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Effects of dietary non-phytate phosphorus and fine-to-coarse limestone ratio on performance, egg quality, and bone traits in laying hens of different ages

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Doctor Scientiae

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Thesis submitted to the Animal Science Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

Adviser: Arele Arlindo Calderano

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Assent:

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ABSTRACT

PAGNUSSATT, Heloisa, D.Sc., Universidade Federal de Viçosa, November, 2025. **Effects of dietary non-phytate phosphorus and fine-to-coarse limestone ratio on performance, egg quality, and bone traits in laying hens of different ages**. Adviser: Arele Arlindo Calderano.

Two studies were conducted at the Federal University of Viçosa to evaluate the effects of different levels of non-phytate phosphorus (NPP) and fine-to-coarse limestone ratios (FL:CL) on the productive performance, internal and external egg quality, and bone and blood traits of Lohmann Brown laying hens at different production phases. In the first set of experiments, 120 hens at 26 weeks and 96 hens at 66 weeks of age were assigned to a completely randomized design with two NPP levels (high: 3.8 and 3.6 g/kg; and reduced: 2.2 g/kg). Reducing NPP did not affect performance or egg quality but increased tibia calcium and phosphorus contents (26–41 weeks) and enhanced eggshell thickness, breaking strength, and Seedor index (66–81 weeks). In the second set of experiments, 180 hens at 26 weeks and 96 hens at 66 weeks were fed diets containing different FL:CL ratios (60:40, 50:50, and 30:70 in the early phase; 50:50 and 30:70 in the late phase). The FL:CL ratios did not influence performance, egg quality, or blood parameters in either phase but affected bone and physiological traits in young hens, reducing relative tibia weight and tibia breaking strength (30:70) and decreasing renal 1-hydroxylase concentration (50:50). In older hens, the 30:70 ratio increased feed intake and reduced tibia calcium content. In conclusion, dietary NPP can be safely reduced to 2.2 g/kg without impairing laying performance or egg quality, improving bone mineralization and shell strength, while FL:CL ratios primarily affect physiological responses in young hens without compromising productive performance.

Keywords: age effects; renal 1-hydroxylase; seedor index; semi-heavy layers; shell strength

RESUMO

PAGNUSSATT, Heloisa, D.Sc., Universidade Federal de Viçosa, novembro de 2025. **Efeitos do fósforo disponível na dieta e da relação entre calcário fino e grosso sobre o desempenho, a qualidade dos ovos e as características ósseas em galinhas poedeiras de diferentes idades**. Orientador: Arele Arlindo Calderano.

Dois estudos foram conduzidos na Universidade Federal de Viçosa com o objetivo de avaliar os efeitos de diferentes níveis de fósforo não fítico (NPP) e de diferentes proporções entre calcário fino e grosso (CF:CG) sobre o desempenho produtivo, qualidade interna e externa dos ovos, características ósseas e sanguíneas de poedeiras Lohmann Brown em distintas fases de produção. No primeiro conjunto experimental, 120 aves com 26 semanas de idade e 96 aves com 66 semanas foram distribuídas em delineamento inteiramente casualizado com dois níveis de NPP (alto: 3,8 e 3,6 g/kg; e reduzido: 2,2 g/kg). Em ambos os ensaios, a redução de NPP não afetou o desempenho nem a qualidade dos ovos, mas melhorou o teor de cálcio e fósforo na tíbia (26 a 41 semanas) e aumentou a espessura e a resistência da casca e o índice de Seedor (66 a 81 semanas). No segundo conjunto experimental, 180 poedeiras com 26 semanas e 96 aves com 66 semanas receberam dietas contendo diferentes proporções CF:CG (60:40, 50:50 e 30:70 na fase inicial; 50:50 e 30:70 na fase final). As proporções de CF:CG não influenciaram o desempenho, a qualidade dos ovos e os parâmetros sanguíneos em nenhuma das fases, mas afetaram características ósseas e fisiológicas em aves jovens, reduzindo o peso relativo e a resistência da tíbia (30:70) e a concentração renal de 1-hidroxilase (50:50). Em aves mais velhas, a relação 30:70 aumentou o consumo de ração e reduziu o teor de cálcio tibial. Conclui-se que a redução do NPP para 2,2 g/kg não compromete o desempenho ou a qualidade dos ovos, podendo melhorar a mineralização óssea e a qualidade da casca, enquanto as proporções de CF:CG influenciam respostas fisiológicas principalmente em poedeiras jovens, sem afetar o desempenho produtiv

Palavras-chave: efeitos da idade; índice de seedor; poedeiras semipesadas; resistência da casca; renal 1-hidroxilase

Summary

General Introduction

Economic efficiency and environmental sustainability have become priorities in poultry and livestock farming. Phosphorus (P) stands out as one of the most expensive nutrients in poultry diets, being fundamental for physiological processes and eggshell formation (JING et al., 2021).

The production of one egg every 24 hours requires high metabolic coordination, supported by the interaction between the mechanisms that regulate vitamin D_3 metabolism, calcium (Ca) and P homeostasis, and intestinal absorption of these minerals. This integration is essential for supplying the components necessary for adequate shell calcification and maintenance of bone mineralization (SINCLAIR-BLACK et al., 2023).

Limestone particles with coarser granulometry have been associated with improved eggshell quality and greater bone mineralization. However, there is wide variability in the birds' responses to this factor, highlighting the need for a more precise characterization of the modulating elements related to coarse particles that can explain this variation in results (HERVO et al., 2022).

In this context, the laying hen industry has set a goal of achieving 500 eggs per hen by 100 weeks of age, as a way to optimize resources and reduce emissions (SHAO et al., 2025). Thus, understanding the role of P and Ca in the bird organism and their complex physiological interactions becomes fundamental for the development of more efficient and sustainable nutritional strategies.

P is the second most required mineral by birds, second only to Ca, with approximately 80% stored in the skeleton in the form of hydroxyapatite, in a 1:2 ratio with Ca. The remainder circulates as inorganic phosphate, free or bound to organic compounds. Its intra- and extracellular levels are rigorously controlled, as variations can compromise functions such as energy metabolism, cell signaling, and bone integrity (MARKS et al., 2010).

Abbasi et al. (2019) add that P acts in skeletal mineralization, regulates key metabolic enzymes, and participates in various genomic and physiological processes. Studies indicate that, along with Ca and parathyroid hormone, vitamin D3 contributes to the regulation of intestinal absorption of P (BERTECHINI, 2012).

According to Bertechini (2012), in severe P deficiency, a reduction in feed intake, bone fragility, and mortality are observed. Moderate deficiencies can result in rickets, slow growth in young birds, osteomalacia, low fertility, and eggshell deformities in adult laying hens. P is also essential for maintaining bone tissue.

Dietary Ca plays an important role in P absorption in the small intestine of birds (AL-MASRI, 1995). The adequate presence of these minerals in the diet influences the expression of proteins such as calbindin in the duodenum (LI et al., 2021). In addition, genes such as NAPIIb, calbindin D28k, and the vitamin D receptor are involved in the regulation of Ca and P absorption (NIE et al., 2013). Once absorbed, P is transported through the bloodstream under hormonal regulation (LEESON and SUMMERS, 2001).

Ca and P are deposited in the organic matrix forming hydroxyapatite crystals, which are fundamental for bone mineralization and strength (BERNER and SHIKE, 1988). Adedokun and Adeola (2013) emphasize that, unlike amino acids, the metabolism of these minerals is strongly regulated by vitamin D3 and parathyroid hormone, with the action of vitamin D3 being crucial for the efficiency of their utilization.

Studies show that the partial replacement of fine limestone with coarser particles, as a source of Ca for laying hens, can benefit bone quality. Coarse limestone dissolves more slowly, releasing Ca continuously and contributing to the maintenance of adequate serum levels during the night, when shell calcification is more intense and the birds do not have access to feed (CUFADAR et al., 2011; FLEMING et al., 1998; GUINOTTE & NYS, 1991; KORELESKI & ŚWIĄTKIEWICZ, 2004; SAUNDERS-BLADES et al., 2009).

Rao et al. (1992) observed that particles of at least 1.0 mm favor the selective retention of limestone in the gizzard of laying hens. However, data on the interaction between limestone particle size and dietary Ca concentration on bone quality in modern, high-producing laying hens are still limited.

Ca and P are the two most abundant and essential macrominerals to ensure adequate growth, production, and performance in poultry (ADEDOKUN and ADEOLA, 2013). According to the authors, plant ingredients commonly used in feed do not fully meet the requirements for these minerals, which justifies supplementation with inorganic sources in diets.

P supply in laying hen diets has been higher than recommended, with averages close to 4.5 g/kg — almost double the requirement indicated by the NRC (1994), of 2.5 g/kg of available P. This oversupply is related to the variability in the non-phytatic P content of the ingredients (Slominski, 2011), which increases formulation costs and increases environmental excretion of the mineral (KNOWLTON et al., 2004).

The requirements reported in the literature vary according to the genotype and performance of the birds, generally ranging between 2.5 to 3.0 and 3.72 to 3.82 g/kg of available P (LEESON & SUMMERS, 2001; ROSTAGNO et al., 2017). Genetic companies, in turn, adopt safety margins, adjusting the supply of P according to age, feed consumption, and temperature (SAKOMURA et al., 1995; SNOW et al., 2004). However, high levels of available P, above 4.0–4.5 g/kg, can interfere with intestinal calcium absorption, compromising eggshell formation (Hossain & Bertechini, 1998; USAYRAN et al., 2001; WALDROUP et al., 2005; SILVERSIDES et al., 2006).

On the other hand, Keshavarz (1986) observed that diets with lower levels of available P reduced mineral excretion without impairing performance or bone integrity. However, very low levels of P can compromise bone metabolism and production, especially when there is an imbalance in the Ca:P ratio (SCHEIDELER & AL-BATSHAN, 1994; XIE et al., 2009).

Since the skeleton acts as the main calcium reserve for shell mineralization during the dark period, inadequate intake of this mineral by laying hens not only reduces eggshell quality but also increases bone fragility and susceptibility to fractures (SWIATKIEWICZ et al. 2015). In view of this, several authors (Bar et al., 2002; Castillo et al., 2004; Costa et al., 2008; Lichovnikova, 2007; Safaa et al., 2008) suggest that the dietary calcium requirement in laying hens may be higher than the value recommended by the NRC (1994), of 3.25% in the diet. On the other hand, excessive calcium concentrations can impair the retention of other essential minerals (PASTORE et al., 2012).

One strategy to reduce shell quality loss and bone fragility with aging laying hens is to increase the use of coarse limestone particles (>0.8 mm). There is no consensus on the ideal ratio between coarse and fine limestone throughout the cycle, although the use of 75% coarse: 25% fine is common in older birds (HERVO et al., 2022).

The age of laying hens is a critical factor in determining nutritional requirements, especially regarding P. With 3.8% Ca in the diet, Shao et al. (2025) observed that reducing available P from 0.32% to 0.27% did not compromise shell or tibia quality, meeting the requirements of older laying hens. In addition, birds fed three times a day with 0.27% P showed better performance and shell quality, demonstrating that this strategy can be applied at the end of the production cycle to minimize economic losses from soft or broken eggs, especially in intensive systems with laying hens in cages (SHAO et al., 2025).

In young laying hens, adequate P levels are essential for bone growth and a healthy start to laying. Pongmanee et al. (2020) observed that diets with reduced P did not compromise performance between 25 and 37 weeks, although signs of bone demineralization were detected, indicating that maintaining production may mask subtle mineral imbalances.

Another strategy to reduce eggshell quality loss and bone fragility with aging laying hens is to increase the use of coarse limestone particles (>0.8 mm). There is no consensus on the ideal ratio between coarse and fine limestone throughout the cycle, although the use of 75% coarse:25% fine is common in older birds (Hervo et al., 2022).

Eggshell quality is a fundamental parameter for the commercial value of the product, ensuring protection of the internal contents and facilitating transport and marketing (ZHANG et al., 2016). This quality is generally evaluated by characteristics such as thickness and resistance (ZHANG et al., 2019).

As a consequence of these genetic and productive advances, the proportion of laying hens in the late laying phase has increased significantly throughout the production cycle, making the challenge related to the performance and physiology of these birds at this stage more evident (REN et al., 2018). In this scenario, Shao et al. (2025) highlight the importance of maintaining shell quality, especially during the final phase of the laying cycle, when birds are more susceptible to structural losses in the egg.

Feeding strategies that optimize P availability, especially at night, favor mineral deposition and improve the structural quality of the shell. Such evidence justifies special attention to the dynamics of P release and utilization, considering its importance for product quality and production profitability.

Studies demonstrate positive effects of coarse limestone on shell quality (JARDIM-FILHO et al., 2005a; GUO & KIM, 2012) and bone health (JARDIM-FILHO et al., 2005b; DE ARAUJO et al., 2011). RAO and ROLAND (1990) highlight that coarse particles remain longer in the gizzard, releasing soluble calcium in a prolonged manner during the dark period, while Fleming et al. (2006) observed that this continuous release reduces bone calcium mobilization.

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Chapter 1: Effects of dietary non-phytate phosphorus on performance, egg quality, and bone traits in laying hens of different ages

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ABSTRACT

In diets for laying hens, excessive concentrations of non-phytate phosphorus (NPP) have been associated with disrupted calcium homeostasis, reduced eggshell quality, and an increased risk of osteoporosis. Thus, two experiments were conducted to evaluate the productive performance, egg quality, and bone characteristics of laying hens at different ages fed diets with reduced NPP levels. A total of 120 Lohmann Brown hens at 26 weeks of age (Experiment 1) and 96 hens at 66 weeks of age (Experiment 2) were assigned to a completely randomized design with two dietary treatments: a high NPP level (3.8 and 3.6 g/kg in Experiments 1 and 2, respectively) and a reduced NPP level (2.2 g/kg in both experiments). Experiment 1 consisted of ten replicates with six hens per experimental unit, while Experiment 2 consisted of eight replicates with six hens per experimental unit. Both experiments lasted 102 days. Performance, egg quality, and blood and bone traits were evaluated and analyzed by ANOVA at a 5% significance level. Dietary NPP levels did not affect performance or egg quality in either experiment. In Experiment 1, the 2.2 g/kg NPP reduced NPP intake and serum phosphorus (P), and improved tibia calcium (Ca) and P content ($p < 0.05$). In Experiment 2, the reduced NPP level reduced NPP intake and improved eggshell thickness, breaking strength, and Seedor index ($p < 0.05$). In conclusion, dietary NPP can be reduced to 2.2 g/kg without impairing the productive performance or egg quality of laying hens. Moreover, this reduction increases tibia Ca and P content in hens between 26 and 41 weeks of age and enhances eggshell thickness and breaking strength in hens between 66 and 81 weeks of age.

Keywords: mineral; nutrition; poultry

1. Introduction

Phosphorus (P) is an essential and costly nutrient in poultry nutrition, playing a key role in eggshell formation, bone development, and mineralization (Abbasi et al., 2019; Ren et al., 2023b). Consequently, a consistent dietary supply of P is necessary to ensure optimal productivity in laying hens. Common ingredients such as corn and soybean meal contain approximately two-thirds of their total P bound to phytic acid, an unavailable form for birds (Rostagno et al., 2024). Therefore, dietary P requirements for laying hens primarily focus on non-phytate P (NPP) (Mirabile et al., 2020). NPP requirements vary significantly across different physiological stages of hens. This is especially important for maintaining bone health. Cortical bone quality declines during extended egg-laying periods (Yamada et al, 2021). According to Lohmann (2022), NPP levels should be adjusted based on the hen's age, ranging from 3.2 to 4.0 g/kg of diet.

In commercial diets, NPP concentrations are often elevated beyond recommended levels to prevent deficiency symptoms. However, excessive dietary NPP levels offer no added benefits, as shown by Rodehutscord et al. (2023), who reported no improvements in laying performance or eggshell quality when NPP exceeded 2.2 g/kg. Moreover, excessive dietary P has been associated with disrupted calcium homeostasis, reduced eggshell quality, and an increased risk of osteoporosis in laying hens (Jing et al., 2018; Gloux et al., 2020; Magnuson et al., 2024), as well as increased feed costs and elevated P excretion into the environment. Therefore, formulating diets with elevated NPP levels does not ensure optimal productivity and may negatively impact on the economic and environmental sustainability of egg production.

This study hypothesizes that reducing dietary NPP to 2.2 g/kg, without phytase supplementation, does not impair productive performance, egg quality (internal and external), or bone mineralization. Therefore, the objective of this study was to evaluate the productive performance, egg quality, and bone characteristics of laying hens of different ages fed diets with reduced NPP levels.

2. Materials and Methods

2.1 Ethical approval and experimental design

Two experiments were conducted at the Department of Animal Science, Federal University of Viçosa, Viçosa, Minas Gerais, Brazil, following the rules issued by the Brazilian National Council for Animal Experimentation Control (CONCEA), and approved by the Ethics Commission on the Use of Farm Animals of UFV (CEUAP-UFV) under protocol nº 133/2023. The experiments differed only in the phase of the production cycle evaluated.

In Experiment 1, 120 Lohmann Brown laying hens, 26 weeks old with an average body weight of 1.748 ± 0.006 kg and an egg production rate of 98.21% ± 0.41, were assigned to a completely randomized design with two treatments (3.8 and 2.2 g/kg dietary NPP), ten replicates, and six hens per experimental unit. In Experiment 2, 96 Lohmann Brown laying hens, 66 weeks old with an average body weight of 1.876 ± 0.017 kg and an egg production rate of 88.22% ± 1.13, were assigned to the same design with two treatments (3.6 and 2.2 g/kg dietary NPP), eight replicates, and six hens per experimental unit.

Both experiments lasted 102 days and were divided into four 28-day data collection cycles. The hens were housed in metal cages measuring 34 × 47 cm, each equipped with trough feeders and nipple drinkers, providing feed and water *ad libitum*. Each experimental unit consisted of three cages, with two hens per cage, totaling six hens per unit. A 16-hour photoperiod lighting program was used throughout the experiments.

2.2 Experimental diets

The experimental diets were formulated to meet the nutritional requirements of laying hens according to Rostagno et al. (2024), except for the NPP levels. Diets were divided into phases based on hen age: 26 to 33 weeks and 34 to 41 weeks in experiment 1, and 66 to 81 weeks in experiment 2 (Table 1). The treatments consisted of two dietary NPP levels: 3.8 g/kg and 2.2 g/kg in experiment 1, and 3.6 g/kg and 2.2 g/kg in experiment 2. The NPP levels of 3.8 and 3.6 g/kg were selected according to the recommendations of the Lohmann Brown-Classic management guide (Lohmann, 2022). The Ca and P levels in the dicalcium phosphate used for both experiments were previously analyzed and found to be 290.5 g/kg Ca and 187.5 g/kg P.

Table 1. Ingredients and calculated composition of experimental diets for experiment 1 (26 to 33 weeks and 34 to 41 weeks) and experiment 2 (66 to 81 weeks).

	26 to 33 weeks		34 to 41 weeks		66 to 81 weeks	
Ingredients	**3.8 g/kg**	**2.2 g/kg**	**3.8 g/kg**	**2.2 g/kg**	**3.6 g/kg**	**2.2 g/kg**
Corn	615.46	615.46	622.43	622.43	662.67	662.67
Soybean meal, 46%	237.38	237.38	231.29	231.29	207.88	207.88
Soybean oil	21.43	21.43	17.91	17.91	4.34	4.34
Salt	4.72	4.72	4.72	4.72	4.52	4.52
DL-Methionine, 99%	3.06	3.06	3.08	3.08	2.72	2.72
L-Lysine HCl, 78.8%	0.82	0.82	0.95	0.95	0.94	0.94
L-Threonine, 98.5%	0.78	0.78	0.82	0.82	0.67	0.67
L-Valine, 96.5%	0.60	0.60	0.66	0.66	0.50	0.50
L-Tryptophan, 98.5%	0.15	0.15	0.17	0.17	0.16	0.16
Mineral premix[1]	1.10	1.10	1.10	1.10	1.10	1.10
Vitamin premix[2]	1.00	1.00	1.00	1.00	1.00	1.00
Choline chloride, 60%	1.00	1.00	1.00	1.00	1.00	1.00
Dicalcium phosphate	15.84	7.31	15.89	7.36	15.00	7.53
Limestone	96.66	103.04	98.98	105.36	97.50	103.08
Inert	0.00	2.15	0.00	2.150	0.00	1.89
Calculated Composition of nutrients (g/kg) and metabolizable energy (kcal/kg)						
Metabolizable energy	2,820	2,820	2,800	2,800	2,760	2,760
Crude protein	160.0	160.0	158.0	158.0	150.0	150.0
Calcium	43.0	43.0	43.9	43.9	43.0	43.0
No phytate phosphorus	3.80	2.20	3.80	2.20	3.60	2.20
Sodium	1.96	1.96	1.96	1.96	1.88	1.88
Digestible lysine	8.10	8.10	8.06	8.06	7.53	7.53
Digestible methionine + cysteine	7.45	7.45	7.42	7.42	6.92	6.92
Digestible threonine	6.24	6.24	6.21	6.21	5.80	5.80
Digestible valine	7.29	7.29	7.26	7.26	6.77	6.77
Digestible tryptophan	1.86	1.86	1.85	1.85	1.73	1.73

[1] Mineral premix, guaranteed levels per kg of product: manganese, 58.36 g; zinc, 54.21 g; iron, 41.68 g; copper, 8.31 g; iodine, 843.00 mg; selenium, 250.0 mg. [2] Vitamin premix, guaranteed levels per kg of product: vitamin A, 9,638,000 IU; vitamin D$_3$, 2,410,000 IU; vitamin E, 36,100 IU; vitamin B$_1$, 2.600 mg; vitamin B$_2$, 6.450 mg; vitamin B$_6$, 3.610 mg; vitamin B$_{12}$, 15.9 mg; vitamin K$_3$, 1.936 mg; pantothenic acid, 12.95 g; niacin, 39.2 g; folic acid, 903.0 mg; and biotin, 89.8 mg.

2.3 Layer performance

In both experiments, hens were weighed at the beginning and end of the trial, and average weight gain (WG, g/hen) was calculated as the difference between final and initial body weights. Egg production was recorded daily to calculate the egg production rate (EPR, %), determined as the ratio of the total number of eggs produced to the number of hens. At the end of each 28-day cycle, feed intake (FI, g/hen/day) was calculated by subtracting the remaining feed from the total amount supplied. The feed conversion ratio (FCR, g/g) was calculated by dividing FI by the total egg mass produced. Additionally, NPP intake (mg/hen/day) was estimated based on dietary NPP concentration and FI. The percentage of noncommercial eggs (broken or defective) was also recorded.

2.4 Egg quality traits

Egg quality traits were evaluated at the end of each 28-day cycle by collecting all intact eggs over five consecutive days. Eggs collected during the first two days were used to measure eggshell breaking strength (EBS, kgf) with a texture analyzer (GR Electrical Manufacturing Company, Manhattan, KS). Eggs collected during the remaining three days were used to determine average egg weight (EW, g), egg mass (EM, g/hen/ day), and to conduct internal and external quality analyses. Eggs were weighed, cracked onto a glass table, and immediately assessed for albumen and yolk quality. Albumen quality was assessed using the Haugh unit (HU), calculated according to Eq. (1), with albumen height (mm) measured using a digital micrometer. Yolk quality was evaluated based on the yolk index (YI), calculated as the ratio of yolk height (mm) to yolk diameter (mm), measured with a digital micrometer and a digital caliper, respectively.

$$(2)\ SWSA, mg\ cm^{-2} = \left(\frac{ESW}{3.9782\ \times EW^{0.7056}}\right) \times 1000$$

Where H is albumen height (mm) and W is egg weight (g).

Additionally, yolk color was assessed using a DSM Yolk Color Fan scale (DSM, São Paulo, SP, Brazil). Yolk weight (YW) and eggshell weight (ESW) were then recorded, and albumen weight (AW) was calculated by subtracting the sum of YW and ESW from the total EW. Shell weight was measured after washing the samples to remove excess albumen and air-drying them for 48 hours. The proportions of egg components were also calculated as percentages of the total EW.

Eggshell thickness (EST, mm) was measured using a digital micrometer at three equidistant points along the equatorial zone of the shell, and the arithmetic mean of these measurements was used. Shell density was estimated as shell weight per surface area (SWSA, mg/cm^2), according to Eq. (2), as proposed by Abdallah et al. (1993):

$$(2)\ SWSA, mg\ cm^{-2} = \left(\frac{ESW}{3.9782\ \times EW^{0.7056}}\right) \times 1000$$

Where, ESW represents eggshell weight and EW represents egg weight.

2.5 Blood and bone parameters

At the end of each experimental period, blood samples were collected from one bird per experimental unit via brachial vein puncture. Blood samples were analyzed for serum levels of Ca, P, and total alkaline phosphatase (ALP) using a Cobas c 311 analyzer (Roche Diagnostics GmbH, Basel, Switzerland), following the manufacturer’s instructions. The same bird was then euthanized for tibia collection. The breaking strength of the right tibia was measured using a texture analyzer (GR Electrical Manufacturing Company, Manhattan, KS). The left tibia was used to determine Ca, P, and ash content. Additionally, relative tibia weight (RTW) was calculated as the ratio of tibia weight (g) to body weight (kg), and the Seedor index (SI) was calculated as bone mass (mg) divided by bone length (mm), as described by Seedor (1991).

2.6 Statistical analysis

Data were analyzed using the GLM procedure of SAS (Statistical Analysis System, version 9.4). Three cages, with two hens per cage (six hens in total), constituted the experimental unit. The Shapiro–Wilk test assessed residual normality, and the Hartley test evaluated homogeneity of variances. After confirming these assumptions, analysis of variance was conducted with a $p < 0.05$ level of significance.

3. Results

Overall, dietary NPP levels did not influence laying hen performance in both experiments, as no significant differences ($p > 0.05$) were observed in WG, FI, FCR, EPR, or the percentage of noncommercial eggs (Tables 2 and 3). Similarly, egg quality traits, including EM, EW, YI, yolk color, SWSA, HU, and the relative percentages of yolk, albumen, and shell, were not affected ($p > 0.05$) by dietary NPP levels in both experiments (Tables 2 and 3). However, in Experiment 2, corresponding to the late production phase (66–81 weeks), hens fed 2.2 g/kg of NPP showed higher EST ($p = 0.014$) and EBS ($p = 0.003$) values, whereas these traits were not influenced ($p > 0.05$) by dietary NPP in Experiment 1. As expected, NPP intake was higher ($p < 0.001$) in hens receiving the higher dietary NPP levels in both experiments (Tables 2 and 3).

Table 2. Productive performance, egg quality, blood, and bone traits of laying hens fed different levels of npp from 26 to 41 weeks of age (experiment 1).

Traits	**Dietary NPP**		**SEM**	***p*-value**
	3.8 g/kg	**2.2 g/kg**		
Weight gain (kg/hen)	0.194	0.213	0.009	0.322
Feed intake (g/hen/day)	110.6	111.5	0.800	0.562
NPP intake (mg/hen/day)	420.1	245.4	20.20	<0.001
Egg production rate (%)	98.08	98.28	0.230	0.670
Noncommercial eggs (%)	0.470	0.350	0.080	0.453
Feed conversion ratio (g/g)	1.940	1.94	0.010	0.971
Egg mass (g/hen/day)	57.15	57.70	0.300	0.336
Egg weight (g)	58.05	58.73	0.310	0.287
Yolk index	0.422	0.423	0.001	0.892
Yolk color	5.865	5.935	0.040	0.407
Yolk percentage (%)	24.96	25.11	0.120	0.539
Albumen percentage (%)	64.94	64.78	0.130	0.532
Eggshell percentage (%)	10.09	10.11	0.060	0.898
Shell weight per surface area (mg/cm^2)	83.94	84.26	0.370	0.679
Eggshell thickness (mm)	0.374	0.376	0.001	0.518
Eggshell breaking strength (kgf)	5.040	5.000	0.050	0.671
Haugh unit	82.42	81.02	0.530	0.195
Serum calcium (mg/dL)	28.58	29.36	0.510	0.456
Serum phosphorus (mg/dL)	7.520	6.010	0.350	0.029
Serum alkaline phosphatase (U/L)	283.1	291.3	28.60	0.890
Relative tibia weight (%)	0.563	0.547	0.012	0.540
Seedor index (mg/mm)	99.57	98.82	2.000	0.856
Tibia breaking strength (kgf)	21.82	21.50	1.640	0.925
Tibia calcium content (%)	19.72	21.53	0.300	0.008
Tibia phosphorus content (%)	7.900	8.630	0.130	0.001
Tibia ash content (%)	57.03	58.74	0.590	0.149

NPP: non-phytate phosphorus. SEM: standard error of the mean.

Table 3. Productive performance, egg quality, blood, and bone traits of laying hens fed different levels of npp from 66 to 81 weeks of age (experiment 2).

Traits	**Dietary NPP**		**SEM**	***P* value**
	3.6 g/kg	**2.2 g/kg**		
Weight gain (kg/hen)	0.137	0.138	0.008	0.994
Feed intake (g/hen/day)	112.0	110.8	0.600	0.357
NPP intake (mg/hen/day)	403.5	243.7	20.70	<0.0001
Egg production rate (%)	94.67	94.09	0.380	0.466
Noncommercial eggs (%)	0.650	0.600	0.220	0.919
Feed conversion ratio (g/g)	1.430	1.410	0.010	0.370
Egg mass (g/hen/day)	56.61	56.27	0.300	0.597
Egg weight (g)	60.35	60.15	0.460	0.839
Yolk index	0.414	0.411	0.001	0.154
Yolk color	6.037	6.106	0.027	0.227
Yolk percent (%)	25.48	25.56	0.180	0.852
Albumen percent (%)	64.83	64.56	0.210	0.548
Shell percent (%)	9.670	9.870	0.060	0.145
Shell weight per surface area (mg/cm^2)	81.30	82.88	0.430	0.067
Egg shell thickness (mm)	0.363	0.372	0.001	0.014
Egg shell breaking strength (kgf)	4.220	4.540	0.060	0.003
Haugh unit	77.71	76.38	0.410	0.106
Serum calcium (mg/dL)	27.35	28.57	0.580	0.308
Serum phosphorus (mg/dL)	5.610	6.380	0.240	0.123
Serum alkaline phosphatase (U/L)	241.6	215.0	28.90	0.661
Relative tibia weight (%)	0.530	0.574	0.014	0.131
Seedor index (mg/mm)	95.96	106.8	2.350	0.015
Tibia breaking strength (kgf)	23.41	23.45	1.480	0.987
Tibia calcium content (%)	23.09	22.42	0.380	0.404
Tibia phosphorus content (%)	8.910	8.860	0.130	0.860
Tibia ash content (%)	60.41	60.22	0.450	0.838

NPP: non-phytate phosphorus. SEM: standard error of the mean.

Regarding blood and bone parameters, serum Ca and ALP concentrations were not influenced ($p > 0.05$) by dietary NPP levels in both experiments (Tables 2 and 3). However, serum P was higher ($p = 0.029$) in hens fed 3.8 g/kg of NPP in Experiment 1 (Table 2), but it was not affected ($p = 0.123$) by dietary NPP levels in older hens (Experiment 2) (Table 3). No significant differences ($p > 0.05$) were observed for RTW and TBS in both experiments. In the younger birds (Experiment 1), hens fed 2.2 g/kg of NPP showed higher tibia Ca ($p = 0.008$) and tibia P ($p = 0.001$) contents (Table 2), whereas these variables were not influenced by dietary NPP levels in older hens (Experiment 2) (Table 3). In contrast, SI was higher ($p = 0.015$) in aged hens (Experiment 2) fed 2.2 g/kg of NPP (Table 3), whereas SI did not differ ($p = 0.856$) between treatments in Experiment 1 (Table 2). Tibia ash content was not influenced by dietary NPP levels in both experiments ($p > 0.05$) (Tables 2 and 3).

4. Discussion

The results of the present study contribute to understanding how dietary NPP levels influence laying hen performance and egg quality, showing that reducing NPP from 3.8 or 3.6 g/kg to 2.2 g/kg did not impair these parameters, as previously expected. These findings agree with those reported by Bello and Korver (2019), in which NPP reduction around 3.8 to 2.3 g/kg did not affect performance, feed efficiency, and eggshell quality of laying hens from 30 to 70 weeks of age. Additionally, our results are consistent with previous studies showing that dietary NPP reductions ranging from 1.5 to 2.3 did not impair layer performance or egg quality (Nie et al., 2013; Jing et al., 2018; Pongmanee et al., 2020; Sommerfeld et al., 2020).

In contrast, Boling et al. (2000) assessed the reduction of dietary NPP from 4.5 to 1.0 g/kg in laying hens from 20 to 70 weeks of age. The authors reported that egg production and egg quality were maintained at 2.0 g/kg NPP, whereas 1.5 g/kg sustained egg production but led to a reduction in body weight from 41 weeks of age. However, when they supplied 1.0 g/kg NPP, there were considerable reductions in egg production from 28 weeks, in addition to an increase in mortality compared to other treatments (19 vs.

5.5%), with hens becoming lethargic and unwilling to stand. Impairments in egg production rates or shell quality have also been observed in other studies when layers were fed diets containing approximately 1.2 g/kg to 1.5 g/kg NPP (Liu et al., 2007; Wei et al., 2022). Therefore, the indication of P deficiency affecting performance or egg quality in laying hens has been associated with dietary NPP levels lower than 1.5 g/kg, reinforcing that the tested level of 2.2 g/kg is sufficient to meet the requirements of layers without compromising their productivity.

Reductions in dietary NPP have been associated with upregulation of the type IIb sodium-dependent phosphate cotransporters (NaPi-IIb) in the intestine of piglets (Saddoris et al., 2010) and broiler chickens (Yan et al., 2007). Li et al. (2018) fed 25-week-old laying hens diets containing three levels of NPP and reported that the mRNA expression of NaPi-IIb in the ileum was upregulated in the 1.5 g/kg NPP group compared with both the 4.1 g/kg and 8.2 g/kg NPP groups. NaPi-IIb is mostly found in the brush-border membranes of the small intestine and is the main form of Pi uptake (Tang et al., 2021). In addition, studies have reported that 25- and 69-week-old laying hens fed lower NPP levels, ranging from 0.7 to 1.5 g/kg, exhibited higher mRNA expression of sodium-dependent phosphate cotransporters type IIa (NaPi-IIa) in the kidney than those fed higher NPP levels, ranging from 4.1 to 8.2 g/kg (Li et al., 2018; Ren et al., 2023a). NaPi-IIa mediates renal P reabsorption and plays a critical role in maintaining P homeostasis in hens (Huber et al., 2015). Therefore, the lack of differences observed in performance and egg quality, even with reduced NPP levels, may be attributed to an increased expression of these cotransporters, although not evaluated in the present study, indicating that P absorption may have been enhanced in hens fed diets with lower NPP concentrations.

Although overall results indicated that NPP reductions did not impair egg quality, reports regarding EST remain inconsistent. Jing et al. (2018) fed laying hens diets containing 1.5 to 4.5 g/kg NPP and reported thicker and thinner eggshells at 2.0 g/kg and 4.5 g/kg, respectively. The authors attributed the increased EST associated with lower NPP levels to enhanced Ca absorption and retention. Similarly, Dijkslag et al. (2023) reported that EST of laying hens was affected by dietary Ca and P levels, but without a consistent pattern across the assessed ages (30 to 89 weeks). Thicker eggshells were only observed at 45 weeks, when layers were fed with lower Ca and P levels, whereas at other ages the response varied and interacted with feed form. In the present study, the reduction in NPP levels also increased EST during the later laying phase (66 to 81 weeks), leading to an improvement in EBS. However, during the early phase, neither EST nor EBS was affected by dietary NPP levels. These results are particularly relevant in the later stages of lay, when eggshell thickness typically declines with advancing age, and 2.2 g/kg NPP may help attenuate this effect. The explanation for the observed effect of NPP levels on EST in older hens, but not in younger ones, may be related to age-dependent changes in the expression of intestinal cotransporters. Lv et al. (2022) demonstrated that the expression of several P transporters, including NaPi-IIb, follows a quadratic pattern as broiler chickens age. In this context, older hens tend to exhibit reduced NaPi-IIb expression. Given that a linear increase in NaPi-IIb expression has been reported when dietary NPP levels are reduced in laying hens after 41 weeks of age (Nie et al., 2013), the reduction of NPP from 3.6 g/kg to 2.2 g/kg in the present study may have positively modulated NaPi-IIb expression in older hens, thereby compensating for the natural age-related decline in this transporter.

Serum P concentrations have been reported to increase with dietary phosphorus supplementation in a dose-dependent manner (Jing et al., 2018; Wang et al., 2018). This effect is primarily attributed to enhanced intestinal absorption, as higher NPP intake increases the amount of P absorbed, resulting in increased P in the blood, even when FI remains unchanged. In the present study, serum P concentrations were higher in hens fed 3.8 g/kg NPP during the early laying phase, as NPP intake was also higher. However, this pattern was not observed in the late laying phase, where, despite higher NPP intake, serum P concentrations did not differ between treatments. The lack of a serum P response to increased dietary NPP observed in older hens may be explained by age-related changes in phosphorus metabolism. It has been reported that intestinal P absorptive capacity declines with advancing age, as NaPi-IIb expression decreases in older birds (Lv et al., 2022). Thus, even if it is hypothesized that reducing dietary NPP levels could induce an upregulation of NaPi-IIb expression, thereby improving P absorption in older hens, the additional absorbed P would be rapidly directed towards eggshell calcification and medullary bone replenishment, resulting in unchanged serum P concentrations.

Tibia quality is considered a key parameter for evaluating phosphorus uptake in poultry. However, results regarding the influence of dietary NPP levels on tibia mineralization are inconsistent. While some studies have reported a linear increase in tibial Ca and P content with increasing dietary NPP levels from 1.2 to 4.2 g/kg (Nie et al., 2018; Ren et al., 2023b), others found no significant effect (Jing et al., 2018; Park et al., 2024), suggesting that laying hens can maintain mineral homeostasis and bone integrity through compensatory mechanisms. In the present study, we observed an unexpected result in the early laying phase, where lower dietary NPP levels increased tibia Ca and P content, indicating a prioritization of bone deposition. Notably, tibia breaking strength was not affected, despite higher Ca and P content. In the late phase, dietary NPP reduction did not affect tibia Ca or P content, nor bone strength, suggesting that lower NPP levels may be safely applied in layer nutrition. However, bone density improved, as indicated by a higher SI. These results suggest that lower dietary NPP levels may be beneficial during both early and late laying phases. These results may be explained by the enhanced efficiency in the utilization of Ca and P, related to the lower dietary NPP levels, associated with the requirement of younger laying birds for intense bone remodeling to establish Ca and P reserves. Studies in broilers have shown that reducing dietary NPP levels (2.5 g/kg vs. 4.5 g/kg) upregulated the mRNA expression of 25-hydroxylase in the liver, PMCA1b in the duodenum, and CaBP-D28k in both the duodenum and kidney (Ma et al., 2025). The enzyme 25-hydroxylase plays a pivotal role in converting vitamin D_3 into its active form, $1,25(OH)_2D_3$, whereas PMCA1b and CaBP-D28k are key components of transcellular Ca transport. Additionally, younger laying hens exhibit a greater capacity for bone deposition due to the intense remodeling of medullary bone at the onset of lay (Garcia-Mejía et al., 2024). Therefore, lower dietary NPP levels, by upregulating the expression of both Ca- and P-related cotransporters, may have contributed to the increased tibia Ca and P content observed in younger hens in the present study.

5. Conclusion

The present study provides valuable insights into the effects of dietary NPP reduction across two laying phases. Overall, reducing dietary NPP from 3.8 or 3.6 to 2.2 g/kg did not impair performance or egg quality. Moreover, lower dietary NPP levels were beneficial by increasing tibia Ca and P content between 26 and 41 weeks of age and enhancing the thickness and breaking strength of eggshell between 66 and 81 weeks of age. Therefore, reducing dietary NPP represents a viable strategy to decrease environmental P excretion and potentially reduce diet formulation costs without compromising laying hens' productivity.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability statement

All data used in this manuscript may be provided by the corresponding author upon request.

Declaration of use of AI Technologies

The authors state that no AI technologies were used to produce this manuscript.

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Chapter 2: Effects of fine-to-coarse limestone ratio on performance, eggshell quality, bone traits, and renal 1α-hydroxylase concentration of peak and aged laying hens

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Abstract

Two trials were conducted at Federal University of Viçosa to evaluate the productive performance, internal and external egg quality, blood and bone traits, and renal 1α-hydroxylase concentration in laying hens fed different fine to coarse limestone ratios (FL:CL) during two production phases. A total of 180 Lohmann Brown hens at 26 weeks (trial 1) and 96 hens at 66 weeks of age (trial 2) were assigned to three dietary FL:CL ratios (60:40, 50:50, and 30:70) in trial 1, and to two dietary FL:CL ratios (50:50 and 30:70) in trial 2. The data were submitted to ANOVA at a 5% significance level, and the Tukey Test was used to identify differences among treatments. Dietary FL:CL ratios did not affect performance, internal and external egg quality, and blood parameters in both trials. In trial 1, the 30:70 FL:CL ratio reduced relative tibia weight and tibia breaking strength ($p < 0.05$). Additionally, the intermediate 50:50 FL:CL ratio showed lower renal 1α-hydroxylase concentration ($p < 0.05$). In trial 2, the 30:70 FL:CL ratio increased feed intake, whereas it decreased the tibial Ca content of layers. In conclusion, dietary FL:CL ratios do not influence performance, eggshell, and bone quality, but influence physiological responses in the early phase.

Keywords: calcium homeostasis, vitamin D, particle size, eggshell formation, calcium kinetics.

1. Introduction

In the laying hens, eggshell formation occurs daily in the uterus and represents one of the most rapid biomineralization processes known. To deposit approximately 6 g of shell as calcium carbonate (CaCO3), the hen exports 2.4 g of calcium (Ca) per day, which corresponds to nearly 10% of her total body calcium (Nys et al., 2022). Although dietary sources supply approximately two-thirds of the Ca requirement, there is a desynchronization between Ca intake during the day and egg formation, which mainly takes place during the night, leading to bone mobilization (Nys, 2017; Nys et al., 2022).

To support optimal egg production, strategies including different limestone particle sizes have been demonstrated effective in improving eggshell thickness and strength (Hervo et al., 2022, 2023), internal egg quality (Khosht et al., 2020), and bone content and strength (Molnár et al., 2017). These beneficial effects are attributed to the slower and gradual release of soluble Ca by coarse limestone (CL), when compared to fine limestone (FL), reducing the desynchronization between Ca supply and demand (Bueno et al., 2016; Hervo et al., 2022). However, there is no consensus regarding the optimal FL to CL ratio (FL:CL), and findings across studies remain inconsistent.

In addition, as hens age, the efficiency in absorbing Ca declines, thereby increasing their dependence on bone-sourced calcium for adequate shell formation. In this context, increasing the proportion of CL particles may represent a promising strategy to support Ca homeostasis and maintain adequate egg production in older hens.

We hypothesized that increasing the dietary proportion of CL would promote more stable calcium homeostasis in laying hens, thereby enhancing eggshell and bone quality. Thus, the objective of this study was to evaluate productive performance, internal and external egg quality, blood parameters, bone characteristics, and renal 1α-hydroxylase concentration in laying hens during the early production phase (26 to 41 weeks of age) and the late production phase (66 to 81 weeks of age).

2. Materials and Methods

Two trials were carried out at the Department of Animal Science of the Federal University of Viçosa (UFV), located in Viçosa, Minas Gerais, Brazil. All procedures complied with the guidelines established by the Brazilian National Council for the Control of Animal Experimentation (CONCEA) and were approved by the Ethics Committee on the Use of Production Animals of UFV (CEUAP-UFV), under protocol number 133/2023. The trials assessed different phases of the production cycle.

Trials 1 and 2 were conducted in a completely randomized design, and both lasted 102 days. The birds used in both trials were selected based on average body weight (BW) and laying rate to ensure initial uniformity between experimental groups. In trial 1, a total of 180 Lohmann Brown laying hens, 26 weeks of age, with an average BW of 1.81 kg and an egg production rate of 98.37%, were housed in 34 × 47 cm metallic cages following three treatments, with ten replicates and six hens per experimental unit. In trial 2, 96 Lohmann Brown laying hens, 66 weeks of age, with an average BW of 1.860 kg and an egg production rate of 89.66%, were assigned to two treatments with eight replicates and six hens per experimental unit. Each unit consisted of three cages, with two birds per cage. All cages were equipped with trough feeders and nipple drinkers, allowing hens *ad libitum* access to feed and water. Furthermore, the temperature inside the shed was monitored daily by two digital thermometers that measured maximum and minimum temperatures in degrees Celsius (°C), being 29.7° and 18.8°, respectively.

The experimental diets were formulated to meet or exceed the nutritional requirements of laying hens, according to Rostagno et al. (2024), for the phases from 26 to 33 and 34 to 41 weeks of age in trial 1 (Table 1), and from 66 to 81 weeks of age in trial 2 (Table 2). The dietary treatments were based on different inclusion ratios of two limestone particle sizes, both derived from the same source and differing only in the grinding process: fine limestone (FL), with a geometric mean diameter of 0.200 mm and a geometric standard deviation of 2.58 mm, and coarse limestone (CL), with a geometric mean diameter of 3.166 mm and a geometric standard deviation of 1.59 mm. Thereby, the treatments consisted of three FL:CL ratios of 60:40, 50:50, and 30:70 in trial 1 (Table 1), and two FL:CL ratios of 50:50 and 30:70 in trial 2 (Table 2). The Ca and P levels in the dicalcium phosphate used for both experiments were previously analyzed and found to be 290.5 g/kg Ca and 187.5 g/kg P.

Table 1. Ingredients and calculated composition of experimental diets in trial 1(g kg^{-1}).

Ingredients	26 to 33 weeks			34 to 41 weeks		
	60:40	**50:50**	**30:70**	**60:40**	**50:50**	**30:70**
Corn	619.49	619.49	619.49	626.48	626.48	626.48
Soybean meal, 46%	236.67	236.67	236.67	230.57	230.57	230.57
Soybean oil	20.06	20.06	20.06	16.54	16.54	16.54
Dicalcium phosphate	8.05	8.05	8.05	8.05	8.05	8.05
Salt	4.72	4.72	4.72	4.72	4.72	4.72
DL-Methionine, 99%	3.06	3.06	3.06	3.08	3.08	3.08
L-Lysine HCl, 78.8%	0.83	0.83	0.83	0.96	0.96	0.96
L-Threonine, 98.5%	0.79	0.79	0.79	0.83	0.83	0.83
L-Valine, 96.5%	0.60	0.60	0.60	0.66	0.66	0.66
L-Tryptophan, 98.5%	0.15	0.15	0.15	0.17	0.17	0.17
Mineral premix[1]	1.10	1.10	1.10	1.10	1.10	1.10
Vitamin premix[2]	1.00	1.00	1.00	1.00	1.00	1.00
Choline chloride, 60%	1.00	1.00	1.00	1.00	1.00	1.00
Fine limestone	61.48	51.24	30.74	62.90	52.42	31.45
Coarse limestone	41.00	51.24	71.74	41.94	52.42	73.39
Calculated composition of nutrients (g kg^{-1}) and metabolizable energy (kcal kg^{-1})						
Metabolizable energy	2.820	2.820	2.820	2.800	2.800	2.800

Crude protein	160.0	160.0	160.0	158.0	158.0	158.0
Calcium	43.0	43.0	43.0	43.9	43.9	43.9
Nonphytate phosphorus	2.34	2.34	2.34	2.33	2.33	2.33
Sodium	1.96	1.96	1.96	1.96	1.96	1.96
Digestible lysine	8.10	8.10	8.10	8.06	8.06	8.06
Digestible methionine + cysteine	7.45	7.45	7.45	7.42	7.42	7.42
Digestible threonine	6.24	6.24	6.24	6.21	6.21	6.21
Digestible valine	7.29	7.29	7.29	7.26	7.26	7.26
Digestible tryptophan	1.86	1.86	1.86	1.85	1.85	1.85

[1]Mineral premix, guaranteed levels per kg of product: manganese, 58.36 g; zinc, 54.21 g; iron, 41.68 g; copper, 8.31 g; iodine, 843.00 mg; selenium, 250.0 mg. [2]Vitamin premix, guaranteed levels per kg of product: vitamin A, 9,638,000 IU; vitamin D_3, 2,410,000 IU; vitamin E, 36,100 IU; vitamin B_1, 2.600 mg; vitamin B_2, 6.450 mg; vitamin B_6, 3.610 mg; vitamin B_{12}, 15.9 mg; vitamin K_3, 1.936 mg; pantothenic acid, 12.95 g; niacin, 39.2 g; folic acid, 903.0 mg; and biotin, 89.8 mg.

Table 2. Ingredients and calculated composition of experimental diets in trial 2 (g kg-^{1}).

Ingredients	**66 to 81 weeks**	
	50:50	**30:70**
Corn	666.46	666.46
Soybean meal, 46%	207.21	207.21
Soybean oil	3.07	3.07
Dicalcium phosphate	7.74	7.74
Salt	4.52	4.52
DL-Methionine, 99%	2.71	2.71
L-Lysine HCl, 78.8%	0.95	0.95
L-Threonine, 98.5%	0.67	0.67
L-Valine, 96.5%	0.49	0.49
L-Tryptophan, 98.5%	0.16	0.16
Mineral premix[1]	1.10	1.10
Vitamin premix[2]	1.00	1.00
Choline chloride, 60%	1.00	1.00
Fine limestone	51.46	30.88
Coarse limestone	51.46	72.04
Calculated composition of nutrients (g kg^{-1}) and metabolizable energy (kcal kg^{-1})		
Metabolizable energy	2.760	2.760
Crude protein	150.0	150.0
Calcium	43.0	43.0
Nonphytate phosphorus	2.24	2.24
Sodium	1.88	1.88
Digestible lysine	7.53	7.53
Digestible methionine + cysteine	4.85	4.85
Digestible threonine	5.80	5.80
Digestible valine	6.77	6.77

Digestible tryptophan	1.73	1.73

[1] Mineral premix, guaranteed levels per kg of product: manganese, 58.36 g; zinc, 54.21 g; iron, 41.68 g; copper, 8.31 g; iodine, 843.00 mg; selenium, 250.0 mg. [2] Vitamin premix, guaranteed levels per kg of product: vitamin A, 9,638,000 IU; vitamin D_3, 2,410,000 IU; vitamin E, 36,100 IU; vitamin B_1, 2.600 mg; vitamin B_2, 6.450 mg; vitamin B_6, 3.610 mg; vitamin B_{12}, 15.9 mg; vitamin K_3, 1.936 mg; pantothenic acid, 12.95 g; niacin, 39.2 g; folic acid, 903.0 mg; and biotin, 89.8 mg.

At the beginning and end of the experimental period, laying hens were weighed to determine BW gain (BWG, kg bird^{-1}). Egg production was recorded daily to calculate the egg production rate (EPR, %) by dividing the total number of eggs produced by the total number of hens. The experimental period was divided into 28-day cycles, during which the amount of feed offered, and the leftovers were weighed to determine feed intake (FI, g bird^{-1} day^{-1}) and subsequently calculate the feed conversion ratio (FCR, kg kg^{-1}). Additionally, the rate of noncommercial eggs (%) was calculated by dividing the number of broken and defective eggs by the total number of eggs collected.

At the end of each 28-day cycle, all intact eggs were collected over five consecutive days. Eggs collected during the first two days were used to assess eggshell breaking strength (EBS, kgf) using a texture analyzer (GR Electrical Manufacturing Company, Manhattan, KS, USA). Eggs collected over the remaining three days were used to determine average egg weight (EW, g), egg mass (EM, g bird^{-1} day^{-1}), and to perform internal and external quality analyses.

For internal quality assessment, eggs were weighed, cracked on a specialized glass table, and immediately evaluated for albumen and yolk quality. Albumen quality was measured using the Haugh unit (HU), calculated according to Equation 1:

$$(1)\ HU = 100\log(H + 7.57 - 1.7 \times EW^{0.37})$$

Where H is the albumen height (mm), measured using a digital micrometer, and EW is the egg weight (g).

Yolk quality was assessed using the yolk index (YI), calculated by dividing yolk height (YH, mm), measured with a digital micrometer, by yolk diameter (YD, mm), measured with a digital caliper. Additionally, yolk color was evaluated using a DSM colorimetric fan. Subsequently, the yolk and shell were weighed, and albumen weight was calculated by subtracting their combined weight from the total egg weight. The percentages of the egg components were also calculated.

For external quality assessment, the eggshell weight (ESW, g) was recorded after the samples were washed to remove residual albumen and air-dried for 48 hours. Eggshell thickness (EST, mm) was assessed using a digital micrometer. Measurements were taken at three equidistant points along the equatorial zone of the shell, and the arithmetic mean of these values was used to represent EST. Eggshell density was estimated as eggshell weight per surface area (SWSA, mg/cm^2), according to Equation 2, as proposed by Abdallah et al. (1993):

$$(2)\ SWSA, mg\ cm^{-2} = \left(\frac{ESW}{3.9782\ \times EW^{0.7056}}\right) \times 1000$$

Where ESW represents eggshell weight and EW represents egg weight, in grams.

At the end of the experimental period, one hen per experimental unit was selected based on the BW of its respective unit for blood collection via brachial vein puncture. The samples were used to analyze serum levels of Ca (mg dL^{-1}), phosphorus (P, mg dL^{-1}), and total alkaline phosphatase (ALP, U L^{-1}) using an automated biochemical analyzer (Cobas c 311; Roche Diagnostics GmbH, Basel, Switzerland) following the manufacturer's instructions. Subsequently, the same hen was euthanized for gizzard and tibia sampling. The gizzard weight (g) was measured. The breaking strength of the right tibia (kgf) was determined using a texture analyzer. The left tibia was used to determine Ca, P, and ash contents. Additionally, relative tibia weight (RTW, %) was calculated as the ratio of tibia weight (g) to BW, and the Seedor index (SI) was calculated as bone mass (mg) divided by bone length (mm), according to Seedor (1993).

Kidney samples were also collected to evaluate the concentration of 1α-hydroxylase enzyme in renal tissue. Tissue homogenates were prepared by washing kidney fragments in 0.01 M phosphate-buffered saline (PBS) and homogenizing them in a ratio of 0.3 g of tissue to 1.5 mL of PBS. The homogenates were centrifuged at 12,500 G for 10 minutes, and the resulting supernatant was stored at −20 °C until further analysis. The enzymatic activity of 1α-hydroxylase was quantified using a commercial ELISA kit (Chicken 25-hydroxyvitamin D-1 alpha hydroxylase ELISA Kit; MyBioSource, San Diego, CA, USA), based on the double antibody sandwich technique. The assay involved incubation of samples in 96-well plates pre-coated with anti-CYP27B1 monoclonal antibodies, followed by sequential addition of a biotinylated polyclonal antibody, enzyme–avidin–peroxidase conjugate, and chromogenic substrate (TMB). Optical density was read at 450 nm using a microplate spectrophotometer (Multiskan TM Go, Thermo Fisher Scientific Inc., Waltham, MA). Sample concentrations were calculated based on a standard curve prepared with known concentrations of CYP27B1, following the manufacturer's instructions. The renal 1α-hydroxylase was expressed as U mg^{-1}.

Statistical analyses were performed using the GLM procedure of SAS software (version 9.4; SAS Institute Inc., Cary, NC, USA). The assumptions of normality and homogeneity of variances were assessed using the Shapiro–Wilk and Hartley tests, respectively. After confirming these assumptions, the data were subjected to analysis of variance (ANOVA), considering a 5% significance level. In Experiment 3, which included three dietary treatments, treatment means were compared using Tukey's test when significant effects were detected. In Experiment 4, which involved only two treatments, mean comparisons were performed directly through ANOVA, without the need for a post hoc multiple comparison test.

3. Results

In trial 1, across all evaluated parameters, varying the FL:CL ratios had no significant effect on performance, internal and external egg quality, or blood parameters, as no differences were observed among treatments for hens aged 26 to 41 weeks ($p > 0.05$) (Table 3). Regarding bone quality, gizzard weight, SI and tibial Ca, P, and ash contents were also not influenced by FL:CL ratios ($p > 0.05$) (Table 3). However, the highest proportion of CL (30:70) resulted in a reduced RTW ($p = 0.050$) and lower tibia breaking strength ($p < 0.001$) (Table 3). Furthermore, hens fed the intermediate FL:CL ratio (50:50) showed lower renal 1α-hydroxylase concentration compared to those fed 60:40 and 30:70 ratios ($p = 0.018$).

Table 3. Performance, internal and external egg quality, blood parameters, and bone quality of laying hens fed different fine-to-coarse limestone ratios from 26 to 41 weeks of age (trial 1).

Traits	**Fine-to-coarse ratio**			**SEM**	**P-value**
	60:40	**50:50**	**30:70**		
Body weight gain (kg hen^{-1})	0.198	0.186	0.189	0.005	0.674
Feed intake (g hen^{-1} day^{-1})	116.2	113.1	116.4	0.700	0.159
Egg production rate (%)	98.30	98.27	97.99	0.150	0.701
Noncommercial eggs (%)	0.590	0.670	0.640	0.090	0.951
Feed conversion ratio (g g^{-1})	1.410	1.380	1.420	0.010	0.132
Egg mass (g hen^{-1} day^{-1})	58.54	57.55	58.25	0.330	0.476
Egg weight (g)	59.79	58.44	59.42	0.290	0.151
Yolk index	0.423	0.426	0.422	0.001	0.544
Yolk color	5.735	5.840	5.894	0.035	0.177
Yolk percentage (%)	25.28	24.78	25.12	0.120	0.239
Albumen percentage (%)	64.69	65.32	64.97	0.130	0.169
Eggshell percentage (%)	9.980	9.930	9.920	0.030	0.805
Shell weight per surface area (mg cm^{-2})	83.55	82.87	82.96	0.240	0.500

Eggshell thickness (mm)	0.379	0.376	0.372	0.001	0.081
Eggshell breaking strength (kgf)	5.150	5.010	5.050	0.030	0.285
Haugh unit	80.57	82.27	80.56	0.410	0.151
Serum calcium (mg dL^{-1})	27.82	27.74	28.44	0.400	0.759
Serum phosphorus (mg dL^{-1})	5.920	5.530	5.560	0.210	0.721
Serum alkaline phosphatase (U L^{-1})	440.1	343.7	371.0	53.50	0.763
Relative tibia weight (%)	0.564^{ab}	0.583^{a}	0.520^{b}	0.010	0.050
Seedor index (mg mm^{-1})	98.98	100.7	93.18	1.400	0.065
Tibia breaking strength (kgf)	23.11^{a}	26.93^{a}	16.57^{b}	1.140	<0.001
Tibia calcium content (%)	22.26	20.74	20.53	0.340	0.078
Tibia phosphorus content (%)	8.22	8.470	8.190	0.140	0.711
Tibia ash content (%)	58.72	57.77	57.23	0.320	0.163
Gizzard weight (g)	21.64	21.75	22.94	0.360	0.271
Renal 1α-hydroxylase (U mg^{-1})	220.6^{a}	176.3^{b}	221.7^{a}	7.750	0.018

$^{a-b}$Means within the same row followed by different letters differ significantly according to Tukey’s test ($p \leq 0.05$). SEM: standard error of the mean.

In trial 2, FI was affected by limestone particle size ratios, with hens fed the 30:70 FL:CL ratio showing higher intake ($p = 0.030$). In contrast, BWG, FCR, EPR, and the percentage of noncommercial eggs were not influenced by the treatments ($p > 0.05$). Similarly, no significant differences were observed between the 50:50 and 30:70 FL:CL ratios for internal and external egg quality, blood parameters, gizzard weight, and bone quality, except for tibial Ca content, which was higher in the 50:50 treatment ($p = 0.002$) (Table 4).

Table 4. Performance, internal and external egg quality, blood parameters, and bone quality of laying hens fed different fine-to-coarse limestone ratios from 66 o 81 weeks of age (trial 2).

Traits	**Fine-to-coarse ratio** **50:50**	 **30:70**	**SEM**	**P-value**
Body weight gain (kg hen^{-1})	0.103	0.138	0.013	0.218
Feed intake (g hen^{-1} day^{-1})	107.2^{b}	111.2^{a}	0.900	0.030
Egg production rate (%)	93.02	93.98	0.540	0.401
Noncommercial eggs (%)	0.690	0.540	0.110	0.546
Feed conversion ratio (g g^{-1})	1.390	1.420	0.010	0.222
Egg mass (g hen^{-1} day^{-1})	56.03	56.27	0.550	0.838
Egg weight (g)	60.20	59.76	0.520	0.688
Yolk index	0.415	0.415	0.002	0.909
Yolk color	5.905	6.033	0.046	0.177
Yolk percentage (%)	25.84	26.33	0.130	0.064
Albumen percentage (%)	64.46	63.91	0.170	0.121
Eggshell percentage (%)	9.670	9.750	0.080	0.626
Shell weight per surface area (mg cm^{-2})	81.15	81.76	0.610	0.637
Eggshell thickness (mm)	0.361	0.360	0.003	0.872
Eggshell breaking strength (kgf)	4.380	4.410	0.050	0.818
Haugh unit	78.99	76.82	0.960	0.276
Serum calcium (mg dL^{-1})	29.92	29.42	0.320	0.454
Serum phosphorus (mg dL^{-1})	5.880	6.050	0.200	0.710
Serum alkaline phosphatase (U L^{-1})	178.6	181.1	17.00	0.944
Relative tibia weight (%)	0.550	0.578	0.015	0.391
Seedor index (mg mm^{-1})	94.58	101.8	2.290	0.118
Tibia breaking strength (kgf)	17.50	21.03	1.820	0.351
Tibia calcium content (%)	21.38^{a}	20.05^{b}	0.240	0.002
Tibia phosphorus content (%)	8.550	8.400	0.090	0.469
Tibia ash content (%)	60.37	59.27	0.460	0.252
Gizzard weight (g)	21.88	22.84	0.449	0.299
Renal 1α-hydroxylase (U mg^{-1})	193.0	186.3	8.330	0.701

$^{a\text{-}b}$Means within the same row followed by different letters differ significantly according to the F-tests ($p \leq 0.05$). SEM: standard error of the mean.

4. Discussion

Given that approximately two-thirds of the Ca deposited in the eggshell is absorbed directly from the diet, while the remaining one-third is mobilized from bone due to the gap between daytime feeding and nighttime shell calcification (Nys et al., 2022), studies on limestone particle size are particularly important for laying hens. Notably, a meta-analysis encompassing 251 observations across 40 publications reported no significant effects of limestone particle size or its interaction with hen age on egg production or egg weight (Hervo et al., 2022). The authors attributed these findings to the fact that, in all experiments included in the meta-analysis, hens received adequate energy and nutrients to meet their requirements. Our findings are consistent with these results as no differences were observed on productive performance of laying hens in both phases. Other studies also reported that physical form of limestone did not affect laying performance (Świątkiewicz et al., 2015; Hervo et al., 2023). Guo and Kim (2012) have previously suggested that dietary Ca level and genetic strain could affect egg production. In the present study, dietary treatments were formulated to fully meet the hens' nutritional requirements. In addition, the Ca levels did not alter among diets, and the same genetic line was used. Thus, under these conditions, limestone particle size seems not to affect laying performance.

Although the FI was not influenced in the early phase (22-41 weeks of age), the higher proportion of CL in diets increased the intake of older hens (66-81 weeks of age). Previous studies have reported that the large particle size or mixture of calcium sources have beneficial effects on FI, compared to small particles, as finely ground limestone tends to provide insufficient mechanical stimulation in the gizzard, which may negatively affect FI (Guo and Kim, 2012; Saki et al., 2019). Additionally, the increase in FI associated with coarser limestone may be related to the lower immediate availability of Ca for intestinal absorption. According to Gloux et al. (2020), older laying hens show reduced expression of TRPV2 and TRPM7 channels in the jejunum, as well as a reduction in vitamin D receptor expression, limiting the efficient absorption of Ca and leading to compensatory FI. Wistedt et al. (2019) reported that at 70 weeks, hens exhibited a reduction in carbonic anhydrase activity in the duodenum, which results in a lower Ca absorption capacity, requiring greater FI to meet the demands for eggshell calcification and bone maintenance. This compensatory behavior may explain the results observed in this study.

A recent meta-analysis using regression models reported that increasing the dietary limestone particle size improves egg specific gravity, EST, and EBS (Hervo et al., 2022). The physiological mechanisms by which CL particles enhance eggshell traits are multifactorial. Coarse particles are retained longer in the gizzard, resulting in a slower release of Ca, particularly during the nighttime when eggshell calcification occurs and Ca intake has ceased (Hervo et al., 2022). This prolonged Ca availability reduces bone mobilization and helps maintain adequate plasma Ca levels during peak eggshell formation. Furthermore, CL is less likely to form insoluble Ca–phytate complexes in the gastrointestinal tract (Hervo et al., 2023). Consequently, increasing CL proportions, with 75% of coarse particles, has been associated with increased eggshell proportion, weight, thickness, and strength (Khosht et al., 2020; Hervo et al., 2023). However, unexpected results were observed in the present study, as eggshell parameters were not influenced by limestone particle size. Rostagno et al. (2024) recommend dietary Ca levels of 41.0 g kg^{-1}

for laying hens with an estimated BW of 1.800 kg and FI of 114.8 g hen^{-1} day^{-1}, and 40.3 g kg^{-1} for hens with a BW of 1.850 kg and FI of 112.4 g hen^{-1} day^{-1}. According to the Lohmann management guide (Lohmann, 2020), Ca levels of 35.7 g kg^{-1} are recommended for hens aged 19 to 50 weeks with an estimated FI of 115 g hen^{-1} day^{-1}, and 40.9 g kg^{-1} for hens older than 70 weeks with an estimated FI of 110 g hen^{-1} day^{-1}. In the present study, hens during the early laying phase had an average BW of approximately 1.81 kg and FI of 115.2 g hen^{-1} day^{-1}, receiving a dietary Ca level of 43.5 g kg^{-1}. During the late phase, hens had a BW of 1.860 kg and FI of 109.2 g hen^{-1} day^{-1}, with a dietary Ca level of 43.0 g kg^{-1}. These Ca levels were higher than the recommendations, which may explain the absence of effects of limestone particle size on eggshell traits, as the FL proportion in the diets was sufficient to meet the Ca requirements for adequate shell formation. Previous studies, in agreement with our findings, have shown that the physical form of limestone does not affect eggshell quality, even in aged laying hens (Świątkiewicz et al., 2015; Lima et al., 2024). These results reinforce that eggshell quality can be maintained as long as the minimum Ca intake and the balance between solubility and gastrointestinal retention time are ensured.

Results of internal egg quality have been shown to be less sensitive to dietary Ca levels and limestone particle size (Pizzolante et al., 2009; Cordeiro et al., 2017), which is consistent with our findings, as replacing FL with CL did not affect yolk or albumen quality. According to Wilson and Duff (1990), internal egg quality is more related to the physiological state of the hen and the storage time of the egg than directly related to the mineral composition of the diet. Furthermore, Van de Velde et al. (1984) reported that during the laying cycle, the activity of medullary bone cells influences Ca mobilization for eggshell formation without directly affecting the internal egg components. In this context, the results of the present study indicate that, despite changes in FL:CL ratios in the diet, the Ca supply was sufficient to maintain internal quality of the eggs during the evaluated period.

The blood parameters were also not affected by the treatments in the present study, suggesting efficient maintenance of mineral homeostasis and stable osteoblastic stimulation across with different particle sizes. These findings are in line with those reported by Świątkiewicz et al. (2015), where the range of limestone particle size from 0.2–0.6 mm to. 1.0–1.4 mm did not influence serum Ca, P, and ALP concentrations in hens aged 25 to 70 weeks.

Coarse granulometry was expected to improve bone quality as larger particles are retained longer in the gizzard and provide soluble Ca more gradually, reducing the desynchronization between Ca supply and demand (Bueno et al., 2016; Hervo et al., 2022). This delayed release was expected to promote more efficient Ca absorption in the small intestine and, consequently, reduce the need for bone Ca mobilization (Wistedt et al., 2019). However, such effects were not observed in the present study. Increasing the CL proportion tended to reduce tibial Ca content in the early phase, which may have contributed to a lower tibia breaking strength and RTW, whereas it significantly reduced tibial Ca content in the late phase. Consistent with our findings, Pelicia et al. (2010) reported an interaction between dietary Ca levels and limestone particle size on tibial Ca content in 35-week-old laying hens, showing that increases in both dietary Ca and CL levels led to a decrease in tibial Ca. Jardim Filho et al. (2005) observed inconsistent results regarding the effect of limestone granulometry. When the authors supplied a limestone source 1, hens fed coarser particles had higher tibial Ca content at 53 and 61 weeks of age. However, when they used limestone source 2, the opposite trend was observed, in which coarser particles reduced tibial Ca in hens of the same age. Although both sources had similar geometric diameters, the differences were attributed to the origin of limestone, which resulted in distinct Ca solubility and availability.

The assessment of renal 1α-hydroxylase concentration in response to different limestone particle size ratios represents a novel contribution of the present study. Plasma Ca levels are tightly regulated within narrow limits by feedback mechanisms involving parathyroid hormone (PTH), calcitonin (CT), and vitamin D_3. These hormones coordinate Ca fluxes between the small intestine, bone, and kidneys (Pizauro Junior et al., 2017). In the avian kidney, the enzyme 1α-hydroxylase catalyzes the conversion of the inactive vitamin D_3 form (25-hydroxycholecalciferol) to the hormonally active form (1α,25-dihydroxycholecalciferol) (Nys et al., 2022). The active form of vitamin D_3 plays a pivotal role in Ca homeostasis, enhancing Ca absorption through both genomic and non-genomic mechanisms (Pizauro Junior et al., 2017). A decline in plasma Ca is sensed by the calcium-sensing receptor, which stimulates PTH synthesis. PTH, in turn, activates renal 1α-hydroxylase, increasing the production of active vitamin D_3, which enhances intestinal Ca absorption. Once plasma Ca levels are restored, the expression of PTH is downregulated via negative feedback, thereby maintaining homeostasis (Pizauro Junior et al., 2017). In our study, birds fed a 50:50 FL:CL ratio showed lower renal 1α-hydroxylase concentrations compared to those fed 60:40 or 30:70 ratios. This may be due to the balance between fast and slow Ca release, especially under pre-slaughter fasting. Diets with more FL may have caused early Ca absorption, leading to serum Ca decline during fasting and stimulating PTH and 1α-hydroxylase to recover Ca homeostasis. Conversely, higher CL may have delayed Ca availability, producing a similar effect.

Thereby, the 50:50 ratio provided a steady Ca supply, maintaining Ca homeostasis and reducing the need for vitamin D_3 activation.

5. Conclusion

In conclusion, dietary FL:CL ratios did not affect laying performance, blood parameters, or eggshell quality in either young or older hens. The absence of differences in shell traits likely reflects the adequate dietary Ca levels supplied across treatments. Although higher CL inclusion increased feed intake in older hens, it did not result in improvements in eggshell quality. Increased proportions of CL reduced tibia strength and tibial Ca content, whereas the 50:50 FL:CL ratio promoted more stable Ca homeostasis. Overall, when dietary Ca is sufficient, modifying limestone particle size does not improve eggshell or bone quality, regardless of hen age, but may influence Ca regulation. Further studies should investigate eggshell characteristics, tibial Ca deposition, and renal 1α-hydroxylase activity.

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GENERAL CONCLUSION

The present study provides valuable insights into the effects of dietary NPP reduction across two laying phases. Overall, reducing dietary NPP from 3.8 or 3.6 to 2.2 g/kg did not impair performance or egg quality. Moreover, lower dietary NPP levels were beneficial by increasing tibia Ca and P content between 26 and 41 weeks of age and enhancing the thickness and breaking strength of eggshell between 66 and 81 weeks of age. Therefore, reducing dietary NPP represents a viable strategy to decrease environmental P excretion and potentially reduce diet formulation costs without compromising laying hens' productivity.

Dietary FL:CL ratios did not affect laying performance, blood parameters, or eggshell quality. The lack of differences in shell traits likely reflects the adequate Ca levels supplied. Although CL inclusion increased feed intake in older hens, it did not enhance shell parameters. Higher CL proportions reduced tibia strength and Ca content, while the 50:50 ratio showed more stable Ca homeostasis. Overall, when dietary Ca is sufficient, changing limestone particle size does not improve shell or bone quality but can influence

Ca regulation. Further studies should explore eggshell traits, tibial Ca, and renal 1α-hydroxylase.