Dietary zinc supplementation affects eggshell quality and ultrastructure in commercial laying ducks by influencing calcium metabolism

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ABSTRACT This study evaluated dietary Zn supplementation on productive performance, eggshell quality and ultrastructure, and calcium metabolism during eggshell formation in laying ducks. A total of 360 Longyan laying ducks (45-wk) were randomly divided into 5 treatment groups with 6 replicates of 12 birds each and fed for 20 wk. The 6 treatments fed the basal diet supplemented with 0 (control), 20, 40, 80, or 160 mg Zn/kg ($ZnSO_4 \cdot H_2O$). Dietary supplemental level at 80 mg/kg increased egg production (4.3%) and mass (5.7%), and decreased FCR (2.9%) compared to the basal diet, and these indices increased quadratically with increasing Zn supplemental levels ($P < 0.05$). The shell breaking strength (15.8%) and fracture toughness (10.6%) were higher with the supplementation of Zn at 80 mg/kg than the basal diet, and increased quadratically with Zn supplementation ($P < 0.05$). Dietary supplementation of Zn at 80 mg/kg improved shell ultrastructure by increasing total (9.0%) and effective thickness (14.2%) and decreasing mammillary thickness (12.0%), and their responses were quadratic with increasing Zn levels ($P < 0.05$). The supplementation of Zn affected the calcium contents in plasma, tibias and ulna, ulna phosphorus content, and linear and quadratic effects were observed, and higher values were observed with 160 mg/kg Zn supplementation than control ($P < 0.05$). The supplemental Zn level at 80 mg/kg increased shell effective thickness in growth stage ($P < 0.05$), and shell calcium and phosphorus content in initial and growth stages ($P < 0.05$). Dietary Zn supplementation did not affect the gene expression of Ca^{2+} transporters in the eggshell gland, but affected the expression of HCO_3^- exchanger in initial and growth stage ($P < 0.05$). Overall, dietary Zn supplementation could improve productive performance and shell quality in laying ducks at late phase of production, and calcium metabolism and deposition were modulated by Zn influencing HCO_3^- secretion and thus affecting shell ultrastructure and quality. A supplemental level of 80 mg/kg Zn in the diet with a basal content of 34.0 mg/kg was optimal, and higher level (160 mg/kg) decreased shell calcium deposition by depressing its metabolism.

Key words: zinc, mechanical property and ultrastructure, calcium metabolism, duck eggshell

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INTRODUCTION

The eggshell is of great importance to the poultry industry as a primary packaging container and also as a microbial barrier. It is particularly vital to duck eggs as most are processed into preserved or salted eggs, and cracked eggshells caused much economic loss and reduced quality and safety of egg products (Samiulah and Chousalkar, 2014). This problem gets worse with age-related reduction in shell quality, and it has been estimated that the incidence of cracked and broken eggs during the late phase of production could reach as high as 12 to 20% (Travel et al., 2011). Previous studies have explored possibilities for improving eggshell quality, including modulating dietary calcium (Cufadar et al., 2011; Wang et al., 2014), phosphorus (Bello et al., 2020), and micromineral nutrition (Zhang et al., 2017a,b). Zinc (**Zn**) is an essential trace mineral for eggshell formation, and can be involved in calcium deposition and in affecting shell ultrastructure and mechanical properties (Min et al., 2018; Li et al.,

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2019a). On the one hand, Zn plays an important role as a cofactor of carbonic anhydrase (**CA**), which catalyzes the hydration of metabolic CO_2 to HCO_3^- and supplying the precursor of eggshell carbonate (Zhang et al., 2017a). On the other hand, Zn is reported to be involved into the growth step during calcite crystal formation in vitro (Elzinga and Reeder, 2002). In laying hens, some studies have shown the positive effect of Zn in improving eggshell quality, especially in aged layers. Zhang et al (2017a) reported that dietary inorganic Zn supplementation increased eggshell thickness by enhancing CA activity in the plasma and eggshell gland; but the breaking strength did not increase, and similar findings were observed by Abd El-Hack et al. (2018b). However, Li et al. (2019a) observed that dietary addition of zinc-methionine (80 mg/kg) rather than inorganic Zn increased eggshell breaking strength and decreased mammillary knob width. In addition, dietary supplementation with 40 mg/kg methionine hydroxyl analog chelated zinc could improve eggshell quality by promoting Ca deposition and CA activity compared with the inorganic source (Min et al., 2018) and thicker eggshells were found with organic Zn relative to inorganic Zn (Zhang et al., 2017a). It can be concluded that dietary inorganic Zn can increase shell thickness rather than breaking strength in laying hens, and organic Zn can affect breaking strength in relative to inorganic source.

Compared with studies in layers, few studies have reported the Zn application in laying ducks, and its effect on shell quality and supplemental levels needed further study. In duck breeders at 36 wk of age, dietary supplementation of 140 mg Zn/kg increased palisade layer and shell thickness rather than other shell quality indices (Huang et al., 2020). In contrast, Chen et al. (2017) reported that supplementation of Zn (15−90 mg/kg) in the diet did not affect the shell quality of laying ducks at the peak phase of production. Though similar processes of shell formation are observed in laying hens and ducks, unpublished data from our laboratory indicate significant differences between hen and duck eggshell in the contents of phosphorus (0.19 vs. 0.29 %), copper (0.53 vs. 17.7 mg/kg), manganese (0.22 vs. 0.68 mg/kg), magnesium (0.29 vs. 0.09%), and matrix proteins (284 vs. 191 ug/g). Furthermore, the contents of matrix proteins, calcium, phosphorus, and magnesium in eggshells all decreased with aging of laying ducks (Zhang et al., 2021). However, calcium, phosphorus and matrix protein in eggshell were not changed between young and aged laying hens (Feng et al., 2020). These differences indicate a greater influence of these factors in duck eggshells than in chicken eggshells, which imply that the duck rather than hen eggshell quality possibly is easier to be modulated by dietary mineral supplementation at late laying phase. Nevertheless, little is known of the effect of Zn on eggshell quality nor mechanisms in laying ducks at the late phase of production and the optimal Zn level need to confirm.

The eggshell is a composite bioceramic consisting of shell membrane, mammillary knob layer, palisade layer, vertical crystal layer, and cuticle (Arias et al., 1993), and it is formed from the inorganic and organic constituents in uterine fluid simultaneously depositing into calcium carbonate crystals when the egg traverses the oviduct (Nys et al., 2004). The mineral precursors of Ca^{2+} and HCO_3^- ions are continuously supplied during eggshell formation from blood plasma via transepithelial transport taking place across the uterine glandular cells (Bar, 2008). Numerous ion transporters are present and involved in the uterus for calcium secretion, such as CALB1 (intracellular transfer), endoplasmic Ca^{2+} pumps type 2 and 3 (ATP2A2 and 3), Ca^{2+}/Na^+ exchangers (SLC8A1), carbonic anhydrase 2 (CA2), Na^+/HCO_3^- cotransporters (SLC4A4, A5), and HCO_3^-/Cl^- exchanger (SLC26A9) (Brionne et al., 2014). Most of the Ca^{2+} ions in blood are directly transferred from intestinal uptake and some are indirectly mobilized from the medullary bone (Bar, 2009), which mainly composed of tibia, humerus, femur and ulna, and served as a calcium "reservoir" for shell formation (Reynolds, 2003). It was reported that medullary bone could be mobilized to provide 40% of the calcium to the egg shell daily, or up to 60% when fed a low-calcium diet (Nys and Roy, 2018). An added complexity arises from dietary Zn affecting tibial characteristics and Zn deposition in bones of duck breeders (Zhang et al., 2020). Above all, it was hypothesized that dietary Zn supplementation might modulate eggshell quality and ultrastructure, and affect it by regulating calcium metabolism in laying ducks at the late phase of production.

This study, therefore, investigated the effect of different supplemental Zn levels on productive performance, eggshell quality and ultrastructure, and calcium metabolism during eggshell formation in laying ducks. The findings potentially provide practical information relevant to improving eggshell quality in the late laying period and help to provide appropriate application of Zn in laying ducks.

MATERIALS AND METHODS

The use of the ducks and the experimental protocol were approved by the Animal Care and Use Committee of the Animal Science Institute of Guangdong Academy of Agriculture Sciences (No. GAASIAS-2016-017).

Experimental Design and Diets

A total of 360 Longyan laying ducks (45 wk) were randomly divided into 5 treatments, each with 6 replicates of 12 birds, and fed for 20 wk corresponding to the late laying period. The 6 treatments were the basal diet supplemented with 0 (control), 20, 40, 80, or 160 mg Zn/kg in the form of zinc sulfate monohydrate ($ZnSO_4 \bullet H_2O$). Birds were housed singly in cages (42 cm × 30 cm × 50 cm) with a feeder and nipple drinker (Guangzhou Huanan Poultry Equipment, Guangzhou, PRC). Fresh drinking water was available ad libitum, and 80 g of pelleted feed per duck was introduced twice daily at 07:00

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

Ingredients	%	Nutrients	%
Corn (CP, 7.8%)	54.6	AME (MJ/kg)	10.48
Soybean meal (CP, 43.6%)	26.2	Crude protein (CP)	17.00
Wheat bran (CP, 15.7%)	8.0	Calcium	3.58[2]
Limestone	8.2	Methionine	0.43
Dicalcium phosphate	1.5	Lysine	0.89
Salt	0.3	Total phosphorus	0.63[2]
DL-Methionine	0.18	Available phosphorus	0.40
Lysine	0.02	Methionine + cysteine	0.67
Vitamin-mineral Premix[1]	1.0	Zinc (mg/kg)	34.0[2]
Total	100.00		

[1]Provided per kilogram of diet: VA 12,500 IU; VD_3 4,125 IU; VE 15 IU; VK 2 mg; thiamine 1 mg; riboflavin 8.5 mg; calcium pantothenate 50 mg; niacin 32.5 mg; pyridoxine 8 mg; biotin 2 mg; folic acid 5 mg; VB_{12} 5 mg; Zn 90 mg; I 0.5 mg; Fe 60 mg; Cu 8 mg; Se 0.2 mg; Co 0.26 mg; choline chloride 500 mg.

[2]Analyzed value.

and 15:00. The basal diet (Table 1) was composed mainly of corn and soybean meal and was formulated to satisfy the nutritional requirements for Longyan laying ducks with the exception of Zn. The analyzed levels of Zn in the 5 diets were 34.0, 55.9, 78.1, 113 and 183 mg/kg, respectively. The dietary Zn levels in diets were measured by inductively coupled plasma/mass spectrometry (Agilent 7700 series ICP/MS; Agilent Technologies Inc., Alpharetta, GA) as previously described (Zhang et al., 2020).

Sample Collection

At the end of wk 4, 8, 12, 16 and 20 during the treatment period, 5 eggs per replicate were collected for determining eggshell quality; measurements were made on the day of collection.

At the end of wk 20, after the eggshell quality measurements, 2 pieces (~0.5 cm^2) of each eggshell from its equatorial section were examined by scanning electronic microscopy (SEM; FEI Quanta 600, Thermo Fisher Scientific Ltd., Portland, OR) to measure the eggshell ultrastructure.

At the end of wk 20, at 9.5 (initial deposition stage) and 15.5 h (growth deposition stage) post-oviposition (PO) to coincide with the mammillary knobs and palisade layer formation stages, respectively, 2 healthy ducks from each replicate were selected for plasma sampling, and then killed by cervical dislocation. The tibias and ulna of each duck were dissected to measure their contents of calcium and phosphorus. Eggs of the 0, 80, and 160 mg/kg treatments were taken from those ducks to measure the physical properties and ultrastructure of their eggshells. The eggshell glands were taken to measure gene expression. Each treatment had 6 replicates of 2 eggs or ducks each at each stage.

Productive Performance and Eggshell Quality

The number and weight of all oviposited eggs, and feed consumption were recorded daily in each replicate, and then expressed as averages for their corresponding laying period.

Eggshell thickness and breaking strength were separately determined using an Egg Shell Thickness Gauge and Egg Force Reader (Orka Food Technology Ltd., Ramat Hasharon, Israel). The shells with membranes were weighed after drying at 105°C for 6 h. The egg weight and shell weight of the 5 eggs for each treatment replicate were individually recorded, and shell proportion was calculated as the shell weight relative to egg weight. Fracture toughness, or resistance to fracture (**KC**), was derived by the formula $KC = K_{nd}\ (F/T^{3/2})$. In this expression, $K_{nd} = 0.777 \times (2.388 + (2.9934 \times (6/R))$, where R = radius of curvature, F = breaking strength, and T = shell thickness (Zhang et al., 2017b).

Eggshell Ultrastructure

Before imaging, both the inside and outside of each eggshell was washed with distilled water to remove dirt, and then dried overnight. To observe the eggshell ultrastructure by SEM, samples were first mounted onto copper blocks and then coated with gold powder. The effective thickness (combined palisade, vertical crystal, and cuticle sections, μm), mammillary thickness (μm), and width of the mammillary knobs (μm) were measured using the SEM ruler according to Zhang et al (2017b).

Calcium and Phosphorus in Plasma, Shell, Tibias and Ulnar

After shell ultrastructure measurements, the shells were mixed and then crushed into powder. The tibias and ulna were firstly immersed in alcohol for 48 h, then diethyl ether for 48 h, then dried at 105°C for 1 h. Then the tibias and ulna were crushed into powder, and content of calcium and phosphorus were measured. Approximately 0.2 g of shell or tibial or ulnar powder were dissolved in 3 mL nitric acid and 3 mL H_2O_2 and then set aside for 2 h. The samples were then digested with a microwave digestion instrument (MDS-10, Shanghai Xinyi Instrument Technology Co., Ltd, Shanghai, China). The contents of calcium were analyzed using flame atomic absorption spectrophotometry (Zeenit700P, Analytik Jena, Jena, Germany), and the content of phosphorus was measured by ammonium phosphomolybdate colorimetric method using a spectrophotometer (UV-2700, Shimadzu, Kyoto, Japan) (Zhang et al., 2017b).

Gene Expression of Transporters in Eggshell Gland

The RNA samples were reverse transcribed with the Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction to prepare cDNA. The mRNA expression of target genes was examined by qRT-PCR using the CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA) with a 20 μL PCR reaction

mixture (primer concentration: 0.3 μM) according to instructions of the iTaq Universal SYBER Green Supermix (TaKaRa, Tokyo, Japan). Primers used in this study are shown in Additional file 1. The relative mRNA expression levels were normalized to avian β-actin by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Statistical Analysis

A completely randomized experimental design was used in this study. Each replicate was considered as an experimental unit. Data analysis was performed using SPSS 16.0 (Chicago, IL) and subjected to one-way ANOVA with Tukey's honestly significant difference, and then polynomial regressions were fitted. Data are presented as means and pooled SEM, differences were considered significant at $P < 0.05$.

RESULTS

Productive Performance

The effects of dietary zinc supplementation on productive performance of laying ducks at 46 to 65 wk of age are shown in Table 2. Dietary Zn supplemental levels increased egg production (13−16 wk of study, 17−20 wk, and 1−20 wk; $P < 0.05$) of laying ducks, and the responses were linear (13−16 wk, 17−20 wk, and 1−20 wk; $P < 0.05$) and quadratic (13−16 wk, 17−20 wk, and 1−20 wk; $P < 0.05$) with increasing Zn levels. The egg mass was affected (13−16 wk, 17−20 wk, and 1−20 wk; $P < 0.05$) and increased linearly (17−20 wk; $P < 0.05$), and quadratically (13−16 wk, 17−20 wk, and 1−20 wk; $P < 0.05$) with dietary Zn added levels. The FCR was affected (17−20 wk; $P < 0.05$) and responses were linear (17−20 wk, $P < 0.05$) and quadratic (13−16 wk, 17−20 wk, and 1−20 wk; $P < 0.05$) during the laying period from 46 to 65 wk. Dietary Zn supplementation did not affect the average egg weight and daily feed intake (ADFI), except egg weight quadratically increased at 17 to 20 wk ($P < 0.05$) and ADFI quadratically increased at 13 to 16 wk ($P < 0.05$). Through the whole trial period, dietary supplementation with 80 mg/kg Zn, compared to the basal diet, increased egg production (4.3%) and egg mass (5.7%), and decreased FCR (2.9%) ($P < 0.05$).

Eggshell Quality

As shown in Table 3, the supplementation of Zn in the diet affected eggshell breaking strength of laying ducks after feeding for 12, 16, and 20 wk ($P < 0.01$), and it increased linearly (wk 16, $P < 0.01$) and quadratically (wk 16 and 20, $P < 0.001$) with increasing Zn supplemental levels. The fracture toughness was increased ($P < 0.05$) by dietary Zn supplementation at the end of wk 16 and 20, and the responses were linear (wk 16, $P <$

Table 2. Dietary zinc supplementation affected productive performance in laying ducks (46−65 wk).

		Zn supplemental level (mg/kg)						*P*-value		
Variables[1]	Trial period (wk)	0	20	40	80	160	SEM	ANOVA	Linear	Quadratic
Egg production (%)	1−4	84.7	84.0	84.2	83.2	84.7	0.60	0.948	0.979	0.727
	5−8	83.9	85.4	88.2	87.6	88.0	0.67	0.172	0.076	0.073
	9−12	85.9	88.1	87.0	88.7	87.6	0.64	0.729	0.519	0.508
	13−16	79.7	81.1	83.8	86.5	84.4	0.84	0.070	0.047	0.013
	17−20	80.0^{bc}	76.7^{c}	82.1^{ab}	86.4^{a}	85.4^{a}	0.92	0.001	0.001	0.082
	1−20	82.9^{b}	83.1^{b}	85.1^{ab}	86.5^{a}	86.0^{a}	0.46	0.018	0.007	0.004
Average egg weight (g)	1−4	67.4	68.0	67.8	67.1	67.0	0.24	0.645	0.243	0.507
	5−8	67.6	67.2	68.2	67.4	66.5	0.26	0.328	0.151	0.212
	9−12	68.0	68.1	68.8	68.2	67.3	0.24	0.485	0.232	0.238
	13−16	68.0	68.2	68.3	68.5	67.2	0.26	0.547	0.219	0.212
	17−20	67.4	69.1	69.1	69.2	67.5	0.27	0.062	0.444	0.008
	1−20	67.7	68.1	68.4	68.1	67.1	0.23	0.412	0.198	0.153
Egg mass (g/d)	1−4	57.1	57.1	57.2	55.6	56.7	0.48	0.916	0.628	0.770
	5−8	56.1	56.7	59.2	59.1	58.3	0.55	0.269	0.222	0.112
	9−12	58.4	59.7	59.7	60.9	58.9	0.50	0.609	0.884	0.272
	13−16	54.4^{b}	55.1^{b}	57.3^{ab}	59.6^{a}	56.7^{ab}	0.61	0.048	0.156	0.010
	17−20	53.6^{b}	52.9^{b}	57.2^{a}	60.1^{a}	57.8^{a}	0.69	0.001	0.002	0.003
	1−20	55.9^{b}	56.3^{b}	58.1^{ab}	59.1^{a}	57.7^{ab}	0.38	0.033	0.094	0.007
Average daily feed intake (g/duck/d)	1−4	158	156	158	155	158	0.54	0.531	0.986	0.645
	5−8	153	156	158	156	157	0.59	0.112	0.138	0.151
	9−12	156	155	156	155	155	0.50	0.931	0.645	0.901
	13−16	155	156	156	158	155	0.44	0.088	0.777	0.031
	17−20	156	155	156	157	157	0.47	0.734	0.484	0.674
	1−20	156	156	157	157	156	0.35	0.704	0.531	0.559
FCR (g:g)[2]	1−4	2.76	2.74	2.77	2.79	2.77	0.022	0.980	0.752	0.915
	5−8	2.79	2.69	2.65	2.66	2.70	0.019	0.174	0.387	0.073
	9−12	2.66	2.62	2.62	2.59	2.64	0.019	0.849	0.836	0.522
	13−16	2.87	2.82	2.74	2.66	2.75	0.026	0.090	0.108	0.017
	17−20	2.91^{a}	2.93^{a}	2.81^{ab}	2.64^{c}	2.73^{bc}	0.030	0.004	0.003	0.021
	1−20	2.80	2.76	2.72	2.67	2.71	0.016	0.092	0.087	0.017

[a-b]Values within a row with no common superscripts differ significantly ($P < 0.05$).
[1]Mean of 6 replicates (12 ducks per replicate) per treatment.
[2]FCR, feed conversion ratio.
Means within a row with different superscripts differ significantly ($P < 0.05$).

Table 3. Dietary zinc supplementation affected eggshell quality in laying ducks (46−65 wk).

Variables[1]	Time (wk)	Zn supplemental level (mg/kg) 0	20	40	80	160	SEM	*P*-value ANOVA	Linear	Quadratic
Egg weight (g)	4	68.7	68.6	68.6	68.2	68.5	0.28	0.989	0.763	0.883
	8	66.6	67.9	66.7	66.8	66.5	0.24	0.317	0.423	0.692
	12	67.6	67.2	68.5	68.2	67.4	0.30	0.612	0.915	0.470
	16	67.7	68.5	67.8	67.6	66.8	0.31	0.625	0.168	0.371
	20	66.8	68.8	68.0	68.4	67.7	0.34	0.413	0.887	0.419
Eggshell thickness (mm)	4	0.364	0.367	0.367	0.364	0.367	0.001	0.917	0.857	0.954
	8	0.353	0.356	0.358	0.356	0.357	0.001	0.915	0.697	0.811
	12	0.320	0.318	0.317	0.325	0.325	0.001	0.292	0.098	0.259
	16	0.293	0.305	0.297	0.313	0.303	0.002	0.054	0.198	0.074
	20	0.340	0.355	0.350	0.352	0.342	0.002	0.056	0.510	0.064
Eggshell breaking strength (N)	4	44.0	44.6	44.4	45.8	45.2	0.50	0.838	0.396	0.559
	8	41.1	43.6	45.6	43.7	43.4	0.51	0.077	0.510	0.122
	12	40.0^{b}	41.7^{ab}	43.7^{a}	40.4^{b}	40.6^{b}	0.41	0.022	0.546	0.390
	16	33.9^{d}	36.4^{cd}	39.0^{bc}	42.4^{a}	40.7^{ab}	0.72	<0.001	0.001	<0.001
	20	37.9^{c}	40.4^{bc}	43.1^{ab}	43.9^{a}	40.6^{bc}	0.57	0.002	0.256	<0.001
Fracture toughness	4	498	510	495	510	508	5.80	0.897	0.657	0.863
	8	508	500	519	520	501	6.26	0.798	0.858	0.332
	12	569	583	576	571	560	5.36	0.751	0.312	0.599
	16	542^{b}	553^{b}	584^{ab}	621^{a}	628^{a}	9.05	0.001	<0.001	0.065
	20	606^{b}	644^{ab}	660^{a}	670^{a}	632^{ab}	7.44	0.041	0.555	0.007
Eggshell ratio (%)	4	9.63	9.68	9.63	9.62	9.66	0.030	0.976	0.931	0.962
	8	9.32	9.36	9.49	9.40	9.43	0.047	0.853	0.593	0.723
	12	9.09	9.21	9.21	9.22	9.14	0.036	0.748	0.976	0.449
	16	8.86	9.12	9.11	9.38	9.06	0.056	0.058	0.359	0.016
	20	9.12	9.42	9.34	9.50	9.12	0.053	0.063	0.593	0.023

[a-c]Values within a row with no common superscripts differ significantly ($P < 0.05$).
[1]Mean of 6 replicates (5 eggs per replicate) per treatment.
Means within a row with different superscripts differ significantly ($P < 0.05$).

0.001) or quadratic (wk, $P < 0.01$). Dietary Zn supplementation tended to increase eggshell thickness and ratio of duck eggs at wk 16 and 20 ($P < 0.1$), and there was quadratic (wk 16 and 20, $P < 0.05$) responses of thickness and ratio by dietary Zn levels. At wk 16 and 20, the supplementation of Zn at 80 mg/kg increased shell breaking strength (25.1% and 15.8%) and fracture toughness (14.6% and 10.6%) compared with ducks fed the basal diet ($P < 0.05$).

Eggshell Ultrastructure

Dietary Zn supplementation increased the total thickness, effective thickness and its ratio to total thickness, decreased mammillary knob thickness and its ratio to total thickness, and the responses were quadratic ($P < 0.05$, Table 4). Dietary supplementation with 80 mg/kg Zn increased the total (9.0%) and effective thickness (14.2%), decreased mammillary thickness (12.0%) and its ratio relative to total thickness (18.3%, $P < 0.05$).

Calcium and Phosphorus Contents in Eggshell, Plasma, Tibias and Ulna

As observed in Table 5, the calcium content in eggshell, plasma, tibias and ulna were affected by dietary Zn supplementation ($P < 0.05$), and linear and quadratic responses were found in plasma and ulnar calcium content, whereas, quadratic effect was observed in calcium content of tibias ($P < 0.05$). There were no significant differences between phosphorus contents in eggshell, plasma and tibias with the Zn supplementation, however, the ulna phosphorus content was affected ($P < 0.05$), and linearly and quadratically increased with increasing Zn levels ($P < 0.05$). The supplementation of Zn at 160 mg/kg in diet increased the calcium content in plasma (24.2%), tibias (4.5%) and ulna

Table 4. Dietary zinc supplementation affected eggshell ultrastructure in laying ducks (65 wk).

Variables[1]	Zn supplemental level (mg/kg) 0	20	40	80	160	SEM	*P*-value ANOVA	Linear	Quadratic
Total thickness (μm)	410^{b}	431^{ab}	421^{b}	447^{a}	432^{ab}	3.8	0.021	0.079	0.017
Effective thickness (μm)	295^{b}	312^{b}	299^{b}	337^{a}	313^{b}	4.2	0.006	0.100	0.017
Mammillary thickness (μm)	125^{a}	120^{a}	123^{a}	110^{b}	120^{a}	1.5	0.011	0.136	0.015
Mammillary knob width (μm)	110	109	114	104	112	1.3	0.188	0.766	0.594
Effective layer (%)	70.8^{b}	72.2^{b}	70.9^{b}	75.4^{a}	72.4^{b}	0.46	0.004	0.133	0.021
Mammillary layer (%)	30.1^{a}	27.8^{ab}	29.1^{ab}	24.6^{c}	27.6^{b}	0.46	<0.001	0.055	0.002

[a-b]Values within a row with no common superscripts differ significantly ($P < 0.05$).
[1]Mean of 6 replicates (5 eggs per replicate) per treatment.
Means within a row with different superscripts differ significantly ($P < 0.05$).

Table 5. Dietary zinc supplementation affected calcium and phosphorus contents in eggshell, plasma, tibias and ulna in laying ducks (65 wk).

	Zn supplemental level (mg/kg)						*P*-value		
Variables	0	20	40	80	160	SEM	ANOVA	Linear	Quadratic
Shell calcium (%)[1]	35.6	35.9	36.0	36.0	35.8	0.07	0.496	0.843	0.271
Plasma calcium (mmol/L)[2]	8.65[b]	8.75[b]	9.46[ab]	9.84[ab]	10.74[a]	0.240	0.024	0.001	0.003
Tibia calcium (%)[2]	22.1[b]	22.5[ab]	22.2[b]	21.3[c]	23.1[a]	0.160	0.004	0.186	0.009
Ulna calcium (%)[2]	21.4[b]	21.6[b]	22.0[b]	21.3[b]	23.0[a]	0.173	0.003	0.002	0.003
Shell phosphorus (%)[1]	0.220	0.228	0.230	0.224	0.259	0.009	0.693	0.174	0.358
Plasma phosphorus (mmol/L) [2]	4.00	4.06	3.86	3.90	3.86	0.123	0.984	0.668	0.895
Tibia phosphorus (%)[2]	10.4	10.5	10.3	9.93	10.4	0.082	0.211	0.736	0.163
Ulna phosphorus (%)[2]	10.0[b]	10.3[ab]	10.6[a]	10.5[ab]	10.7[a]	0.082	0.045	0.016	0.028

[a-c]Values within a row with no common superscripts differ significantly ($P < 0.05$).
[1]Mean of 6 replicates (5 eggs per replicate) per treatment.
[2]Mean of 6 replicates (2 ducks per replicate) per treatment.
Means within a row with different superscripts differ significantly ($P < 0.05$).

Table 6. Dietary zinc supplementation affected calcified shell mechanical properties and ultrastructure in the growth deposition stage of shell formation in laying ducks (65 wk).

		Zn supplemental level (mg/kg)				
Variables[1]		0	80	160	SEM	ANOVA
Mechanical properties	Calcified shell breaking strength (N)	26.5	28.5	28.4	0.61	0.348
	Calcified shell thickness (mm)	0.273	0.285	0.272	0.003	0.120
	Calcified shell ratio (%)	7.22	7.48	7.22	0.092	0.442
	Egg weight (g)	66.7	66.0	67.4	0.86	0.801
Ultrastructure	Total thickness (μm)	326	338	317	6.2	0.394
	Effective thickness (μm)	220[b]	245[a]	233[ab]	3.95	0.026
	Mammillary knob thickness (μm)	98.3	98.4	88.1	3.23	0.341
	Mammillary knob width (μm)	106	107	106	3.6	0.997
	Effective layer (%)	69.8	72.3	71.7	0.68	0.314
	Mammillary layer (%)	30.2	27.7	28.3	0.68	0.314

[a-b]Values within a row with no common superscripts differ significantly ($P < 0.05$).
[1]Mean of 6 replicates (2 ducks per replicate) per treatment.
Means within a row with different superscripts differ significantly ($P < 0.05$).

(7.5%), and increased ulna phosphorus (7.0%) compared with the ducks fed the basal diet ($P < 0.05$).

Mechanical Properties and Ultrastructure of Calcified Shell in Growth Deposition Stage

In the growth deposition stage (9.5 h postoviposition), equivalent to the formation of the palisade layer of the eggshell, dietary Zn supplementation did not affect the mechanical properties of the uncalcified eggshell (Table 6). However, the effective thickness was influenced by the supplementation with Zn ($P < 0.05$), and higher value was observed with 80 mg/kg (11.4%) added Zn compared with the control.

Calcium and Phosphorus in Plasma and Eggshell in Initial and Growth Deposition Stages

In the initial deposition stage, dietary supplemented with 160 mg/kg Zn decreased the calcium content in plasma (13.4%) and eggshell (3.3%) compared with the control (Table 7. $P < 0.05$), no differences were observed between control and 80 mg/kg Zn-added diet. However,

Table 7. Dietary zinc supplementation affected calcium and phosphorus in plasma and eggshell in initial and growth deposition stage of shell formation in laying ducks (65 wk).

		Zn supplemental level (mg/kg)				
Variables[1]		0	80	160	SEM	ANOVA
Initial deposition stage	Plasma calcium (mmol/L)	9.95[a]	9.40[ab]	8.62[b]	0.204	0.017
	Plasma phosphorus (mmol/L)	3.81	3.60	3.70	0.105	0.748
	Shell calcium (%)	35.9[a]	35.5[a]	34.7[b]	0.144	<0.001
	Shell phosphorus (%)	0.180[b]	0.304[a]	0.246[ab]	0.018	0.007
Growth deposition stage	Plasma calcium (mmol/L)	8.65[b]	9.84[ab]	10.74[a]	0.316	0.015
	Plasma phosphorus (mmol/L)	4.00	3.90	3.86	0.120	0.907
	Shell calcium (%)	32.9[b]	33.9[a]	33.6[ab]	0.149	0.011
	Shell phosphorus (%)	0.15[b]	0.25[a]	0.22[ab]	0.014	0.003

[a-b]Values within a row with no common superscripts differ significantly ($P < 0.05$).
[1]Mean of 6 replicates (2 ducks per replicate) per treatment.
Means within a row with different superscripts differ significantly ($P < 0.05$).

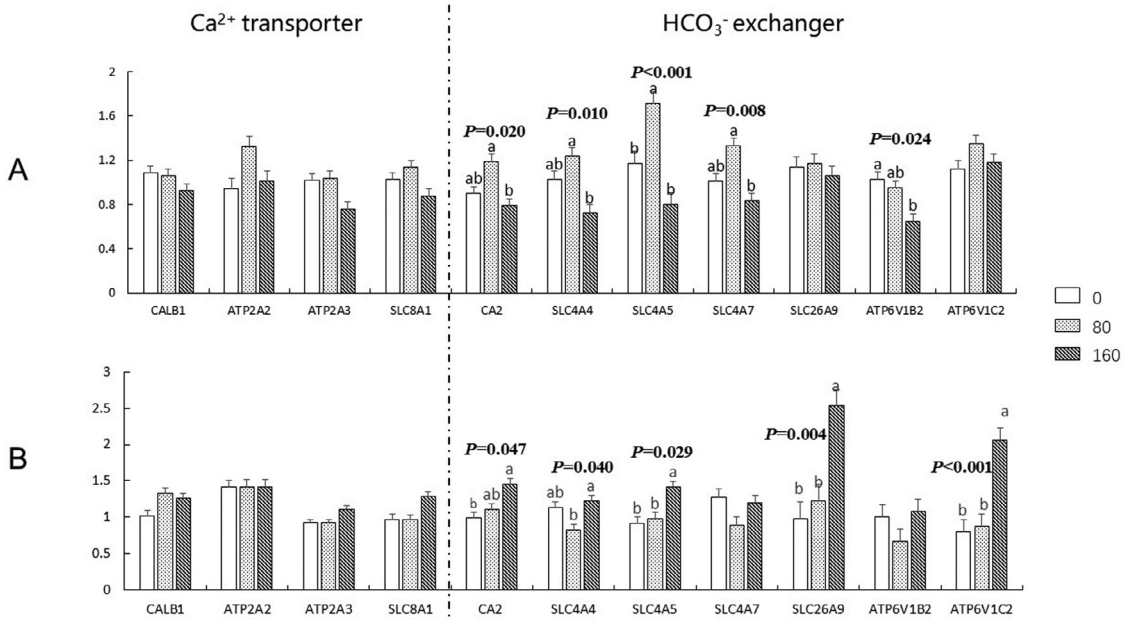

Figure 1. Gene expression of uterine transporters in initial (A) and growth (B) deposition stage of shell formation.

CALB1 (Calbindin D28K): Ca^{2+} intracellular transporter; *ATP2A2* (Endoplasmic reticulum calcium ATPase 2): Ca^{2+} ATPase; *ATP2A3* (Endoplasmic reticulum calcium ATPase 3): Ca^{2+} ATPase; *SCL8A1* (Sodium/calcium exchanger 1), Na^{+}/Ca^{2+} exchanger; *CA2* (Carbonic anhydrase 2): catalyze HCO_3^- formation; *SLC4A4* (Solute carrier family 4 member 4): Na^{+} /HCO_3^- cotransporter; *SLC4A5* (Solute carrier family 4 member 5): Na^{+} /HCO_3^- cotransporter; *SLC4A7* (Solute carrier family 4 member 7): Na^{+} /HCO_3^- cotransporter; *SLC26A9* ((Solute carrier family 26 member 9): HCO_3^- /Cl^- exchanger; *ATP6V1B2* (Vacuolar H ATPase B subunit osteoclast isozyme): H^{+} pump; *ATP6V1C2* (Vacuolar H ATPase B subunit osteoclast isozyme): H^{+} pump.

Mean of 6 replicates (2 ducks per replicate) per treatment.

Means within a row with different superscripts differ significantly ($P < 0.05$).

[a-b] Values within a row with no common superscripts differ significantly ($P < 0.05$).

dietary supplementation with 80 mg/kg Zn increased shell phosphorus (68.9%) content compared with the control ($P < 0.05$). In the growth deposition stage, compared with the control, the plasma calcium content (24.2%) was increased with 160 mg/kg Zn supplementation ($P < 0.05$), and the shell calcium (3.0%) and phosphorus (66.7%) contents were increased with the supplemental Zn level at 80 mg/kg ($P < 0.05$).

Gene Expression of Uterine Transporters in Initial and Growth Deposition Stages

In the initial deposition stage, the gene expression of *CA2, SLC4A4, SLC4A5*, and *SLC4A7* was affected by Zn supplementation (Figure 1. $P < 0.05$), and higher values were observed with the supplementation of 80 mg/kg Zn than the 160 mg/kg diet. Dietary Zn supplementation also affected the expression of *ATP6V1B2*, and it was decreased by 160 mg/kg added Zn compared with the control ($P < 0.05$). In the growth deposition stage, dietary Zn supplementation affected the expression of *CA2, SLC4A4, SLC4A5, SLC26A9* and *ATP6V1C2* in the uterus ($P < 0.05$). Higher values of *SLC4A5, SLC26A9* and *ATP6V1C2* were obtained with 160 mg/kg added Zn compared with the control and 80 mg/kg added Zn ($P < 0.05$). Dietary supplementation with 160 mg/kg Zn increased the expression of *CA2* over the control ($P < 0.05$), and increased *SLC4A4* expression compared to 80 mg/kg added Zn ($P < 0.05$).

DISCUSSION

Consistent with most previous studies in laying hens (Abd El-Hack et al., 2018a; 2018b; Li et al., 2019b; Abedini et al., 2018) and ducks (Chen et al., 2017; Huang et al., 2020; Zhang et al., 2020), dietary Zn supplementation increased egg production, mass, and feed efficiency in the laying ducks during the late phase of production in the present study. Some studies in laying hens, however, failed to show productive performance being affected by Zn supplementation (Zhang et al., 2017a; Han et al., 2020), which possibly reflects the short feeding period (8 wk or 10 wk). The present study also found quadratic responses to supplemental Zn in egg production, mass and FCR, similar to previous studies (Chen et al., 2017; Zhang et al., 2020), where the positive effect of Zn on antioxidant capacity underlay the response in laying performance. Using the egg mass data (Table 2), the quadratic equation ($y = -0.0004x^2+0.0719x+55.595$, $R^2 = 0.929$, $P = 0.033$) enables deriving an optimal supplemental level of Zn in the diet of 85.5 mg/kg. Furthermore, ducks fed 80 mg/kg added Zn maintained the best performance across the trial period. Similarly, zinc methionine or zinc oxide nanoparticles as dietary supplements improved the performance of laying hens, and 80 mg/kg was optimal (Abedini et al., 2018; Li et al., 2019b). Overall, dietary Zn supplementation (40−160 mg/kg) improved productive performance of laying ducks during the late phase of production, and the recommended supplemental level

was 80 mg/kg for the basal diet containing 34.0 mg/kg Zn.

Compared with studies of inorganic Zn in laying hens (Zhang et al., 2017a; Abd El-Hack et al., 2018b), the positive roles of Zn on eggshell quality in laying ducks was more prominent, evidence by increasing shell breaking strength and fracture toughness during the late phase of production, and this positive effect was obviously apparent after feeding for 16 wk. In addition, Zn supplementation tended to increase shell thickness and ratio at wk 16 and 20. In this respect, the beneficial effects of Zn were developed progressively, and more than 12 wk was needed to obtain its full effect. The improved shell mechanical properties with Zn addition in laying ducks was mainly due to its modulating shell ultrastructure, including increasing effective thickness and decreasing mammillary thickness. Moreover, dietary Zn supplementation at 80 mg/kg increased effective thickness in the growth deposition stage in the present study. Shell ultrastructure is the major determinant of shell quality (Park and Sohn, 2018). The shell breaking strength depends on the palisade thickness and the organization of calcite crystals in this layer (Radwan et al., 2010), and it was strongly related to decreased mammillary thickness and improved density of mammillary knobs (Dunn et al., 2012). Additionally, early fusion of the interstitial spaces and their subsequently smaller spaces in the palisade layer led to increased fracture resistance (Fathi et al., 2016). Therefore, the increased shell thickness with Zn addition mostly results from the thicker effective layer, and the increased breaking strength and fracture toughness closely related with the changes in the mammillary layer: decreased mammillary knob thickness and width. The shell ultrastructure is established from the sequential precipitation of mineral carbonate and organic matrix during the mineralization stages (Fernandez et al., 2001), during which large quantities of ions (Ca^{2+} and HCO_3^-) are transferred across the uterus to supply the mineral precursors of the eggshell $CaCO_3$ (Nys and Roy, 2018). In this respect, the improvement of ultrastructure with Zn supplementation here in laying ducks possibly reflects altered calcium metabolism; as reported that dietary zinc-methionine influenced eggshell quality by affecting calcium deposition into eggshell in laying hens (Li et al., 2019a). Above all, the supplementation of Zn in diets could improve shell quality by modulating shell ultrastructure in laying ducks at late laying phase, and this positive effect was more obvious than in laying hens, exactly as the increased shell breaking strength and fracture toughness observed in the present study. Besides, the quadratic responses of shell breaking strength, fracture toughness, shell thickness and its ratio with supplemental Zn showed ~80 mg/kg added Zn to be optimal. Based on fitted quadratic equations, optimal Zn supplementation was 83.9 mg/kg for shell breaking strength, 83.2 mg/kg for fracture toughness, and 86.9 mg/kg for mammillary thickness. This finding helps to provide appropriate application of Zn in laying ducks at late phase of production.

Eggshell biomineralization is closely related to calcium homeostasis in female chickens, involving both the transfer of calcium in the intestine and uterus, along with the resorption and mobilization of medullary bone (Nys and Roy, 2018). Mobilization of medullary bone, a labile calcium store, can provide 40 to 60% of the eggshell calcium (Kerschnitzki et al., 2014). In the current study with ducks, late in the laying cycle, Zn supplementation clearly affected calcium metabolism and deposition into eggshells. In laying hens, Ca deposition was increased by dietary Zn-Met and was mainly due to upregulated expression of *CA* and *CALB1* in the eggshell gland (Li et al., 2019a). The positive effect of Zn on CA expression and shell thickness, was similar to that in laying hens (Zhang et al., 2017a). Inconsistently, here in ducks, dietary Zn supplementation did not affect gene expression of Ca^{2+} transporters, including the Ca^{2+} intracellular transporter, Ca^{2+} ATPase, and Na^+/Ca^{2+} exchanger, which participate in the uterine calcium secretion (Brionne et al., 2014; Nys and Roy, 2018). But Zn affected the expression of genes related to HCO_3^- exchanger in the shell gland in the initial and growth deposition stages in the current study; the gene products are involved in HCO_3^- formation and its transepithelial transfer (Jonchère et al., 2012; Nys and Roy, 2018). It has been suggested that the driving force for calcium secretion might be HCO_3^- secretion and CA because calcium secretion was reduced when CA was inhibited by acetazolamide and HCO_3^- levels influence Ca^{2+} fluxes (Bar, 2009). In this regard, Zn could affect calcium deposition by regulating HCO_3^- generation and secretion although no significant changes in expression of the Ca^{2+} transporters were observed in the present study. This finding indicates that the modulation of Zn on shell Ca deposition is mainly by affecting the exchangers of HCO_3^- rather than the Ca^{2+} transporter, and also provides technical support for eggshell quality improvement in laying ducks at late phase of production.

During eggshell formation, 80 mg/kg supplemental Zn increased shell calcium and phosphorus content in the growth deposition stage, and 160 mg/kg Zn decreased calcium content in plasma and shell; these findings in laying ducks were in accordance with changes in mechanical properties and ultrastructure of the shell in the current study. Dietary supplementation with 160 mg/kg Zn, however, decreased plasma and shell calcium content in the initial stage, but increased them in the growth stage, both corresponding to changes in uterine gene expression of the HCO_3^- exchangers. In this respect, calcium metabolism and deposition were likely to be affected by uterine HCO_3^- secretion in the laying ducks with Zn supplementation. The negative effect of high additional Zn (160 mg/kg) on calcium metabolism is possibly due to the competition among minerals for binders (Sirirat et al., 2012; Ao and Pierce, 2013). However, this finding was not consistent with results in laying hens that dietary Zn addition (35~140 mg/kg) linearly increased shell thickness and higher value was observed in 140 mg/kg Zn-added group (Zhang et al.,

2017a). Overall, supplemental Zn at 80 mg/kg improved shell mechanical properties and ultrastructure by promoting calcium metabolism and deposition in laying ducks, while higher supplementation (160 mg/kg) might decrease shell calcium deposition, probably by depressing calcium metabolism in some extent.

The present study indicated that dietary Zn supplementation improved productive performance and eggshell quality in laying ducks during the late phase of production. Calcium metabolism and deposition were modulated by Zn influencing HCO_3^- secretion and thus affecting shell ultrastructure and quality. A supplemental level of 80 mg/kg Zn in the diet with a basal content of 34.0 mg/kg was optimal, and higher level (160 mg/kg) may even decrease shell calcium deposition by depressing its metabolism.

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DISCLOSURES

The authors declare no conflicts of interest.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2021.101539.

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