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Estimation of calcium requirements for optimal productive and reproductive performance, eggshell and tibial quality in egg-type duck breeders

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*Optimizing the dietary calcium (Ca) level is essential to maximize the eggshell quality, egg production and bone formation in poultry. This study aimed to establish the Ca requirements of egg-type duck breeders from 23 to 57 weeks of age on egg production, eggshell, incubation, tibial, plasma and ovary-related indices, as well as the expression of matrix protein-related genes. Totally, 450 Longyan duck breeders aged 21 weeks of age were allotted randomly into five treatments, each with six replicates of 15 individually caged birds. The data collection started from 23 weeks of age and continued over the following 35 weeks. The five groups corresponded to five dietary treatments containing either 2.8%, 3.2%, 3.6%, 4.0% or 4.4% Ca. The tested dietary Ca levels increased (linear, $P<0.01$) egg production and egg mass, and linearly improved ($P<0.01$) the feed conversion ratio (**FCR**). Increasing the dietary Ca levels from 2.8% to 4.4% increased ($P<0.01$) the eggshell thickness and eggshell content. The tested Ca levels showed a quadratic effect on eggshell thickness and ovarian weight ($P<0.01$); the highest values were obtained with the Ca levels 4.0% and 3.6%, respectively. Dietary Ca levels affected the small yellow follicles (**SYF**) number and SYF weight/ovarian weight, and the linear response ($P<0.01$) was significant vis-à-vis SYF number. In addition, dietary Ca levels increased ($P<0.05$) the tibial dry weight, breaking strength, mineral density and ash content. Plasma and tibial phosphorus concentration exhibited a quadratic ($P<0.01$) response to dietary Ca levels. Plasma calcitonin concentration linearly ($P<0.01$) increased as dietary Ca levels increased. The relative expression of carbonic anhydrase 2 in the uterus rose ($P<0.01$) with the increment of dietary Ca levels, and the highest value was obtained with 3.2% Ca. In conclusion, Longyan duck breeders fed a diet with 4.0% Ca had superior eggshell and tibial quality, while those fed a diet with 3.6% Ca had the heaviest ovarian weights. The regression model indicated that the dietary Ca levels 3.86%, 3.48% and 4.00% are optimal levels to obtain maximum eggshell thickness, ovarian weight and tibial mineral density, respectively.*

Keywords: dietary calcium, laying ducks, ovarian morphology, plasma hormones, gene expression

Implications

The dietary calcium level is a critical factor in poultry production, which plays an essential structural and metabolic function in bone formation, eggshell quality and egg production. The present study determined the dietary calcium requirement for optimal egg production, eggshell quality, bone mineralization and ovarian morphology in laying duck breeders. The obtained results have a significant role, for feed formulators and producers, in enhancing the egg production profits, and health of laying duck breeders.

Introduction

Calcium (Ca) is the critical nutritional factor for egg production, eggshell formation and bone quality of laying birds. Much is known about the Ca requirements of laying hens, but little information is available on the Ca requirements for duck breeders. The U.S. National Research Council (1994) recommended a Ca level of 2.75% for white Pekin

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duck breeders, whereas 3.0% Ca for breeder ducks was suggested in Commercial Poultry Nutrition (2004). Usually, obtaining good egg production with an acceptable shell quality in the laying hens requires sufficient dietary Ca and bones' Ca reservoir. The laying performance, eggshell and tibial quality of hens were affected by the level, source and particle size of dietary Ca (Cufadar *et al.*, 2011; Ganjigohari *et al.*, 2018; Manangi *et al.*, 2018). Wang *et al.* (2014) found that using the large-particle size limestone (0.85 to 2.0 mm) to provide 3.49% dietary Ca facilitates superior productive performance, egg quality and bone characteristics of laying ducks. Castillo *et al.* (2004) showed that the optimal biological level for maximal egg production and egg-specific gravity in Hy-Line W-98 hens are 4.34% and 4.62% Ca, respectively. Roland *et al.* (1996) found that 5% dietary Ca increased bone quality without any adverse effect on the egg production of commercial Leghorns.

The Longyan duck breed is one of the most important egg-type duck breeds, which lays huge amounts of eggs. In southern China, more than 300 million birds are raised from such breed, producing around 80 billion eggs annually (Ruan *et al.*, 2018). In a previous study, we found that diets containing 3.2% to 3.6% Ca exhibited superior egg production and bone quality of Longyan laying ducks during their peak laying period (Xia *et al.*, 2015). The established levels of Ca requirements for duck layers are not adequate to meet the requirements of duck breeders, which have a different genetic capacity for egg production, egg size, egg quality, fertility and hatchability. It is hypothesized that determining the optimal dietary Ca level for the breeding ducks is essential to maximize their reproductive performance. The objective of the current study, therefore, was to estimate the dietary Ca requirements for optimal productive and reproductive performance as well as eggshell and tibial quality for Longyan duck breeders.

Materials and methods

Animals and treatments

In total, 450 egg-laying Longyan-duck breeders with the same genetic background and comparable BW at 21 weeks of age were allotted randomly into five treatments, each with six replicates of 15 birds. The experimental groups had a similar initial BW (1.39 ± 0.02 kg) according to ANOVA test ($P > 0.05$). The data collection started from 23 weeks of age and continued over the following 35 weeks. The experimental ducks were housed in individual galvanized battery cages (having the dimensions: length 27.8 cm × width 40 cm × height 55 cm, purchased from Guangzhou Huanan Poultry Equipment Co., Ltd, Guangzhou, China). The ducks were purchased at the age of 17 weeks, and from 17 to 20 weeks of age, they were fed a diet containing 2600 kcal metabolizable energy/kg, 16.0% CP, 0.90% Ca and 0.40% available phosphorus (P). Each battery cage was equipped with a feeder and nipple drinker. The different groups corresponded to five dietary treatments containing 2.8%, 3.2%, 3.6%, 4.0% or 4.4% Ca. The Ca content was adjusted using finely ground (<0.1 mm) limestone and zeolite as 'filler', and 0.40% available P (0.63% total P) was provided by the inclusion of calcium hydrogen phosphate. The other dietary nutrient levels were covered according to our previous findings for laying ducks (Ruan *et al.*, 2018). Chemical and calculated analyses of the experimental diets are shown in Table 1. Water was available *ad libitum* and 85 g of feed was introduced twice daily at 0700 h and 1500 h throughout the experimental period. In addition to ambient daylight, 4 h of artificial light (15 lx per m^2) was provided from 1830 h to 2230 h to give in total 16 h light: 8 h dark a day.

Table 1 *Composition and nutrient levels in the experimental diets of laying duck breeders (%, as fed basis)*

	Dietary Ca (%)				
Ingredients	2.8	3.2	3.6	4.0	4.4
Corn	52.1	52.1	52.1	52.1	52.1
Soybean meal	23.7	23.7	23.7	23.7	23.7
Wheat middling	7.78	7.78	7.78	7.78	7.78
Corn gluten meal	2.52	2.52	2.52	2.52	2.52
Limestone	6.25	7.37	8.48	9.60	10.7
Dicalcium phosphate	1.66	1.66	1.66	1.66	1.66
DL-Methionine	0.18	0.18	0.18	0.18	0.18
Salt	0.30	0.30	0.30	0.30	0.30
Premix[1]	1.00	1.00	1.00	1.00	1.00
Zeolite powder	4.47	3.35	2.24	1.12	0
Total	100	100	100	100	100
Chemical analysis					
Calcium (%)	2.76	3.19	3.58	4.03	4.41
Total phosphorus (%)	0.56	0.56	0.59	0.58	0.57
Calculated results					
Metabolizable energy (kcal/kg)	2500	2500	2500	2500	2500
CP (%)	18.0	18.0	18.0	18.0	18.0
Calcium (%)	2.80	3.20	3.60	4.00	4.40
Total phosphorus (%)	0.63	0.63	0.63	0.63	0.63
Non-phytate phosphorus (%)	0.40	0.40	0.40	0.40	0.40
Lysine (%)	0.90	0.90	0.90	0.90	0.90
Methionine (%)	0.45	0.45	0.45	0.45	0.45
Methionine + cystine (%)	0.76	0.76	0.76	0.76	0.76

[1] The premix provided the following per kilogram of diet: vitamin A 12 000 IU, vitamin D_3 1800 IU, vitamin E 26 IU, vitamin K 1.0 mg, vitamin B_1 3.0 mg, vitamin B_2 9.6 mg, vitamin B_6 6.0 mg, vitamin B_{12} 0.03 mg, choline 500 mg, D-calcium pantothenate 28.5 mg, folic acid 0.6 mg, biotin 0.15 mg, Fe 50 mg, Cu 10 mg, Mn 90 mg, Zn 90 mg, I 0.50 mg and Se 0.40 mg.

Tissue sampling and storage

At the termination of the study, two birds were randomly selected from each replicate and blood samples were collected from the left-wing vein using 5 ml vacutainer tubes at 1000 h after an overnight fast for 12 h. Within 30 min of collection, the plasma was separated by centrifugation (1200×**g** for 10 min) and stored in 0.5 ml Eppendorf tubes at −20°C until analysis. The birds were then euthanized by cervical dislocation and exsanguinated, and the uteruses, ovaries and tibiae were collected. In addition, samples from the uteruses and ovaries were washed with phosphate-buffered saline, frozen by direct

bluning into liquid nitrogen, and stored at −80°C until analysis.

Productive performance
Throughout the experimental period, the numbers of total broken and shell-less eggs were recorded daily on a replicate basis. All daily oviposited eggs were weighed on an individual basis. The feed intake (the difference between the added and refused feed amounts), egg number, egg weight, egg mass (egg weight/bird/day) and feed conversion ratio (**FCR**, daily feed intake/daily egg mass) were calculated on a per replicate basis, and then expressed as averages for their corresponding laying periods: early laying period (18 weeks, average daily egg production by all ducks ranged from 50% to 80%), peak laying period (17 weeks, average daily egg production by all ducks >80%) and the entire experimental period (35 weeks).

Eggshell quality
In total, 90 eggs were used for evaluating the eggshell quality (three eggs/replicate/four weeks). In each collection time, the treatment average ($N = 18$ eggs) was calculated to determine the eggshell quality traits. The breaking strength of the shell was determined by an Egg Force Reader (model EFR-01, ORKA Food Technology, Ramat HaSharon, Israel). The shell thickness was measured at three different points of the shell without membranes from the blunt, mid-length and pointed ends using a digital micrometer, and the obtained values were then averaged. To calculate the ash content, shells without membranes were washed under a gentle flow of water to remove any adhering albumen, dried in an oven at 65°C to a constant weight, and then ashed overnight in a muffle furnace between 550 and 600°C.

Incubation indices
During the peak laying period, all birds were artificially inseminated twice (with a three-day interval) with 100 μl of diluted fresh semen (diluted with 0.9% saline solution in a proportion of 1:1 volume/volume). The semen samples were collected from drakes belonging to the same breed. Fifty eggs per replicate (egg weight >63 g, with no soft shells, cracks, dirtiness or double yolks) were collected from the third through tenth day following the first insemination. All eggs were incubated in the same incubator (JXB2000; Dezhou Jingxiang Technology Co., Ltd, China) from 36.5°C to 38.4°C and from 45% to 65% relative humidity for 1 to 28 days. Eggs were turned (16 turns/day) throughout the incubation period and sprayed with water once daily from the 15th day of incubation until they hatched. The egg fertility was checked by candling at the seventh day of incubation. After 28 days, the healthy hatched ducklings were counted and recorded, and eggs that failed to hatch were counted. Hatchability was calculated as hatchability of fertile eggs. Healthy ducklings (clean and dry, free of deformities and with bright eyes) were determined macroscopically as described by Tona *et al.* (2004).

Ovary-related indices
The collected ovaries, small yellow follicles (**SYF**; 3 mm < diameter < 8 mm) and large yellow follicles (**LYF**; diameter ≥ 8 mm) were dissected and weighed. The numbers of SYF and LYF were recorded, and their weights were each expressed as proportions of ovarian weight. The relative ovary weight was calculated on the basis of live BW.

Tibial quality
The collected tibiae were trimmed of muscles and tendons, and weighed. Left tibiae then were boiled in water for 6 min and de-fatted by soaking in diethyl-ether for 96 h. Thereafter, they were oven dried to a constant weight and ashed using a muffle furnace (550 to 600°C for 24 h) to determine the tibial ash, which was expressed on the basis of dry-defatted weight (Lin and Shen, 1979). In addition, Ca and P levels in the tibial ash were estimated. Besides, the right tibiae were used in examining the bone mineral density at the Guangzhou Overseas Chinese Hospital with an X-ray osteodensitometer (Lunar Prodigy, General Electric Company, Fairfield, CT, USA). The breaking strength was carried out at half of the tibia length using a materials tester (Instron 4411, Instron Corporation, Grove City, PA, USA). The tibia bone was held by two supporters, spaced 30 mm apart, and the physical power was applied to the midpoint of the bone by a static load cell with a crosshead speed of 30 mm/min. The breaking strength measured the failure point of each loading curve according to Fleming *et al.* (1998).

Chemical analysis of plasma, uterus, eggshells and tibiae
Ca and P concentrations of plasma and uterus were determined spectrophotometrically in duplicates using kits purchased from Jiancheng Bioengineering Institute, Nanjing, China. Approximately 1 g of shell or ash from tibiae was mixed in a solution comprising 15 ml of nitric acid and 2 ml of perchloric acid. The mixture was boiled until white fumes appeared, which was then diluted with distilled water to 100 ml. Ca cotent was determined by titration with EDTA, while P was titrated by ammonium molybdenate according to the procedures of Yoshida *et al.* (1976). Plasma concentrations of progesterone, estradiol-17β (E2), FSH, LH, calcitonin (CT) and parathyrin (PTH) were determined following the procedure of Yang *et al.* (2005) with radio-immunoassays, using kits purchased from Beijing North Institute of Biological Technology, Beijing, China.

Transcript abundance of matrix protein genes in the uterus
To extract the RNA samples from the frozen uteruses, an extraction kit (Invitrogen, Carlsbad, CA, USA) was used. The extracted RNA was then treated with DNAase (Takara, Biotechnology Co. Ltd, Dalian, China) and evaluated with gel electrophoresis. The quality of the test was confirmed via the optical density at 260 and 280 nm ($1.7 < OD_{260:280} < 2.0$).

Thereafter, complementary DNA (**cDNA**) was obtained by reverse transcription using 1.0 μg of RNA in a final volume of 20 μl of reaction mixtures according to the manufacturer's instructions (Promega, Madison, WI, USA). The primer's design

Table 2 *Effects of dietary Ca levels on laying performance of duck breeders aged 23 to 57 weeks*[1]

	Dietary Ca (%)						*P*-value[2]		
Variable	2.8	3.2	3.6	4.0	4.4	SEM	Ca	L	Q
Early-laying period (50% < egg production < 80%, 23 to 40 weeks of age)									
Feed intake (g/day)	160	161	161	159	160	0.54	0.11		
Egg production (%)	54.2	56.0	67.9	77.6	80.0	2.12	<0.01	<0.01	0.92
Egg weight (g)	60.4	60.4	61.6	61.1	61.7	0.49	0.22		
Egg mass (g/day)	32.7	33.8	41.7	47.6	49.6	1.29	<0.01	<0.01	0.94
Feed conversion (g/g)	4.90	4.76	3.85	3.35	3.22	0.13	<0.01	<0.01	0.38
Peak-laying period (egg production >80%, 41 to 57 weeks of age)									
Feed intake (g/day)	163	163	164	163	163	0.34	0.29		
Egg production (%)	82.9	80.4	82.8	83.4	84.5	2.22	0.76		
Egg weight (g)	66.3	66.8	66.9	65.8	66.5	0.42	0.38		
Egg mass (g/day)	54.6	53.7	55.3	55.7	56.3	1.42	0.75		
Feed conversion (g/g)	2.98	3.04	2.96	2.92	2.89	0.08	0.71		
Whole laying period (23 to 57 weeks of age)									
Feed intake (g/day)	161	162	162	160	161	0.52	0.14		
Egg production (%)	74.7	74.7	78.2	81.9	82.3	2.01	<0.05	<0.01	0.89
Egg weight (g)	64.7	65.1	65.3	64.2	65.2	0.38	0.28		
Egg mass (g/day)	48.2	48.9	51.2	52.2	54.5	1.15	<0.01	<0.01	0.64
Feed conversion (g/g)	3.35	3.31	3.17	3.06	2.96	0.07	<0.01	<0.01	0.75

SEM = standard error of the mean.
[1] Data are means for $n = 6$ replicates (15 birds/replicate).
[2] Ca = treatment effect; L = linear; Q = quadratic.

was prepared according to the GenBank sequences using Primer Premier 6.0, which was manufactured by Shanghai Shenggong Biological Company (Shanghai, China). The primer sequences for the examined genes were as follows:

carbonic anhydrase 2 (*CA2*, GenBank: XM_013096806.2), 5′-CGAGACTATTGGACATACCCTGGCT-3′ and 5′-GTG GCACACGGGCTCATTCTC-3′;

calbindin 1 (*CALB1*, GenBank: XM_013108520.2), 5′-TGGTATTTGTGAATGAGTCTCTGCC-3′ and 5′-CCTGC CTTCTTCCTCGCCT-3′;

secreted phosphoprotein 1 (*SPP1*, GenBank: XM_ 005012779.3), 5′-CAGTTCTTTGCTTATGCCTTATCAG-3′ and 5′-GCCAGG TCATTCTGCGGGT-3′;

ovocalyxin-32 (*OCX-32*, GenBank: XM_005027579.3), 5′-CCTTTGCGTTTGCTGTTCG-3′ and 5′-CAGGTAATACT TCTGACCCATGCC-3′;

ovocalyxin-116 (*OC-116*, GenBank: XM_005012815.1), 5′-GAGGAACCAGACGCAGATAAAGAAG-3′ and 5′-GTT TTCAGGCTTGGGGCTGTA-3′; and *β-actin* (GenBank: EF667345.1), 5′-GCTATGTCGCCCTGGATTT-3′ and 5′-GGATGCCACAG GACTCCATAC-3′.

Amplified volumes of cDNA were generated by PCR standard protocol, which started with an initial denaturation at 94°C for 5 min, followed by 35 cycles at 94°C for 30 s, 30 s at 59°C or 60°C and 30 s at 72°C, with a final extension for 10 min at 72°C. The evaluation of PCR products was performed using electrophoresis on 1.5% agarose gel. These products were then excised from the gels and sequenced in order to verify their authenticity.

Quantitative real-time PCR was done by MXPro 3500 system (Stratagene, La Jolla, CA, USA), using 1 μl of the cDNA product in a total volume of 20 μl, which contained 10 μl of SYBR-green PCR master Mix (Takara, Biotechnology Co. Ltd, Dalian, China) and 0.5 μL (10 mmol) of each primer. The reaction accuracy was examined by evaluating the melting curve of the product. The following method was used: denaturation for 30 s at 95°C, followed by 35 cycles of 20 s at 95°C, 30 s at 60°C and 20 s at 72°C. The transcript quantification was assayed based on a standard curve derived from tenfold serial dilutions of cDNA. The samples were measured in triplicates with a standard deviation lower than 0.5.

The ΔCt method ($R = 2^{-\Delta\Delta\ Ct}$), described previously by Chen *et al.* (2015), was used to determine the relative abundance of each target gene, where *R* represents the relative expression level of the target gene and ΔCt reflects the value resulting from subtracting the Ct of *β-actin* mRNA from the Ct value of the target mRNA.

Statistical analysis

Data were statistically analyzed following the general linear model method, and using the statistical software SAS version 9.1 (Copyright (c) 2002–2012, SAS Institute Inc., Cary, NC, USA). Polynomial contrasts were used to identify the linear and quadratic responses to the dietary Ca treatments. A quadratic regression equation, predicted on 95% of the maximum replication, was employed to identify the optimal Ca requirement whenever the quadratic response ($P < 0.05$) was significant (Corzo *et al.*, 2006).

Table 3 *Effects of dietary Ca levels on eggshell quality of duck breeders aged from 23 to 57 weeks*[1]

Item	Dietary Ca (%)					SEM	*P*-value[2]		
	2.8	3.2	3.6	4.0	4.4		Ca	L	Q
Breaking strength (N)	39.1	39.1	40.6	42.0	40.3	1.22	0.45		
Eggshell thickness[3] (mm)	0.327	0.334	0.340	0.344	0.341	0.002	<0.01	<0.01	<0.05
Eggshell content (%)	8.97	9.02	9.19	9.36	9.24	0.06	<0.01	<0.01	0.12

SEM = standard error of the mean.
[1] Data are means for $n = 6$ replicates (3 eggs/replicate).
[2] Ca = treatment effect; L = linear; Q = quadratic.
[3] Regression equation based on dietary Ca level (%); quadratic equation: Y (eggshell thickness) = 0.17 + 0.08 (Ca) − 0.01 (Ca)^2; $R^2 = 0.58$; *P*-value <0.01 yielded the optimized dietary Ca value of 3.86%.

Table 4 *Effects of dietary Ca levels on the incubation indices of duck breeders aged 41 weeks*[1]

Item	Dietary Ca (%)					SEM	*P*-value[2]		
	2.8	3.2	3.6	4.0	4.4		Ca	L	Q
Fertility of set eggs (%)	91.0	88.5	87.6	88.0	88.4	1.12	0.27		
Hatchability of fertile eggs (%)	69.2	66.8	70.2	72.4	71.3	2.29	0.50		
Healthy ducklings (%)	96.2	97.3	96.0	95.6	96.7	0.90	0.73		
Hatchling weight (g)	34.8	34.2	35.0	34.6	34.7	0.34	0.62		

SEM = standard error of the mean.
[1] Data are means for $n = 6$ replicates (50 eggs/replicate).
[2] Ca = treatment effect; L = linear; Q = quadratic.

Results

Productive performance

As shown in Table 2, the egg production and egg mass of breeder ducks linearly increased as dietary Ca levels increased from 2.8% to 4.4%, and a linear (P <0.01) improvement in FCR was obtained during the early- and entire laying periods. There were no differences in the laying performance of laying duck breeders during the peak laying period. In addition, the different Ca levels had no significant effects on the feed intake throughout the experimental periods.

Eggshell quality

The effects of dietary Ca levels on the eggshell quality of duck breeders are shown in Table 3. Eggshell thickness and eggshell content increased (linear, P <0.01) as the dietary Ca levels increased, and a quadratic response (P <0.05) was observed in the eggshell thickness. According to the regression model of eggshell thickness in relation to dietary Ca levels, the optimal Ca level of 3.86% was reached.

Incubation indices

The incubation indices of Longyan-duck breeders were unaffected by the tested dietary Ca levels (Table 4).

Ovary-related indices

As shown in Table 5, a quadratic effect of ovarian weight was observed in response to the graded dietary Ca levels, and the highest ovarian weight was obtained with 3.48% Ca according to the regression model. The SYF number and SYF weight/ovarian weight were affected by dietary Ca levels, and the linear response was significant in relation to the SYF number.

Tibial quality

The tibial quality indices are summarized in Table 6. Of the examined indices, the tibial dry weight, breaking strength, and ash content were affected by dietary Ca levels, and the highest values were obtained with the level 4.0% Ca. The tibial mineral density linearly (P <0.01) increased as dietary Ca levels increased, and the optimal Ca level for tibial mineral density was obtained with 4.00% according to the regression model.

Chemical analysis of plasma, uterus, eggshells and tibiae

Plasma P concentration and tibial P content exhibited a quadratic (P <0.01) response to dietary Ca levels. According to the regression models, the highest plasma and tibial P concentrations were obtained with 3.47% and 3.49% Ca, respectively. The Ca concentration in the uterus linearly (P <0.01) decreased as dietary Ca levels increased. In addition, the plasma CT concentration linearly (P <0.01) increased as dietary Ca levels increased (Table 7).

Transcript abundance of matrix protein genes in the uterus

With the exception of *CA 2* relative expression in the uterus, no differences were observed in the matrix protein gene expression indices due to the dietary Ca levels. There were both linear and quadratic effects of *CA 2* relative expression in response to dietary Ca levels, and the highest *CA 2* relative expression was obtained with 3.2% Ca.

Discussion

The findings of the present study confirm that the tested dietary Ca levels affect egg production, eggshell quality, ovary development and tibial quality of duck breeders. Certainly, numerous studies demonstrated that Ca-deficient diets impaired the reproductive organs and decreased the

Table 5 *Effects of dietary Ca on reproductive organ indices for duck breeders aged 57 weeks*[1]

	Dietary Ca (%)						*P*-value[3]		
Variable[2]	2.8	3.2	3.6	4.0	4.4	SEM	Ca	L	Q
LYF number	4.40	4.60	4.58	3.60	4.33	0.36	0.31		
SYF number	26.0	28.1	23.0	22.0	22.7	1.34	<0.05	<0.01	0.79
Ovarian weight[4] (g/kg BW)	37.1	43.7	47.2	40.2	41.1	1.62	<0.01	0.38	<0.01
LYF weight/ovarian weight	57.5	72.3	80.4	61.2	70.3	6.88	0.18		
SYF weight/ovarian weight	4.01	4.37	2.90	3.89	3.88	0.28	<0.05	0.41	0.11

SEM = standard error of the mean.
[1] Data are means for $n = 6$ replicates (2 birds/replicate).
[2] LYF = large yellow follicles, diameter > 8 mm; SYF = small yellow follicles, diameter = 3 to 8 mm.
[3] Ca = treatment effect; L = linear; Q = quadratic.
[4] Regression equation based on dietary Ca level (%); quadratic equation: Y (ovarian weight) = −86.1 + 71.7 (Ca) − 9.80 (Ca)^2; $R^2 = 0.29$; *P*-value = 0.01 yielded the optimized dietary Ca value of 3.48%.

Table 6 *Effects of dietary Ca levels on tibial quality of duck breeders aged 57 weeks*[1]

	Dietary Ca (%)						*P*-value[2]		
Item	2.8	3.2	3.6	4.0	4.4	SEM	Ca	L	Q
Tibial dry weight (g)	2.81	2.75	2.94	3.16	2.85	0.13	<0.05	0.08	0.16
Tibial length (mm)	95.1	93.3	93.2	94.3	91.8	0.80	0.06		
Breaking strength (N)	126	125	126	141	127	3.4	<0.05	0.10	0.40
Mineral density[3](g/cm^3)	0.21	0.22	0.23	0.24	0.24	0.006	<0.01	<0.01	0.28
Ash content (g)	1.73	1.71	1.79	2.00	1.73	0.06	<0.05	0.11	0.12

SEM = standard error of the mean.
[1] Data are means for $n = 6$ replicates (2 birds/replicate).
[2] Ca = treatment effect; L = linear; Q = quadratic.
[3] Regression equation based on dietary Ca level (%); broken linear equation: $Y = 0.24 - 0.025 \times (4.00 - \text{Ca})$, Ca ≤4.00; $Y = 0.24$, Ca >4.00; $R^2 = 0.99$; *P*-value <0.01 yielded the optimized dietary Ca value of 4.00%.

Table 7 *Effects of dietary Ca levels on chemical analysis of plasma, uterus, eggshell and tibiae of duck breeders aged 57 weeks*[1]

	Dietary Ca (%)						*P*-value[3]		
Item[2]	2.8	3.2	3.6	4.0	4.4	SEM	Ca	L	Q
Plasma									
Calcium (mmol/l)	1.07	1.02	0.97	0.98	1.06	0.03	0.13		
Phosphorus[4] (mmol/l)	2.76	3.08	3.57	3.70	2.72	0.19	<0.01	0.37	<0.01
Parathyrin (pg/ml)	54.4	49.2	53.7	46.4	49.9	4.48	0.66		
Calcitonin (pg/ml)	13.3	14.6	14.5	14.5	17.7	0.61	<0.01	<0.01	0.06
Progesterone (pg/ml)	348	324	278	218	317	46.2	0.26		
E_2 (pg/ml)	79.5	80.0	76.0	93.0	85.8	11.5	0.83		
FSH (mIU/ml)	1.62	1.78	1.83	1.60	1.84	0.13	0.50		
LH (mIU/ml)	5.04	4.59	4.47	4.04	4.87	0.25	0.06		
Uterus									
Calcium (mmol/g protein)	0.10	0.11	0.08	0.05	0.06	0.01	<0.01	<0.01	0.98
Phosphorus (mmol/g protein)	0.19	0.18	0.19	0.17	0.17	0.01	0.40		
Eggshell									
Calcium (% of DM)	33.0	33.2	33.4	34.0	34.4	0.34	0.06		
Phosphorus (% of DM)	0.20	0.21	0.24	0.34	0.31	0.04	0.08		
Tiabiae									
Calcium (% of ash)	36.1	36.1	36.0	35.9	36.5	0.20	0.18		
Phosphorus[4] (% of ash)	15.4	16.4	16.2	16.1	15.9	0.15	<0.01	0.16	<0.01

SEM = standard error of the mean.
[1] Data are means for $n = 6$ replicates (2 birds/replicate).
[2] E_2 = estradiol-17β; FSH = follicle-stimulating hormone; LH = luteinizing hormone.
[3] Ca = treatment effect; L = linear; Q = quadratic.
[4] Regression equation based on dietary Ca level (%); quadratic equation: Y (plasma phosphorus) = −14.0 + 9.62 (Ca) − 1.32 (Ca)^2, $R^2 = 0.29$, *P*-value <0.01; Y (tiabial phosphorus) = 2.16 + 7.72 (Ca) − 1.05 (Ca)^2, $R^2 = 0.28$, *P*-value <0.01 yielded the optimized dietary Ca values of 3.47% and 3.49%, respectively.

egg production of hens (de Bernard *et al*., 1980; Abdallah *et al*., 1993). Chen *et al*. (2015) showed that the egg production and the egg mass of laying ducks were reduced to 84% and 47% of control (3.6% Ca) for 1.8% and 0.38% Ca, respectively. In laying hens, Safaa *et al*. (2008) found that Lohmann Brown laying hens (58 weeks of age) that were fed a diet containing 4.0% Ca had higher egg production, egg mass and better FCR than those fed 3.5% Ca. In addition, similar to our results (Table 2), the latter authors found that the daily feed intake was not affected by 3.5% to 4% dietary Ca levels. The findings of Cufadar *et al*. (2011) suggest that molted brown laying hens required 3.6% Ca in the diet to maintain their normal reproductive performance. These latter studies indicate that the optimal dietary Ca level is well established for the maximum productive performance of laying birds. In the current study, the results of egg-type duck breeders showed that increasing the dietary Ca levels from 2.8% to 4.4%, which was accompanied with a linear increase in plasma CT concentration, linearly increased the egg production, egg mass and SYF number, with linearly decreased FCR (Table 2, Table 5 and Table 7). This result, therefore, suggests that the diets with <4.4% Ca level yielded an inferior productive performance in duck breeders. However, the maximal ovarian weight, which is one of the most important indices of the reproductive system, was obtained with 3.48% Ca according to the regression model (Table 5), thereby indicating that 3.48% Ca facilitates follicles development in duck breeders. This goes in line with the recommendation of 3.5% Ca for breeder hens in Ministry of Agriculture of the People's Republic of China (2004) and exceeds the US National Research Council (1994) suggestion with a Ca level of 2.75% for breeder Pekin ducks.

In the 60 g shelled egg of laying hens, approx. 60% to 75% of Ca is obtained from the diet and the remainder Ca (25% to 40%) stems from the body reserves (Comar and Driggers, 1949; Mueller *et al*., 1964). It is known that medullary bone is a woven bone that acts as a labile source of Ca for eggshell formation (Whitehead, 2004). In laying hens, the results of Roland *et al*. (1996) indicated that increasing the dietary Ca level up to 5% increased tibial density and breaking strength without any adverse effect on egg quality indices. A similar result was obtained in the present study, where the highest tibial dry weight, breaking strength and ash content were achieved when duck breeders fed a diet containing 4.0% Ca (Table 6). Ca and P are important for bone development and mineralization in the form of hydroxyapatite (99% Ca, 80% P) (Veum, 2010). Bone mobilization occurs and Ca ion releases to fulfill Ca requirement when Ca supplied from feed is insufficient, with increasing concentration of plasma P during the time of shell formation (Hurwitz and Bar, 1965). In the present study, the highest plasma and tibial P concentrations were reached when the birds fed the diets containing 3.47% and 3.49% Ca according to the regression model (Table 7), respectively. This finding suggests that using 3.47% Ca is insufficient for maximum eggshell formation in egg-type duck breeders. Furthermore, the results indicated that the dietary Ca level (3.86%) is optimal to maximize eggshell thickness. The best relative eggshell weight was reached with 4.0% and 4.4% dietary Ca, but there were no beneficial effects of the levels higher than 4.0% Ca on the other shell quality indices. When the Ca supply is excessive and concentrations in the gut exceed 1 mmol/L, the homeostatic mechanism is reversed by the secretion of CT; this inhibits the intestinal activation of VD_3, which lead to reduction in Ca absorption in the intestine (Beckman *et al*., 1994). This is consistent with the remarkable increase of plasma CT with 4.4% Ca in the current study; however, the high egg mass obtained in this group (4.4% Ca) implies that higher amounts of Ca and P, either from dietary supplementation or bone mobilization, have been used in egg and eggshell formation versus the other groups. In addition, the increased tibial ash content could be associated with increased contents of other minerals (e.g. Mn, Mg, Zn). Significant linear improvements in eggshell strength and thickness were observed with increasing dietary Ca levels from 3.5% to 4.7% in 70-week-old Hy-Line Brown layers (An *et al*., 2016). In molted laying hens, Cufadar *et al*. (2011) noted that the level of dietary Ca had no significant effect either on eggshell breaking strength or on eggshell thickness. Much amount of Ca ion is usually excreted from the shell gland combined with the bicarbonate ion in the form of Ca carbonates, which are deposited in the shell matrix (Hunton, 2005). This could imply why increasing the dietary Ca level from 2.8% to 4.4% linearly decreased free Ca ion concentrations in the uterus (Table 7).

The formation of $CaCO_3$ predominantly occurs in the presence of carbonic anhydrase (*CA*) enzyme, where the bicarbonate ions are mainly formed in the glandular cells from metabolic CO_2 in a reaction catalyzed by *CA*: $CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$ (Nys *et al*., 1999). In the present study with egg-type duck breeders, a quadratic effect was observed on the *CA 2* relative mRNA expression in the uterus in response to increased dietary Ca levels (Table 8). In a recent study, Chen *et al*. (2015) found that using calcium-deficient diets, containing 1.8% or 0.38% Ca, decreased the relative expression of *CA 2* in the uteruses of laying ducks. Considering the latter result along with the increasing tendency of Ca content in eggshell, this indicates that the deposition of $CaCO_3$

Table 8 *Effects of dietary Ca levels on the mRNA expression levels of matrix protein genes in the uterus of duck breeders aged 57 weeks*[1]

	Dietary Ca (%)						*P*-value[3]		
Item[2]	2.8	3.2	3.6	4.0	4.4	SEM	Ca	L	Q
CA 2	0.95	2.03	1.33	0.98	0.95	0.11	<0.01	<0.01	<0.01
CALB1	0.82	0.88	1.10	1.11	0.94	0.17	0.72		
SPP1	1.28	1.25	0.86	0.84	0.90	0.13	0.06		
OCX-32	0.99	1.11	1.02	0.88	0.91	0.09	0.38		
OC-116	0.79	1.07	1.18	1.07	1.14	0.12	0.37		

SEM = standard error of the mean.
[1] Data are means for $n = 6$ replicates (2 birds/replicate).
[2] *CA 2* = carbonic anhydrase 2; *CALB1* = calbindin 1; *SPP1* = secreted phosphoprotein 1; *OCX-32* = ovocalyxin-32; *OC-116* = ovocalyxin-116.
[3] Ca = treatment effect; L = linear; Q = quadratic.

crystallites in eggshell is dependent on the optimal dietary Ca level, which goes in harmony with the obtained results of eggshell thickness (Table 3). *SPP1* (also named osteopontin) is a glycosylated and highly phosphorylated protein that is characterized by Ca ion transport in bone, kidney and uterus. As one of the major phosphorylated proteins of the avian eggshell matrix localized specifically in the palisade and mammillae layers (Mann *et al.*, 2007; Fernandez *et al.*, 2003), *SPP1* expression in chicken uterus is stimulated by the presence of the egg (Brionne *et al.*, 2014) and mechanical distension of the uterine wall (Lavelin *et al.*, 1998). The results of the current study point out that the levels of *SPP1* relative mRNA expression in the uterus could be associated with the plasma LH levels, where both of them showed a tendency to decrease with an increase in the dietary Ca level (Table 8).

It is concluded that, in egg-type duck breeders, the egg production and egg mass linearly increased as dietary Ca levels increased from 2.8% to 4.4%. In addition, 4.4% Ca seems to be high for the duck breeders, where the birds fed a diet with 4.0% Ca showed superior eggshell and tibial quality. The birds fed a diet with 3.6% Ca obtained the heaviest ovarian weight. The regression analysis revealed that the optimal dietary Ca requirements for maximum eggshell thickness, ovarian weight and tibial mineral density were 3.86%, 3.48% and 4.00%, respectively.

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Declaration of interest

No potential conflict of interest declared.

Ethics statement

All of the procedures employed in this study were approved by the Animal Care and Use Committee of Guangdong Academy of Agricultural Sciences.

Software and data repository resources

None of the data were deposited in an official repository.

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