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Dietary supplementation with sodium bicarbonate or sodium sulfate affects eggshell quality by altering ultrastructure and components in laying hens

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ABSTRACT

Dietary sodium (Na) levels were related to the content of the eggshell matrix. We therefore speculated that dietary Na supplementation as sodium bicarbonate ($NaHCO_3$) or sodium sulfate (Na_2SO_4) may improve eggshell quality. Additionally, dietary $NaHCO_3$ or Na_2SO_4 supplementation may further affect eggshell quality in different ways due to differences in anions. This study investigated and compared the effects of dietary Na supplementation in either $NaHCO_3$ or Na_2SO_4 form on laying performance, eggshell quality, ultrastructure and components in laying hens. A total of 576 29-week-old Hy-Line Brown laying hens were randomly allocated to 8 dietary treatments that were fed a Na-deficient basal diet (0.07% Na, 0.15% Cl) supplemented with Na_2SO_4 or $NaHCO_3$ at 0.08, 0.18, 0.23 or 0.33% Na for 12 weeks. No differences were observed in laying production performance with dietary Na supplementation. Dietary Na supplementation resulted in quadratic increases of eggshell breaking strength in both Na_2SO_4 and $NaHCO_3$ added groups ($P < 0.05$), and Na_2SO_4-fed groups had a quadratic increase in the eggshell ratio at week 12 ($P < 0.05$). Compared with supplementing 0.08% Na, dietary supplementation of 0.23% Na increased the effective thickness ($P < 0.05$) in both Na_2SO_4 and $NaHCO_3$ added groups, but decreased the thickness and knob width of the mammillary layer ($P < 0.05$). A linear increase on the calcium content of the shell was only observed with Na supplementation from $NaHCO_3$ ($P < 0.05$). No differences were observed in Na contents of the shell with dietary Na supplemented by both sources. Dietary Na addition had a quadratic increase on uronic acid contents of shell membrane in $NaHCO_3$-fed groups ($P < 0.05$). Moreover, the sulfated glycosaminoglycan (**GAG**) contents of shell membranes increased linearly with dietary Na supplementation ($P < 0.05$). Dietary supplementation of 0.23% Na from Na_2SO_4 increased the sulfated GAG contents of calcified eggshell ($P < 0.05$). Additionally, compared with $NaHCO_3$-fed groups, Na_2SO_4-fed groups had higher eggshell breaking strength, thickness, eggshell weight ratio, effective thickness and the sulfated GAG contents of calcified eggshell at week 12. Overall, dietary supplementation of $NaHCO_3$ or Na_2SO_4 could increase eggshell breaking strength, which may be related to increased sulfated GAG contents in eggshell membranes and improved ultrastructure. Higher eggshell breaking strength, thickness and eggshell ratio could be obtained when the diet was supplemented with 0.23% Na from Na_2SO_4.

Implications

Chloride-free sodium supplementation may improve the eggshell quality in laying hens. Sodium bicarbonate and sodium sulfate are common chloride-free sodium sources and are expected to elevate eggshell quality. Therefore, it is important to explore and compare the effect and mechanism of dietary sodium supplementation in sodium bicarbonate or sodium sulfate form on eggshell quality. This study showed that dietary supplementation with sodium bicarbonate or sodium sulfate improves eggshell quality by altering ultrastructure and components, and better eggshell quality was obtained in sodium sulfate groups. These findings will provide a reference for layers choosing the dietary sodium level and modulating eggshell quality.

Introduction

Cracked and damaged eggs result in substantial economic loss to the egg industry (Dunn, 2011). It has been reported that the components of the eggshell can determine the eggshell mechanical quality through regulating the eggshell ultrastructure (Nys et al., 2004). The eggshell consists of 95% inorganic mineral (mainly $CaCO_3$) and 3.3–3.5% organic

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material (Nys and Guyot, 2011). Sulfated glycosaminoglycan (**GAG**) is the fundamental component of shell organic matrices (Arias et al., 1992) and is reported to modulate $CaCO_3$ crystal nucleation, crystal growth and morphology of shell (Liu et al., 2016). Sulfated GAG is a negatively charged macromolecule, predominantly bounds to positively charged ions. Sodium (Na), involved in the acid–base equilibrium in the body, is an essential positively charged macro-mineral element for birds. Laying hens were tolerant of high Na levels in the feed, evidenced by no significant effects on laying performance when birds were fed diets supplementing 0.15 to 0.39% of Na (Junqueira et al., 1984; Wei et al., 2015). It has also been shown that the total content of GAG correlates with dietary Na intake, especially in tissues rich in GAG (Machnik et al., 2009; Sugar et al., 2018). Thus, we speculate that dietary Na supplementation may have a positive effect on the content of GAG in the eggshell, which is beneficial to eggshell quality.

The recommended levels for laying hens of Na and chloride (Cl) in diets are 0.15% (NRC, 1994), generally provided by sodium chloride (NaCl) supplementation at ~0.3% in practice. However, in addition to NaCl, the use of hydrochloride additives such as lysine hydrochloride or choline chloride may contribute to excessive Cl in practical diets. This is considered to be unfavorable for eggshell formation as it provides an acidic condition (Austic, 1984) that reduces the supply of HCO_3^- in the eggshell gland (Balnave et al., 1989) during shell calcification. Previous studies have shown that a Cl-free Na source may be more suitable for supplementing Na to improve eggshell quality in certain situations (Faria et al., 2000). Sodium bicarbonate ($NaHCO_3$) and sodium sulfate (Na_2SO_4) are common Cl-free sources of Na in poultry production. It has been previously reported that dietary $NaHCO_3$ or Na_2SO_4 addition improved eggshell quality and reduced the percentage of rough-shelled eggs (1% $NaHCO_3$, Balnave and Muheereza, 1997; 0.60% Na_2SO_4, Wei et al., 2015). However, to the best of our knowledge, the effects of dietary Na supplementation using $NaHCO_3$ or Na_2SO_4 on eggshell quality were limited to the correlation with dietary electrolyte balance (**dEB**, dEB = Na + K − Cl) values in previous studies (Gezen et al., 2005; Nobakht et al., 2007). There has been no clear information regarding the positive effects of dietary Na supplementation on eggshell quality. More work is needed to investigate the effects of dietary Na supplementation with $NaHCO_3$ or Na_2SO_4 on eggshell quality, and further explore its mechanism.

Dietary supplementation of Na as $NaHCO_3$ or Na_2SO_4 may further affect eggshell quality in different ways due to differences in anions. Dietary HCO_3^- addition was speculated to benefit $CaCO_3$ synthesis by buffering the hydrogen ions produced during eggshell formation (Balnave and Muheereza, 1997), while dietary SO_4^{2-} is involved in the synthesis of the sulfated GAG in the hen oviduct (Suzuki and Strominger, 1960). Both $CaCO_3$ and the sulfated GAG are necessary for shell formation and composition, which determine the structural and mechanical properties of the shell. Thus, comparing the differences between these two Na additives may reveal their specific ways of improving the eggshell. The objective of this study was therefore to investigate and compare the effects of dietary supplementation with Na at various levels of $NaHCO_3$ or Na_2SO_4 on eggshell mechanical quality, ultrastructure and components in laying hens. This study explored the possible mechanism by which Na improved eggshell quality and also provided reference data for dietary Na supplementation in laying hen diets.

Material and methods

Experimental design and diets

The management of birds and the experimental program in this study were approved by the Animal Care and Use Committee of the Feed Research Institute of the Chinese Academy of Agricultural Sciences. Five hundred and seventy-six 28-week-old Hy-Line Brown laying hens were fed a pre-trial diet (Supplementary Table S1) which contained 0.33% NaCl (the commercial level) for 1 week and then randomly allocated to one of 8 dietary treatments for 12 weeks. The experimental diets consisted of 8 Na-added diets with supplemental levels of 0.08, 0.18, 0.23 or 0.33% Na from Na_2SO_4 (≥32.4%, [wt/wt] Na, Nafine Chemical Industry Group Co., Ltd., Yuncheng, Shanxi, China) or $NaHCO_3$ (≥27.1%, [wt/wt] Na, Xilinguole Sonid Alkali Industry Co., Ltd., Xilinguole, Inner Mongolia, China) in addition to the basal diet (Table 1). The Cl level of basal diets was set at 0.15% according to Chinese Feeding Standard of Chicken (Ministry of Agriculture of China, 2004) and the nutrient requirements of the National Research Council (1994). The total Na levels of 8 experimental diets were 0.15, 0.25, 0.30 and 0.40% Na in Na_2SO_4 or $NaHCO_3$ added groups (Table 2). Each treatment had 6 replicates with 12 hens, and 3 hens were assigned to 1 cage (45 cm × 45 cm × 45 cm). Each experimental unit consisted of 4 consecutive cages. All hens were fed in a fully enclosed chicken house with an automated ventilation system and necessary insulation measures. The temperature in the hens' house was controlled at 15–23 °C, and the humidity was controlled at 60–65%. All light sources were fixed to a light density of 20 lx and applied for 16 h/day. The drinking water for hens came from municipal water supply during the experiment period, which contained 64.7 mg/L Na, 2.81 mg/L Cl and 0.592 mg /L potassium.

Sample collection

For 3 consecutive days, 6 eggs per replicate were collected each day for determining eggshell quality in weeks 0, 4, 8 and 12 of the feeding trial. Eggshells of week 12 from 1 replicate were dried and mixed as a sample to measure eggshell components in the calcified eggshell and membrane. Since the eggshell strength increased quadratically with Na supplementation levels and reached its peak at supplemental 0.23% Na (according to results of '*Laying performance and eggshell quality*'), additional 6 eggs per replicate from the 0.23% Na supplemental group and the group of supplemental 0.08% Na (close to the Na supplemental level of practical diets) were collected to measure the eggshell ultrastructure at the end of the trial.

Table 1
Dietary composition and nutrient level of the basal diet for laying hens (29 to 40 weeks of age) (as-fed basis).

Ingredient	%	Nutrient level[4]	%
Corn	58.71	AME[5] (MJ/kg)	11.24
Soybean meal	25.60	CP	16.50
Soybean oil	2.00	Calcium[6]	3.52
Limestone	9.00	Methionine	0.36
Salt	0.13	Lysine	0.83
DL-Methionine	0.11	Total phosphorus	0.53
50% choline chloride	0.12	Available phosphorus	0.33
Calcium hydrogen phosphate	1.30	Methionine + Cysteine	0.65
Yeast culture using distiller's grains	1.50	Magnesium (Mg)	0.33
Montmorillonite[1]	0.10	Sodium (Na)	0.07
Vitamin and mineral premix[2]	0.23	Chloride (Cl)	0.15
Sodium supplement[3]	1.20	Potassium (K)	0.63
Total	100.00	dEB[7] (Na + K − Cl) (mEq/kg)	149.31

[1] An aluminosilicate mineral clay.
[2] Provided per kilogram of diet: vitamin A 12 500 IU; vitamin D_3 4 125 IU; vitamin E 15 IU; vitamin K 2 mg; thiamine 1 mg; riboflavin 8.5 mg; pantothenate (as calcium pantothenate) 50 mg; niacin 32.5 mg; pyridoxine 8 mg; biotin 2 mg; folic acid 5 mg; VB12 5 mg; choline 500 mg; Zn 66 mg; Mn 65 mg; I 1 mg; Fe 60 mg; Cu 8 mg; Se 0.3 mg.
[3] Sodium bicarbonate or sodium sulfate was used as separate dietary sodium supplement, and the remainder of the 1.20% was zeolite.
[4] The nutrient levels are calculated based on Feeding standard of chicken (NY/T 33–2004) and Tables of Feed Composition and Nutritive Value in China (2017, 28th edition).
[5] AME, apparent metabolic energy.
[6] Analyzed value of calcium was 3.50%.
[7] dEB, dietary electrolyte balance.

Table 2
Dietary sodium (Na) supplementation and the concentration of Na in diets of laying hens (29 to 40 weeks of age) (%).[1]

Dietary Na supplementation			Na content in diets (%)	
Na source	Supplementation level (%)	Na addition (%)	Calculated value[2]	Analyzed value[3]
Na_2SO_4	0.24	0.08	0.15	0.14
	0.55	0.18	0.25	0.25
	0.71	0.23	0.30	0.31
	1.01	0.33	0.40	0.42
$NaHCO_3$	0.29	0.08	0.15	0.14
	0.65	0.18	0.25	0.25
	0.84	0.23	0.30	0.31
	1.20	0.33	0.40	0.42

Na_2SO_4 = sodium sulfate; $NaHCO_3$ = sodium bicarbonate.
[1] Zeolite powder was used to replenish the remaining components of sodium supplement.
[2] Sodium contents in the diets were calculated according to Tables of Feed Composition and Nutritive Value in China (2017, 28th edition).
[3] Sodium contents in the diets were determined by national standard recommendation method (GB/T 13885–2003).

Laying performance and eggshell quality

The number of dead hens was recorded daily. The number and total weight of eggs in each experimental unit were recorded daily. Feed intake and water consumption were calculated every 2 weeks during the experimental period. Eggshell quality was measured as described by Zhang et al. (2017b). The analyses of eggshell breaking strength and thickness were conducted using Egg Force Reader and Egg Shell Thickness Gauge (Ramat Hasharon, Israel Orka Food Technology Ltd., Ramat Hasharon, Israel), respectively. Eggshell breaking strength was measured at room temperature with the following parameters: the speed of the cross head, 0.8 mm/S; rated load capacity, 50 N; overload tolerance, 100 N; accuracy, 0.001 kgf. Eggshell thickness was defined as the calcified shell including the cuticle, but without the shell membrane in this trial. Eggshell thickness was measured at the equator and both poles, and then eggshell thickness was determined by the average measurement of 3 points. The eggshell was dried and weighed after removing the dirt and egg albumen. Eggshell ratio was defined as the percentage of eggshell weight to egg weight.

Sodium and calcium contents in eggshell

Eggshell Na and Calcium (Ca) contents were identified using established methods (Zhang et al., 2017a) with modifications as described below. At the end of the week 12, 6 eggshells per replicate were cleaned and dried and then mixed and crushed as a sample to measure the contents of Na and Ca in the calcified eggshell. HNO_3 (3 ml) and H_2O_2 (3 ml) were added to a digestive tube containing 0.5 g eggshell powder and digested with a microwave dissolution instrument (MDS-10, Shanghai Xinyi Instrument Technology co., Ltd., Shanghai, China) after standing for 2 h. Flame atomic absorption spectrophotometry (Z2000, Hitachi, Tokyo, Japan) was performed to determine the concentrations of shell Na and Ca .

Uronic acid and sulfated glycosaminoglycans in shell membranes and calcified eggshell

At the end of week 12, 6 eggshells (the same as samples from '*Sodium and calcium contents in eggshell*') from each replicate were mixed as a sample; approximately 1 g calcified eggshell and 0.1 g membrane were removed from the sample to measure the contents of sulfated GAG and uronic acid in the eggshell membranes or calcified eggshells. Uronic acid contents were determined using the method described by Bitter and Muir (1962) and Zhang et al. (2017a). The dye 1,9-dimethylmethylene blue method, as reported by Farndale et al. (1982) and Zhang et al. (2017a), was used to determine the contents of sulfated GAG. More details are described in the Methods paragraph in the Supplementary Material S1.

Eggshell ultrastructure

The eggshell piece of approximately 0.5 cm^2 was first cleaned and dried without affecting the vertical profiles of eggshells, then fixed in the copper block with conductive glue, sprinkled gold powder and examined by scanning electronic microscopy (FEI Quanta 600, Thermo Fisher Scientific Ltd., Portland, OR). The effective thickness (including cuticle, vertical crystal layer and palisade layer), mammillary thickness and the mammillary knobs width were determined and calculated using the scanning electronic microscopy ruler (Dunn et al., 2012; Zhang et al., 2017b). Mammillary thickness was defined as the length from the outer membrane to the base of the palisade columns (Zhang et al., 2017b). The average width of the mammillary knobs was calculated as the length of the top of mammillary layer/the number of mammillary knobs. All of the scanning electronic microscopy images were taken on 200× magnification.

Statistical analysis

All analyses were performed using SPSS 19.0 for windows (SPSS Inc., Chicago, IL). The homogeneity of variances was tested at first, and the Shapiro–Wilk test was used to analyze normality of the data. Then, the data were analyzed using one-way ANOVA, and the means were compared using Tukey's multiple range test. The linear effect (L) and the quadratic effect (Q) of dietary Na supplementation were assessed using regression analysis (statistical model was described in the Statistical analysis paragraph in the Supplementary Material S1). The interactions between egg weight, the supplemental level and the source of Na were analyzed with a covariance analysis using GLM in SPSS software. Then, the statistical test of comparisons between the Na_2SO_4 and $NaHCO_3$ forms was further assessed using covariance analysis if the interaction between egg weight, the supplemental level and source of Na was not significant. Data are expressed as the mean and pooled SEM. The differences were considered statistically significant at $P < 0.05$ and considered as tendency at $0.05 < P < 0.10$.

Results

Laying performance and eggshell quality

No hens died before the end of the trial. Egg production, average egg weight, average daily feed intake, feed conversion ratio and water consumption were not affected by the dietary Na supplemental levels or sources during weeks 29 to 40 of age ($P > 0.10$, Table 3). The effects of dietary Na supplementation on eggshell mechanical properties are listed in Table 4. No significant differences in eggshell breaking strength were observed in response to supplemental dietary Na in either Na_2SO_4 or $NaHCO_3$ form at week 4 or 8. However, dietary Na supplementation resulted in quadratic increases of eggshell breaking strength at week 12 of the treatment period ($P < 0.05$). In Na_2SO_4-added groups, dietary supplementation of 0.23% Na significantly increased shell breaking strength and thickness compared with other levels ($P < 0.05$). Na_2SO_4-fed hens had increased eggshell breaking strength and thickness at week 12 compared with the $NaHCO_3$-fed hens ($P < 0.05$).

No significant differences were observed in eggshell weight among the different levels and sources of Na supplementation in the diet (Table 5). However, compared with the $NaHCO_3$-fed groups, Na_2SO_4-fed groups tended to have increased on shell weight at week 12 ($P = 0.067$). In Na_2SO_4-added groups, dietary Na addition resulted in a quadratic increase in the eggshell ratio at week 12 ($P < 0.05$). Furthermore, the eggshell ratio increased significantly in response to supplementing

Table 3
Effects of dietary sodium (Na) supplemental levels and sources on performance of laying hens (29 to 40 weeks of age).[1]

Items	Source	Supplemental Na (%)				SEM	P-value			Covariance analysis	
		0.08	0.18	0.23	0.33		ANOVA	L[4]	Q[4]	Egg weight × Level × source	Source
Egg production (%)	$NaHCO_3$	92.12	94.38	94.28	93.63	0.43	0.32	0.28	0.17	0.22	0.14
	Na_2SO_4	92.72	93.60	92.80	92.05	0.53	0.81	0.60	0.67		
Average egg weight (g)	$NaHCO_3$	62.01	61.72	60.93	61.84	0.19	0.19	0.56	0.24	0.20	0.30
	Na_2SO_4	61.50	62.07	61.82	62.20	0.18	0.56	0.21	0.45		
ADFI (g/d per hen)[2]	$NaHCO_3$	119.53	119.84	119.16	118.11	0.49	0.64	0.28	0.44	0.57	0.70
	Na_2SO_4	120.09	119.54	118.92	120.52	0.32	0.34	0.75	0.25		
FCR (feed,g/egg,g)[3]	$NaHCO_3$	2.06	2.03	2.06	2.02	0.01	0.16	0.16	0.29	0.21	0.16
	Na_2SO_4	2.08	2.06	2.06	2.10	0.02	0.80	0.66	0.60		
Average daily water consumption (mL/d per hen)	$NaHCO_3$	200.49	204.72	220.21	222.99	6.20	0.55	0.14	0.36	0.74	0.93
	Na_2SO_4	200.15	193.40	198.89	228.61	6.68	0.25	0.13	0.12		

$NaHCO_3$ = sodium bicarbonate; Na_2SO_4 = sodium sulfate.
[1] Means of 6 replicates (12 hens per replicate) per treatment.
[2] ADFI, average daily feed intake.
[3] FCR = feed conversion ratio.
[4] L, linear effect; Q, quadratic effect.

0.23% Na in Na_2SO_4 form compared with other groups ($P < 0.05$). Na_2SO_4-fed hens presented a higher eggshell ratio at week 12 compared with the $NaHCO_3$-fed hens ($P < 0.05$). The effects of age on eggshell quality are shown in Table S2 in the Supplementary Material S1. With the increase of age, eggshell breaking strength significantly decreased ($P < 0.05$) and eggshell thickness first increased and then decreased ($P < 0.05$). There was an interaction between age and experimental treatment on the effects of eggshell weight and eggshell weight ratio ($P < 0.05$).

Eggshell components

The effects of supplementing dietary Na in Na_2SO_4 and $NaHCO_3$ forms on eggshell components in the end of the trial are listed in Table 6. In $NaHCO_3$-added groups, dietary Na supplementation linearly increased the Ca content of the shell ($P < 0.05$). Additionally, linear and quadratic variation trends in the shell Ca contents were observed with dietary Na supplementation using Na_2SO_4 ($L = 0.060$; $Q = 0.083$), and a quadratic trend was also observed in $NaHCO_3$-added groups ($P = 0.058$). In Na_2SO_4 supplemental groups, compared with the dietary 0.33% Na supplementation, supplementing 0.23% Na increased the eggshell Ca content ($P < 0.05$). In addition, in $NaHCO_3$-added groups, higher Ca contents of the shell were observed in response to dietary supplementation of 0.23% and 0.33% Na compared with dietary supplementation of 0.18% Na ($P < 0.05$). However, no differences were observed in the shell Na contents with dietary Na addition in either Na_2SO_4 or $NaHCO_3$ form. $NaHCO_3$-fed hens had higher Ca contents of the eggshell at week 12 compared with the Na_2SO_4-fed hens ($P < 0.05$).

Dietary Na addition showed quadratic effects on the uronic acid contents of the shell membrane (Na_2SO_4, $P = 0.073$; $NaHCO_3$, $P = 0.024$), but no significant differences were observed in the calcified eggshell. Compared with the dietary supplementation of 0.23% Na, supplementing 0.33% Na in both Na_2SO_4 and $NaHCO_3$ forms decreased uronic acid contents of the shell membrane ($P < 0.05$). Linear increases on the sulfated GAG contents of the shell membrane were observed in response to dietary Na addition ($P < 0.05$). In addition, dietary supplementation of 0.23% Na significantly increased the sulfated GAG contents of calcified eggshell compared with other levels in Na_2SO_4-added groups ($P < 0.05$). However, no significant differences were observed in the sulfated GAG contents of the calcified eggshell with dietary Na supplementation in the $NaHCO_3$ form. $NaHCO_3$-fed groups had a tendency to increase sulfated GAG contents of the calcified eggshell compared with the Na_2SO_4-fed groups ($P = 0.075$).

Table 4
Effects of dietary sodium (Na) supplemental levels and sources on breaking strength and thickness of laying hens (29 to 40 weeks of age).[1]

Items	Time (wk)	Source	Supplemental Na (%)				SEM	P-value			Covariance analysis	
			0.08	0.18	0.23	0.33		ANOVA	L[2]	Q[2]	Egg weight × Level × source	Source
Eggshell breaking strength (N)	0	$NaHCO_3$	47.54	47.16	47.35	47.24	0.43	0.99	0.83	0.97	0.90	0.82
		Na_2SO_4	47.57	47.55	47.80	47.09	0.32	0.90	0.66	0.79		
	4	$NaHCO_3$	45.67	46.10	46.79	46.15	0.26	0.53	0.43	0.45	0.62	0.56
		Na_2SO_4	45.62	45.26	47.21	45.52	0.32	0.13	0.76	0.58		
	8	$NaHCO_3$	43.60	44.82	45.67	44.15	0.41	0.32	0.50	0.20	0.52	0.12
		Na_2SO_4	45.64	45.39	46.67	44.08	0.41	0.17	0.28	0.20		
	12	$NaHCO_3$	42.85	44.56	45.07	43.66	0.33	0.067	0.34	0.028	0.72	0.008
		Na_2SO_4	44.16[b]	45.11[b]	48.02[a]	44.37[b]	0.39	<0.001	0.50	0.005		
Eggshell thickness (μm)	0	$NaHCO_3$	446.57	453.28	450.28	448.83	1.22	0.24	0.76	0.22	0.68	0.33
		Na_2SO_4	449.80	451.08	450.90	448.47	1.09	0.66	0.63	0.45		
	4	$NaHCO_3$	441.91	441.25	445.10	443.70	1.20	0.54	0.27	0.52	0.44	0.17
		Na_2SO_4	442.75	441.20	445.62	443.12	0.85	0.33	0.61	0.85		
	8	$NaHCO_3$	433.18	440.93	436.90	440.23	1.34	0.15	0.11	0.20	0.27	0.49
		Na_2SO_4	434.80	436.75	439.68	433.42	1.25	0.33	0.82	0.26		
	12	$NaHCO_3$	430.97	435.03	430.51	436.30	1.41	0.38	0.29	0.55	0.39	0.048
		Na_2SO_4	434.58[b]	435.45[b]	443.66[a]	435.05[b]	1.12	0.003	0.53	0.079		

$NaHCO_3$ = sodium bicarbonate; Na_2SO_4 = sodium sulfate; wk = week.
[a,b]Values within a row with different superscripts differ significantly at $P < 0.05$.
[1] Means of 6 replicates (12 hens per replicate) per treatment.
[2] L, linear effect; Q, quadratic effect.

Table 5
Effects of dietary sodium (Na) supplemental levels and sources on eggshell weight and shell ratio of laying hens (29 to 40 weeks of age) during 12 weeks of the treatment period.[1]

Items	Time (wk)	Source	Supplemental Na level (%)				SEM	*P*-value			Covariance analysis	
			0.08	0.18	0.23	0.33		ANOVA	L[2]	Q[2]	Egg weight × Level × source	Source
Eggshell weight (g)	0	$NaHCO_3$	6.20	6.38	6.18	6.12	0.05	0.33	0.44	0.38	0.61	0.67
		Na_2SO_4	6.30	6.34	6.44	6.28	0.05	0.69	0.98	0.61		
	4	$NaHCO_3$	6.04	6.00	6.00	5.97	0.03	0.84	0.37	0.67	0.090	0.96
		Na_2SO_4	6.08	5.97	6.14	6.08	0.03	0.15	0.61	0.81		
	8	$NaHCO_3$	6.01	5.97	5.96	5.98	0.03	0.93	0.70	0.79	0.79	0.13
		Na_2SO_4	6.01	6.09	6.05	6.07	0.03	0.86	0.64	0.81		
	12	$NaHCO_3$	6.00	6.05	5.91	6.10	0.03	0.16	0.45	0.43	0.71	0.067
		Na_2SO_4	6.09	6.03	6.21	6.06	0.03	0.23	0.91	0.81		
Eggshell ratio (%)	0	$NaHCO_3$	10.14	10.45	10.18	10.09	0.07	0.30	0.63	0.34	0.39	0.61
		Na_2SO_4	10.32	10.30	10.47	10.24	0.08	0.77	0.85	0.79		
	4	$NaHCO_3$	9.55	9.74	9.77	9.74	0.05	0.47	0.21	0.28	0.080	0.31
		Na_2SO_4	9.82	9.72	9.87	9.72	0.03	0.13	0.39	0.60		
	8	$NaHCO_3$	9.47	9.55	9.74	9.62	0.08	0.69	0.42	0.60	0.66	0.53
		Na_2SO_4	9.55	9.71	9.78	9.58	0.06	0.54	0.79	0.36		
	12	$NaHCO_3$	9.55	9.90	9.57	9.83	0.06	0.12	0.26	0.51	0.32	0.032
		Na_2SO_4	9.77^{b}	9.82^{b}	10.13^{a}	9.81^{b}	0.04	<0.001	0.37	0.042		

$NaHCO_3$ = sodium bicarbonate; Na_2SO_4 = sodium sulfate; wk. = week.
[a,b]Values within a row with different superscripts differ significantly at $P < 0.05$.
[1] Means of 6 replicates (12 hens per replicate) per treatment.
[2] L, linear effect; Q, quadratic effect.

Eggshell ultrastructure

Scanning electronic microscopy images of vertical profiles of eggshells are shown in Fig. 1 (A-D). Dietary supplementation of 0.23% Na increased the effective thickness significantly ($P < 0.05$; Fig. 2 E) and decreased the thickness and knob width of mammillary layer ($P < 0.05$; Fig. 2 F, G) compared with dietary supplementation of 0.08% Na in $NaHCO_3$ and Na_2SO_4 forms. Trends to increased effective thickness ($P = 0.062$; Fig. 2 E) and mammillary knob width ($P = 0.072$; Fig. 2 G) were observed in Na_2SO_4-added groups compared with $NaHCO_3$-added groups. However, no differences were observed in the mammillary thickness between the $NaHCO_3$ and Na_2SO_4 supplemental groups.

Discussion

Depressed laying performance was observed in laying hens when dietary Na supplementation was more than 0.45% (Junqueira et al., 1984). In the current study, we failed to observe significant effects of dietary supplementation of 0.08–0.33% Na on laying performance during weeks 29 to 40 of age. Similar results were reported that there were no significant effects on feed efficiency, feed consumption and egg weight with dietary 0.15–0.45% Na addition in Hy-Line Brown laying hens (Junqueira et al., 1984). Moreover, no physiological symptoms caused by excessive Na were observed in our study, such as acute increases in manure moisture (Davison and Wideman, 1992). These findings indicated that dietary Na supplemental levels (0.08–0.33%) were within the accepted range for hens' rations in our study.

The quadratic increases in shell breaking strength with dietary Na supplementation in $NaHCO_3$ or Na_2SO_4 forms were observed in this study at week 12, which reached the peak of breaking strength at supplemental 0.23% Na. In fact, dietary Na addition also improved the eggshell breaking strength at week 10 (Na_2SO_4, $Q = 0.044$; $NaHCO_3$, $Q = 0.074$, data not shown), but failed to modify eggshell quality in the first 8 weeks. These results indicate that a period longer than 8 weeks may be needed to examine the effects of dietary Na addition on eggshell breaking strength. Elevations of blood pH and HCO_3^- concentrations were observed when Na was added to the diet, which could provide an alkaline condition for eggshell formation (Cohen and Hurwitz, 1974). In addition, eggshell quality was reported to be improved by feeding a diet with moderately higher dEB value (256 mEq/kg) in laying hens (Gezen et al., 2005). The dEB value was 252 mEq/kg in the present trial diets supplemented with 0.23% Na. However, no additional benefits

Table 6
Effects of dietary sodium (Na) supplemental levels and sources on eggshell components of laying hens (40 week of age) at the end of the treatment period.[1]

Items		Source	Supplemental Na (%)				SEM	*P*-value			Covariance analysis	
			0.08	0.18	0.23	0.33		ANOVA	L[2]	Q[2]	Egg weight × Level × source	Source
Eggshell	Ca (%)	$NaHCO_3$	34.91^{ab}	34.64^{b}	35.40^{a}	35.35^{a}	0.09	0.001	0.020	0.058	0.020	–
		Na_2SO_4	34.50^{ab}	34.12^{ab}	34.67^{a}	33.54^{b}	0.16	0.044	0.060	0.083		
	Na (mg:kg)	$NaHCO_3$	1402.34	1454.79	1389.78	1458.43	13.38	0.15	0.27	0.53	0.53	0.93
		Na_2SO_4	1437.42	1429.58	1389.70	1454.12	13.00	0.36	0.82	0.39		
Calcified eggshell (mg:kg)	Uronic acid	$NaHCO_3$	0.28	0.28	0.29	0.27	0.011	0.96	0.47	0.89	0.38	0.42
		Na_2SO_4	0.26	0.28	0.27	0.21	0.016	0.75	0.24	0.27		
	GAG[3]	$NaHCO_3$	0.19	0.20	0.20	0.19	0.002	0.14	0.81	0.073	0.46	0.075
		Na_2SO_4	0.20^{b}	0.20^{b}	0.21^{a}	0.20^{b}	0.001	0.010	0.14	0.29		
Membrane (mg:kg)	Uronic acid	$NaHCO_3$	1.80^{ab}	1.90^{ab}	2.16^{a}	1.63^{b}	0.06	0.012	0.54	0.024	0.57	0.57
		Na_2SO_4	1.83^{ab}	1.91^{ab}	2.19^{a}	1.75^{b}	0.06	0.029	0.87	0.073		
	GAG[3]	$NaHCO_3$	2.06	2.10	2.30	2.34	0.05	0.17	0.037	0.12	0.80	0.96
		Na_2SO_4	2.04	2.05	2.31	2.35	0.06	0.12	0.035	0.11		

$NaHCO_3$ = sodium bicarbonate; Na_2SO_4 = sodium sulfate.
[a,b]Values within a row with different superscripts differ significantly at $P < 0.05$.
[1] Means of 6 replicates (12 hens per replicate) per treatment.
[2] L, linear effect; Q, quadratic effect.
[3] GAG, Glycosaminoglycan.

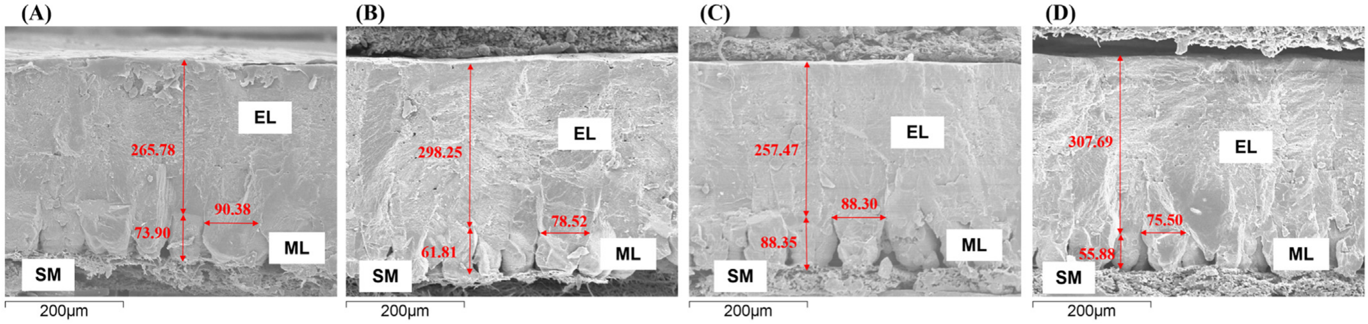

Fig. 1. Scanning electron microscope images of vertical profiles of laying hens' eggshells. A, B represent the vertical ultrastructure with dietary sodium (Na) supplementation at 0.08% and 0.23% as sodium bicarbonate ($NaHCO_3$) form, respectively. C, D represent the vertical ultrastructure with dietary Na supplementation at 0.08% and 0.23% as sodium sulfate (Na_2SO_4) form, respectively. EL = effective layer; ML = mammillary layer; SM = shell membrane.

were observed on eggshell quality with dietary Na supplementation at the level of 0.33% (dietary total Na, 0.40%); it was previously reported that eggshell quality was no longer improved when the total Na level of the diet reached approximately 0.39% (Gezen et al., 2005).

The specific ways in which dietary $NaHCO_3$ and Na_2SO_4 supplementation improved eggshell mechanical quality appeared to be different. Higher eggshell breaking strength, thickness and eggshell ratio were observed only among Na_2SO_4-added groups when the diet was supplemented with 0.23% Na. In contrast, no significant effects of $NaHCO_3$ supplementation were observed on eggshell thickness and eggshell ratio. Similarly, no significant differences were found with dietary supplementation of 0.3% $NaHCO_3$ in eggshell thickness from 25 week to 50 week of age in laying hens (Jiang et al., 2015). In addition, a quadratic increase in shell ratio with dietary Na supplementation was observed only in Na_2SO_4-fed groups. These changes in eggshell ratio were similar to those observed for eggshell thickness. We also observed that eggshell ratio was positively correlated with eggshell thickness ($r = 0.818$, $P < 0.001$; data not shown). Moreover, no significant effects were found in shell weight in response to dietary Na supplementation from $NaHCO_3$ or Na_2SO_4. Other studies also did not observe any significant change in eggshell weight with dietary supplementation of Na from 0 to 0.5% (Junqueira et al., 1984; Özturk, 1999). Although quadratic increases of shell breaking strength with dietary Na supplementation were observed in both $NaHCO_3$ and Na_2SO_4 forms, higher breaking strength was observed in Na_2SO_4-fed groups, accompanying the higher eggshell thickness and shell ratio, which suggests better eggshell quality could be obtained from Na_2SO_4-fed groups than $NaHCO_3$-fed groups.

The improvements of eggshell induced by Na supplementation with Na_2SO_4 or $NaHCO_3$ may result from changes in eggshell components. The eggshell breaking strength is related to the content of GAG in shell membranes (Ha et al., 2007). In the current study, dietary supplementation of Na_2SO_4 or $NaHCO_3$ increased GAG contents linearly in shell membranes, which may be related to the redistribution of body fluids after high Na intake, ultimately leading to more GAG deposition in GAG-rich tissues (Sugar et al., 2018). Glycosaminoglycans are involved in the formation of eggshell membranes and mineralization processes and thus benefit shell ultrastructure and eggshell breaking strength (Fernandez et al., 2001). However, eggshell breaking strength was not further improved when the Na supplemental level of the diet reached 0.33%, even though the group had higher GAG contents. Liu et al. (2016) have reported that the effects of GAG in shell membranes on eggshell ultrastructure and breaking strength were not in a dose-response manner and were also related to the composition of GAG. There are four kinds of GAG in the eggshell, all of which can be broken down into uronic acid (except for keratan sulfate) and hexosamine (Liu et al., 2014). In our study, uronic acid showed a quadratic increase with increased Na supplementation level, which was different from the linear increase in the GAG content, suggesting a change in the composition of GAG, at least in 0.33% Na supplementation groups.

These two Na sources may further alter the eggshell composition in their specific ways, which may be a fundamental reason for the differences in eggshell mechanical quality. Higher GAG contents in calcified eggshells were only observed in Na_2SO_4-added group when the diet was supplemented with 0.23% Na, consistent with the higher Ca contents in eggshell. Besides, compared with $NaHCO_3$-fed groups, a tendency for increased sulfated GAG in calcified shell was observed in Na_2SO_4-fed group ($P = 0.075$). These findings meant the synthesis of sulfated GAG in calcified shells was promoted in Na_2SO_4-added groups, which could be beneficial for the regulation of mineral deposition, crystal growth and orientation (Panheleux et al., 1999), leading to better

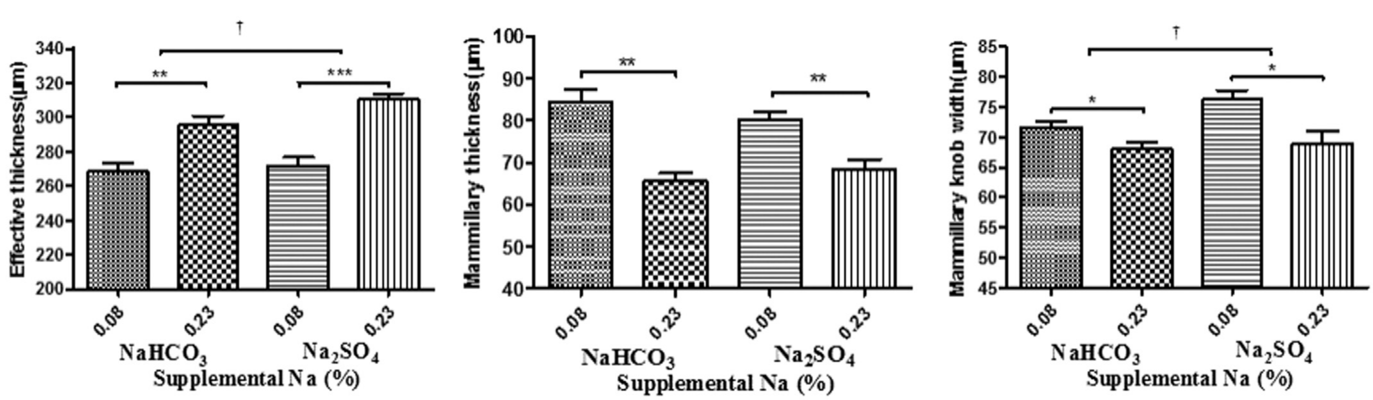

Fig. 2. Effects of dietary Na supplemental level and source on eggshell ultrastructure of laying hens (40 week of age) at the end of the treatment period. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, and †$P \leq 0.10$.

eggshell quality. Consistently, positive correlations between eggshell breaking strength ($R = 0.734$, $P < 0.001$), thickness ($R = 0.500$, $P = 0.025$) and weight ratio ($R = 0.496$, $P = 0.026$) and the level of GAG in calcified shell were observed in Na_2SO_4 groups; however, no similar results were found in the $NaHCO_3$ groups. However, $NaHCO_3$ may enhance eggshell formation by promoting $CaCO_3$ deposition, as Ca contents in eggshell increased linearly with $NaHCO_3$ supplementation. This increase induced by $NaHCO_3$ supplementation was partly related to HCO_3^- which can buffer the hydrogen ions generated during $CaCO_3$ formation (Makled and Charles, 1987).

Eggshell matrices and $CaCO_3$ crystals interweave and form the eggshell ultrastructure which is considered to be the major determinant of eggshell mechanical characteristics (Van Toledo et al., 1982; Ahmed et al., 2005). More ordered mineralized structures were found in 0.23% Na supplementation groups, contributing to stronger eggshells to resist external force (Radwan, 2016). In our current study, decreased width of mammillary cones and thickness of mammillary layer, but increased thickness of effective layers were observed in both $NaHCO_3$ and Na_2SO_4 supplemental groups when the diets were supplemented with 0.23% Na. The size of the mammillary cones and the thickness of effective layer are determined by the spacing of nucleation sites on the eggshell membrane (Dunn et al., 2012). Each palisade column grows from the nucleation site with the competitive growth of $CaCO_3$ columns for spaces and ultimately forms the compact layer of the shell (Solomon, 2010). The decreased width of the mammillary cones indicated an eggshell ultrastructure with closely spaced mammillae, which could increase the bonding strength between mammillary cones and have a positive effect on mammillary layer strength (Zhang et al., 2017a). Effective thickness of eggshell, which comprises palisade layer, surface crystal layer and cuticle, forms two-thirds of total shell thickness and is crucial for eggshell breaking strength (Fathi et al., 2007). The increased thickness of the effective layer may be related to early fusion of the mammillae, evidenced by the decreased thickness of the mammillary layer (Dunn et al., 2012) and increased GAG contents in shell membranes (Fernandez et al., 2001). These observations indicate the roles for $NaHCO_3$ or Na_2SO_4 in the modulation of shell ultrastructure, which could partly explain the improvements observed in eggshell breaking strength.

Thicker effective layer was observed in Na_2SO_4-added groups than $NaHCO_3$-added groups in the current study ($P = 0.06$), which was consistent with higher GAG contents in calcified eggshells in Na_2SO_4-added groups, suggesting higher eggshell thickness and breaking strength may be the result of increased effective layer thickness in Na_2SO_4-added groups. Notably, greater increases in the thickness of the effective layer were observed in Na_2SO_4-added groups than $NaHCO_3$ groups in the current study (36 μm vs 29 μm, $P = 0.060$, *t*-test), while $NaHCO_3$ groups had numerical decreases in the thickness of the mammillary layer (12 μm vs 20 μm, $P = 0.12$, *t*-test). This compensation could result in no significant effects of $NaHCO_3$ supplementation on eggshell thickness in general.

In conclusion, dietary supplementation of Na with $NaHCO_3$ or Na_2SO_4 could increase eggshell breaking strength, which may result from increased sulfated GAG contents in eggshell membranes and improved ultrastructure. Higher eggshell breaking strength, thickness and eggshell ratio could be obtained when the diet (0.07% Na) was supplemented with 0.23% Na from Na_2SO_4.

Supplementary materials

Supplementary data to this article can be found online at https://doi.org/10.1016/j.animal.2020.100163.

Ethics approval

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

Data and model availability statement

None of the data were deposited in an official repository.

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Declaration of interest

No potential conflict of interest declared.

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