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Effect of Feeding Dietary Calcium on Egg Production, Egg Defects, Tibia, and Femur Bone Status during Extended Laying Cycle in Commercial Layers

Apoorva K. Shinde[1], Vijaysinh D. Lonkar[1]*, Avinash S. Kadam[1], Vitthal S. Dhaygude[2], Sanjay M. Bhalearao[3], and Satyawan M. Agivale[4]

[1]Department of Poultry Science, KNP College of Veterinary Science, Shirwal, Dist. Satara, MAFSU, Maharashtra, INDIA
[2]Department of Veterinary Pathology, KNP College of Veterinary Science, Shirwal, Dist. Satara, MAFSU, Maharashtra, INDIA
[3]Department of Animal Nutrition, KNP College of Veterinary Science, Shirwal, Dist. Satara, MAFSU, Maharashtra, INDIA
[4]Department of Veterinary Surgery & Radiology, KNP College of Veterinary Science, Shirwal, Dist. Satara, MAFSU, Maharashtra, INDIA

*Corresponding Author: vijulvet1982@gmail.com

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Abstract

A total of 192, 80-week-old commercial layers (BV-300) were randomly assigned to four groups viz., A, B, C, and D (48 hens/group; 4 replicates/group). Laying hens from groups A, B, C, and D were fed with 3.85%, 4.00%, 4.20%, and 4.40% Ca (65% coarser and 35% fine powder form) in their diets, respectively, from 81 to 100 weeks of age. Hen Day Egg Production (HDEP) and egg defect parameters were recorded at four-week intervals. Weight, biometry, relative bone index, histomorphometry, ash content of tibia and femur bones, and keel bone scoring were performed at the end of 100 weeks. Results indicated that the overall HDEP was significantly (p<0.05) higher in layers fed with 4.00 (B) and 4.40% (D) Ca than 3.85 (A) and 4.20% (C). Feeding 4.00%, 4.20%, and 4.40% Ca levels significantly (p<0.05) reduced total egg defects (%) and thereby increased normal egg (%) production than 3.85% Ca level. Among egg defects, 4.00%, 4.20%, and 4.40% Ca levels decreased significantly (p<0.05) percent pimpled eggs, and numerically percent wrinkled, corrugated, misshapen, and cracked egg defects than 3.85% Ca levels. Various dietary Ca levels did not influence the tibia and femur's biometrical parameters (length and width), weight, ash, and relative bone index. The 4.00% Ca-diet led to significant increases in tibia and femur medullary cavity width and femur cortical thickness (p<0.05), while tibia trabecular thickness decreased (p<0.05). Additionally, a better keel bone score suggests improved efficiency in 4.00% Ca-fed laying hens. It concludes that the 4.00% dietary Ca during the extended laying cycle helps to improve egg production and bone status with minimum egg defects.

Keywords: Extended Laying Cycle, Femur, Keel Bone, Layers, Tibia.

Introduction

The poultry industry targets modern layers to get 500 eggs in a 100-week (extended) laying cycle (Bain *et al.,* 2016). Maintaining production persistency, egg quality, and skeletal health throughout the last phase of the production period will make the extended laying cycle profitable. Osteoporosis in high egg-producing laying hens, especially during the extended laying cycle, can be avoided by bone mineralization through nutrition. Calcium (Ca) is an essential mineral crucial in maintaining eggshell quality and bone strength (Ahmed *et al.*, 2013). The incidence of downgraded or defective eggs represents an economic loss for the poultry industry (Roberts, 2004; Wolc *et al*., 2012). The incidence of egg abnormalities, egg cracking, and poor shell quality increases with the hen age (Zita *et al*., 2009). During later stages of egg production, an adequate Ca supply can maintain the egg quality (Whitehead and Fleming, 2000). Insufficient dietary Ca during laying adversely affects the eggshell quality (Hartel, 1990; Leeson and Summers, 2005; Duran *et al*., 2018) and bone strength (Whitehead, 2004). The Ca requirement in the extended laying cycle is not fully exploited. Inclusion of adequate dietary Ca during the extended laying cycle would help minimize egg defects, and bone problems, resulting in good performance and quality.

Throughout the extended laying cycle, it is essential to monitor bone health, with particular attention to medullary bones such as the tibia and femur. Laying hens have cortical, cancellous, and medullary bones (Riczu *et al*., 2004). Medullary bone is a highly labile woven bone lying in the marrow cavities and acts as Ca storage for eggshell formation. Bone quality is closely related to egg production and eggshell quality (Kim *et al.*, 2012). This experiment was designed to study the effect of feeding dietary calcium on egg production, egg defects, tibia, and femur bone status during extended laying cycles in commercial layers.

Materials and Methods

Experimental Design

The study was conducted in Completely Randomized Design (Snedecor and Cochran, 1994) at the Department of Poultry Science, KNP College of Veterinary Science Shirwal, Satara, Maharashtra, India. A total of 192, BV-300 strains of commercial layers were assigned to four groups A, B, C, and D, during the extended laying cycle (81 - 100 weeks). Each group consisted of 48 hens, further subdivided into four replicates containing 12 hens per replicate. Iso-caloric and iso-nitrogenous basal layer diets were formulated as per the nutrient requirement of the BV - 300 commercial strain. These iso-caloric and iso-nitrogenous diets contained 3.85%, 4.00%, 4.20%, and 4.40% Ca in groups A, B, C, and D, respectively, from 81 - 100 weeks of age. The diet included calcium in the proportion 65% coarser and 35% fine powder form. The layers were reared in California individual cages under identical management conditions. The ingredient and nutrient composition of different layer diets is mentioned in Table 1.

Parameters

Hen Day Egg Production

The percent Hen-Day Egg Production (HDEP) was recorded (Number of eggs produced/number of hens present x 100) at four weekly intervals from 81 to 100 and for an overall period of 81 -100 weeks.

Egg Defects

The daily eggs laid from each group were examined for defects viz., calcium-deposited eggs, cracked eggs, shell-less eggs, wrinkled eggs, pimpled eggs, misshapen eggs, and corrugated eggs to obtain the percentage of respective egg defects. The daily eggs laid by the laying hens were visually inspected for respective egg defects as per Plate 1. The average percentage of each egg defect (number of defective eggs/number of eggs produced x 100) was calculated at four weekly intervals and the data was presented for an overall period of 81 - 100 weeks.

Table 1: Ingredient and nutrient composition of different experimental layer diets fed during extended laying cycle.

Treatment	A (3.85% Ca)	B (4.00%Ca)	C (4.20%Ca)	D (4.40%Ca)
Ingredients (%)				
Maize	52.250	52.600	53.150	53.500
Soybean meal deoiled cake	5.120	5.300	5.520	5.380
Deoiled Rice Bran	22.460	21.550	20.140	18.850
Rapeseed Meal	3.200	3.200	3.320	3.270
Groundnut Cake	6.000	6.000	6.000	6.600
Limestone Powder	3.465	3.598	3.780	4.030
Marble Grit	6.435	6.682	7.020	7.300
Mineral Premix [1]	0.140	0.140	0.140	0.140
Vitamin Premix[2]	0.050	0.050	0.050	0.050
Monocalcium Phosphate	0.100	0.100	0.100	0.100
Salt	0.150	0.150	0.150	0.150
Sodium Bi-carbonate	0.180	0.180	0.180	0.180
L - Lysine	0.060	0.060	0.060	0.060
DL-Methionine	0.040	0.040	0.040	0.040
Liver tonic	0.050	0.050	0.050	0.050
Choline chloride (60%)	0.150	0.150	0.150	0.150
Toxin binder	0.100	0.100	0.100	0.100
Phytase 5000	0.010	0.010	0.010	0.010
NSP zyme 2000	0.013	0.013	0.013	0.013
Acidifiers	0.030	0.030	0.030	0.030
Total	100.0	100.0	100.0	100.0
Nutrient composition				
ME (kcal/kg)	2456.10	2455.97	2456.80	2456.17
CP (%)	13.72	13.70	13.70	13.71
Ca (%)	3.85	4.00	4.20	4.40
Available phosphorus (%)	0.30	0.30	0.29	0.28
Crude fat (%)	2.18	2.19	2.21	2.22
Crude fibre (%)	4.50	4.39	4.22	4.04
Acid insoluble ash (%)	1.56	1.54	1.51	1.48
Digestible lysine (%)	0.50	0.50	0.50	0.50
Digestible methionine (%)	0.24	0.24	0.24	0.24

Each kg of Trace mineral[1] contain: Copper- 15.00g, Iodine- 1.00g, Iron-60.00g, Manganese-80.00g, Selenium- 0.30g, Zinc-80.00g and Cobalt-0.50g; Each kg of vitamin[2] contain: Vit.A - 22.50 MIU, Vit.D_3 - 4.50 MIU, Vit.E -60.00g, Vit.K - 8.00g, Vit.B_1 - 4.00g, Vit.B_2 - 20.00g, Vit.B_6 - 6.00g, Vit.B_{12}- 0.03g, Niacin - 60.00g, Calcium D Pantothenate - 30.00g, Folic Acid - 4.00g, Biotin - 0.20g and Vit.C - 100.00g

a) Pimpled egg

b) Shell less egg

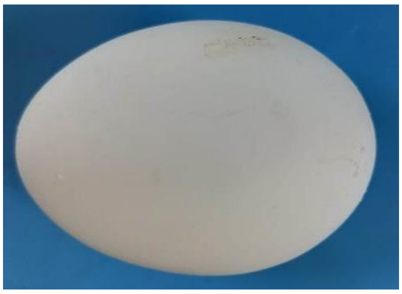
c) Wrinkled egg

d) Cracked egg

e) Corrugated egg

f) Calcium deposited egg

g) Misshapen egg



h) Normal egg

Plate 1. Variour types of egg defects (a to g) and normal egg (h) photographs used in the study for visual inspection

Tibia And Femur Weight, Biometry, And Relative Bone Index

At the end of the 100th week of age, a total of 32 apparently healthy laying hens were selected (8 laying hens per treatment) and slaughtered using cervical dislocation (Physical method). The birds selected for slaughter were close to the average body weight of the treatment. The intact femur and tibia were carefully dissected and de-fleshed from each slaughtered hen. The tibia and femur bones (right and left) were weighed and stored for further bone biometry and histomorphometry. The length and width of the Right (Rt) and Left (Lt) tibia and femur bones were measured Groupwise by performing the X-ray on the machine (Siemens model No. 10092611, 1000 mA, Optitop) to obtain the average length and width of femur and tibia. The film focal distance was adjusted to 90cm. Width of the bone was taken at three points (At the proximal one-third region, mid-diphysial region, and at the distal extremity of bone), and the length of the bone was taken at lateral condyle to cnemial crest for tibial and from trochanter major to lateral condyle for the femur. The relative bone index was calculated based on the body weight of laying hen and the weight of bone as per Song *et al.* (2022).

Relative bone index = bone weight (g) / BW (g) ×100

Bone Histomorphometry

Only the right tibia (n = 8 per treatment) and femur (n = 8 per treatment) were processed for histological examination. The tibia and femur bones were fixed in a 10% neutral buffered formalin and decalcified. Decalcification was done in 10% formic acid for 20 days (Luna, 1968). The bone tissues, after decalcification, were processed by routine paraffin embedding technique, blocks were prepared, and sections were cut and stained with the Hematoxylin and Eosin (H & E) staining method (Luna, 1968). The sections were examined under a microscope (4x, 10x, and 40x power of objective lenses). Four slides each from the tibia and femur were used to measure cortical and trabecular thickness. In each slide, cortical thickness was measured at three different sites and the trabecular width was measured at three different areas from six trabeculae.

Bone Ash

Only the left tibia (n = 8 per treatment) and femur (n = 8 per treatment) bones were used for ash estimation. The bones were defatted by soaking in petroleum ether for 48 hrs (Ramarao *et al.*, 2019) and then dried in a hot air oven at 100^0 for 3 hrs. The bone ashing was done in a muffle furnace at $600^0 \pm 20$ ˚C for 2 hrs (Ramarao *et al.*, 2019). The material left in the silica crucible after ashing was the total ash.

Keel Bone Scoring

The keel bone scoring was performed at the end of the 100 weeks of age from the slaughtered hens. The scoring was done on 8 birds per treatment. The keel bone scoring was performed as Normal (Score 1), Mild keel curvature condition (Score 2), Moderate keel curvature condition (Score 3), and Severe keel curvature (Score 4).

Statistical Analysis

The data was analyzed by one-way ANOVA with the help of IBM SPSS Software-20. The "Duncan's Multiple Range Test" (MRT) post-hoc analysis was done to test the significant mean differences between the groups with significance levels defined at $p < 0.05$.

Ethical Approval

Institutional Animal Ethics Committee of KNP College of Veterinary Science Shirwal, Satara, Maharashtra Animal & Fishery Sciences University, Nagpur, India approved this research work (Reg No of establishment: 309/GO/Re/SL/2000/CPCSEA). The birds were treated following the guidelines of the local ethics committee.

Results and Discussion

Hen Day Egg Production (HDEP)

During 81 - 84 weeks of age, the HDEP was significantly ($p<0.05$) higher in groups B and D than in A and C (Table 2). During 85 - 88 weeks of age, there was a significantly ($p<0.05$) highest HDEP in Group D, followed by B, A, and C. The HDEP during 89 - 92, 93 - 96, and 97 - 100 weeks of age did not differ significantly among different groups. However, groups B and D recorded numerically higher HDEP. The HDEP for an overall period of 81 - 100 weeks of age differed significantly ($p<0.05$) among different groups. Significantly ($p<0.05$), higher HDEP was recorded in groups B and D than in groups A and C. The results indicated that the dietary Ca influenced HDEP during the extended laying cycle. In accordance with the present results, **previous studies** of El Boushy and Papadopoulos (1979), Keshavarz (1986), Bar *et al.* (2002), Liu *et al.* (2007), Safaa *et al.* (2008) and Zhao *et al.* (2020) demonstrated that dietary Ca influences egg production in laying hens. The dietary Ca levels of 4.00% and 4.40% improved HDEP during the extended laying cycle in the present study. Safaa *et al.* (2008) recommended more than 3.50% dietary Ca (4 g Ca/day/hen) in the late phase of egg production. In the present study, dietary Ca between 4.00 to 4.40% increased egg production, but the 4.40% Ca group recorded higher defective eggs. Hence, by considering higher egg production with low total egg defect, it is recommended to use 4.00 or 4.20 % dietary Ca during the extended laying cycle rather than 4.40% Ca.

Table 2: Hen day egg production (%) of layers fed with different levels of calcium during the extended laying cycle

Groups	Age in weeks					
	81-84	85-88	89-92	93-96	97-100	81-100
A (3.85%Ca)	67.86^{a} ± 2.68	72.25^{ab} ± 2.49	63.99 ± 3.92	57.66 ± 1.55	45.98 ± 3.68	61.55^{a} ± 1.67
B (4.00% Ca)	80.14^{b} ± 2.91	77.16^{bc} ± 3.23	72.17 ± 4.55	61.38 ± 3.40	51.11 ± 3.07	68.39^{b} ± 3.02
C (4.20% Ca)	65.96^{a} ± 1.52	68.20^{a} ± 1.55	62.35 ± 2.01	58.90 ± 2.32	51.81 ± 4.97	61.44^{a} ± 1.52
D (4.40% Ca)	77.61^{b} ± 2.13	80.29^{c} ± 2.14	72.62 ± 0.88	62.35 ± 1.51	52.16 ± 2.66	69.01^{b} ± 1.39
SEm	1.90	1.61	1.87	1.15	1.78	1.29
SD	7.756	6.530	7.481	4.576	6.978	5.198
p-value	0.002	0.020	0.084	0.484	0.622	0.028

Means bearing different superscripts within a column differ significantly (p<0.05). SD: Standard Deviation, SEm: Standard Error Mean

Egg Defects

The data on various egg defects recorded are depicted in Table 3. The laying hens from groups B, C, and D laid significantly (p<0.05) higher percentage of normal eggs than control A. The percent pimpled eggs laid by hens fed from groups B, C, and D were significantly (p<0.05) deceased than the control A. Including higher levels of Ca (4.00, 4.20, and 4.40%) during the extended laying cycle helps decrease the percentage of pimpled eggs. The percentage of wrinkled, corrugated, shell-less, and cracked eggs was comparable among all groups. Compared to control A, there was a numerical reduction in the corrugated, shell-less, and cracked eggs in the B, C, and D groups. Elaroussi *et al.* (1994) suggested that the increase in cracked eggs seen in aged layers could be a result of disturbances related to Ca homeostasis. An *et al.* (2016) found that increasing dietary Ca decreased the incidence of cracked eggs and was associated with improvement in eggshell strength and thickness. The percentage of misshapen eggs was significantly higher in group D than in groups A, B, and C. The percentage of eggs having calcium deposits was comparable among all groups but found to be numerically increased in group D than in others. This indicated level of 4.40% Ca increased the percentage of eggs with calcium deposits compared to 3.85, 4.00, and 4.20% Ca in laying hens during the extended laying cycle. The percentage of total egg defects was significantly (p < 0.05) higher in control group A than the groups B, C, and D. Al-Batshan *et al.* (1994) found a decrease in eggshell quality of aged laying hens attributed to reduced intestinal Ca uptake. Carrillo *et al.* (2020) reported that defective eggshell production has been mainly linked to a decrease in gastrointestinal absorption of calcium. Alfonso-Carrillo *et al.* (2021) conducted a study using 100 Brown Nick (H&N) laying hens from 100 -105 weeks of age and found that hens with a high production and good eggshell quality have a greater capacity to mobilize the calcium needed for eggshell formation. However, among the higher dietary Ca groups (4.00, 4.20, and 4.40% Ca), the inclusion of 4.40% Ca level in group D significantly increased the percentage of total egg defect. The significant increase in total egg defects in groups A (lowest Ca-diet: 3.85%) and D (highest Ca-diet: 3.85%) might be due to an imbalance in calcium homeostasis or reduced Ca calcium uptake (Al-Batshan *et al.*, 1994; Carrillo *et al.*, 2020). Laying hens fed with 3.8% Ca increased the rate of broken, soft-shelled, and unmarketable eggs than 4.0% Ca reported by Salajegheh *et al.* (2020) corroborates with present findings. Molnar *et al.* (2016) reported that the aging of layers affected most egg quality traits but was still acceptable at the end of lay (74 to 92 weeks) indicating that the commercial layers could extend the laying cycle. They reported variability of egg deformation at the end of the production cycle.

It was found that 4.00 and 4.20% Ca levels were beneficial for increasing normal egg production with minimum egg defects than 3.85 and 4.40% Ca. An *et al.* (2016) found a significant (p < 0.01) linear reduction in cracked eggs with the increase in dietary Ca from 3.50 to 4.70% and concluded that aged laying hens require relatively higher Ca. Safaa *et al.* (2008) found that the birds fed the high-Ca diet (4.00%) had a lower rate of damaged eggs than those fed the low-Ca diet (3.50%). Zhao *et al.* (2020) found a significant (p<0.05) increase in the rate of broken eggs at low dietary Ca (1.5%) than normal (3.70%). These earlier workers' findings indicated the need for higher dietary Ca during extended laying cycles to reduce egg defects and increase the number of normal egg production.

Table 3: Overall (81-100 week) egg defects (%) in layers fed with different dietary calcium

Groups	Normal egg	Pimpled	Wrinkled	Corrugated	Misshapen	Shell-less	Cracked	Calcium Deposited	Total Defects
A (3.85%Ca)	71.43[a] ± 1.16	15.42[c] ± 0.67	9.50 ± 1.45	0.41 ± 0.27	0.29[a] ± 0.10	0.75 ± 0.22	3.93 ± 0.48	0.40 ± 0.17	28.58[b] ± 1.16
B (4.00% Ca)	78.64[b] ± 2.10	11.53[ab] ± 1.66	7.11 ± 0.55	0.22 ± 0.09	0.30[a] ± 0.09	0.60 ± 0.25	3.27 ± 0.82	0.21 ± 0.05	21.36[a] ± 2.10
C (4.20% Ca)	77.55[b] ± 0.94	10.27[a] ± 0.34	9.91 ± 0.69	0.09 ± 0.03	0.47[ab] ± 0.13	0.29 ± 0.03	2.58 ± 0.51	0.40 ± 0.24	22.46[a] ± 0.94
D (4.40% Ca)	76.25[ab] ± 2.14	14.65[bc] ± 0.98	6.63 ± 1.70	0.13 ± 0.05	0.81[b] ± 0.14	0.39 ± 0.10	2.10 ± 0.34	0.77 ± 0.28	23.75[ab] ± 2.14
SEm	1.03	0.72	0.65	0.07	0.08	0.09	0.31	0.11	1.03
SD	4.130	2.876	2.609	0.286	0.301	0.365	1.241	0.420	4.130
p-value	0.046	0.012	0.184	0.431	0.026	0.311	0.169	0.314	0.046

Means bearing different superscripts within a column differ significantly (p<0.05), SD: Standard Deviation, SEm: Standard Error Mean

Tibia And Femur Weight, Biometry, And Relative Bone Index

The right and left femur and tibia weights and their relative bone index (Table 4) did not differ significantly among different groups. The different levels of Ca in layer diet during the extended laying cycle did not influence femur and tibia bone biometrical parameters concerning length and width. Keshavarz *et al.* (1993), did not find the effect of 3.5 to 4.05 dietary Ca (Ca intake was increased from 3.85 to 4.40 g/hen per day) on the tibia weight of 62-week-old laying hens. Lee *et al.* (2021) reported that tibia characteristics like weight, length, width, and breaking strength are not influenced by different Ca sources fed to old layers after 73 weeks of age. The shorter tibial and femoral bone length was reported by Zhao *et al.* (2020) when the laying hens were fed with that low-Ca diet (1.50%) than the normal Ca group (3.7%) during 22-34 weeks. Similarly, Jiang *et al.* (2013) also found poor bone quality at lower dietary Ca (2.62%) than 3.70 and 4.40% Ca. They reported that higher Ca benefits bone homeostasis than low Ca. The range of Ca level included in the present study was 3.85 to 4.40%, which did not affect the femur and tibia bone biometrical parameters.

Bone Histomorphometry

Significantly (p<0.05) higher femoral cortical thickness was observed in group C than others, while for tibial bone it was comparable (Table 5). In the present study, 4.00% dietary Ca during the extended laying cycle was adequate to maintain the cortical thickness of the femur and tibia bone. The trabecular thickness of the tibia bone was significantly (p<0.05) decreased in group C than others while it was comparable for the tibia bone among different groups. Medullary cavity width of tibia and femur bones was significantly (p<0.05) increased in groups A and B than in C and D during the extended laying cycle.

Feeding of 4.00% Ca during the extended laying cycle significantly increased femur bone cortical thickness, decreased tibia bone trabecular thickness, and increased tibia and femur bone medullary cavity width compared to other dietary Ca levels included in the experiment. This indicated that the laying hens are efficiently utilizing 4.00% Ca. Zhao *et al.* (2020) reported that a low-Ca diet (1.5%) significantly (p<0.05) resulted in thinner cortical thickness of femurs and tibias than normal Ca (3.70%). Yamada *et al.* (2021) found similar tibial trabeculae area, perimeter, number, or spacing in 25-week-old hens (4.94% Ca-diet) than in 52-week-old hens (4.40% Ca-diet) of Hy-Line W36. The findings of decreased trabecular thickness in good egg-producing hens were supported by Kerschnitzki *et al.* (2014), who studied the rapid alterations of avian medullary bone material during the daily egg-laying cycle and reported that during eggshell calcification, the mineral content and the size of trabeculae of medullary bone decrease markedly. However, Zhao *et al.* (2020) found a thinner cortex with more cavities in the cortex and cancellous bone; the trabecular bone network was fewer, and the trabeculae were less well-connected in the low-Ca group (1.50%) as compared to normal calcium (3.70%). Hence, the present study's 4.00% Ca level was sufficient to maintain the cortical and trabecular thickness.

Table 4: Average weight, length, width, relative bone index, and ash content of the femur and tibia bones at the end of 100 weeks of layers fed with different levels of calcium

Groups	Femur					Tibia				
	Weight (g)	Relative bone index	Length (cm)	Width (cm)	Ash (%)	Weight (g)	Relative bone index	Length (cm)	Width (cm)	Ash (%)
A (3.85%Ca)	6.58 ± 0.10	0.50 ± 0.01	7.85 ± 0.03	0.76 ± 0.00	51.56 ± 0.59	8.86 ± 0.15	0.67 ± 0.01	11.44 ± 0.08	0.60 ± 0.02	52.25 ± 0.57
B (4.00% Ca)	6.68 ± 0.22	0.48 ± 0.01	7.89 ± 0.07	0.77 ± 0.01	52.00 ± 0.64	8.91 ± 0.25	0.64 ± 0.02	11.45 ± 0.10	0.60 ± 0.01	52.31 ± 0.62
C (4.20% Ca)	6.89 ± 0.18	0.50 ± 0.01	7.95 ± 0.04	0.77 ± 0.01	51.37 ± 0.43	8.92 ± 0.24	0.64 ± 0.02	11.49 ± 0.10	0.59 ± 0.008	52.00 ± 0.59
D (4.40% Ca)	6.86 ± 0.21	0.49 ± 0.02	7.98 ± 0.05	0.76 ± 0.01	51.62 ± 0.67	9.12 ± 0.14	0.65 ± 0.01	11.48 ± 0.04	0.59 ± 0.009	52.81 ± 0.84
SEm	0.090	0.006	0.028	0.004	0.284	0.097	0.007	0.041	0.005	0.324
p-value	0.599	0.793	0.340	0.592	0.898	0.808	0.528	0.971	0.957	0.856

Table 5: Histomorphometrical observations of femur and tibia bones of layers fed with different levels of calcium in the extended laying cycle

Groups	Femur			Tibia		
	Cortical thickness (mm)	Trabecular thickness (mm)	Medullary bone cavity width (cm)	Cortical thickness (mm)	Trabecular thickness (mm)	Medullary bone cavity width (cm)
A (3.85%Ca)	0.366^{a}±0.02	0.157±0.02	0.48^{bc} ± 0.02	0.446±0.01	0.245^{c}±0.08	0.36^{b} ± 0.02
B (4.00% Ca)	0.508^{c}±0.01	0.144±0.00	0.50^{c} ± 0.02	0.451±0.02	0.182^{a}±0.06	0.36^{b} ± 0.01
C (4.20% Ca)	0.428^{b}±0.01	0.154±0.00	0.38^{a} ± 0.03	0.469±0.03	0.217^{b}±0.01	0.28^{a} ± 0.03
D (4.40% Ca)	0.398^{ab}±0.01	0.145±0.00	0.40^{ab} ± 0.05	0.397±0.03	0.199^{ab}±0.06	0.28^{a} ± 0.03
SEM	0.010	0.005	0.017	0.014	0.005	0.014
p-value	0.000	0.758	0.024	0.329	0.000	0.030

Means bearing different superscripts within a column differ significantly ($p<0.05$)

Bone Ash

The percent femur and tibia bone ash (100th week) from different groups are shown in Table 4. The femur and tibia ash did not differ significantly among different groups. The results indicated that feeding different Ca levels in the layer diet during an extended laying cycle did not influence the ash status of the femur and tibia bones. Bone ash weight is a fundamental measure of bone mineral content. Similar to the present findings, Keshavarz (1986) found that the tibia ash was not significantly affected by different dietary Ca levels (3.50, 4.50, and 5.50%) from 56 to 72 weeks of age in layers. An *et al.* (2016) also found no significant differences in the tibia ash in aged laying hens (70 - 80 weeks of age) fed with varying Ca levels (3.50, 3.80, 4.10, 4.40, or 4.70%). In contrast, El Boushy and Papadopoulos (1979), and Frost and Roland (1991) found a significant correlation between the level of dietary Ca and tibial ash content in the first laying cycle. However, dietary Ca during the extended laying cycle did not influence the bone ash weight in the present study. The nonsignificant difference in the bone ash weight of femur and tibial bones in the present study indicated that increasing supplementation of Ca from 3.85 to 4.40% did not help to increase the medullary bone mineralization as bone ash weight is a direct verification of the degree of mineralization of the bone tissue itself (Kim *et al.*, 2012).

Keel Bone Scoring

The percentage of keel bone score is mentioned in Table 6. It was observed that 75% of laying hens had normal keel (score 1) in group B, followed by groups A, C (50%), and D (25%). The laying hens from groups A, B, and D recorded 12.5% mild curvature of the keel (score 2), while it was not evident in group C. A higher incidence of moderate curvature keel (score 3) was observed in group D, followed by C (25%), A, and B (12.5%). The laying hens from groups A, C, and D recorded 25% severe curvature of the keel bone (score 4), while severe curvature was not evident in group B. The overall results of the study on keel bone scoring indicated that the 75% laying hens supplemented with a moderate level of Ca (4.00%) had 1 score (normal keel), 12.5% hens had 2 and 3 scores (mild and moderate curvature) and no hens with 4 score (severe curvature). A higher percentage (25%) of severe curvature keel bone was observed in groups A, C, and D. The higher percentage (37.5% and 25%) of moderate curvature keel bone was observed in groups D and C, respectively. Moderate Ca (4.00%) during the extended laying cycle was found to decrease the keel bone deformity as compared to lower (3.85%) and higher levels (4.20 and 4.40%) of Ca in commercial laying hens. Cufadar *et al.* (2011) reported that 76-week-old H&N Brown Nick laying hens (molted at 60 weeks of age) should be fed 3.60% Ca to maintain bone quality. Yamada *et al.* (2021) reported prolonged egg production affects skeletal integrity and structural bone loss because structural bone is not restored during the laying period. Habig *et al.* (2021) reported that the keel bones were more often broken in hens of the layer lines with a high laying rate than the lines with a moderate laying rate.

Table 6: Keel bone scoring of laying hens slaughtered at 100 weeks of age

Groups	Normal keel (Score 1)	Mild curvature (Score 2)	Moderate curvature (Score 3)	Severe Curvature (Score 4)
A (3.85%Ca)	4 (50%)	1 (12.5%)	1 (12.5%)	2 (25%)
B (4.00% Ca)	6 (75%)	1 (12.5%)	1 (12.5%)	0 (0.0%)
C (4.20% Ca)	4 (50%)	0 (0.0%)	2 (25%)	2 (25%)
D (4.40% Ca)	2 (25%)	1 (12.5%)	3 (37.5%)	2 (25%)

Conclusion

In conclusion, feeding 4.00% dietary Ca in commercial layers during the extended laying cycle (81 - 100 weeks age) was beneficial for improving egg production, medullary bone status with minimum egg defect, and reducing keel bone deformities.

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Contribution by Authors

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Conflict of Interests

There is no conflict of interest.

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