duodenum of HL group, same as Cry1 and Per3 at 0,800 h in eggshell gland of HL group, Cry2 at 1600h in eggshell gland of LH group. By contrast, at 1,600 h, BMAL1 in duodenum of LH group was significantly suppressed. This was consistent with that peripheral circadian oscillator may be coupled to SCN primarily through rhythmic behaviors, such as feeding behavior (Stokkan et al., 2001), and dynamic feeding schedule may act on peripheral oscillator via chemical cue that can signal to peripheral tissue (Damiola et al., 2000). It suggested that Zn feeding time may be served as Zeitgeber to entrain peripheral, intestine, and eggshell gland oscillators, resulting that a connection between circadian genes regulation and Zn homeostasis. The present study showed that upregulation of duodenum CLOCK, Cry2, Per2, and Per3 at 1,600 h in HL group led to serum Zn increase at 1,600 h in HL group, mRNA expression level of NCX1, CaBP, and ALP at 1,600 h of duodenum in HL group upregulation corresponding increase. It was speculated that dynamic Zn time feeding schedule affected expression levels of circadian clock genes. These observations suggested that the upregulation of clock genes speed up the expression levels of NCX1, CaBP, and ALP. There were possible interactions between circadian clock and Zn feeding time, which the high Zn intake in morning and low Zn intake afternoon may be more sensitive. It also suggested clock-controlled genes regulate Ca homeostasis during laying period, which led to low Ca deposition in egg yolk in HL group. Calcium deposition in egg yolk was affected by high or low level Zn supplementation at daily limited time, which was possibly connected to physiological process of Ca and Zn metabolism. This was regulated by circadian rhythm. In summary, the findings indicated that dynamic Zn feeding time schedule had effects on altering egg yolk and tibia minerals deposition, mRNA expression level of clock, Ca and Zn transporter, and biological enzyme genes in duodenum and eggshell gland. It was also in accordance with the suggestion that circadian regulation may be linked to metabolic homeostasis (Green et al., 2008). Feeding schedule may be involved into generation of circadian rhythm (Hotchkiss and Jerome, 1998). However, precise mechanism of study like physiological process needs to be elucidated through further studies.

CONCLUSIONS

In conclusion, circadian feeding regime with low and high Zn supplement diets affected gene expression levels of circadian clock including CLOCK, BMAL1, Cry1, Cry2, Per2, and Per3, Zn transporters including Zn1 and Zn5, Ca transporters including NXC1 and CaBP, and biological enzymes including CA, ALP, and CuZn-SOD in present study. It indicated that high Zn intake in morning and low Zn intake afternoon affected the interrelationship of Ca and Zn metabolism possibly through regulating laying hens' circadian rhythms. This led to increase of Zn concentration in serum and tibia and decrease of Ca deposition in egg yolk. Present study suggested that circadian feeding diet regime with low and high Zn diets was beneficial to improvement of FCR without negative effects on egg quality. The regulation of antioxidant capacity and interaction of Zn and Ca metabolism in laying hen was related to laying hens' circadian rhythms, which may contribute to the improvement of the hens' laying performance.

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SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at <https://doi.org/10.1016/j.psj.2020.06.086>.

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