



Article

Limestone Granulometry and Vitamin D Supplementation in Brown Laying Hens: Implications for Performance, Egg Quality, Bone Health, Thermoregulation, and Behavior in a Hot Environment

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Abstract

This study evaluated the effects of limestone particle size and 25-hydroxycholecalciferol supplementation on performance, egg quality, digestive organ biometrics, bone characteristics, thermoregulatory responses, and behavior of brown laying hens reared under hot environmental conditions. The trial lasted five periods of 28 days. A total of 270 Lohmann Brown Lite hens (48 weeks old) were allocated in a completely randomized design with a $2 \times 2 + 1$ factorial arrangement, comprising two limestone particle sizes (MGD 0.568 mm and MGD 1.943 mm) and two supplementation levels of vitamin D (2760 IU and 1380 IU), plus a control diet, totaling five treatments with six replicates each. The dietary treatments were as follows: (1) control diet without vitamin D supplementation; (2) 250 g vitamin D with 100% fine limestone; (3) 125 g vitamin D with 100% fine limestone; (4) 250 g vitamin D with 50% fine + 50% coarse limestone; and (5) 125 g vitamin D with 50% fine + 50% coarse limestone. Productive performance, egg quality, organ biometrics, bone traits, thermoregulatory variables, and behavioral indicators were measured. Data were analyzed by ANOVA, followed by Tukey test (5%), considering the factorial model. There was no interaction between the factors for any parameter evaluated. There was an effect of shift on thermoregulatory variables. The other variables were not influenced by the treatments. These findings indicate that the tested limestone particle sizes can be incorporated in the diets of brown laying hens without affecting performance, egg quality, bone integrity, thermoregulation, or behavior, regardless of vitamin D supplementation, under hot climatic conditions.

Keywords: limestone particle size; vitamin D; egg quality; thermoregulation; behavior; heat stress



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1. Introduction

Birds are homeothermic animals, capable of maintaining their body temperature within a narrow range, even under variable environmental conditions. When exposed to heat stress, they undergo behavioral and physiological changes or develop adaptations to achieve thermoregulation and restore homeostasis [1].

Heat stress occurs when the amount of heat generated by the animal exceeds its ability to dissipate it into the environment. This can be influenced by factors such as thermal radiation, sunlight, humidity, air temperature, and air circulation [2,3]. This imbalance negatively affects breeding performance, reducing egg production and quality [4,5].

Calcium sources vary in particle size and solubility. Coarse limestone remains longer in the gizzard, gradually releasing calcium and extending its availability to the birds, especially at night when feed intake ceases [6]. However, this characteristic requires greater mechanical effort from the gizzard, which can increase heat production and exacerbate heat stress [7].

High-performance laying hens require careful nutritional management. In this context, mineral nutrition plays a decisive role in bone health and eggshell quality, with calcium (Ca) being the most critical mineral [8]. In addition to Ca, the biologically active form of vitamin D is essential for calcium homeostasis and bone integrity, regulating intestinal Ca and phosphorus (P) absorption, modulating parathyroid hormone secretion, and participating in bone mineralization and remodeling [9,10]. Adequate interaction between Ca and vitamin D is crucial for optimizing calcium deposition in the eggshell and can modulate the caloric increase generated by gizzard metabolism, especially under heat stress conditions. Thus, balanced nutrition prevents bone metabolic disorders and ensures skeletal development and maintenance, directly influencing production performance and eggshell quality. From a commercial perspective, eggshells must be strong enough to prevent contamination and cracking from farm to consumer [11]. Calcium and phosphorus deficiencies result in poor shell quality, causing considerable losses in the poultry industry [12].

Thus, we hypothesize that the use of coarse limestone associated with greater vitamin D3 supplementation improves the performance, egg quality, bone quality and thermoregulation of laying hens subjected to heat stress. This study aimed to evaluate the effect of limestone particle size and vitamin D supplementation on performance, egg quality, digestive organ biometrics, bone characteristics, thermoregulatory responses, and behavioral responses of commercial brown laying hens raised in tropical environments.

2. Material and Methods

2.1. Study Location

The experiment was conducted at the layer sector of the university's farm located in Sobral, Ceará, Brazil (3°36' S, 40°18' W, and 56 m above sea level), over 140 days, divided into five periods of 28 days. The region's climate is classified as BSh (B—Dry, S—Semi-arid, h—Low latitude and altitude) according to the Köppen climate classification [13].

2.2. Bird Housing

The hens used in this study were donated by Planalto Farm. The animals were reared under commercial conditions in accordance with the company's standard housing and welfare practices before being transferred to the experimental facilities of the Universidade Estadual Vale do Acaraú (UVA), where the trial was conducted.

The birds were housed in conventional laying sheds equipped with galvanized wire cages measuring 90 cm in length, with three subdivisions of 30 cm × 45 cm × 45 cm, arranged in a pyramidal system. The cages were fitted with front trough-type feeders and nipple-type drinkers at the top. The shed was manually operated, built with masonry,

a concrete floor, ceramic roof tiles, wire mesh, and oriented East–West. The birds were subjected to 12 h of natural light and 4 h of artificial light per day.

2.3. Animals, Experimental Design and Diets

A total of 270 Lohmann Brown Lite laying hens, 48 weeks old, weighing $1.723 \text{ kg} \pm 0.063$ and producing $76.56\% \pm 3.38$ of eggs, were used. The birds were weighed and selected to ensure uniformity in weight and egg production prior to starting the experiment, as recommended by Sakomura and Rostagno [14].

A completely randomized design was adopted in a $2 \times 2 + 1$ factorial scheme, consisting of diets with two limestone particle sizes (MGD fine: 0.568 mm or MDG coarse: 1.943 mm), two vitamin D3 supplementation levels (2760 IU or 1380 IU), and a control diet (fine limestone without vitamin D supplementation), totaling five treatments, six replicates, and nine birds per replicate. The vitamin D metabolite supplemented in the diets was 25-hydroxycholecalciferol (25-OHD3). The vitamin–mineral premix contained 2500 IU/kg of feed. The limestone particle sizes were classified based on mean geometric diameter (MGD): fine (MGD 0.568 mm) and coarse (MGD 1.943 mm). Coarse limestone replaced fine limestone by 50%.

The diets were isonutritional and isoenergetic (Table 1), mashed, formulated according to the nutritional recommendations of the Lohmann Brown Lite manual [15] and included inert material and the nutritional composition of the ingredients was obtained from Rostagno et al. [16].

Table 1. Calculated percentage and nutritional composition of the experimental diet.

Ingredient	Control	Fine + 2760 IU Vit. D	Fine + 1380 IU Vit. D	Coarse + 2760 IU Vit. D	Coarse + 1380 IU Vit. D
Corn grain	62.49	62.49	62.49	62.49	62.49
Soybean meal (45%)	23.52	23.52	23.52	23.52	23.52
Fine limestone	9.44	9.44	9.44	4.72	4.72
Coarse limestone	-	-	-	4.72	4.72
Meat meal	3.53	3.53	3.53	3.53	3.53
Vitamin–mineral premix *	0.300	0.300	0.300	0.300	0.300
Common salt	0.325	0.325	0.325	0.325	0.325
Vitamin D	-	0.025	0.0125	0.025	0.0125
Enramycin	0.0125	0.0125	0.0125	0.0125	0.0125
Soybean oil	0.277	0.277	0.277	0.277	0.277
DL-Methionine	0.080	0.080	0.080	0.080	0.080
Inert	0.0250	-	0.0125	-	0.0125
Metabolic Energy (Cal/kg)	2780	2780	2780	2780	2780
Crude Protein (%)	17.50	17.50	17.50	17.50	17.50
Calcium (%)	4.10	4.10	4.10	4.10	4.10
Available phosphorus (%)	0.46	0.46	0.46	0.46	0.46
Sodium (%)	0.17	0.17	0.17	0.17	0.17
Digestible Met + Cis (%)	0.60	0.60	0.60	0.60	0.60
Digestible methionine (%)	0.35	0.35	0.35	0.35	0.35
Digestible lysine (%)	0.79	0.79	0.79	0.79	0.79
Digestible threonine (%)	0.57	0.57	0.57	0.57	0.57
Digestible tryptophan (%)	0.17	0.17	0.17	0.17	0.17

* PX POSTURE 0.3% E300 TEC 08.7434: GUARANTEE LEVELS: Iron (min) 13.33 g/kg; copper (min) 3333.33 mg/kg; manganese (min) 25 g/kg; zinc (min) 25 g/kg; iodine (min) 277.33 mg/kg; selenium (min) 100 mg/kg; vitamin A (min) 2666,667 IU/kg; vitamin D3 (min) 833,333 IU/kg; vitamin E (min) 4000 IU/kg; vitamin K3 (min) 527.90 mg/kg; vitamin B1 (min) 333.33 mg/kg; vitamin B2 (min) 1200 mg/kg; niacin (min) 6701 mg/kg; pantothenic acid (min) 2407 mg/kg; vitamin B6 (min) 334 mg/kg; folic acid (min) 99 mg/kg; biotin (min) 7 mg/kg; vitamin B12 (min) 3200 mg/kg; phosphatidylcholine (min) 690.13 mg/kg; methionine (min) 100 g/kg; xylanase (min) 4,000,000 U/kg; phytase (min) 100,000 FTU/kg during the day.

2.4. Productive Performance Evaluation

The performance parameters of the layers were evaluated based on feed intake and egg production, which were recorded daily during each 28-day period. The following performance variables were calculated: feed intake (g/bird/day), egg production (%), egg weight (g), egg mass (g/bird/day), feed conversion per egg mass (kg/kg) and feed conversion per dozen eggs (kg/dz).

2.5. Egg Quality Analysis

At the end of each 28-day period, the following were analyzed: percentages of white, yolk and shell, shell thickness (mm) and specific gravity (g/cm³). Four eggs per replicate were selected over two consecutive days, two for specific gravity determined by the saline flotation method [17], and two for the remaining quality analyses.

The eggs were manually broken in the lab, and the components were separated and weighed using a precision scale (± 0.01 g, max capacity: 500 g; MH-267-5; China). The percentage of each component was calculated by dividing its weight by the total egg weight and multiplying by 100. Shells were air-dried for 24 h (no forced ventilation), weighed, and their thickness measured at three points (equator, top, bottom) using a digital caliper (± 0.01 cm, max: 150 cm). The average shell thickness was then calculated for each replicate.

2.6. Meteorological Variables and Thermal Comfort Indices

Meteorological data were measured on the last two days of the three experimental periods, in the morning (6 to 11 a.m.) and afternoon (1 to 5 p.m.), during the dry season. Measurements of black globe temperature (BGT, °C), dry bulb temperature (DBT, °C), and wet bulb temperature (WBT, °C) were taken every 30 min using thermometers placed at the birds' center of mass, ~1 m from the ground. Wind speed (m/s) was obtained from the FUNCEME (Ceará Foundation for Meteorology and Water Resources) database. The Thermal Comfort Indices were calculated from this data: Black Globe-Humidity Index (BGHI) and Radiant Heat Load (RHL) according to the formulas below [18,19]:

$$\text{BGHI} = \text{BGT} + 0.36 \times \text{DPT} + 41.5$$

In which:

BGT—Black Globe Temperature, °C

DPT—Dew Point Temperature °C

$$\text{RHL} = \sigma (\text{MRT})^4$$

In which:

σ —Stefan–Boltzmann constant ($5.67 \times 10^{-8} \text{ W m}^{-2} \text{ K}^{-4}$)

MRT⁴—Mean Radiant Temperature, K.

$$\text{MRT} = 100^4 \sqrt{(2.51 \sqrt{W_s} (\text{BGT} - \text{DBT}) + (\text{BGT}/100)^4)}$$

In which:

W_s—Wind Speed, m/s

BGT—Black Globe Temperature, K

DBT—Dry Bulb Temperature, K.

2.7. Biometric Analysis of Digestive Organs

At the end of the trial, 5 birds per treatment (30 hens in total) were euthanized by the cervical dislocation method (according to Normative Resolution No. 37/2018—CONCEA) [20]. The proventriculus, gizzard, liver, pancreas, and intestines were collected for biometric anal-

ysis of the digestive organs. The intestines and gizzard were emptied, and all organs were weighed on a precision scale (accuracy of 0.01 g). The length of the intestines was measured with a tape measure. The relative weight of each organ was calculated using the formula:

$$\text{Relative organ weight} = (\text{organ weight/body weight}) \times 100$$

2.8. Bone Quality Analysis

The right and left legs of the euthanized hens were collected, identified, and stored under refrigeration until dissection. For dissection, the legs were thawed at room temperature for 6 h before processing. They were then scalded in hot water to facilitate removal of muscle and cartilage, freeing the tibias (right and left). These bones were identified, placed in plastic bags, and frozen for later bone analysis.

Right tibias were used for mineral matter (g/kg) and Seedor index (mg/mm) analysis. For mineral matter, bones were placed in labeled aluminum containers, and pre-dried in a forced-air oven at 65 °C for 72 h. Then, they were ground using a mortar and pestle. A 2 g sample (in triplicate) was weighed and placed into crucibles, which were incinerated in a muffle furnace at 600 °C for 4 h [21]. The resulting ash weight was recorded.

Bone density was assessed using the Seedor index, calculated as the ratio of bone length (mm) to ash weight (mg), expressed in mg/mm [22]. Left tibias were used to assess bone strength (kgf/cm²) and deformity (mm). These tests were conducted at the Soil Mechanics Laboratory of the Federal University of Ceará (UFC) using a mechanical press (Ronald Top Ltda., Rio de Janeiro, Brazil). The tibias were placed horizontally, and compression force was applied at the midpoint until fracture. Maximum force at break was recorded using a digital extensometer (Engetotus, 704.020, Contagem, Brazil) and deformity was measured using an analog extensometer (Engetotus, 704.010, Contagem, Brazil).

2.9. Thermoregulatory Responses

Physiological responses were evaluated on the penultimate day of the last period, at the same time as meteorological measurements. One bird per replicate was randomly selected, identified with non-toxic paint on the back, and ringed.

Respiratory rate (RR, breaths/min) was determined by counting visible respiratory movements for 15 s, then multiplying the count by four to obtain breaths per minute, according to the methodology of Farias et al. [23].

Next, each bird was temporarily removed from its cage to obtain body temperatures. The Cloaca Surface (CST, °C), Crest (CRST, °C), Paw (PST, °C), Dewlap (DST, °C), Back (BST, °C), Wing (WST, °C) and Head (HST, °C) temperatures were measured. A digital infrared thermometer (STHT 77365, Stanley, Seattle, WA, USA) with an accuracy of ±0.2 °C was used for these measurements, without contact with the skin at a distance of approximately 15 cm from the birds' bodies.

Cloacal temperature (CT, °C) was measured with a digital thermometer (accuracy ±0.1 °C, G-Tech, Brazil) inserted 3 cm into the cloaca until stabilization. The thermal gradient (TG) was calculated by subtracting the average skin surface temperature from the cloacal temperature, as used by Farias et al. [23], and adapted in this study to include the surface temperatures of different regions of the body, using the following formula:

$$\text{TG} = \text{CT} - ((\text{HST} + \text{BST} + \text{DST} + \text{PST}) \div 4)$$

In which:

TG = Thermal gradient, °C; CT = Cloacal temperature, °C; HST = Head surface temperature, °C; BST = Back surface temperature, °C; DST = Dewlap surface temperature, °C; PST = Paw surface temperature, °C.

2.10. Behavioral Responses

Behavioral responses were assessed on the last day of each period alongside meteorological data collection. The frequencies of the following behaviors were recorded: sitting, feeding, drinking, exploring feathers, non-aggressive pecking and object pecking, comfort movements, and idling. The methodology and ethogram adapted from [24] were used. Observations were performed by four trained observers, each monitoring 8 to 10 birds from 6 a.m. to 5 p.m., using a truncated focal sampling method, with 5 min of observation followed by 10 min of rest.

2.11. Statistical Analysis

Data was analyzed as two-way ANOVA using the Dunnett test at a 5% significance level, considering the effect of limestone particle size and vitamin D³ supplementation, as well as their interactions. In interactions, the means were compared using the Tukey test ($p < 0.05$). Tukey's test was used to analyze environmental data ($p < 0.05$). The cage with 9 birds was considered the experimental unit. The data presented are cumulative from the 5 collection periods. The procedures were performed using SAS version 10, 2000 (SAS Institute Inc., Cary, NC, USA).

Canonical discriminant analysis (CDA) was performed to verify the dynamics of animal classification based on their respective feeding plans. If the discriminant functions were significant ($p < 0.005$), the most influential variables responsible for differentiating the animals' behavior based on diet were identified. The general CDA model is described in Equation:

$$Z_n = \alpha + \beta_1 X_1 + \beta_2 X_2 + \dots + \beta_n X_n$$

In which:

Z_n : is the dependent variable (data sets),

α : is the intercept,

X_i : are the explanatory variables,

β_i : are the discriminant coefficients for each explanatory variable.

A simultaneous method was used, which is recommended when there is a large number of variables to be included in the function. Discriminant power was evaluated using the percentage of variance, Wilks' Lambda statistic, and standardized coefficients. Data were processed and analyzed using the Statistical Package for the Social Sciences—SPSS, version 20 (2010) (SPSS[®] Inc., Chicago, IL, USA).

Additionally, graphs based on the raw data collected were generated to visualize the main behaviors exhibited by the birds during different periods of the day using Microsoft Excel[®] software, version 16, 2016.

3. Results

The meteorological variables were not influenced by the time of day, except for radiant heat load (RHL), which was higher in the afternoon (Table 2).

No effects of the treatments were observed on any of the evaluated performance, egg quality, biometric, or bone characteristics variables, regardless of the calcium source or vitamin D supplementation. There were similarly no interactions between the factors for any of the evaluated variable (Tables 3–6).

Regarding thermoregulatory responses, no treatment effects were observed for physiological variables (respiratory rate, cloacal temperature, skin surface temperatures, and thermal gradient) (Table 7). However, the time of day affected all these variables: respiratory rate, cloacal temperature, and both feathered and unfeathered surface temperatures were higher in the afternoon; in contrast, the thermal gradient (TG) was greater in the morning.

Table 2. Environmental variables and thermal comfort indices of brown layers fed diets supplemented with vitamin D3 reared in a hot environment.

Variable	Day Shift	Mean	Error	Maximum	Minimum	p-Value
Ws ¹ m/s	Morning	0.75	0.57	0.10	1.71	0.164
	Afternoon	0.60	0.58	0.10	1.71	
DBT ² °C	Morning	31.44	2.30	26.90	35.50	0.766
	Afternoon	31.61	4.10	24.40	36.70	
BGHI ³ °C	Morning	81.35	2.51	76.67	86.49	0.116
	Afternoon	82.41	4.84	74.10	88.04	
RHL ⁴ (W/m ²)	Morning	480.57 ^a	18.27	448.09	535.30	0.001
	Afternoon	496.65 ^b	32.87	440.61	549.13	
RH ⁵ (%)	Morning	68.07	7.73	50.63	83.57	0.689
	Afternoon	68.84	13.44	52.08	94.16	

¹ Wind speed in meters per second; ² Dry bulb temperature; ³ Black globe and humidity index; ⁴ Radiant Heat Load, ⁵ Relative humidity. ^{a,b}—Means followed by different capital letters in the same column differ from each other using Tukey's test at 5% probability.

Table 3. Performance of brown layers fed diets containing two limestone particle sizes and two vitamin D supplements in a hot environment.

Variables						
Diet	Intake (g/bird/day)	Production (%)	Egg Weight (g)	Egg Mass (g/ave/dia)	CM ¹ (kg/k)	CDZ ² (kg/Dz)
Control	104.06	85.08	55.81	47.49	2.194	1.469
FL ³ + 2760 IU	95.12	80.01	56.51	45.21	2.118	1.436
FL + 1380 IU	99.30	80.27	55.90	44.85	2.213	1.485
FL + CL ⁴ + 2760 IU	104.34	80.07	57.20	48.07	2.169	1.490
FL + CL + 1380 IU	97.11	78.62	56.51	44.72	2.252	1.526
SEM ⁵	1.587	0.989	0.251	0.594	0.034	0.024
<i>p</i> -value						
Diet	0.2545	0.0722	0.4298	0.0869	0.8223	0.8631
Particle size (PS)	0.3301	0.9378	0.2851	0.6152	0.5890	0.4377
Supplementation D ₃ (D ₃)	0.6687	0.0973	0.2836	0.0565	0.2949	0.4826
PS × D ₃	0.1208	0.0770	0.9522	0.0969	0.9393	0.9214
Particle size						
Fine	97.21	80.14	56.20	45.03	2.165	1.461
Fine + Coarse	100.72	80.30	56.85	45.68	2.211	1.508
Supplementation D ₃						
2760 IU	99.73	82.04	56.85	46.64	2.143	1.463
1380 IU	98.20	78.40	56.20	44.07	2.323	1.506

¹ Conversion per egg mass; ² Conversion per dozen eggs; ³ Fine Limestone (0.568 mm); ⁴ Coarse Limestone (1.943 mm); ⁵ Standard error of the mean; Dunnett's test at 5% probability.

Table 4. Internal and external quality of eggs from brown laying hens fed diets containing two limestone particle sizes and two vitamin D supplements in a warm environment.

Diet	Variables				
	Albumen (%)	Yolk (%)	Shell (%)	ST ¹ (%)	SG ² (g/cm ³)
Control	60.77	26.19	10.24	0.384	1.098
FL + 2760 IU	61.83	25.44	10.45	0.393	1.099
FL + 1380 IU	61.62	25.32	10.49	0.394	1.098
FL ³ + CL ⁴ + 2760 IU	61.87	25.62	10.14	0.385	1.099
FL + CL + 1380 IU	61.63	25.33	10.51	0.394	1.098
SEM ⁵	0.185	0.155	0.051	0.001	0.002
<i>p</i> -value					
Diet	0.3429	0.3855	0.0755	0.0808	0.8434
Particle size (PS)	0.9467	0.8014	0.1982	0.0817	0.6212
Supplementation D ₃ (D ₃)	0.5835	0.5882	0.0780	0.0739	0.4271
PS × D ₃	0.9685	0.8282	0.1492	0.1257	0.6756
Particle size					
Fine limestone	61.72	25.38	10.47	0.394	1.099
Fine + coarse limestone	61.75	25.48	10.32	0.388	1.098
Supplementation D ₃					
2760 IU	61.85	25.53	10.29	0.388	1.099
1380 IU	61.62	25.33	10.50	0.394	1.098

¹ Shell thickness; ² Specific gravity; ³ Fine Limestone (0.568 mm); ⁴ Coarse Limestone (1.943 mm); ⁵ Standard error of the mean; Dunnett's test at 5% probability.

Table 5. Relative weight of digestive organs and intestinal length of brown layers fed diets containing two limestone particle sizes and two vitamin D supplements in a hot environment.

Diet	Variables					Int. Length ² (cm)
	Pro. ¹ (%)	Gizzard (%)	Liver (%)	Pancreas (%)	Intestine (%)	
Control	0.399	1.16	2.30	0.21	2.25	161.50
FL + 2760 IU	0.400	1.25	2.65	0.19	2.12	167.83
FL + 1380 IU	0.419	1.24	2.09	0.21	2.22	167.17
FL ³ + CL ⁴ + 2760 IU	0.412	1.15	2.18	0.18	2.14	163.33
FL + CL + 1380 IU	0.360	1.29	2.32	0.18	2.17	161.33
SEM ⁵	0.011	0.023	0.071	0.006	0.035	1.664
<i>p</i> -value						
Diet	0.464	0.077	0.072	0.277	0.565	0.627
Particle size (PS)	0.430	0.581	0.171	0.200	0.430	0.199
Supplementation D ₃ (D ₃)	0.507	0.058	0.440	0.372	0.190	0.736
PS × D ₃	0.232	0.070	0.105	0.409	0.311	0.866
Particle size						
Fine limestone	0.408	1.24	2.46	0.20	2.23	167.50
Fine + coarse limestone	0.386	1.22	2.25	0.18	2.15	162.33
Supplementation D ₃						
2760 IU	0.406	1.20	2.42	0.18	2.13	165.58
1380 IU	0.388	1.27	2.30	0.19	2.25	164.25

¹ Proventriculus; ² Intestine length; ³ Fine Limestone (0.568 mm); ⁴ Coarse Limestone (1.943 mm); ⁵ Standard error of the mean; Dunnett's test at 5% probability.

Table 6. Bone characteristics of the tibias of layers fed diets containing two limestone particle sizes and two vitamin D supplements in a hot environment.

Variables				
Diet	Seedor Index (mg/mm)	Strength (kgf/cm ²)	Deformity (mm)	MM ¹ (%)
Control	42.03	11.89	3.58	39.14
FL ² + 2760 IU	38.00	11.20	3.22	39.64
FL + 1380 IU	38.68	11.23	3.43	37.80
FL + CL ³ + 2760 IU ⁴	41.31	10.96	3.68	37.27
FL + CL + 1380 IU	39.38	11.72	3.53	37.90
SEM ⁵	0.780	0.417	0.136	0.516
p-value				
Diet	0.4375	0.9488	0.8525	0.8667
Particle size (PS)	0.2813	0.8765	0.3656	0.9621
Supplementation D3 (D3)	0.1954	0.4021	0.9780	0.2432
PS x D3	0.7969	0.4205	0.5318	0.6697
Particle size				
Fine limestone	39.10	11.21	3.34	48.33
Fine + Coarse limestone	40.34	11.08	3.60	48.29
Supplementation D3				
2760 IU	40.53	10.76	3.47	48.83
1380 IU	39.03	11.47	3.48	47.79

¹ Mineral Matter; ² Fine Limestone; ³ Fine Limestone (0.568 mm); ⁴ Coarse Limestone (1.943 mm); ⁵ Standard error of the mean; Dunnett’s test at 5% probability.

The canonical discriminant analysis biplot for behavioral analysis showed no behavioral changes based on the feeding plans when considering all variables combined (Discriminant function 1: $p = 0.148$) (Figure 1).

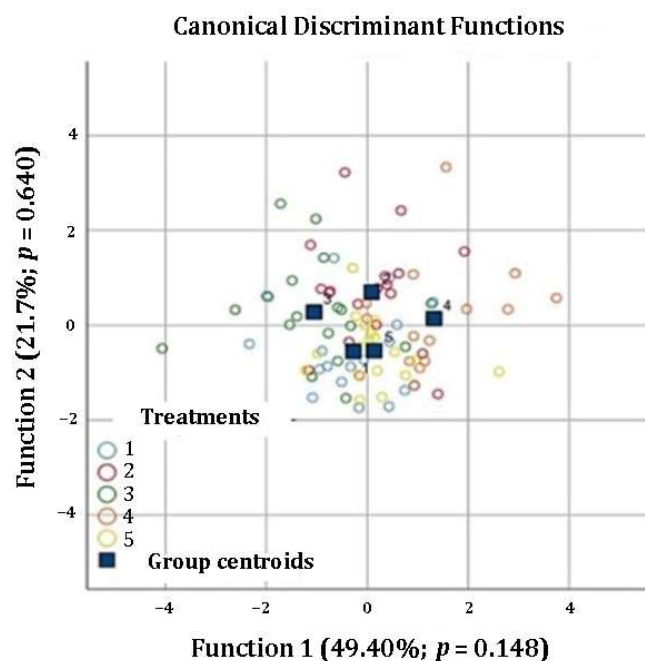


Figure 1. Biplot of the canonical discriminant analysis of the behavioral responses of brown laying birds fed diets supplemented with vitamin D3 reared in a hot environment. 1: Control diet; 2: Fine Limestone + 2760 IU; 3: Fine Limestone + 1380 IU; 4: Fine Limestone + Coarse Limestone + 2760 IU; 5: Fine Limestone + Coarse Limestone + 1380 IU.

Table 7. Thermoregulatory responses of brown layers fed diets supplemented with vitamin D3 and thermal gradient reared in a hot environment.

Thermoregulatory Responses					
Diet	RR ¹ (mov.min)	CT ² (°C)	TG ³ (°C)	FST ⁴ (°C)	FLST ⁵ (°C)
Control	106.50	41.22	6.45	34.47	35.17
FL + 2760 IU	103.44	41.24	6.54	34.43	35.08
FL + 1380 IU	108.36	41.20	6.57	34.51	34.93
FL ⁶ + CL ⁷ + 2760 IU	99.72	41.11	6.38	34.75	35.08
FL + CL + 1380 IU	112.33	41.17	6.60	34.30	34.97
SEM ⁸					
<i>p</i> -value					
Diet	0.4446	0.9487	0.9200	0.4874	0.8566
Particle size (PS)	0.9795	0.6705	0.7396	0.7619	0.8812
Supplementation D ³ (D ³)	0.0780	0.7061	0.4692	0.2930	0.4237
Day shift (DS)	0.0001	0.0001	0.0001	0.0001	0.0001
PS × D ³	0.4319	10,000	0.5834	0.1538	0.9143
PS × DS	0.1920	0.5959	0.8090	0.9029	0.9321
D ³ × DS	0.7305	0.4310	0.9366	0.4398	0.9566
PS × D ³ × DS	0.7263	0.0529	0.3375	0.9644	0.6107
Particle size					
Fine limestone	105.90	41.22	6.55	34.47	35.00
Fine + Coarse limestone	106.03	41.20	6.49	34.53	35.03
Supplementation D ₃					
2760 IU	101.58	41.24	6.46	34.59	35.08
1380 IU	110.35	41.19	6.59	34.41	34.95
Day shift					
Morning	38.39B	40.82B	8.25A	32.38B	32.85B
Afternoon	173.54A	41.61A	4.79B	36.61A	37.18A

¹ Respiratory Rate (mov. min-1); ² Cloacal Temperature (°C); ³ Thermal Gradient; ⁴ Feather surface temperature; ⁵ Featherless Surface Temperature; ⁶ Fine Limestone (0.568 mm); ⁷ Coarse Limestone (1.943 mm); and ⁸ Standard error of the mean AB Averages followed by different capital letters in the same column differ by Dunnet test at 5% probability.

4. Discussion

Air temperature, relative humidity, and wind speed are typically indirect measures that define characteristics of the microclimatic environment in the barn [25]. It is essential to correlate environmental factors with performance, physiological, and behavioral variables to achieve optimal balance to better understand these interactions.

Frequent genetic advances in laying hen strains have made them increasingly productive; however, as a consequence of high performance, these birds are more sensitive to heat. According to the Lohmann Brown Lite management guide [15], the thermoneutral temperature range is between 18 °C and 20 °C. Therefore, both times of day exceeded the birds' thermal comfort zone (Table 2). It is essential that the birds are raised in thermoneutral zones to express their full genetic potential and achieve maximum performance [26,27], which remains a major challenge in tropical regions characterized by high temperatures.

Although air temperature was above the thermal comfort zone, relative humidity (RH) remained within the ideal range recommended by the Lohmann Brown Lite manual [15], between 60 and 70% (Table 2). According to Ok et al. [28], lower RH is important to compensate for high air temperatures, aiding heat dissipation in birds. Wind speed is

a commonly used variable in poultry houses, as it reduces the birds' heat sensation by increasing heat removal from the facilities [29]. As shown in Table 2, the values observed were within the recommended standards to ensure thermal comfort, possibly contributing to maintaining homeothermy.

Radiant Heat Load (RHL) is an indicator of thermal environment and under steady-state conditions represents radiation received by the black globe from all surrounding surfaces. Another indicator, the Black Globe-Humidity Index (BGHI), is currently considered the most suitable index for indicating thermal comfort in hot regions [30].

The highest values for these thermal comfort indices were observed in the afternoon. BGHI values exceeded the danger threshold, with values from 76 to 81 indicating emergency situations [31]. The RHL averages remained within the acceptable limit (Table 2), which for commercial layers is 500 W/m^2 [32]. Opposite results were found by Sena et al. [33] and Angelim et al. [34], who recorded averages above the limit, possibly due to seasonal variations.

As observed in the zootechnical parameters (Table 3), feed intake, egg production, egg weight, egg mass, feed conversion per egg mass and per dozen eggs were not influenced by the diet, regardless of limestone particle size or vitamin D supplementation. Although the birds were exposed to heat stress, all performance variables were within the limits described in the management guide. Since the thermal environment directly affects animal performance, it can be inferred that the birds were well adapted to their rearing environment, maintaining homeothermic balance and productivity.

One strategy birds use to reduce endogenous heat production and maintain homeostasis is reducing feed intake. This behavior often negatively impacts performance and eggshell quality. According to the management guide, coarse-textured diets increase feed intake in layers, while finer textures reduce it [15]. Thus, by changing the feed texture, coarse limestone may positively stimulate feed intake even under heat stress.

However, it is important to emphasize that no differences were observed in this study, probably because brown layer hens adapt quickly to physical changes in diet [35]. Therefore, the different DGM of limestone used were not sufficient to affect the performance variables. Despite this, the birds showed good zootechnical performance.

In turn, none of the factors evaluated had an effect on egg production. This may be due to the fact that the laying hens were in the post-peak production phase, a phase characterized by lower nutritional requirements, and also to the isoenergetic and isonutritive diets, which provided an adequate supply of nutrients in all treatments, resulting in the observed results.

The diets did not affect egg weight, as observed. It is noteworthy that, both in terms of granulometry and vitamin D supplementation, the egg weights were within the marketing standard, falling into the 'large eggs' category (48 g to 57.99 g), according to SDA Ordinance No. 747, dated February 6, 2023 [36]. This demonstrates that the treatments are suitable for use in industrial production.

A nutritional strategy that has been adopted in industrial poultry farming is to supplement vitamin D in broiler diets due to its important role in calcium absorption and phosphorus in the intestines, favoring bone and muscle development [12]. Higher vitamin D levels in feed are needed to prevent bone abnormalities and ensure proper development, which are essential for good performance.

According to the strain manual [15], the Vitamin D3 requirement for the production phase is 2500 IU/kg of feed. However, in this study we used both supplements (2760 IU and 1380 IU) and 2500 IU per kg of feed (contained in the vitamin premix). As can be seen (Table 3), the two supplements did not influence the performance variables, probably

because the birds already had their digestive capacity formed and were therefore able to absorb the nutrients, as well as the absence of field challenges.

The results found in this work for the egg components (Table 4) showed that increasing the limestone particle did not significantly influence its constituents, constituting results which confirm those of Murata et al. [37].

Eggshell quality is influenced by dietary calcium levels, feeding time and the particle size of calcium sources [38]. Thus, it was expected that the coarse-grained limestone would favor the gradual release of calcium for shell formation by promoting a longer retention time in the gizzard, thereby improving shell quality parameters, which was not observed. However, even though the diets had no effect on egg quality parameters, these results are relevant considering that shell thickness and specific density are required characteristics when it comes to product shelf life and did not differ from the control treatment.

The higher the specific gravity of the eggs, the better the shell quality [39]. From this perspective, measurements equal to or greater than 1080 g/cm³ indicate good quality eggs [40], and the specific gravity was between 1098 and 1099 g/cm³. Thus, it can be inferred that the particle sizes and vitamin D3 supplementation used favored the use of minerals in the diet, resulting in the egg characteristics found.

Since diet influences the morphology and physiology of the gastrointestinal system of birds [41], biometric analysis of the digestive organs becomes an important tool for identifying changes caused by nutritional management and, consequently, for validating new products. Thus, changes in feed particle size may be a factor inducing the remodeling of these organs.

Brown layers fed coarse-textured diets show changes in the digestive organs, with an increase in the weight of the gizzard [42] as a result of the increased activity of this organ. In this study, changes in the physical form of the feed were not enough to influence the biometry of the organs evaluated (Table 5). Likewise, Pacheco et al. [43] also observed no difference in organ weight with the granulometries studied in analyzing the same biometric variables using fine 0.222 mm and coarse 1.922 mm limestone at 32 weeks of age.

In turn, the results found for the two limestone granulometries offered probably indicate that the calcium requirements were adequately met without impairing the functioning of the digestive organs, making it possible to use these calcium sources. There was no effect of the diet on the layers' bone characteristics (Table 6). Calcium is an essential mineral for maintaining bone integrity [44]; however, bone strength is negatively affected in situations where the dietary calcium supply is deficient [45].

Furthermore, the limestone granulometry did not influence the bone parameters of the birds, indicating the animals' ability to efficiently assimilate the calcium necessary for bone integrity, regardless of the limestone size used.

The results regarding vitamin D supplementation in layer diets were contrary to those found in broiler production, where increasing vitamin D3 in diets improved ash content and bone strength [46]. Thus, it can be inferred that the amount of vitamin D present in the vitamin and mineral premix was effective in ensuring bone quality, making it unnecessary to use extra vitamin D at the production stage the layers were in.

The increase in respiratory rate is directly correlated with the increase in ambient temperature. As can be seen in Table 7, the highest value for respiratory rate (106.07 mov/min) was observed during the afternoon shift, the period with the highest ambient temperatures (Table 2). This was probably due to the birds' inefficiency in losing heat to the environment through perspiration because of the absence of sweat glands. Therefore, one of the most efficient ways of stabilizing temperature is through evapotranspiration, where heat is lost through the evaporation of water in the respiratory tract. This process occurs through panting, and these movements are observed and counted to obtain the respiratory

rate [47]. It should be noted that the values observed for respiratory rate in this experiment are within the range determined by Garcia et al. [30], and they can vary from 23 mov/min to 273 mov/min.

Similarly, cloacal temperature was also higher (41.19 °C) during the afternoon shift (Table 7). The main mechanism for regulating the birds' body temperature in thermoneutral zones is through sensible heat loss mechanisms. As the air temperature rises, other pathways are activated (latent heat loss) in an attempt to maintain homeothermy. However, when the extremes of environmental temperatures go beyond the birds' thermal comfort range, they may find it difficult to sustain the homeostatic balance, causing an increase in cloacal temperature [48].

The thermal gradient (Table 7) showed the highest values in the morning (8.25 °C). This variable shows a range of variation between two temperatures. Thus, the greater the difference between the temperatures observed, the greater the thermal gradient. As the air temperature was milder in the morning (31.44 °C), The thermal gradient between the cloaca (body core temperature) and the surface of the birds was shown to be higher. On the other hand, the high ambient temperatures in the afternoon make it difficult for the birds to dissipate heat, which depends on temperature variation to occur. As a result, both temperatures are close to each other, resulting in a smaller thermal gradient [49].

It is known that the feather cover of animals plays an important role in the homeostatic balance, acting as a thermal insulator (for example). However, in situations of heat stress, feathers hinder heat loss to the environment through sensitive mechanisms such as conduction, radiation and convection. Therefore, regions without feathers are essential because they favor heat exchange processes with the environment [27]. According to Castillo et al. [48], skin surface temperatures are directly influenced by climatic conditions. This was clearly observed in Table 7, where both surface temperatures showed higher values as the ambient temperature increased.

Analyzing the behavioral variables, the biplot resulting from the canonical discriminant analysis (Graph 1) indicates that the central points (centroids) of the behavioral responses are close. Their location is important because it shows the effect of the treatments on the animals' behavior. In turn, it can be said that regardless of vitamin D3 supplementation or the grain sizes of limestone used, the diets did not influence the birds' behavioral characteristics. This pattern is reinforced by the discriminant functions which were not significant.

As also observed by Cordeiro et al. [50], the birds spend most of their time feeding. This high frequency is a result of the confinement and limited space in which they live. As can be seen in Graph 2, the birds showed an increase in feeding frequency in the afternoon, a period which triggered changes in the birds' thermoregulatory responses (Table 7).

It is known that when birds are outside the thermoneutrality zone, there is an increase in metabolic processes to dissipate heat and, at the same time, birds seek to reduce thermogenesis [49], with a reduction in feed consumption being one of the ways to mitigate metabolic heat production. However, this was not observed, since the birds increased their search for food in the afternoon. This is probably related to the time the birds are handled, as feeding takes place in the afternoon, encouraging feed consumption despite the heat stress.

In addition, water consumption behavior was also more frequent in the afternoon (Figure 2). This increase may be related to the higher radiant heat load during this shift (Table 2), which leads birds to drink more water to maintain homeothermy [51]. Higher water intake during the hottest hours of the day helps to cool the body and minimizes dehydration resulting from heat loss through panting [52].

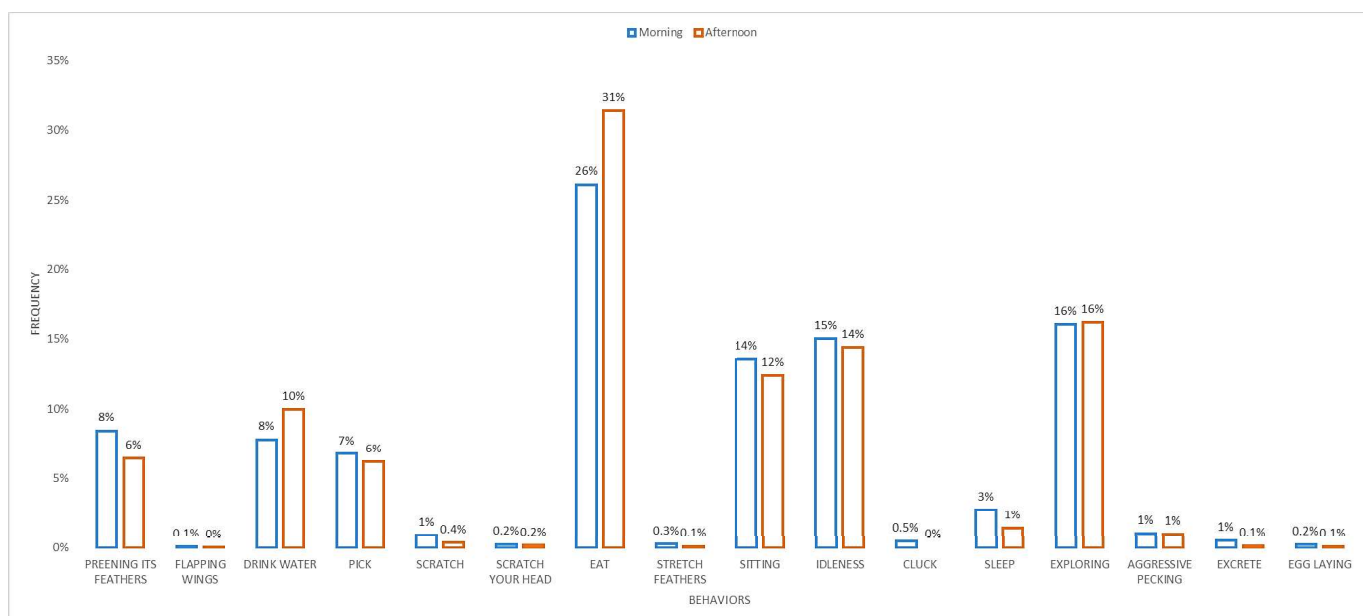


Figure 2. Behavior of brown layers fed diets supplemented with vitamin D₃, according to day shifts in a hot environment.

Both “sitting” and “idleness” behaviors showed similar frequencies in the morning and afternoon shifts. As can be seen in Table 2, both shifts showed no significant differences in terms of air temperature and relative humidity. These variables are essential for determining heat dissipation in animals, so it is understood that the environment is not conducive to thermoregulation when both are high [28]. As a result, sitting behavior may be an attempt by the birds to lose heat to the cage through conduction, when direct contact between surfaces with different temperatures promotes thermal exchange [53]. In contrast, “idle” behavior at certain times of the day probably indicates that these birds were trying to save energy and reduce heat production.

5. Conclusions

Neither limestone particle size nor 25-hydroxycholecalciferol supplementation influenced performance, bone characteristics, or thermoregulatory and behavioral responses of brown layers reared under natural heat stress conditions. However, the high radiant heat load observed during the afternoon altered the birds’ thermoregulatory profile, compromising their welfare. These findings suggest that, under high-temperature environments, nutritional effects may be masked by the intensity of heat stress, highlighting the need for further studies under milder environmental conditions or with heat-mitigation strategies to better understand the interactions among diet, mineral metabolism, and bird welfare.

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