Effects of feeding strategies on eggshell quality of laying hens during late laying period

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ABSTRACT Insufficient calcium supply during the dark period is an important reason for deteriorated eggshell quality in laying hens. In the present study, the feeding time of hens was altered in order to investigate whether the changes in feeding time and feed consumption could influence the laying performance and eggshell quality of hens. A total of 192, 60-wk-old Hy-line Brown hens with similar body weight and laying rate were obtained. The hens were randomly divided into 4 groups and subjected to the following feeding strategies: feeding 3 times a day (control group, **CON**), or feeding once a day in the morning at 08:00 (**MF**), in the noon at 12:00 (**NF**), or in the afternoon at 16:00 (**AF**), respectively. The feeding strategies had no significant effect ($P > 0.05$) on laying rate, egg weight, and egg mass. Although the feed intake did not differ among treatments, the time phase of feed consumption was changed. From 15:00 to 21:00 h, hens consumed 49.7%, 42.4%, 49.1%, and 70.8% of daily feed intake in the CON, MF, NF, and AF groups, respectively. Feeding strategy had no detectable influence ($P > 0.05$) on egg shape index, eggshell strength, and eggshell percentage. Compared to CON, AF hens tended to have a higher eggshell thickness ($P = 0.053$). In MF and NF treatments, plasma calcium (**Ca**), phosphorus (**P**) levels, and alkaline phosphatase (**ALP**) activity did not differ ($P > 0.05$) compared with CON. In contrast, AF-hens had lower Ca and P levels, but a higher ALP activity than CON ($P < 0.01$). The AF hens had higher uterine fluid Ca than MF and NF hens ($P < 0.05$). Compared to CON, the expression level of *CaBP-D28K* was increased in the shell gland mucosa of MF-hens. Also, MF-, NF-, and AF-hens had higher *Osteopontin* (**OPN**) expression level ($P < 0.05$), whereas NF had a higher expression of *OC-116* ($P < 0.01$). In conclusion, the results indicated that feeding in the afternoon changed the pattern of feed consumption and exerted a positive influence on eggshell thickness.

Key words: laying hens, feeding strategy, eggshell quality, calcium, uterine fluid

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INTRODUCTION

Deterioration in eggshell quality with flock age is a major challenge with hens producing 500 eggs in a laying cycle of 100 weeks. In laying hens, egg quality decreases with age, especially during the late laying cycle. Egg weight increases, whereas the percentage of eggshell decreases with the age of hens (Silversides and Scott, 2001; Park and Sohn, 2018; Feng et al., 2020; El-Tarabany et al., 2021; Huang et al., 2022). Eggshell quality parameters such as eggshell thickness and breaking strength gradually decrease during the production cycle (Benavides-Reyes et al., 2021).

A laying hen requires about 2.2 g of calcium (**Ca**) to produce an egg. About two-thirds of the needed Ca is obtained from the diet, whereas one-third comes from the medullary bone (Bain et al., 2016; Gloux et al., 2020). During the laying cycle, Ca ingestion and deposition in bone occurs during the day, whereas it is immobilized from bone and mineralized into the eggshell during the night. It is speculated that providing hens with adequate Ca during the period of eggshell formation will be beneficial for eggshell quality. In a previous study, providing hens with most of the daily Ca need in the afternoon had no favorable effect on the shell quality, compared to the control group that received a diet with 3.5% Ca in the morning and afternoon. More so,

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inadequate Ca intake during the afternoon adversely affected the eggshell quality (Keshavarz, 1998). Molnár et al. (2018) evaluated the effects of split-feeding system on the eggshell quality of aged laying hen between 75 and 92 wk of age and they found that the optimal dietary combinations were a morning diet with fine limestone and an afternoon diet with coarse limestone, which both provided ~50% of the total daily Ca intake. Hence, increasing dietary Ca supply to the intestinal tract during eggshell formation may have a favorable effect on eggshell quality.

Recently, it was shown that prolonged scotophase from 8 h to 15 h increased the eggshell strength, the contents of Ca and phosphorus (**P**) in the eggshell, and the blood Ca and P concentration (Xin et al., 2021). The commercial laying hen habitually performs dusk feeding, the behavior of feeding more at night to satisfy nutrient requirements (Scanes et al., 1987). Dusk feeding is observed from 5 h before scotophase and can be synchronously shifted with an altered lighting program (Wang et al., 2021a). In laying hens, about 50% of the daily feed intake was consumed from 05:00 to 15:00 h and the next 50% from 15:00 to 21:00 h, showing a peak from 17:00 to 21:00 h (Keshavarz, 1998). Hence, we hypothesized that changing the feeding strategy to augment Ca supply for eggshell formation during scotophase via increasing feed consumption in the afternoon may exert a favorable influence on the eggshell quality.

The aim of the present study was to investigate whether delayed feeding time from morning to afternoon could effectively improve the eggshell quality of laying hens during the late laying phase. The laying performance, egg quality, blood Ca and P levels, and mRNA expression level of genes related to Ca transportation in the uterus of laying hens were examined.

MATERIALS AND METHODS

All test protocols used in this study were approved by the Animal Care Committee of Shandong Agricultural University.

Animals

A total of 192 healthy 60-wk-old Hy-line Brown laying hens with similar body weights were selected. Two or 3 hens were reared in an individual cage (60 cm length × 45 cm width × 50 cm height), equipped with a nipple drinker and a feeder (15 cm/hen). The light regime was 16-h light and 8-h dark (16 L: 8 D). The experimental hens were divided into 4 treatments, 6 groups of 8 hens each. The treatment groups were feeding 3 times a day at 8:00, 12:00, and 16:00 h, respectively (**CON**), feeding once in the morning at 08:00 h (**MF**), feeding once at noon, 12:00 h (**NF**), or feeding once in the afternoon at 16:00 h (**AF**). The experiment lasted for 8 wk from 61 to 68 wk of age. A corn-soybean meal-based diet was formulated (Table 1). During the experimental period, feed intake, egg production, egg weight, and the number of broken eggs were recorded daily.

Table 1. Composition and nutrient levels of the experimental diets (air-dry basis) (%).

Ingredients	%	Nutrient levels[3]	%, MJ/Kg
Corn (8.5% CP)	59.75	Crude protein	16.50
Soybean meal (43% CP)	25.69	Metabolic energy	11.30
Limestone	9.00	Calcium[4]	3.30
Wheat bran	2.06	Total phosphorus[4]	0.48
Soybean oil	1.50	Lysine	0.78
Calcium hydrogen phosphate	1.21	Methionine	0.36
Salt	0.35	Threonine	0.67
Choline chloride (50%)	0.10	Tryptophan	0.22
Methionine (99%)	0.09	Met + Cys	0.65
Vitamin premix feed[1]	0.05		
Trace element feed[2]	0.20		

[1]Vitamin premix provides the following per kg of diet: vitamin A, 3.96 IU; vitamin D3, 0.06 IU; vitamin E, 20 IU; vitamin K, 3 mg; thiamin mononitrate, 1.70 mg; riboflavin, 5.50 mg; pantothenic acid, 9.40 mg; niacin, 28 mg; pyridoxine, 6.60 mg; vitamin B12, 3.30 mg; biotin, 0.10 mg; folic acid, 0.60 mg; choline chloride, 470 mg.
[2]Premix provided the following per kg of diet: Fe, 80 mg; Zn, 75 mg; Mn, 88 mg; Cu, 10 mg; I, 0.4 mg; Se, 0.3 mg.
[3]Calculated values.
[4]Measured values.

At the end of the experiment, 6 hens (1 per group) were randomly selected from each treatment, and blood sample was collected at 6 time points: 04:00, 08:00, 12:00, 16:00, 20:00, and 24:00 h. Furthermore, another 12 hens (2 per group) were randomly selected from each treatment and allocated in single cages. At 16 to 18 h after ovulation, the period of rapid calcification of eggshell (Stapane et al., 2020), a blood sample was collected from the brachial vein into heparinized tubes. Plasma samples were obtained after centrifugation at 3000 × *g* at 4°C for 15 min and stored at -20°C for further analysis. Thereafter, the hens were sacrificed by exsanguination. Eggshell gland was obtained and the uterine fluid was collected and immediately diluted with PBS (1v:1v) according to Riou et al. (2019). After centrifuging at 3000 rpm, 4°C for 10 min, the supernatant was separated, snap-frozen in liquid nitrogen, and stored at - 80°C for subsequent determination. The mucosa of eggshell gland was scraped, snap frozen in liquid nitrogen and store at -80°C for further analysis according to Aurélien et al. (2014).

Eggshell Quality

Egg quality was measured every 2 wk (62, 64, 66, and 68 wk of age). All the eggs on the last day of the measuring week were collected in each group. Egg length and egg width were measured using vernier caliper (LXZ919160, Shenzhen, China) and the egg shape index was calculated by dividing the egg width by the egg length. Eggshell thickness was measured by averaging 3 locations on the egg (air cell, equator, and sharp end) using an eggshell thickness tester (ETG-1061, Tokyo, Japan). Eggshell strength was measured using an egg force reader (EFG-0503, Tokyo, Japan). Yolk color, Haugh unit, and the height of the thick albumen were

measured using the egg quality analyzer (EMT-5200, Tokyo, Japan). Yolk and albumen were separated and weighed using a sensitive weighing balance, and their relative proportions (% egg weight) were determined according to Hussein et al. (1992).

Calcium, Phosphorus, and Alkaline Phosphatase Measurement

Plasma calcium (**Ca**), phosphorus (**P**), and the activity of alkaline phosphatase (**ALP**) were measured by an automatic biochemical analyzer with commercially available kit (Maccura Biotechnology Co. Ltd., Chengdu, Sichuan, China).. The Ca content in the uterine fluid was determined using ELISA Kit (Shanghai Enzyme Linked Biology Co., Ltd., Shanghai, China).

Total RNA Extraction and Real-Time PCR Analysis

The expression of calbindin-D28k (***CaBP-D28k***), plasma membrane ca-atpase-1b (***PMCA 1b***), milk fat globule-EGF factor 8 protein (***MFGE8***), EGF like repeats and discoidin domains 3 (***EDIL3***), ovalbumin (***OVAL***), osteopontin (***OPN***), ovocalyxin-32 (***OCX-32***), ovocalyxin-36 (***OCX-36***), and ovocleidin-116 (***OC-116***) in the eggshell gland mucosa was detected. Total RNA was extracted with Trizol reagent (TransGen Biotech, Beijing, China) and the RNA concentration and purity were tested using the DS-11 spectrophotometer (DeNovix, Wilmington, DE). RNA transcription was done with the HiFiScript cDNA synthesis kit (CWBIO, Jiangsu, China) and Real-time qRT-PCR was performed on ABI QuantStudio five Real-Time PCR Instrument (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA). Using β-actin as an internal reference, the relative mRNA expression level was calculated using the $2^{-\triangle\triangle CT}$ method (Xin et al., 2021). The primers were designed using Primer 5.0 software, as shown in Table 2

Statistical Analysis

The results were expressed as mean ± standard error (**SE**). For variables laying performance, a 2-way ANOVA model was used to estimate the main effects of feeding treatment, age, and their interaction with each group as replicate (n = 6) by SAS statistical software (version V8, SAS Institute, Cary, NC). For variables gene expression and blood parameters during the rapid calcification period, a 1-way ANOVA model was used to estimate the main effect of feeding treatment with each hen as replicate (n = 12). For egg quality, a 2-way ANOVA was used to estimate the main effect of feeding treatment, time, and their interaction with each group as replicate (n = 6). The dynamic changes of plasma parameters within a day were analyzed with repeated measurement analysis with each group as replicate (n = 6). When the main effect of the treatment was significant, the differences between means were assessed by Duncan's multiple comparisons test. $P < 0.05$ was considered to be statistically significant.

RESULTS

Feeding treatment had no significant effect ($P > 0.05$) on laying rate, egg weight, egg mass, feed efficiency, and egg broken rate, whereas decreased ($P < 0.05$) feed intake (Table 3). Feed intake significantly changed with age ($P < 0.01$). Compared to CON, feeding strategy changed

Table 2. Gene-specific primer of detected genes.

Target gene	Primer sequence, 5'-3'	Accession no.
CaBP-D28k	F: TGTTATGGAGTGCAGGATGG R: TAGAGCGAACAAGCAGGTGA	NM_205513.2
PMCA 1b	F: TTCAGGTACTCATGTGATGGAAGG R: CAGCCCCAAGCAAGGTAAAG	XM_046906440.1
TRPV6	F: GGCTGTGGTGATACTAGGCTTTGC R: AAAGGTGGTGAACAAGGACATGGG	XM_040661661.2
MFGE8	F: GCTCGCTCGCCTCAACAACC R: ACCACTCAGCCTCATCTTCCTCAG	NM_001277110.2
EDIL3	F: GCGTACCGAGGCGACACATTC R: TACATTGTGCTGGCAGTGAATCCC	XM_046936088.1
OVAL	F: CAATCTGTCTGGCATCTCCTCAGC R: CGTTGGTTGCGATGTGCTTGATAC	NM_205152.3
OPN	F: AGGTGGACGGAGGAGACA R: ACGGGTGACCTCGTTGTT	NM_204535.5
OCX-32	F: TGCCCATGACCATCTTCAACTGTG R: TGCCAATAAGTAACCCGTGTGCTC	NM_204534.5
OCX-36	F: CCTGAAGCCACACCTCACTAAGC R: ATCGCCAACAGTCCCAACAAGATC	XM_046930818.1
OC-116	F: AAGGCATAGATGAGTTCGCAT R: ATGGTGACAGTGGCATCGC	XM_046915459.1
β-actin	F: TGCGTGACATCAAGGAGAAG R: TGCCAGGGTACATTGTGGTA	NM_205518.2

Abbreviations: *CaBP-D28k*, calbindin-D28k; *EDIL3*, EGF like repeats and discoidin domains 3; *MFGE8*, milk fat globule-EGF factor 8 protein; *PMCA 1b*, plasma membrane ca-atpase-1b; *OC-116*, ovocleidin-116; *OCX-32*, ovocalyxin-32; *OCX-36*, ovocalyxin-36; *OPN*, osteopontin; *OVAL*, ovalbumin; *TRPV6*, transient receptor potential vanilloid 6.

Table 3. Effects of feeding strategy (FS) on laying performance of hens.

Item	Feed intake (g/d)	Laying rate (%)	Egg mass (g)	Egg weight (g)	Feed efficiency (g/g)	Egg broken rate (%)
Feeding strategy						
CON	110.0 ± 0.7[a,b]	77.5 ± 1.4	46.7 ± 0.8	60.3 ± 0.1	2.38 ± 0.04	3.85 ± 0.55
MF	110.4 ± 0.6[a]	80.3 ± 1.2	48.3 ± 0.7	60.2 ± 0.4	2.31 ± 0.04	3.45 ± 0.44
NF	108.0 ± 0.7[b,c]	78.3 ± 1.0	47.1 ± 0.7	60.1 ± 0.4	2.32 ± 0.04	2.97 ± 0.39
AF	107.7 ± 0.9[c]	75.6 ± 1.0	45.9 ± 0.7	60.6 ± 0.3	2.37 ± 0.03	3.70 ± 0.45
Week of age						
61 wk	105.9 ± 1.1[c]	78.9 ± 1.6	47.8 ± 1.1	60.5 ± 0.4	2.35 ± 0.04[c]	4.95 ± 0.72
62 wk	106.7 ± 1.2[c]	78.1 ± 1.7	47.1 ± 1.1	60.3 ± 0.4	2.36 ± 0.05[b,c]	3.96 ± 0.69
63 wk	109.9 ± 1.0[a]	74.7 ± 1.6	44.9 ± 1.0	60.1 ± 0.4	2.39 ± 0.05[a]	3.97 ± 0.71
64 wk	110.0 ± 1.0[a]	78.1 ± 1.6	47.1 ± 1.0	60.3 ± 0.4	2.30 ± 0.06[a,b,c]	4.17 ± 0.57
65 wk	110.1 ± 0.9[a]	77.8 ± 1.6	47.1 ± 0.9	60.6 ± 0.4	2.23 ± 0.04[a,b,c]	2.53 ± 0.61
66wk	108.6 ± 0.9[a,b,c]	77.3 ± 1.7	46.6 ± 1.1	60.3 ± 0.6	2.29 ± 0.04[a,b,c]	2.93 ± 0.65
67 wk	111.6 ± 0.8[a]	78.4 ± 1.7	47.1 ± 1.0	60.1 ± 0.5	2.47 ± 0.05[a,b]	2.66 ± 0.52
68 wk	109.5 ± 1.0[a,b]	79.9 ± 1.9	48.2 ± 1.1	60.3 ± 0.4	2.35 ± 0.04[b,c]	2.79 ± 0.59
P value						
FS	0.018	0.055	0.178	0.776	0.340	0.539
Week of age	0.002	0.624	0.596	0.994	0.042	0.070
FS × week of age	0.998	1.00	1.00	0.999	0.998	0.457

AF, fed one time at 16:00 h; CON, fed ad libitum; MF, fed one time at 08:00 h; NF, fed one time at 12:00 h.
Data were presented at mean ± SE (n = 6).
[a,b,c]Means in the same column with different superscripts differ significantly ($P < 0.05$).

($P < 0.05$) the distribution of feed intake and the peak intake occurred immediately after feeding time in MF at 09:00 h, in NF at 13:00 h, and in AF at 17:00 h (Figure 1A). The hens consumed 49.7%, 42.4%, 49.1%, and 70.8% of daily feed intake in the CON, MF, NF, and AF groups from 15:00 to 21:00 h, respectively (Figure 1B).

Feeding strategy had no detectable influence on egg shape index, eggshell strength, and eggshell percentage

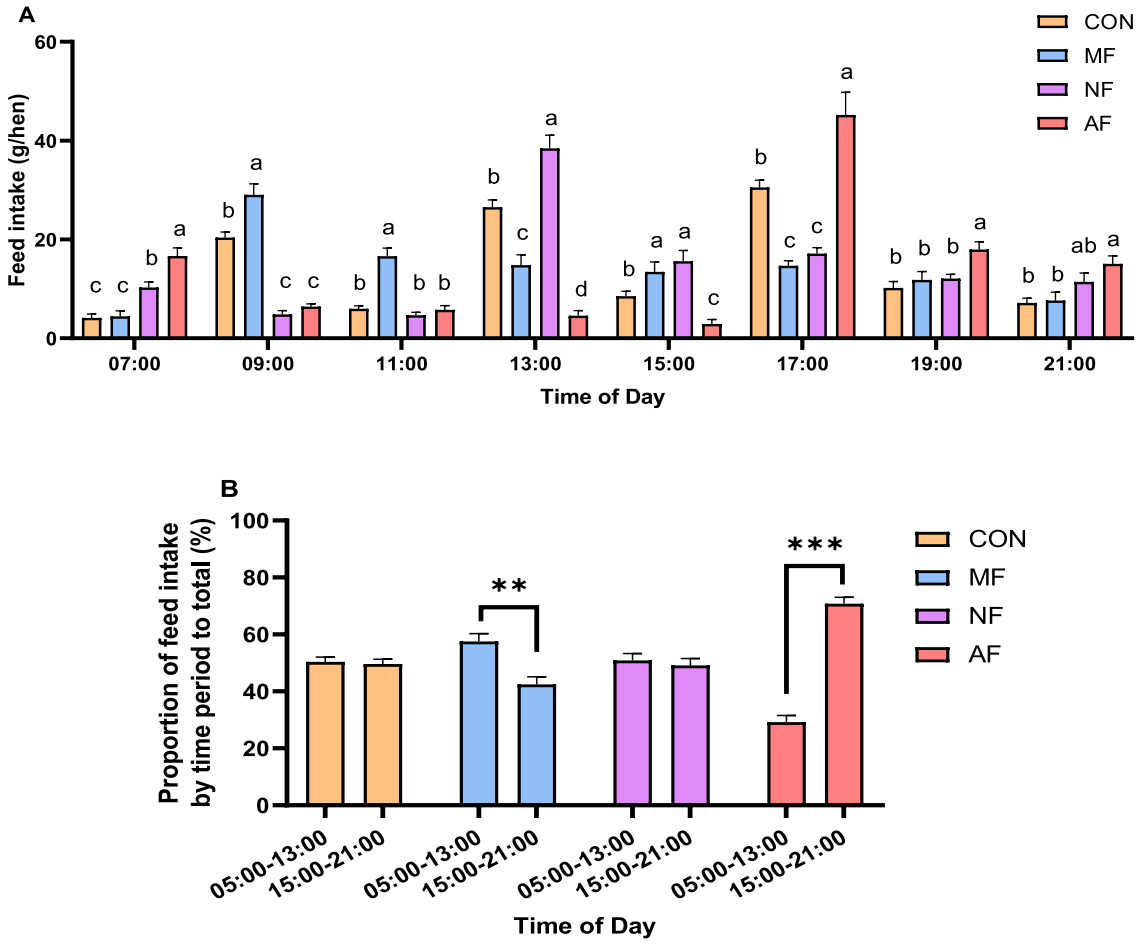

Figure 1. Effects of different feeding strategies on photoperiodic feed intake. (A) feed intake of hen every 2 h during the 16-h light cycle; (B) the proportion of feed intake during the periods of 05:00−13:00 h and 15:00−21:00 h. [a−d], means with different letter differ significantly *$P < 0.05$; ** $P < 0.01$, *** $P < 0.001$ (n = 6).

Table 4. Effects of feeding strategy (FS) on egg quality of laying hens.

Item	Egg weight (g)	Egg shape index (%)	Eggshell strength (Kg·f)	Percentage of eggshell (%)
Feeding strategy				
CON	60.2 ± 0.24	76.39 ± 0.21	3.79 ± 0.06	10.1 ± 0.1
MF	60.8 ± 0.58	76.92 ± 0.32	3.59 ± 0.07	10.0 ± 0.1
NF	60.5 ± 0.47	76.22 ± 0.17	3.66 ± 0.09	10.1 ± 0.1
AF	60.6 ± 0.58	76.20 ± 0.26	3.74 ± 0.09	10.1 ± 0.1
Week of age				
62 wk	59.9 ± 0.59	76.58 ± 0.20	3.65 ± 0.09	10.1 ± 0.1[a,b]
64 wk	60.9 ± 0.49	76.48 ± 0.18	3.78 ± 0.07	10.0 ± 0.1[b]
66 wk	60.4 ± 0.43	76.23 ± 0.32	3.72 ± 0.06	9.9 ± 0.1[b]
68 wk	60.8 ± 0.39	76.43 ± 0.28	3.63 ± 0.08	10.3 ± 0.1[a]
P value				
FS	0.859	0.145	0.297	0.646
Week of age	0.516	0.788	0.491	0.026
FS × week of age	0.900	0.433	0.646	0.387

AF, fed one time at 16:00 h; CON, fed ad libitum; MF, fed one time at 08:00 h; NF, fed one time at 12:00 h.
Data were presented at mean ± SE (n = 6).
[a,b]Means in the same column with different superscripts differ significantly ($P < 0.05$).

Table 5. Effects of feeding strategy (FS) on eggshell thickness (mm) of laying hens.

Weeks	CON	MF	NF	AF
62	0.33 ± 0.00	0.32 ± 0.01	0.32 ± 0.01	0.33 ± 0.01
64	0.33 ± 0.00	0.32 ± 0.01	0.33 ± 0.00	0.33 ± 0.00
66	0.31 ± 0.00	0.32 ± 0.00	0.33 ± 0.00	0.33 ± 0.00
68	0.31 ± 0.00	0.32 ± 0.00	0.32 ± 0.00	0.33 ± 0.00
Means	0.32 ± 0.00	0.32 ± 0.00	0.32 ± 0.00	0.33 ± 0.00
P value		CON vs. MF	CON vs. NF	CON vs. AF
FS		0.487	0.276	0.053
Week of age		0.108	0.003	0.009
FS × Week of age		0.091	0.018	0.173

AF, fed one time at 16:00 h; CON, fed ad libitum; MF, fed one time at 08:00 h; NF, fed one time at 12:00 h.
Data were presented at mean ± SE (n = 6).

(Table 4). No interaction between feeding strategy and week of age was observed ($P > 0.05$). Compared to the CON, AF hens tended to have a higher eggshell thickness ($P = 0.053$), whereas no influence ($P > 0.05$) was detected between CON and MF or NF group (Table 5). In CON, there was an age effect on eggshell thickness that declined with age, which was not observed in MF, NF, and AF treatments ($P > 0.05$).

In MF and NF treatments, plasma Ca, P levels, and ALP activity showed no differences ($P > 0.05$) when compared with CON group (Tables 6–8). In contrast, AF-hens had lower Ca and P levels, but higher ALP activity than CON ($P < 0.01$). Plasma Ca and ALP were not influenced by time ($P > 0.05$), whereas the P level significantly changed with time ($P < 0.001$). No feeding treatment and time was observed ($P > 0.05$).

The plasma Ca, P levels, and ALP activity were further determined during the rapid calcification period. Plasma Ca was increased in MF treatment ($P < 0.05$) compared to CON, whereas P and ALP levels were not changed ($P > 0.05$) by feeding treatment (Figures 2A, 2C, and 2D). In contrast, the AF hens had higher uterine luminal Ca than that MF- and NF-hens ($P < 0.05$; Figure 2B).

Compared to CON, the expression level of *CaBP-D28K* was increased ($P < 0.05$) in the shell gland mucosa of MF treatment (Figure 3A). MF, NF, and AF hens had higher expression levels of OPN ($P < 0.01$), whereas NF had a higher expression level of *OC-116* ($P < 0.01$), compared to CON (Figures 3E and 3I). However, the expression of *PMCA 1b, MFGE8, EDIL3, OVAL, OCX-32*, and *OCX-36* were not changed ($P > 0.05$) by feeding treatments (Figures 3B–3D, 3F, 3G, and 3H).

DISCUSSION

In laying hens, the reproductive system produces an egg in approximately 26 h. Hence, the nutrients needed

Table 6. Effects of feeding strategy (FS) on circadian rhythm of plasma calcium level within a day (mmol/L).

Time of day (h)	CON	MF	NF	AF
04:00	8.50 ± 1.15	7.11 ± 0.55	7.11 ± 0.55	5.74 ± 0.49
08:00	8.48 ± 0.98	8.25 ± 0.91	8.25 ± 0.91	7.47 ± 0.68
12:00	7.51 ± 0.70	7.50 ± 1.15	7.50 ± 1.15	6.95 ± 0.38
16:00	7.87 ± 0.89	7.97 ± 0.58	7.97 ± 0.58	6.25 ± 0.68
20:00	7.72 ± 0.75	9.25 ± 0.80	9.25 ± 0.80	6.84 ± 0.60
24:00	8.45 ± 1.05	8.74 ± 0.59	8.74 ± 0.59	5.61 ± 0.50
Means	8.09 ± 0.36	8.14 ± 0.32	7.85 ± 0.24	6.43 ± 0.26**
P value		CON vs. MF	CON vs. NF	CON vs. AF
FS		0.924	0.600	0.001
Time		0.776	0.938	0.829
FS × time		0.710	0.641	0.612

AF, fed one time at 16:00 h; CON, fed ad libitum; MF, fed one time at 08:00 h; NF, fed one time at 12:00 h.
Data were presented at mean ± SE (n = 6).
**There was a significant difference ($P < 0.01$) compared with control (CON).

Table 7. Effects of feeding strategy (FS) on circadian rhythm of plasma phosphorus level within a day (mmol/L).

Time of day (h)	CON	MF	NF	AF
04:00	1.92 ± 0.39	1.50 ± 0.15	2.03 ± 0.23	1.52 ± 0.13
08:00	1.96 ± 0.15	1.76 ± 0.26	1.66 ± 0.20	1.63 ± 0.09
12:00	1.48 ± 0.20	1.51 ± 0.13	1.41 ± 0.20	0.97 ± 0.25
16:00	1.46 ± 0.17	1.55 ± 0.11	1.34 ± 0.07	1.10 ± 0.12
20:00	2.22 ± 0.17	2.22 ± 0.16	2.15 ± 0.15	1.58 ± 0.15
24:00	2.35 ± 0.29	2.43 ± 0.25	2.27 ± 0.25	2.04 ± 0.26
Means	1.90 ± 0.11	1.84 ± 0.10	1.83 ± 0.10	1.50 ± 0.09**
P value		CON vs. MF	CON vs. NF	CON vs. AF
FS		0.679	0.582	0.002
Time		0.0003	0.0002	0.0002
FS × time		0.841	0.974	0.969

AF, fed one time at 16:00 h; CON, fed ad libitum; MF, fed one time at 08:00 h; NF, fed one time at 12:00 h.
Data were presented at mean ± SE (n = 6).
**There was a significant difference ($P < 0.01$) compared with control (CON).

Table 8. Effects of feeding strategy (FS) on circadian rhythm of plasma alkaline phosphatase (ALP) activity within a day (U/L).

Time of day (h)	CON	MF	NF	AF
04:00	435.7 ± 96.1	569.8 ± 141.5	446.0 ± 101.1	730.0 ± 140.6
08:00	476.7 ± 100.4	572.0 ± 111.5	412.4 ± 117.3	497.7 ± 120.9
12:00	350.3 ± 50.1	247.0 ± 87.6	407.5 ± 93.4	377.4 ± 89.7
16:00	418.7 ± 78.6	539.0 ± 138.4	425.3 ± 73.7	597.4 ± 210.3
20:00	506.2 ± 41.3	683.3 ± 180.6	499.5 ± 96.1	908.0 ± 220.2
24:00	447.3 ± 102.8	589.3 ± 168.9	537.7 ± 109.9	814.5 ± 198.5
Means	439.1 ± 32.0	475.4 ± 49.9	458.9 ± 38.5	519.8 ± 40.3**
P value		CON vs. MF	CON vs. NF	CON vs. AF
FS		0.133	0.708	0.005
Time		0.275	0.764	0.185
FS × time		0.880	0.973	0.753

AF, fed one time at 16:00 h; CON, fed ad libitum; MF, fed one time at 08:00 h; NF, fed one time at 12:00 h.
Data were presented at mean ± SE (n = 6).
**There was a significant difference ($P < 0.01$) compared with control (CON).

for different egg components change with time in the egg cycle. After feeding in the morning, the Ca in the feed is absorbed into the blood through the intestines, then sent to the bones for storage, and mobilized from the bones for the formation of eggshell (Dacke et al., 1993; Kim et al., 2012; Oliveira et al., 2012). Chickens exhibit the feeding behavior of eating more at dusk (Scanes et al., 1987; Wang et al., 2021a). Therefore, it is speculated that feeding in the evening is facilitated to provide more Ca from the digestive tract for eggshell formation. In the present study, feeding time altered the time phase of feed intake, and the peak time appeared at 1 h after feeding. In the CON group, the hens consumed almost the same amount of feed between the periods of 05:00 to 15:00 h (50.9%) and 15:00 to 21:00 h (49.1%). This finding is in line with the work of Keshavarz (1998), who reported that about 50% of the daily feed intake was consumed from 05:00 to 15:00 h and the next 50% from 15:00 to 21:00 h. However, in the AF group, hens consumed 70.8% of daily feed intake from 15:00 to 21:00 h, which was 28.4%, 21.7%, and 21.1% higher than that in MF (42.4%), NF (49.1%), and CON (49.7%) group during the same period. The results indicated that the feed consumption pattern was changed by the feeding strategy. Dusk feeding is observed from 5 h before scotophase (Wang et al., 2021a). Feed consumption is relatively constant up to 17:00 h, and then drastically increased from 17:00 to 21:00 h (Keshavarz, 1998). The results indicate that changes to the feeding time could alter the distribution of feed intake within the daytime. Thus, feeding laying hens once per day in the afternoon is a useful strategy to increase feed intake before light off. Compared to control, AF hens consumed less feed (-2.28 g/d) and the NF group showed a similar tendency (-2.0 g/d). MF-hens, however, consumed comparable amount of feed to that of control. Hence, the effect of feeding time on the appetite of hens needs to be studied further.

It is well known that aging has a significant effect on egg quality. Eggshell quality traits decrease with age, such that the shell thickness is 0.23 μm thinner every week (Molnár et al., 2016). A lot of studies have been conducted to develop strategies to overcome the deterioration in eggshell quality with age. Increasing dietary Ca from 24−25 to 36−40 g/kg improved egg production, shell weight, and shell thickness in 401- to 650-day-old laying hens (Bar et al., 2002). In another study, increasing dietary Ca level from 3.5% to 4.0% enhanced intestinal Ca absorption and improved the eggshell quality of laying hens in the late phase of production (72−79 wk of age) (Wang et al., 2021b). A split feeding system has also been developed to supply fine and coarse limestone in different ratios (Molnár et al., 2017) or to decrease the amount of Ca in the morning and increase it in the afternoon (Molnár et al., 2018). Compared with the traditional feeding mode, separate feeding may be conducive to the formation of high-quality eggshells (Molnár

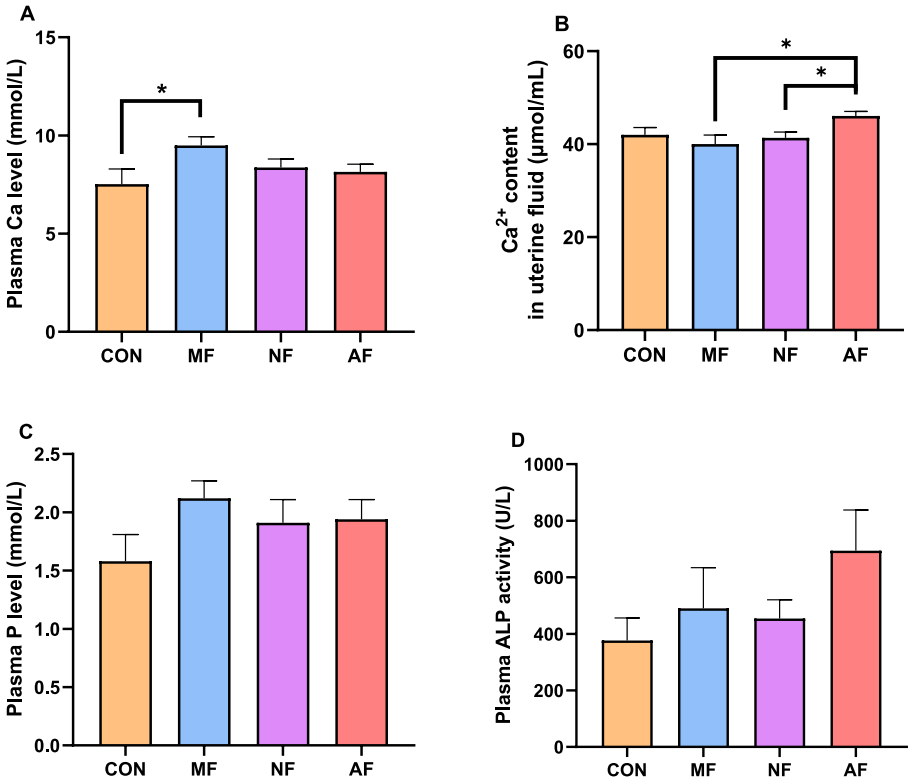

Figure 2. Effects of different feeding strategies on plasma calcium (A), calcium ion content in uterine fluid (B), plasma phosphorus (C), and alkaline phosphatase (D) levels in laying hens during rapid calcification period (16 h p.o.). * $P < 0.05$ (n = 12).

et al., 2016; Molnár et al., 2018). In the present study, AF hens showed a greater tendency for improved eggshell thickness compared to the CON group. The increased eggshell thickness was in accordance with the dynamic changes in plasma Ca and P levels, which were significantly lowered in AF-hen, compared to CON. The relatively low circulating Ca and P may be a result of enhanced eggshell deposition. This speculation was supported by the result that the calcium ion content in the uterine fluid of group AF was relative higher than that of control hens. Eggshell formation involves the complete biomineralization and precipitation of calcium and carbonate ions in the uterus of hens. Calcium is continuously supplied from the blood into the uterus (Gautron et al., 1997). However, further studies are needed to validate whether the low plasma Ca level of AF hens was due to the increased Ca export to the uterine fluid to support eggshell formation.

Circulating ALP activity is a biomarker of hyperparathyroidism in humans (Iwanaga et al., 2016) or abnormal bone metabolism in laying hens (Wei et al., 2021). Elevated ALP activity in AF-hens may be linked with increased Ca mobilization from the bone (Teng et al., 2020; Wei et al., 2021). Hence, the relatively higher plasma ALP activity and decreased plasma Ca and P levels in AF hens may be as a result of enhanced Ca deposit to the eggshell. During the laying period, there is a profound shift in skeletal calcium reserves at mid-lay, from 31 to 42 wk of age, resulting in decreased femur Ca from 1·05 to 0·93 g per bone (Cransberg et al., 2001). High eggshell thickness is related to high plasma ALP activity (Paul and Snetsinger, 1969). The Ca content of the bone was not detected in this experiment, thus, whether the elevated plasma ALP activity in AF-hens occurred to augmented bone mobilization remains to be elucidated.

Plasma P level changed significantly during the egg-laying cycle, whereas Ca was not altered. This suggests that Ca was maintained within a relatively narrow range whereas plasma P fluctuated profoundly. This result was in line with the work of Frost et al. (1991), who reported that total Ca and P increased during the height of Ca need (during eggshell formation). Singh et al. (1986) reported that the concentration of Ca^{2+} during the egg cycle was higher when egg shell calcification was not occurring but lowered to ∼ 0.10 mmol/L during active shell formation. Zhao et al. (2020) reported that a low calcium diet decreased eggshell quality without any detectable influence on serum Ca and P levels. Similarly, hens that laid shell-less eggs did not decrease their serum calcium or egg production when fed a low-calcium diet (Lennards et al., 1981). This suggests that the circulating Ca is more tightly regulated than P in laying hens.

The Ca transport-related genes were further determined in the uterine membrane of laying hens. *CaBP-D28k* and *PMCA 1b* play an important role in Ca transport in the intestine (Wang et al., 2022) and shell gland (Ieda et al., 1995; Xin et al., 2021). The mRNA expression of *CaBP-D28k* and *PMCA1b* were unchanged by AF treatment suggesting that Ca transportation was unaffected. At the initial stage of calcification, Ca^{2+} is targeted to specific mineralization sites for crystal

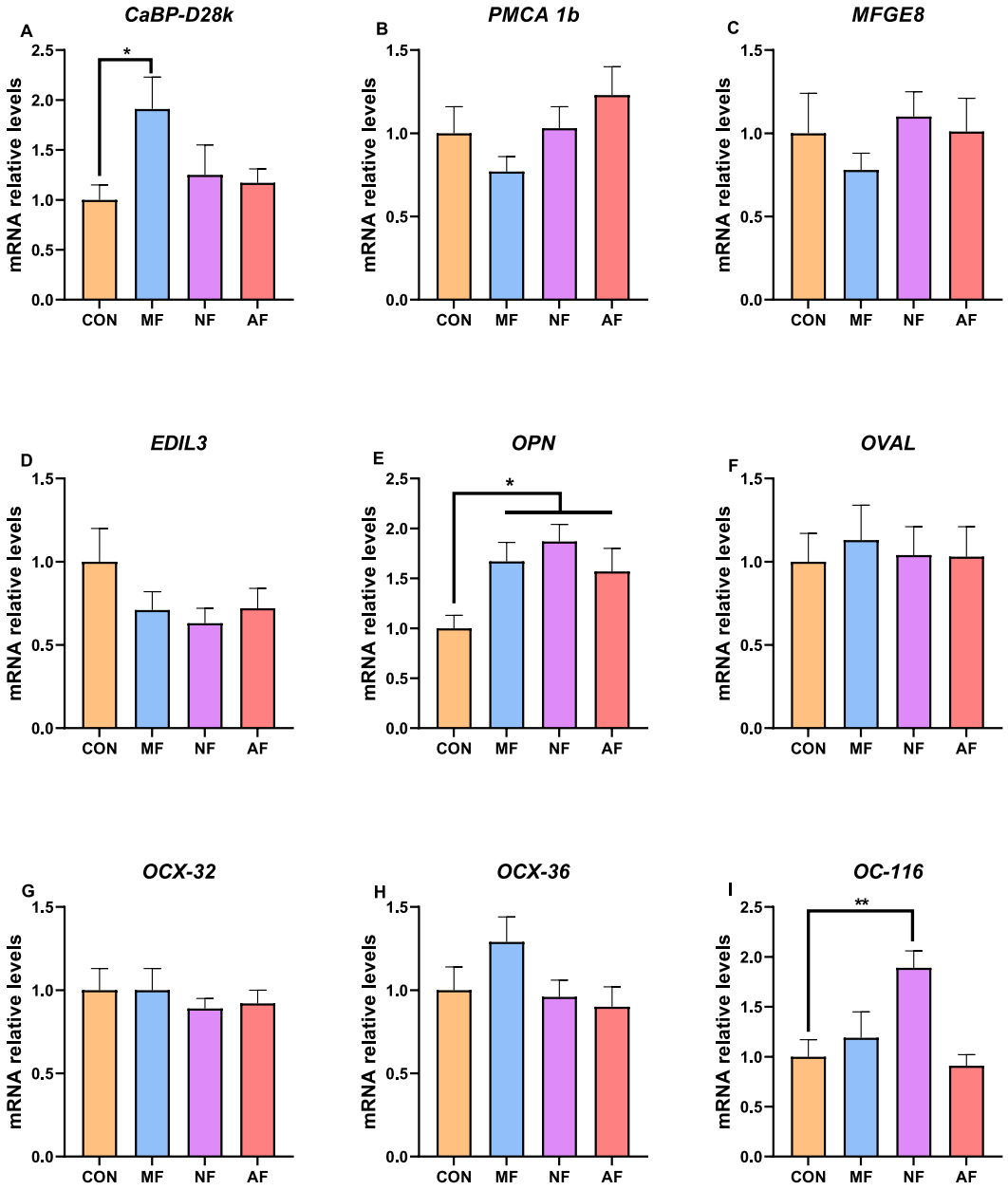

Figure 3. Effects of different feeding strategies on the mRNA expression of Ca transport genes in the eggshell gland mucosa during rapid calcification (16 h p.o.). * $P < 0.05$, $P < 0.01$ (n = 12). (A) *CaBP-D28k.* (B) *OMCA 1b.* (C) *MFGE8.* (D) EDIL3. (E) *OPN.* (F) *OVAL.* (G) *OCX-32.* (H) *OCX-36*, (I) *OC-116.*

deposition through the function of matrix proteins (Wang et al., 2021c). Several matrix proteins are involved in eggshell mineralization such as *EDIL3, MFGE8, OCX-36, OCX-32, OC-116,* and *OVAL* (Dowa et al., 2021). Compared to CON, the expression of *EDIL3, MFGE8, OCX-36, OCX-32, OVAL, OPN*, and *OC-116* were not changed by AF treatment, suggesting that these genes were not involved in the beneficial effects of the AF feeding strategy.

In conclusion, the results indicated that afternoon feeding changed the pattern of feed consumption and had a favorable influence on eggshell thickness. In addition, compared with the traditional feeding mode of 3 times a day, reducing the feeding time had no adverse impact on the production performance and eggshell quality of aged laying hens. The calcium metabolism of hens fed in the afternoon remains to be elucidated in future studies.

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DISCLOSURES

The authors declare that they have no conflicts of interest.

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