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Original Article

Different Calcium Levels and Two Limestone Granulometries in the Diet of Laying Hens: Performance and Bone Characteristics

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Shell, calcium sources, macromineral, posture.

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ABSTRACT

The effect of different calcium levels and two limestone granulometries on performance, egg quality, biometry of digestive organs and bone characteristics of light laying hens were evaluated. A total of 270 laying hens were used during 112 days, distributed in a completely randomized design in a 3 x 2 factorial scheme, with 3 levels of calcium (3.8, 4.0 and 4.2%) and 2 limestone granulometries (0.222 and 1.922 mm), totaling 6 treatments with 5 repetitions each. The parameters evaluated were: feed intake, egg production, egg weight and mass, feed conversion per mass and per dozen eggs, albumen, yolk and shell percentage, specific gravity of eggs and shell thickness. In addition, the relative weight of the heart, proventricle, gizzard, liver, pancreas, intestine weight and length, and bone characteristics were also evaluated. A significant interaction was observed for tibia mineral matter. Egg production was influenced by the limestone granulometry, presenting greater value in the treatments which contained fine granulometry limestone. Higher calcium levels of 4.0 and 4.2% provide greater specific density of eggs. Biometric variables and bone characteristics were not influenced by the treatments. In conclusion, it is recommended to use fine-grained limestone (0.222 mm) and a 4% calcium level in diets for light commercial hens, as they improve performance and quality of eggs, without interfering in biometrics of digestive organs and bone characteristics.

INTRODUCTION

The mineral nutrition assumes relevant importance in the development and production of birds, being that calcium and phosphorus can be considered the main minerals involved in bone development and egg quality (Cordeiro *et al.*, 2017).As a consequence of constant advances in nutrition, genetics, ambience and management, and due to the greater sensitivity of modern hens to variations in nutritional levels in diets (Costa *et al.*, 2008), it is necessary to adjust the calcium requirements for laying hens.

The elevated egg production has caused a concern related to the quality of the eggs produced from the perspective of preserving its nutritional value and commercialization. The eggshell has the biological function to form a chamber for embryonic development, however from a commercial point of view it is packaging which protects the noble content of the egg, and must be resistant enough to prevent possible microbial contamination and cracks during transportation from the farm to the consumer (Gherardi & Vieira, 2018).

Calcium is the main component in the formation of egg shells. Its source and the dietary levels used can especially affect the eggshell quality. Calcium sources have great variation in particle size and solubility; therefore, research in this area is necessary in order to improve egg quality and the bones of the birds.

The shell formation process usually occurs at night, being a period when birds do not have a source of labile calcium in their digestive tract (Bueno *et al.*, 2016) due to being partially withdrawn from the bones. Thus, it is argued that limestone of greater granulometry prolongs the residence time of these particles in the gizzard, making calcium ions available in sufficient quantities for absorption (Wang *et al.*, 2014), and in turn allowing its slow release making it available for forming the shell at the time when the birds do not feed. Thus, Araújo *et al.* (2011) state that different granulometries of calcium sources can influence the availability of this mineral.

In view of the above, several calcium sources have been investigated in order to maintain more constant blood calcium levels at the time of shell formation, asthis situation is essential for obtaining good productivity in the squads of laying hens. In this context, the objective of this study was to evaluate the effect of different calcium levels and two limestone granulometries on performance, egg quality, biometry of digestive organs and bone characteristics of light laying hens.

MATERIAL AND METHODS

This project was approved by the Ethics Committee on the Use of Animals (CEUA) under protocol No. 002.04.017. UVA.504.03.

The experiment was carried out in the posture sector of the Experimental Farm at the State University of Acaraú Valley - UVA, in Sobral-CE. A total of 270 laying hens of the Hy-line White lineage were used in the study at 32 weeks old, weighing 1.465 ± 0.061 kg, and with an average production of 85.5% eggs during 112 days. The birds were weighed and selected before beginning the experiment to obtain experimental plots with medium weight and uniform egg production, according to the recommendations proposed by Sakomura & Rostagno (2016).

The birds were housed in a conventional shed for laying, provided with galvanized wire cages of 90 cm in length, with 3 subdivisions of 30 x 45 x 45 cm, being arranged in a pyramid system equipped with front gutters and a nippledrinker at the top of the cages. A 16-hour light program was adopted (12 hours of natural light and 4 hours of artificial light).

A completely randomized experimental design was adopted in a 3 x 2 factorial scheme with three calcium levels (3.8, 4.0 and 4.2%) and two limestone granulometries (fine with DGM 0.222 mm, and coarse with DGM 1.922 mm), totaling 6 treatments and 5 repetitions of 9 birds each. The experimental diets were isonutrient and isoenergetic (Table 1), formulated according to the nutritional recommendations suggested by the lineage manual (Hy-Line, 2016). The nutritional composition of the ingredients used in the diets followed the recommendations of Rostagno *et al.* (2017).

Daily egg production and at the end of each 28-day period were recorded. Together with feed consumption, these data were used to evaluate the following performance parameters: feed consumption (g/bird/day), eggs (%), egg weight (g), egg mass (g/bird/day), conversion by egg mass (kg/kg), and conversion by dozen eggs (kg/dz).

Egg quality was also analyzed at the end of each 28-day period by: albumen, yolk and shell percentage, shell thickness (mm) and specific gravity of eggs (g/cm^3). Four eggs were selected per repetition, with two eggs for determining specific gravity using the salt fluctuation method, and the other two for the other quality analyzes (Bezerra *et al.*, 2015).

The eggs were broken manually and their components weighed separately. The egg shells were dried for 24 hours at room temperature, weighed and three measurements were taken in the equatorial region of the egg and extremities using a digital caliper in order to calculate the average value of the shell thickness.

At the end of the field experiment, 24 birds were euthanized consisting of 4 birds from each treatment using the universal cervical dislocation method (according to Normative Resolution no. 37/2018 - CONCEA) and then the digestive organs (proventricle, gizzard, liver, pancreas and intestines) and heart were removed to perform the biometrics analysis. These organs were weighed on a 0.01g precision (Maximum capacity: 500 g; MH-267-5; China) scale and all weight data were expressed as a percentage of body weight. The length of the birds' intestines was also measured.

The tibiae were removed, boned and used to analyze the following bone characteristics: weight (g), length (mm), resistance to breaking (kgf/cm^2), bone deformity (mm), Seedor Index (mg/mm) and mineral matter (g/kg). The bone length was measured using a digital caliper and the weight was obtained on an electronic scale with an accuracy of 0.01g. The Seedor

Table 1 – Percentage and nutritional composition calculated from the experimental diet.

Ingredients	Fine Limestone			CoarseLimestone		
	3.8%	4.0%	4.2%	3.8%	4.0%	4.2%
Grain corn	54.44	54.44	54.44	54.44	54.44	54.44
Soybean meal 45%	24.33	24.33	24.33	24.33	24.33	24.33
Fine Limestone (0.222 mm)	7.80	8.33	8.86	-	-	-
Coarse Limestone (1.922 mm)	-	-	-	7.80	8.33	8.86
Meat and bone meal	6.38	6.38	6.38	6.38	6.38	6.38
Vitamin-mineral supplement*	0.400	0.400	0.400	0.400	0.400	0.400
Common salt	0.289	0.289	0.289	0.289	0.289	0.289
Soy oil	4.50	4.50	4.5	4.5	4.5	4.5
DL methionine	0.150	0.150	0.150	0.150	0.150	0.150
Inert	1.71	1.18	0.65	1.71	1.18	0.65
Metab Energy (Cal/kg)	2950	2950	2950	2950	2950	2950
Crude protein (%)	18.50	18.50	18.50	18.50	18.50	18.50
Calcium (%)	3.8	4.0	4.2	3.8	4.0	4.2
Available phosphorus (%)	0.45	0.45	0.45	0.45	0.45	0.45
Sodium (%)	0.18	0.18	0.18	0.18	0.18	0.18
Digestable Met + Cis (%)	0.694	0.694	0.694	0.694	0.694	0.694
Digestible methionine (%)	0.443	0.443	0.443	0.443	0.443	0.443
Digestible lysine (%)	0.849	0.849	0.849	0.849	0.849	0.849
Digestible threonine (%)	0.598	0.598	0.598	0.598	0.598	0.598
Digestible tryptophan (%)	0.179	0.179	0.179	0.179	0.179	0.179

* PX POSTURA 0.4% 500 TEC - Product warranty levels (composition per kg of product): Iron:10.00 g, Copper: 2.500.00 mg, Zinc: 20.00 g, Manganese: 20.00 g, Iodine: 208.00 mg, Selenium: 75.15 mg, Retinol (Vit A): 600.00 mg, Cholecalciferol (Vit. D):15.63 mg, Tocopherol (Vit. E): 3370.79 mg, Menadione sodium: 395.92 mg, Folic acid: 74.25 mg, Choline: 100.00 g, Niacin: 5.025.74 mg, Pantothenic acid: 1.805.16 mg, Thiamine (Vit. B1): 250.09 mg, Riboflavin (Vit. B2): 1.000.00 mg, Pyridoxine (Vit. B6): 250.1 mcg, Cyanocobalamin (Vit. B12): 2.400.00 mcg, Methionine: 125.00 g, Colistin: 1.750.00 mg.

index was obtained by dividing the bone ash value by the length of the evaluated bone to evaluate the bone density (Seedor *et al.*, 1991). Bone resistance and deformity analyzes were performed using a mechanical press, a Testo/Ronald Triaxial mechanical press (Ronald Top Ltda., Rio de Janeiro, RJ, Brazil) with a 150-kg capacity, in which the left tibiae were placed in a horizontal position and a compression force was applied in the center of each position. The maximum amount of force applied to the bone until its rupture was considered the resistance to breaking as measured through a digital extensometer. The deformity was observed through an analog extensometer until the moment of bone rupture.

The right tibiae were weighed and placed in a forced ventilation greenhouse at 105ºC for 72h to determine the mineral matter. Next, they were weighed and crushed with a mortar and pistil. The crushed samples were identified for determining the mineral matter according to the methodology described by Silva &Queiroz (2002).

The data were submitted to ANOVA analysis of variance and the means compared by the Tukey test at 5% probability considering the factorial model, in which the effects of calcium levels and limestone granulometry were included, and the interaction between these factors.

RESULTS AND DISCUSSION

There was no interaction between the factors for any of the performance variables (p>0.05). Similarly, there was no effect of treatments on performance variables with the exception of egg production which was influenced by the limestone granulometry (p<0.05) and presented greater values in treatments with fine granulometry limestone without a difference between dietary calcium levels (Table 2).

The calcium levels used in the experimental diets did not influence the performance variables (Table 2), being adequate to maximize the productive parameters. Therefore, there is no additional effect with the use of 4.0 and 4.2% levels in the diets. According to Lin *et al.* (2018), birds generally have the ability to regulate Ca consumption in order to meet their requirement. However, the dietary calcium levels in this study did not influence feed intake, resulting in an average intake of 3.47, 3.65 and 3.87 g of Ca/bird/day. Thus, the lowest value was sufficient to support the production and weight of eggs, as well as the other parameters derived from them.

Table 2 – Mean feed intake, production, egg weight, egg mass, egg mass conversion and egg conversion dozens of commercial laying hens fed diets containing different levels of dietary calcium and two limestone granulometries.

Factors	Intake (g/bird/day)	Production (%)	Egg weight (g)	Egg mass (g/bird/day)	CM[1] (kg/kg)	CDZ[2] (kg/dz)
Calcium levels						
3.8 %	91.30	84.29	61.21	51.57	1.749	1.298
4.0 %	91.23	84.26	61.33	51.65	1.742	1.297
4.2 %	92.23	83.12	61.40	51.01	1.792	1.331
Granulometry						
Fine (0.222 mm)	92.32	85.11A	61.33	52.17	1.747	1.299
Coarse (1.922 mm)	90.85	82.67B	61.29	50.65	1.774	1.317
CV[3] (%)	3.52	3.33	2.43	4.06	4.07	3.68
Mean	91.58	83.88	61.31	51.41	1.761	1.308
ANOVA[4]			*p*-value			
Calcium levels (C)	0.7452	0.5791	0.9597	0.7568	0.2589	0.2225
Granulometry (G)	0.2226	0.0254	0.9394	0.0564	0.3132	0.3132
C x G	0.5906	0.4996	0.7292	0.8785	0.7699	0.5516

[1]Conversion by egg mass; 2Conversion by dozen eggs; 3Coefficient of Variation; 4analysis of variance. Means followed by different capital letters in the same column differ by the Tukey's test at 5% probability.

Similar results were reported by Vellasco *et al.* (2016) in working with three calcium levels (3.9, 4.2 and 4.5%) and three calcium:phosphorus ratios (3.22, 3.46 and 3.71 g/bird/day) in light laying hens diets at 24 weeks of age, also finding no effects of calcium levels on performance parameters.

An increase in feed consumption due to the change in the granulometry of the diets caused by the addition of coarse limestone was expected, but did not occur. After an initial moment of adaptation by the birds, the size of the limestone particles was probably not sufficient to cause increased consumption.

The coarse grinding of the limestone makes it less soluble, while the fine granulometry limestone promptly releases calcium for absorption (Xavier *et al.*, 2015). In this study, a reduction in egg production was observed in treatments with coarse granulometry limestone, so probably the latter may have caused a reduction in calcium absorption, and therefore the lowest egg production observed, while fine granulometry limestone was able to maintain adequate levels of calcium, being an essential nutrient for egg production (An *et al.*, 2016) and ensuring the good laying rate found.

In working with increasing levels of coarse granulometry limestone (0, 25, 50, 75 and 100%) in laying diets at 56 weeks of age, Garcia *et al.* (2012) reported that coarse limestone had a negative influence on feed consumption, production and egg mass, in addition to worsening feed conversion.

Different results were reported by Xavier *et al.* (2015) in working with five proportions of coarse limestone in relation to fine limestone (0, 25, 50, 75 and 100%) in laying diets (18 weeks of age), concluding that performance studies were not influenced by the limestone granulometries.

There was no significant interaction between the factors for any of the egg quality variables evaluated ($p>0.05$). The albumen, yolk, and shell percentages and shell thickness were not influenced by the treatments ($p>0.05$). However, the specific gravity of the eggs was influenced by the calcium levels ($p<0.05$), presenting a higher value in the treatments with 4.0 and 4.2% of calcium and a lower value in the treatments with 3.8% (Table 3).

According to Zhang *et al.* (2017), the shell quality is influenced by calcium levels, feeding time and the particle size of the calcium sources. However, only calcium levels influenced the shell quality in this study.

Coarse-grained limestone was expected to improve the shell quality parameters, however this was not observed. Such results are in conflict with the concept that the increase in the limestone particle size promotes a longer retention time in the gizzard, so that gradual absorption of calcium occurs during the night time when the birds are not feeding in order to assist in the shell formation process (Sousa *et al.*, 2017). From the results, it can be inferred that the granulometry used did not impact the calcium absorption or consequently the eggshell formation (Pizzolante *et al.*, 2011), which probably occurred due to the fact that once the calcium requirement was met, the size of the limestone particle is not essential for its supply, and does not affect the quality of the shell (Cordeiro *et al.*, 2017).

Different results were found by Swiatkiewicz *et al.* (2015) in working with three levels (0, 25 and 50%) of substituting fine limestone (0.2-0.6 mm) for coarse limestone (1.0-1.4 mm) in Isa Brown laying hens in

Table 3 – Albumen, yolk and shell percentage, shell thickness and specific gravity of laying hens' eggs fed diets containing different levels of dietary calcium and two limestone granulometries.

Factors	Albumen (%)	Yolk (%)	Shell (%)	ST[1](mm)	SG[2](g/cm3)
Calcium levels					
3.8 %	61.38	27.15	9.17	0.360	1.090B
4.0 %	61.61	27.00	9.23	0.367	1.092A
4.2 %	61.94	26.79	9.30	0.368	1.092A
Granulometry					
Fine (0.222 mm)	61.73	26.96	9.26	0.367	1.092
Coarse (1.922 mm)	61.55	27.00	9.20	0.363	1.091
CV[3] (%)	1.54	2.88	2.23	2.68	0.15
Mean	61.64	26.98	9.23	0.365	1.091
ANOVA[4]					
Calcium levels (C)	0.4202	0.5896	0.4027	0.1889	0.0098
Granulometry (G)	0.5973	0.8705	0.4165	0.2737	0.5539
C x G	0.7304	0.5565	0.1111	0.6761	0.2241

[1]Shell Thickness; [2]Specific Gravity; [3]Coefficient of Variation; [4]Analysis of variance. Means followed by different capital letters in the same column differ by the Tukey's test at 5% probability.

the period of 25 to 70 weeks of age. The authors concluded that the replacement of fine limestone by coarse limestone by 25 and 50% improved the eggshell quality.

The specific gravity of the eggs is one of the parameters used to assess the quality of the shells. According to Henriques *et al.* (2018), greater specific gravity results in better shell quality. Thus, treatments containing 4.0 and 4.2% (1.092 g/cm3) of calcium provided better quality shells, although the 3.8% Ca level has also resulted in good quality shells, based on the fact that specific gravity values equal to or greater than 1.080 g/cm3 indicate good quality eggs (Zhang *et al.*, 2017).

These results corroborate those of Maciel *et al.* (2015) in working with five calcium levels (2.5, 3.0, 3.5, 4.0 and 4.5%) in light laying hens at 56 weeks of age, reporting an improvement in the specific gravity of the eggs with the increased calcium levels. On the other hand, Swiatkiewicz *et al.* (2015) reported that calcium levels (3.2, 3.7 and 4.2%) did not influence the quality parameters of Isa Brown commercial laying eggs (25 to 70 weeks of age).

There was no interaction between the factors for any of the biometric variables ($p>0.05$). Similarly, none of the biometric variables were influenced by the treatments studied (Table 4).

The type of diet has a direct effect on the development, morphometry and physiology of the birds' gastrointestinal system (Zaefarian *et al.*, 2016). Thus, the biometric analysis of digestive organs enables identifying changes arising from the nutritional management to which the birds were subjected.

Changes in digestive organs, mainly the proventricle, gizzards and intestines were expected when using different granulometries since they directly participate

Table 4 – Relative weight of digestive organs and intestine length of laying hens fed diets containing different dietary calcium levels and two limestone granulometries.

Factors	Heart (%)	Provent.[1] (%)	Gizzard (%)	Liver (%)	Pancreas (%)	Intestines (%)	Int. Length[2] (cm)
Calcium levels							
3.8 %	0.323	0.343	0.934	1.810	0.155	1.725	143.37
4.0 %	0.317	0.337	0.901	1.964	0.152	1.670	142.37
4.2 %	0.316	0.306	0.916	1.943	0.151	1.613	138.75
Granulometry							
Fine (0.222 mm)	0.327	0.325	0.944	1.859	0.153	1.669	141.25
Coarse (1.922 mm)	0.310	0.332	0.890	1.952	0.152	1.670	141.75
CV[3] (%)	14.04	13.57	7.92	10.99	11.71	13.26	4.81
Mean	0.319	0.328	0.917	1.906	0.152	1.669	141.50
ANOVA[4]				*p*-value			
Calcium levels (C)	0.9564	0.2300	0.6680	0.3076	0.9023	0.6050	0.3801
Granulometry (G)	0.3727	0.6997	0.0848	0.2896	0.9400	0.9906	0.8593
C x G	0.9480	0.3292	0.1643	0.7639	0.6404	0.4307	0.2165

[1]Proventricle; [2] Intestines length; [3]Coefficient of Variation; [4]Analysis of variance.

in the digestion process and adapt quickly to the bromatological and structural conditions of the diet (Svihus, 2014). The gizzard stands out for having a quick response to diet changes, with it being reported that the increase in the size of the feed particles results in a greater weight of the gizzard (Abdollahi *et al.*, 2019), but which was not observed in this study. The coarse granulometry non-influence on the biometry of digestive organs is possibly due to differences in the hardness of calcium sources and sizes of limestone particles found in different regions (Pizzolante *et al.*, 2011). Thus, it can be inferred that the calcium levels and the limestone granulometry adequately provided the necessary nutrients for the good biometric quality of the organs, not affecting the biometrics of the digestive tract of the birds.

Different results were found by Araújo *et al.* (2011) when evaluating three calcium levels (3.92, 4.02 and 4.12%) and two limestone granulometries (0.60 and 1.00 mm) in laying hens, in which the authors observed that the use of coarse limestone (1.00 mm) promoted greater gizzard weight, and this result was associated with greater retention of coarse limestone by the organ.

A significant interaction between the factors for the mineral matter variable was observed ($p<0.05$). However, there was no effect of treatments on bone quality variables ($p>0.05$) (Table 5).

Table 5 – Weight, length, Seedor Index, resistance, deformity and mineral matter of the tibiae of commercial laying hens fed diets containing different dietary calcium levels and two limestone granulometries.

Factors	Weight (g)	Length (mm)	SI[1] (mg/mm)	BR[2] (kgf/cm2)	BD[3] (mm)	MM[4] (%)
Calcium levels						
3.8 %	7.05	115.04	30.27	8.55	3.09	49.43
4.0 %	7.21	114.92	30.87	8.69	2.98	49.16
4.2 %	7.05	115.23	29.86	8.35	3.13	47.77
Granulometry						
Fine (0.222 mm)	7.08	115.36	29.67	8.66	3.15	48.44
Coarse (1.922 mm)	7.12	114.77	30.99	8.39	2.98	49.14
CV[5] (%)	9.47	2.96	8.32	19.40	15.97	3.81
Mean	7.10	115.06	30.33	8.52	3.07	48.79
ANOVA[6]			*p*-value			
Calcium levels (C)	0.8571	0.9836	0.7262	0.9214	0.8273	0.1899
Granulometry (G)	0.8811	0.6740	0.2133	0.7080	0.4208	0.3655
C x G	0.0619	0.3169	0.1407	0.9640	0.5687	0.0252

[1]Seedor Index; [2]Bone Resistance; [3]Bone Deformity; [4]Mineral Matter; [5]Coefficient of Variation; [6]Analysis of variance.

When analyzing the interaction of factors for the mineral material of the tibiae (Table 6), it was found that the level of 3.8% of dietary calcium in the treatments with the fine-grained limestone provided better result of mineral matter in the tibiae, with no significant difference between the dietary calcium levels for coarse granulometry. It was also observed that the 4% level of dietary calcium was better when associated with coarse granulometry limestone.

Table 6 – Analysis of the interaction (calcium vs. grain size) for the mineral material of the tibiae of commercial laying hens fed diets containing different dietary calcium levels and two limestone grain sizes.

Granulometry	Calcium			Mean
	3.8 %	4.0 %	4.2 %	
Fine (0.222 mm)	50.54Aa	47.47Bb	47.30Ab	48.44
Coarse (1.922 mm)	48.32Aa	50.86Aa	48.25Aa	49.14
Mean	49.43	49.17	47.78	

Different letters, uppercase in the column and lowercase in the row, indicate differences between the mean by the Tukey test at 5%.

The increase in level of calcium associated with fine granulometry resulted in a reduction in tibial MM. This may possibly be related to the fact that the smaller particles on limestone becomes more soluble in the digestible tracts of the hens, releasing the calcium readily to be absorbed (Bueno *et al.*, 2016), together with the increase in level of calcium which cause excess of calcium in the intestinal lumen. This negatively affects the mineral absorption process, both by excessive levels of calcium and by antagonism with other minerals such as magnesium, which is present in the calcium sources (Pelícia *et al.*, 2011). Thus, since the hen mobilizes a large amount of calcium from the bone matrix to form egg shells (Zhang *et al.*, 2019) and the replacement of this mineral in done via diet, the insufficient supply of dietary calcium has negative effects on bone mineralization, resulting in weaker bones (Bain *et al.*, 2016).

In working with laying hens (23 weeks old) fed diets containing three levels of calcium (3.5, 3.75 and 4.5%)

and two limestone granulometries (0.18 and 3.90 mm), Pelícia *et al.* (2011) observed that the increase in calcium levels reduced tibial ash and that this result was associated with excessive Ca availability, which resulted in less intestinal Ca absorption and tibial Ca mobilization.

Coarse granulometry was expected to improve bone quality parameters as a result of the longer retention of limestone particles in the gizzard and gradual calcium release into the small intestine (Bueno *et al.*, 2016), in turn promoting more efficient absorption of calcium and consequently reduce the removal of bone calcium (Wistedt *et al.*, 2019), but this was not observed in this study.

In conclusion, it is recommended to use fine-grained limestone (0.222 mm) and a 4% calcium level in diets for light commercial hens, as they improve performance and quality of eggs, without interfering in biometrics of digestive organs and bone characteristics.

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