



## Calcareous Algae (*Lithothamnium calcareum*) as an Alternative Source of Calcium in Commercial Layer Diets

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### ABSTRACT

Calcareous algae (CA) are an alternative renewable source of organic minerals that are used in poultry feed. However, the optimal CA concentration that can be included in poultry diets and its effect on egg production requires further investigation. The aim of this study was to determine the effects of diets containing varying CA (*Lithothamnium calcareum*) levels and particle sizes on the performance, mineral balance, quality, and composition of the eggs of commercial laying hens. A completely randomized experimental design with a  $3 \times 2$  factorial arrangement (CA level  $\times$  particle size) was employed in this study. A total of 210 Dekalb White laying hens were distributed to the following experimental units, with six birds each and five replicates per unit: the control (diet without CA addition) and treatment groups, which were fed with diets containing varying CA levels (1%, 2%, and 3%) and particle sizes (0.128 and 1.114 mm). Productive performance, external egg quality, and percentage of non-viable eggs were not affected by the inclusion of CA in the diets. The inclusion of 2% fine-particle CA reduced ( $p < 0.05$ ) calcium excretion (%) and, consequently, improved calcium retention. Similarly, the inclusion of 2% fine-particle CA decreased phosphorus excretion ( $p < 0.05$ ). The inclusion of 2% and 3% fine-particle CA increased mineral matter retention ( $p < 0.05$ ). Therefore, the inclusion of CA in the diets of lightweight laying hens did not affect productive variables or egg quality. However, 2% fine-particle CA reduced calcium and phosphorus excretion, thereby improving the retention of these minerals.

**Keywords:** calcium source; egg quality; mineral balance; organic minerals; particle size

### INTRODUCTION

In the rearing of laying hens, the physical quality of eggs is crucial for all stakeholders involved in the production chain—from producers to end consumers. Shell quality issues represent the highest percentage of egg-related losses, as eggs are not marketed or their prices are lowered, resulting in significant economic losses during the production phase (Pereira *et al.*, 2021).

Certain minerals play important roles in egg production and quality. For example, calcium (Ca) not only actively participates in all metabolic pathways in an animal (Sousa *et al.*, 2024a) but is also one of the main components of the eggshell, which represents 10% of the total egg weight and is composed of 95% minerals on average (Gautron *et al.*, 2022). To ensure the integrity and quality of eggshells throughout the productive life of laying hens, ingredients with higher Ca availability, which can maintain higher plasma levels, especially during eggshell formation, are being explored.

Calcium can be supplied from different sources in the diet of laying hens, with limestone and dicalcium phosphate being the most common (Sousa *et al.*, 2024b). Inorganic minerals are widely available and

can be more cost-effective. However, they originate from non-renewable sources, and their extraction can have a significant environmental impact (Rezende *et al.*, 2024). Currently, there is a growing demand for more sustainable animal production with a lower environmental footprint. In this context, alternative feed ingredients for mineral supplementation, such as those from organic sources, are emerging in the market due to their higher bioavailability. This allows for the use of smaller amounts in diets compared to inorganic minerals while also reducing excretion and, consequently, the potential for environmental pollution (Zafar & Fatima, 2018).

Among these alternative feed ingredients, calcareous algae (CA), such as *Lithothamnium calcareum*, have gained interest in egg production due to their skeletal composition, which contains up to 99% minerals, such as calcium (32%) and phosphorus (0.034%), as well as various trace elements (Moraleco *et al.*, 2024). Brazil holds the world's largest reserve of calcareous algae; however, it remains largely underutilized. Free forms of CA, such as rhodoliths, allow for extraction through dredging, as they are unconsolidated sedimentary deposits. This process

enables the preservation of existing algae reserves without causing their depletion (Dias, 2000).

The inclusion of calcareous algae (CA) in the diet of laying birds was previously reported by Rezende *et al.* (2024), who replaced 0.5% of limestone with CA but observed no effects on growth or productive performance in quails. On the other hand, Spanivello *et al.* (2022) found that replacing up to 30% of limestone resulted in increased egg weight and shell thickness without harming the animals. Additionally, Badeca *et al.* (2022) reported that egg albumen weight and height were higher when limestone was replaced with CA. There is still no consensus on the optimal amount or level of CA substitution in the diet of laying hens, highlighting the need for further research on this ingredient.

Another important consideration is that the provision of Ca sources with coarser particles in the diet can contribute to the maintenance of the skeletal system of hens and the formation of eggshells. When particles are larger and generally less soluble, the digestive tract of birds can maintain the available Ca levels in the blood for eggshell formation at night, thereby improving eggshell quality (Hervo *et al.*, 2022). However, no studies have yet compared different particle sizes of calcareous algae in animal nutrition.

With these considerations, the aim of this study was to evaluate the effects of diets containing varying calcareous algae (*L. calcareum*) concentrations and particle sizes on the performance, mineral balance, quality, and composition of the eggs of commercial laying hens.

## MATERIALS AND METHODS

### Ethics Statement

The study was performed in accordance with Brazil's National Council for the Control of Animal Experimentation guidelines and was authorized by the Ethics Committee for Animal Use of the State University of Mato Grosso do Sul, the protocol number (004/2015). The experiment was conducted at the Poultry Sector and Laboratory of Quality of Animal-Origin Products, State University of Mato Grosso do Sul, Aquidauana, Brazil.

### Animals, Diets, and Housing Conditions

A total of 210 53-week-old Dekalb White laying hens with an average body weight of 1,693 g were analyzed for 112 days, divided into four 28-day cycles. A completely randomized experimental design was employed in a 3 × 2 factorial arrangement (CA level × particle size) + control (without CA inclusion); there were five replicates and six birds per experimental unit. Control and treatment diets were provided, consisting of varying CA concentrations (1%, 2%, and 3%) and two particle sizes (fine: 0.128 mm; coarse: 1.114 mm) per each concentration. The CA was purchased from Oceana Minerals (Jundiaí, São Paulo, Brazil). The chemical composition of CA is presented in Table 1. The diets were formulated based on corn and soybean meal supplemented with vitamins and minerals, according to

the guidelines recommended by Rostagno *et al.* (2017) (Table 2).

The birds were housed in galvanized wire cages, which were equipped with trough-type feeders and nipple drinkers (Artabas, Bastos, Brazil), in a conventional laying shed with clay tile roofing. Food and water were provided *ad libitum*, and the hens were fed twice daily (8:00 a.m. and 4:00 p.m.). Lighting (natural and artificial lighting) was provided for 17 h per day. The temperature inside the shed was monitored daily using maximum ( $29.95 \pm 5.4$  °C) and minimum thermometers ( $19.79 \pm 3.8$  °C), and the relative humidity (62.33%) was measured using a thermohygrometer that was positioned at bird height.

### Evaluation of Egg Quality

Feed intake (FI) was determined on the last day of each cycle. The egg mass (EM) was recorded daily. During egg collection, cracked (CRK), broken (BRK), deformed (DEF), and shell-less (SL) eggs were counted, and the percentage of non-viable eggs at the end of each cycle was calculated by dividing the number of eggs in each category by the total number of intact eggs. Feed conversion (FC) was calculated using egg weight (kg/kg) and a dozen eggs (kg/dozen).

During the last three days of each cycle, 30 eggs from each treatment were used for quality analyses. The eggs were weighed individually and then immersed in saline solution with different densities (1.070–1.098) to determine their specific gravity (SG, g/cm<sup>3</sup>), according to the method developed by Hamilton (1982).

The albumen height, yolk height, and yolk diameter were measured using a digital caliper ( $\pm 0.01$

Table 1. Chemical composition of calcareous algae (*Lithothamnium calcareum*)

Chemical composition	Unit	Results	Limit of quantification
Mineral matter	g/kg	964.8	0.01
Moisture	g/kg	4.5	0.01
Calcium	g/kg	320.7	0.1
Cobalt	mg/kg	<0.1	0.1
Copper	mg/kg	<0.1	0.1
Iron	mg/kg	3700	0.1
Magnesium	g/kg	32	0.1
Manganese	mg/kg	360	0.1
Sodium	g/kg	1.6	0.1
Nickel	mg/kg	<0.1	0.1
Zinc	mg/kg	<0.1	0.1
Cadmium	mg/kg	<0.1	0.1
Chromium	mg/kg	0.2	0.1
Lead	mg/kg	0.9	0.1
Boron	mg/kg	<0.001	0.001
Iodine	%	0.2	0.1
Vanadium	mg/kg	<0.01	0.01
Mercury	mg/kg	<0.1	0.1
Arsenic	mg/kg	<0.1	0.1
Selenium	mg/kg	<0.1	0.1
SiO <sub>2</sub>	%	7.9	-

Table 2. Proximate and nutritional composition of the experimental diets

Ingredients (%)	Inclusion of <i>Calcareous algae</i>			
	0%	1%	2%	3%
Corn, 8.5%	64.55	64.55	64.55	64.55
Soybean meal, 45%	22.76	22.76	22.76	22.76
Calcareous Algae	-	1.00	2.00	3.00
Dicalcium phosphate	1.06	1.06	1.06	1.06
Limestone	9.23	8.37	7.52	6.66
Soybean oil	1.31	1.31	1.31	1.31
Salt	0.30	0.30	0.30	0.30
DL- Methionine	0.19	0.19	0.19	0.19
L- Lysine HCl	0.013	0.013	0.013	0.013
Mineral/vitamin premix*	0.15	0.15	0.15	0.15
BHT	0.01	0.01	0.01	0.01
Inert (kaolin)	0.43	0.28	0.14	0.00
Total	100.00	100.00	100.00	100.00
	Calculated values			
Crude protein (%)	16.02			
Met. energy (kcal/kg)	2,850			
Digestible Methionine+cystine (%)	0.65			
Digestible lysine (%)	0.72			
Calcium (%)	3.90			
Available phosphorus (%)	0.29			
Sodium (%)	0.15			
Potassium (%)	0.61			
Chlorine (%)	0.21			
Linoleic acid (%)	2.11			
Mongin number (mEq/kg)	163.31			
Mineral matter (%)	2.34			

Note: \*Composition per kg of feed: Vitamin A, 7,000 IU; Vitamin D3, 1,600 IU; Vitamin E, 8 IU; Vitamin K3, 1.0 mg; Nicotinic acid, 20 mg; Pantothenic acid, 7 mg; Vitamin B6, 1.0 mg; Vitamin B12, 0.010 mg; Biotin, 0.02 mg; Cu, 10 mg; Fe, 50 mg; I, 0.83 mg; Mn, 65 mg; Se, 0.30 mg; Zn, 60 mg; Mongin number: It is the calculation of the electrolyte balance of the feed. It is the result of the sum of the Na<sup>+</sup> and K<sup>+</sup> ions, subtracting the negative Cl<sup>-</sup> ion. Corn 8.5%: Corn contains 8.5% crude protein. Soybean meal 45%: Soybean meal contains 45% crude protein.

mm). The Haugh unit (HU) was determined using the equation described by Silversides and Budgell (2004):

$$HU = 100 \log (H + 7.75 - 1.7 W_{0.37})$$

where H is the albumen height (mm), and W is the egg weight (g).

The yolk index (YI) was calculated based on the ratio between the yolk height and diameter. The DSM YolkFan™ (2016) colorimetric fan, ranging from light yellow (1) to deep orange (15), was used for yolk color determination. The percentages of yolk (PY) and albumen (PA) were obtained after separating and individually weighing the yolk and albumen.

To determine the percentage of shell (PS) and shell thickness (ST), the shells were washed with tap water and dried at room temperature for 48 h. Afterward, PS was obtained by weighing the eggs, and ST was determined by measuring four regions of the shell using a digital micrometer (± 0.01 mm). Afterward, the shells were ground and then subjected to dry matter (DM) and mineral matter (MM) analyses, according to the methodology described by Silva and Queiroz (2009).

Five days after the period of egg collection and analysis, total excreta were collected to determine Ca, P, and MM balances. Ferric oxide (2%) was added to the samples to mark the start and end of the collection period. To avoid possible fermentation, total excreta was collected twice daily using trays lined with plastic.

Excreta was collected from each experimental unit after feathers, feed residues, and other contaminants were removed and stored in a horizontal freezer. At the end of the collection period, FI was recalculated to determine the intake of Ca and P. The excreta samples were homogenized, pre-dried, and then subjected to the analyses of DM, MM, Ca, and P content, according to the methodology described by Silva and Queiroz (2009). Ca and P levels were determined using the colorimetric method.

### Statistical Analyses

The data were tested for normality, and only normally distributed data were subjected to analysis of variance to verify the effects of the factors studied (isolated or interactions). When no interaction occurred, the variables were analyzed independently. Tukey's test (p<0.05) was performed for mean comparisons, while Dunnett's test (p<0.05) was used to compare the levels of CA inclusion with the control.

The variables studied were subjected to analysis of variance, according to the statistical model:

$$\hat{Y}_{ijk} = \mu + N_i + T_j + N*T + e_{ijk}$$

Where  $\hat{Y}_{ijk}$  was the studied variables;  $\mu$  was general constant;  $N_i$  was the effect of calcareous algae levels, being i was 1%, 2%, and 3%;  $T_j$  was the effect of particle

size j (fine: 0.128 mm; coarse: 1.114 mm); NxT was the interaction between algae levels and particle size; and  $e_{ijk}$  was random error associated with each observation.

For the data that did not exhibit a normal distribution, such as the percentage of non-viable eggs, a binomial distribution with a logit link function was adopted. The difference between the means of the treatment and control groups was evaluated by performing the chi-square test ( $p < 0.05$ ) in the Genmod program of SAS (2009).

### RESULTS

The variables related to layer performance showed no significant differences between the CA levels and particle sizes, nor did it affect the interaction between

factors (Table 3). For the percentage of CRK, BRK, SL, or DEF eggs (Table 4), no significant results were observed among the diets evaluated, with the percentage of almost all parameters evaluated below 1%.

The variables associated with egg quality (Table 5), such as egg weight, YI, HU, SG, ST, and shell MM, did not significantly differ between the eggs of the hens fed with diets containing fine or coarse CA. On the other hand, the yolk color of the eggs produced by the hens fed with diets containing 1% fine CA was more pigmented ( $p < 0.05$ ) than that of the eggs produced by the hens fed with the control diet.

Regarding egg composition (Table 6), there was no interaction between factors in the variables studied. PS did not differ among the tested diets or from the control diet. However, a higher PA was observed in the eggs

Table 3. Layer performance of light-laying hens (53–68 weeks) fed with varying levels of *Calcareous algae* with different particle sizes (fine: 0.128 mm; coarse: 1.114 mm)

Variables	Particle size	<i>Calcareous algae</i>				Means	SEM	p value
		Control	1%	2%	3%			
FI (g/bird/day)	Fine	0.109	0.108	0.108	0.108	0.108	0.001	0.507
	Coarse		0.108	0.107	0.107	0.107	0.001	
	Means	0.108	0.107	0.107				
	SEM	0.001	0.001	0.001				
	p value			0.728				
FC (kg/kg)	Fine	1.842	1.776	1.805	1.840	1.807	0.017	0.183
	Coarse		1.815	1.849	1.847	1.837	0.014	
	Means	1.795	1.837	1.843				
	SEM	0.016	0.022	0.017				
	p value			0.200				
FC (kg/dz)	Fine	1.438	1.427	1.386	1.436	1.417	0.016	0.188
	Coarse		1.413	1.405	1.403	1.407	0.008	
	Means	1.420	1.396	1.420				
	SEM	0.018	0.010	0.016				
	p value			0.199				
LP (%)	Fine	92.798	92.30	94.27	91.91	92.83	0.845	0.226
	Coarse		92.24	92.04	89.90	91.39	0.724	
	Means	92.27	93.15	90.90				
	SEM	0.922	0.674	1.173				
	p value			0.293				
EM (g/bird)	Fine	60.51	59.92	61.21	58.78	59.97	0.798	0.336
	Coarse		59.60	58.79	58.12	58.84	0.721	
	Means	59.76	59.99	58.45				
	SEM	0.722	0.909	1.102				
	p value			0.509				

Note: SEM, standard error of the mean; FI, feed intake; FC, feed conversion ratio; LP, laying percentage; EM, egg mass.

Table 4. Percentage of non-viable eggs produced by light-laying hens (53–68 weeks) fed with varying levels of *Calcareous algae* with different particle sizes (fine: 0.128 mm or coarse: 1.114 mm)

Treatments	Variables							
	CRK	p value	BRK	p value	SL	p value	DEF	p value
Control	0.28	-	0.3	-	0.33	-	0.16	-
1% CA Fine	0.21	0.97	0.25	0.12	0.5	0.37	0.17	0.44
1% CA Coarse	0.45	0.46	0.04	1.07	0.62	0.09	0.13	0.73
2% CA Fine	0.20	0.55	0.08	0.21	0.28	0.60	0.26	0.98
2% CA Coarse	0.29	0.99	0.45	0.60	0.56	0.22	0.33	0.73
3% CA Fine	0.41	0.75	0.12	0.24	0.39	0.48	0.04	0.23
3% CA Coarse	0.17	0.82	0.49	0.08	0.30	0.27	0.22	0.35

Note: CA, *Calcareous algae*; CRK, cracked eggs; BRK, broken eggs; SL, shell-less eggs; DEF, deformed eggs.

Table 5. Quality of the eggs produced by light-laying hens (53–68 weeks) fed with varying levels of *Calcareous algae* with different particle sizes (fine: 0.128 mm; coarse: 1.114 mm)

Variables	Particle size	<i>Calcareous algae</i>				Means	SEM	p value
		Control	1%	2%	3%			
EW (g)	Fine	65.06	66.25	64.40	64.59	65.08	0.419	0.123
	Coarse		64.75	63.18	64.41	64.12	0.447	
	Means		65.50	63.77	64.50			
	SEM		0.419	0.606	0.473			
	p value			0.087				
YI (mm)	Fine	0.416	0.423	0.424	0.419	0.422	0.002	0.880
	Coarse		0.419	0.425	0.420	0.421	0.002	
	Means		0.421	0.424	0.420			
	SEM		0.002	0.002	0.002			
	p value			0.349				
HU	Fine	93.71	92.37	92.20	90.60	91.72	0.485	0.627
	Coarse		91.72	90.56	91.80	91.36	0.567	
	Means		92.04	91.38	91.20			
	SEM		0.681	0.592	0.639			
	p value			0.620				
Color	Fine	5.289	5.389*	5.231	5.311	5.102	0.030	0.879
	Coarse		5.303	5.269	5.339	5.304	0.033	
	Means		5.346	5.250	5.325			
	SEM		0.026	0.045	0.035			
	p value			0.168				
SG (g/cm <sup>3</sup> )	Fine	1.087	1.085	1.087	1.087	1.087	0.001	0.305
	Coarse		1.087	1.088	1.087	1.807	0.001	
	Means		1.086	1.088	1.087			
	SEM		0.001	0.001	0.001			
	p value			0.091				
ST (mm)	Fine	0.284	0.281	0.286	0.281	0.280	0.003	0.417
	Coarse		0.281	0.289	0.279	0.285	0.004	
	Means		0.284	0.284	0.280			
	SEM		0.004	0.005	0.004			
	p value			0.837				
MM (%)	Fine	85.76	88.36	87.65	88.63	88.22	0.532	0.663
	Coarse		89.07	87.79	88.78	88.55	0.434	
	Means		88.71	87.72	88.71			
	SEM		0.475	0.447	0.760			
	p value			0.477				

Note: \* Differences from the control by Dunnett’s test (p<0.05). SEM, standard error of the mean; EW, egg weight; YI, yolk index; HU, Haugh unit; color, yolk coloration; SG, specific gravity; ST, shell thickness; MM, shell mineral matter.

produced by the hens fed with 1% CA than in those produced by the hens fed with 2% CA. Only the average PY related to particle size differed (p<0.05); a higher value was observed with the inclusion of coarse CA in the diet.

The results for Ca consumption, retention, and excretion are presented in Table 7. Ca consumption was not influenced by experimental diets. The amount of Ca excreted (g) by the hens fed with the diet containing 2% coarse CA was significantly higher (p<0.05) than that of hens fed with the control diet. Similarly, a higher amount of Ca was excreted (g) by the hens fed with diets containing coarse CA than those fed with diets containing fine CA. There was an interaction between particle size and algae levels (p<0.05) in retained and excreted Ca (%). Compared with the control diet (p<0.05), the diets containing 3% fine CA and 1% and 2% coarse CA promoted higher excretion (%) and, consequently, lower Ca retention (g and %).

Data on P consumption, excretion, and retention are shown in Table 8. The amount of P consumed by the hens fed with diets containing 1% and 2% CA was lower than that of those fed with the control diet (p<0.05). Higher P consumption was observed in the hens fed the diet containing 3% CA than in those fed with 1% CA (p<0.05). Similarly, the P consumption of the hens fed with diets containing fine CA was higher than that of hens fed with diets containing coarse CA (p<0.05). The amount of excreted P (g) did not differ among the treatment diets. There was an interaction between particle size and algae levels (p<0.05) in excreted P (%) and retained P (g and %). The excreted P (%) was significantly higher, and retained P (g and %) was significantly lower (p<0.05) in the hens fed with diets containing 1% fine CA and 2% and 3% coarse CA than those in the control hens. The excreted P (%) of the hens fed with diets containing 1% fine CA was significantly higher (p<0.05) than that of the hens fed with 2% and 3%

Table 6. Composition of the eggs of light-laying hens (53–68 weeks) fed with varying levels of *Calcareous algae* with different particle sizes (fine: 0.128 mm; coarse: 1.114 mm)

Variables	Particle size	<i>Calcareous algae</i>				Means	SEM	p value
		Control	1%	2%	3%			
PA (%)	Fine	59.99	60.93	59.40	59.80	60.03	0.246	0.543
	Coarse		59.94	59.52	60.31			
	Means		60.43 <sup>A</sup>	59.46 <sup>B</sup>	60.04 <sup>AB</sup>			
	SEM		0.202	0.199	0.213			
	p value		0.004					
PY (%)	Fine	26.17	25.44	26.33	25.82	25.86 <sup>b</sup>	0.174	0.004
	Coarse		26.63	26.79	26.34			
	Means		26.03	26.56	26.08			
	SEM		0.262	0.203	0.144			
	p value		0.137					
PS (%)	Fine	9.29	9.09	9.40	9.11	9.20	0.09	0.104
	Coarse		9.40	9.43	9.46			
	Means		9.24	9.41	9.29			
	SEM		0.127	0.125	0.107			
	p value		0.577					

Note: Uppercase letters in rows and lowercase letters in columns indicate significant differences according to Tukey's test (p<0.05) and F test (p<0.05), respectively. \* Differences from the control by Dunnett's test (p<0.05). SEM, standard error of the mean; PA, percentage of albumen; PY, percentage of yolk; PS, percentage of shell.

Table 7. Amount of calcium consumed, excreted, and retained by light-laying hens (53–68 weeks) fed with varying levels of *Calcareous algae* with different particle sizes (fine: 0.128 mm; coarse: 1.114 mm)

Variables	Particle size	<i>Calcareous algae</i>				Means	SEM	p value
		Control	1%	2%	3%			
Ca intake (g/bird/day)	Fine	4.037	3.759	3.802	3.797	3.786	0.063	0.456
	Coarse		3.846	3.862	3.868			
	Means		3.803	3.832	3.824			
	SEM		0.077	0.055	0.101			
	p value		0.590					
Ca excreted (g/bird/day)	Fine	1.391	1.373	1.402	1.734	1.503 <sup>b</sup>	0.070	0.004
	Coarse		1.831	1.905 <sup>*</sup>	1.689			
	Means		1.602	1.653	1.712			
	SEM		0.113	0.112	0.077			
	p value		0.663					
Ca excreted (%)	Fine	34.49	36.53 <sup>Bb</sup>	36.99 <sup>Bb</sup>	45.63 <sup>A*</sup>	39.72	1.705	0.005
	Coarse		47.39 <sup>a*</sup>	49.12 <sup>a*</sup>	44.63			
	Means		41.96	43.06	44.87			
	SEM		2.531	2.670	2.086			
	p value		0.599					
Ca retained (g/bird/day)	Fine	2.646	2.386	2.399	2.063 <sup>*</sup>	2.283 <sup>a</sup>	0.076	0.040
	Coarse		2.016 <sup>*</sup>	1.957 <sup>*</sup>	2.179			
	Means		2.201	2.178	2.121			
	SEM		0.093	0.100	0.108			
	p value		0.825					
Ca retained (%)	Fine	65.51	63.41 <sup>Aa</sup>	63.01 <sup>Aa</sup>	54.67 <sup>B*</sup>	60.26	1.705	0.005
	Coarse		52.61 <sup>b*</sup>	50.88 <sup>b*</sup>	55.89			
	Means		58.01	59.94	55.13			
	SEM		2.531	2.670	2.086			
	p value		0.599					

Note: Uppercase letters in rows and lowercase letters in columns indicate significant differences according to Tukey's test (p<0.05) and F test (p<0.05), respectively. \* Differences from the control by Dunnett's test (p<0.05). SEM: standard error of the mean.

fine CA; consequently, retained P (g and %) was lower for the hens fed with 1% fine CA than in those fed with diets containing other fine CA concentrations. In terms of particle size, the inclusion of 2% and 3% coarse CA in the diets resulted in lower P retention (g and %).

## DISCUSSION

The performance variables were not influenced by the experimental diets, consistent with the findings of previous studies that no differences were observed in egg production between the varying levels of CA

Table 8. Amount of phosphorus consumed, excreted, and retained by light-laying hens (53–68 weeks) fed with varying levels of *Calcareous algae* with different particle sizes (fine: 0.128 mm; coarse: 1.114 mm)

Variables	Particle size	<i>Calcareous algae</i>				Means	SEM	p value
		Control	1%	2%	3%			
P intake (g/bird/day)	Fine	0.721	0.665	0.69	0.753	0.703 <sup>a</sup>	0.015	0.001
	Coarse		0.646*	0.640*	0.657	0.648 <sup>b</sup>	0.008	
	Means		0.655 <sup>B</sup>	0.665 <sup>AB</sup>	0.705 <sup>A</sup>			
	EPM		0.013	0.012	0.021			
	p value			0.026				
P excreted (g/bird/day)	Fine	0.360	0.375	0.346	0.369	0.363	0.009	0.991
	Coarse		0.350	0.367	0.372	0.363	0.009	
	Means		0.362	0.357	0.370			
	EPM		0.009	0.011	0.012			
	p value			0.683				
P excreted (%)	Fine	59.89	67.52 <sup>A*</sup>	60.30 <sup>Bb</sup>	58.81 <sup>Bb</sup>	62.21	1.444	0.001
	Coarse		65.20	68.70 <sup>a*</sup>	70.14 <sup>a*</sup>	68.01	1.223	
	Means		66.36	64.50	64.48			
	EPM		1.013	2.059	2.255			
	p value			0.554				
P retained (g/bird/day)	Fine	0.289	0.215 <sup>B*</sup>	0.275 <sup>Aa</sup>	0.310 <sup>Aa</sup>	0.267	0.014	0.001
	Coarse		0.226	0.199 <sup>b*</sup>	0.190 <sup>b*</sup>	0.205	0.009	
	Means		0.220	0.237	0.250			
	EPM		0.008	0.017	0.023			
	p value			0.200				
P retained (%)	Fine	40.11	32.48 <sup>B*</sup>	39.70 <sup>Aa</sup>	41.19 <sup>Aa</sup>	37.79	1.444	0.001
	Coarse		34.80	31.30 <sup>b*</sup>	29.86 <sup>b*</sup>	31.99	1.223	
	Means		33.64	35.50	35.52			
	EPM		1.013	2.059	2.255			
	p value			0.554				

Note: Uppercase letters in rows and lowercase letters in columns indicate significant differences according to Tukey's test (p<0.05) and F test (p<0.05), respectively. \* Differences from the control by Dunnett's test (p<0.05). SEM: standard error of the mean.

included (maximum of 4%) in the diet of laying hens as a replacement for limestone (Sousa *et al.*, 2024). Our results are partially similar to those reported by Badeca *et al.* (2022). They replaced limestone in quail diets with different CA levels and observed a quadratic effect with a maximum reduction in FI after replacing limestone with 16.24% CA; however, the inclusion of CA also altered other productive variables. The reduction in feed consumption without altering productive variables could be explained by the higher nutrient availability of CA, which provides sufficient nutrient levels to meet the nutrition requirements of hens (Badeca *et al.*, 2022). On the other hand, the use of *L. calcareum* has also been investigated for its potential to support poultry production when diets are contaminated with fungi due to its ability to adsorb mycotoxins even at low concentrations (Perali *et al.*, 2020).

Another factor influencing feed consumption is the Ca level in the diet. However, the diets used in this experiment had uniform Ca content, which, when in excess, can negatively affect diet palatability and additionally compromise the absorption of other essential minerals, such as P, manganese, and zinc (Velasco *et al.*, 2016). The lack of significant results between diets may occur because the birds have the ability to absorb calcium similarly from the sources provided (Rezende *et al.*, 2024). Furthermore, birds may exhibit adaptability to this type of feed, even at higher inclusion levels (7.5%), without altering their productive parameters (Oliveira *et al.*, 2023).

Considering that the parameters of feed intake, egg production, feed conversion (kg and dozen), and EM showed similar results among the experimental diets, it can be inferred that the concentration of minerals used was aligned with the nutritional requirements of the hens, without any detriment associated with the use of CA (Melo *et al.*, 2008). On the other hand, the inclusion of 2% red algae positively affected the reduction of feed conversion in layers, with an average value of 1.7 (Kulshreshtha *et al.*, 2014). According to the authors, variations between results can be attributed to differences in bird age, type of algae, experimental conditions, temperature, and humidity. The substitution of 2% limestone with CA, performed by Sousa *et al.* (2024b), increased egg weight in a non-climatized environment compared to the other tested levels, possibly due to the associative effect of limestone with the algae, in a ratio of 76:30. This suggests that this would be the most advantageous ratio for this variable, although it did not differ from the standard diet. The effect of CA on FI of hens is still poorly elucidated in the existing literature.

The integrity of the eggshell is a crucial factor for preventing the contamination of the egg's interior and preserving its quality. Additionally, thicker shells provide greater resistance to breakage during handling and transportation (Pereira *et al.*, 2021). Factors such as nutrition, genetics, management practices, environmental conditions, and the age of hens influence eggshell quality (Bairros *et al.*, 2024). The lack of effect

of the concentration or particle size of CA on the percentage of non-viable eggs may be related to the fact that the hens still produced eggs of good shell quality, considering that the hens included in the experiment were 53–68 weeks of age. When completely substituting limestone with algae, Carlos *et al.* (2011) observed no effects on the performance of broiler chickens, bone growth, or bone ash deposition.

Badeca *et al.* (2022) found that the inclusion of CA did not influence the percentage of marketable eggs. However, as the concentration of CA increased as a replacement for limestone in the diet, the eggshells became thicker, which promoted higher resistance to breakage. On the other hand, Fan *et al.* (2021) found no differences in egg production or quality with the inclusion of up to 5% *Sargassum* meal in Leghorn layers. Younger birds can assimilate Ca more efficiently regardless of the source if the Ca levels in the diet are adequate (Rezende *et al.*, 2024). Another factor is the higher bioavailability of micronutrients adsorbed in the cell wall of the algae, which are easily assimilated by the birds due to the high porosity of the algae, providing a greater specific surface area for action. Thus, algae can be added to the diet without causing harm to the animals or their production (Melo e Moura, 2009).

Regarding internal and external egg quality, Murata *et al.* (2009) also found no influence of diets with different limestone particle sizes; when Ca consumption and availability are adequate, improvements in egg properties may not be evident. In contrast, when 25% fine limestone was replaced with coarse limestone, Ege *et al.* (2019) observed that particle size was less important than the form in which the feed was provided, but it did not affect egg quality. It is worth noting that the HU values recorded in this study were above 90, considered of excellent quality (Bairros *et al.*, 2024). The solubility levels of Ca sources can also be an important factor for the discrepancy in the results of previous studies (Hervo *et al.*, 2022).

The results of this study are partially similar to the findings of Spanivello *et al.* (2022), who observed that increasing the concentration of algae in the diet did not influence the weight and specific gravity of their eggs in quails. Carrillo *et al.* (2008) also found no differences in ST with the use of three types of algae. However, the use of the algae *Enteromorpha spp.* reduced yolk color intensity, possibly because some pigments in red algae may be more sensitive than those in brown algae, which was not observed in our study. A trend also suggests that *Sargassum* meal supplementation could enhance yolk redness and yellowness (Fan *et al.*, 2021).

The results of Spanivello *et al.* (2022) corroborate those of this study, as they did not observe an increase in yolk and shell percentages with the inclusion of CA in quail diets. However, the weight of eggshells increased as the CA concentration increased, which could be related to the higher deposition of Ca in the shell. Egg composition was also unaffected by the inclusion of 0.25% and 0.50% CA (Melo *et al.*, 2008). On the other hand, the PY in the eggs produced by semi-heavy laying hens decreased as the CA concentration in the diet increased up to 4%, which could be related to a slight

decrease in P intake, as P significantly contributes to yolk formation (Sousa *et al.*, 2024). The inclusion of 1% of the red algae *C. crispus* and *S. gaudichaudii* increased egg and yolk weight, which was attributed to the higher synthesis of yolk proteins and the increased availability of water and minerals (Kulshreshtha *et al.*, 2014).

The presence of larger-sized particles can increase the retention time of the feed and allow for a greater action of digestive enzymes on nutrients in the gastrointestinal tract, improving absorption and digestibility (Hervo *et al.*, 2022), which could provide greater nutrient availability for yolk formation. In the present study, the PY in the eggs produced by the hens fed with coarse CA particles was higher than in eggs produced by those fed with fine CA. However, there are no studies in the literature comparing the effects of CA particle sizes on the egg quality of laying hens; only studies on the effects of varying CA inclusion levels are available, and as mentioned earlier, varying CA levels do not alter egg composition.

In previous studies, Ca consumption increased only when dietary Ca levels increased (Velasco *et al.*, 2016). However, in this study, the Ca levels were similar, and consumption was not altered by the experimental diets. Manangi *et al.* (2018) did not find differences in calcium excretion when evaluating different particle sizes. As *L. calcareum* is more soluble than limestone (Melo & Moura, 2009), the inclusion of 3% fine CA in the diet of laying hens might have resulted in the higher Ca excretion observed in this study. Diana *et al.* (2023) found no difference in Ca retention in laying hens when different particle sizes of limestone were evaluated. The levels of Ca retention may be related to the solubility of each Ca source, which may cause variations in the study results and an increase or decrease in Ca retention in animals (Hervo *et al.*, 2022).

*L. calcareum* is composed of approximately 0.03% P (Melo & Moura, 2009); thus, as the concentration of *L. calcareum* in the diet increases, greater P consumption may occur, leading to increased excretion. Algae contain carotenoids and are rich in antioxidants (Carrillo *et al.*, 2008). Bioavailable foods, such as those of organic origin, in conjunction with antioxidant activity, may increase the excretion of minerals at certain concentrations, as occurred with 2% and 3% coarse CA in our experiment. Velasco *et al.* (2016) reported that 0.3 g of P per bird was sufficient for adequately maintaining quail egg production, and increasing the inclusion level in the diet also increased its excretion. This can become an environmental pollution problem, which is undesirable for current animal production systems that aim to minimize their environmental impact. In addition, P is the most expensive mineral in the diet and has a finite source.

Algae can be incorporated into the diets of laying hens; however, depending on the algae species, the use of more than 10% is not recommended, as it may compromise feed acceptance and cause a decrease in production and diarrhea due to the high concentration of minerals present in the algae (Carrillo *et al.*, 2008). With *L. calcareum*, Carlos *et al.* (2011) could completely replace limestone with algae without harming the health

of broiler chickens. Nevertheless, further research is still needed regarding the inclusion limit of CA in diets for laying hens. Research on new mineral sources should be encouraged because most are non-renewable, making *L. calcareum* an alternative to poultry feed without detriment to production. However, in the present study, no differences were observed in egg production or quality after the inclusion of CA.

## CONCLUSION

The results of this study revealed that the inclusion of 3% CA (*L. calcareum*), fine or coarse, in the diets of light-laying hens (53–68 weeks) did not influence performance, internal and external egg quality, and percentages of non-viable eggs. However, the inclusion of 2% fine-particle CA in the diet decreased the excretion of Ca and P, improving the retention of these minerals. The economic feasibility of using this mineral source in poultry production should also be analyzed in future studies to verify its profitability.

## CONFLICT OF INTEREST

We certify that there are no conflicts of interest with any financial, personal, or other relationships with other people or organizations related to the material discussed in the manuscript.

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