# PHYSIOLOGY AND REPRODUCTION

# Influences of low level of dietary calcium on bone characters in laying hens

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**ABSTRACT** Cage layer fatigue (**CLF**), which is commonly caused by calcium deficiency in the feed, leads to loss of structural bone and increase of bone fragility. In order to investigate the influence of low-calcium diets on bone quality and strength, histopathology, and egg quality, 72 laying hens were randomly allocated to 2 groups at 22 wk of age and received low calcium and control calcium until 34 wk, respectively. Egg production, feed consumption, BW, and egg quality were measured throughout. Bone mineral density, bone biomechanical properties, and histomorphology of femurs and tibias were assessed after birds were sacrificed in 26, 30, and 34 wk. The results showed that low-calcium (1.5%) diets decreased BW, feed consumption, and egg production. The broken eggs rate increased, and the eggshell strength and thickness were lower in treated birds than those in control birds at 30 wk and 34 wk. Femoral and tibial bone index and bone mineral density were lower, cortical thicknesses were thinner, and bone length were shorter over time when birds are in a low-calcium diet than those in control birds. In biomechanical properties, the values of stiffness, Young's modulus, and breaking strength were lower in both femurs and tibias in low-calcium hens at 30 wk and 34 wk than those in bones of control hens. In histomorphology of bone, the cortex turned thinner and there were more cavities in cortex and cancellous bone; the trabecular bone network was fewer, thinner, less cohesive, and generally fragmented; and trabeculae were less well-connected in low-calcium birds. Some cell nuclei in cancellous bone disappeared, and vacuolation was observed in bone cells. There appeared osteoid in cortex bone and cancellous bone in tibias. It was concluded that low-calcium diets could facilitate the development of osteoporosis characterized by an increase of osteoid and loss of structural bone and decrease the values of bone quality and strength, accompanied with a decrease in egg production and egg qualities, which may elucidate the developing mechanism of CLF.

## INTRODUCTION

Cage layer fatigue (**CLF**) was first noticed after laying hens begin to be housed in cages in the mid-20th century (Couch, 1955). Urist (1960) suggested that CLF should more properly be referred to as "cage layer osteoporosis." Hens producing eggs at a high rate are the ones most susceptible to this disease. CLF in laying hens leads to loss of structural bone and increased incidence of fracture at various skeletal sites by the end of the laying period (Whitehead and Fleming, 2000). Wilson and Hughes (1993) suggested possible changes in genetic factors, and systems of husbandry might have been responsible for the increase of bone fragility. Approximately 30% of hens have one or more broken bones associated with bone weakness during their lifetime (Olgun and Aygun, 2016).

Skeletal health in laying hens is a major welfare and economic problem and seriously damages the public perception of egg production. Furthermore, CLF resulted in chronic pain and distress to birds (Webster, 2004; Swiatkiewicza et al., 2015). Bone is a reservoir for calcium and phosphorus. Calcium is the critical nutritional factor for eggshell formation and bone health. It is well known that the amount of calcium required by laying hens depends on their stage of production. The recommended amount of calcium for laying hens consuming feed at 100 g/day is 3.5% in China (Ministry of Agriculture of the People's Republic of China, 2004). Roland et al. (1996) indicated that increasing the dietary Ca level

(5%) increased bone quality without any adverse effect on egg production. Härtel (1989) proposed that minimum calcium concentration must be restricted to 2.5% (25 g/kg) and that calcium content of the food should not generally exceed 3.0% (30 g/kg). Although hens will overconsume energy when fed a calcium-deficient diets, the extra energy consumed appears to have little or no beneficial effect on egg size or production (Roland and Bryant, 1994). However, calcium deficiency can quickly induce bone loss if a hen has high metabolic need for the element (Elaroussi et al., 1994; Webster, 2004). As low dietary calcium may affect bone metabolism and eggshell quality, the purpose of the present study was to obtain further information on eggshell quality, bones properties, bone mineral density (**BMD**), bone biomechanical properties, and histopathology with low dietary calcium in early laying hens. Furthermore, this study will provide a better basis on the development of CLF from bone quality and strength and histomorphology in femurs and tibias on poultries feeding with low-calcium diets.

## MATERIALS AND METHODS

### Experimental Design

The study was conducted under the guidelines approved by the Animal Care and Use Committee of the Northeast Agricultural University, China. Ethics approval for all procedures to be carried out was obtained from Committee for Ethics in Research of the Northeast Agricultural University, China. Seventy-two Lohmann Write layers at 22 wk of age were randomly allocated into treated group (Ca) and control group (C) and fed in conventional cages for 90 d from June to September in 2018. During the experimental period, hens received light for 16 h/day. All birds were each given 110 g feed per day. Water was provided ad libitum by a nipple drinker. The treated group was fed with low level of calcium (1.5%), and the control group was fed with normal calcium (3.7%) according to the commercial management guide. Other compositions of diets in both groups were same, mainly including protein, energy, vitamins, and minerals.

### Productive Performance Record and Sample Collection

Egg production and broken eggs rate were recorded every day, and egg production per week was calculated from 22 wk. Feed intake and BW were measured and recorded at the end of every week from 22 wk to 34 wk of age. Ten eggs were randomly collected in each group at a day before sacrifice and were stored for 1 d at 4°C for subsequent measurements of egg qualities including egg broken rate in the 26th, 30th, and 34th week.

Twelve hens in each group were sacrificed through cervical dislocation at the last day of the 26th, 30th, and 34th week. The blood samples were obtained from every hen by cardiac puncture before euthanasia. Serum was separated by centrifugation at 1,500 $g$ for 15 min at 4°C and stored at

−80°C until analysis. The intact femurs and tibias were carefully dissected and defleshed from each bird, weighed, and stored at −80°C until further processing.

### Eggshell Traits

Egg weight, eggshell thickness, and eggshell breaking strength were determined using Material Testing Machines (Robomation Corp., Tokyo, Japan).

### Bone Properties

Before analysis, femurs and tibias were cleaned off all tissue and weighed. Relative bone index was calculated using the following formula:

$$\text{Relative bone index} = \text{bone weight(g)/BW(kg)}$$

The length of femurs and tibias were measured, and the cortical thicknesses of femurs and tibias were measured at the breaking location at the mid diaphysis of every femur or tibia, using an electronic slide caliper (Guilin, China).

### Biomechanical Properties

Biomechanical properties in all the birds were determined by using a testing machine (MTS-858; Hampshire, UK). In brief, both femurs and tibias were mounted across a supporting bridge with a gap of 50 mm (femurs) and 80 mm (tibias), respectively. The weight load was applied to the midpoint of the shaft under a crosshead speed of 100 mm/minute until failure. The values for stiffness (N/m), Young's modulus (MPa), flexural rigidity (Nm$^2$), and maximum force (N) were recorded and calculated by using the testing machine.

### Bone Mineral Density

The BMD was determined on the whole femoral or tibial bones by using a Dual-Energy X-ray BMD Determinator (KEDI KORS, Korea). The whole bones positioned on a custom support platform were determined, and the values of BMD were showed on the machine.

### Histomorphometry

Eight femurs and 8 tibias from each group were fixed in 10% formalin phosphate buffer solution and decalcified for 6 wk in 10% EDTA phosphate buffer solution. The samples were dehydrated in different gradients of ethyl alcohol and hyalinized in xylene. Then 3 parts from every bone were chosen, which are proximal diaphysis, mid-diaphysis, and distal diaphysis, to embed in paraffin wax, and 5-μm sections were sliced from each block and mounted on glass slides. A hematoxylin and eosin stain was used on the samples. Sections were scanned using an optical microscope (BX46; Olympus, Japan) at various magnifications, and 3 photomicrographs at every part of femoral and tibial bone were produced using 200× magnification.
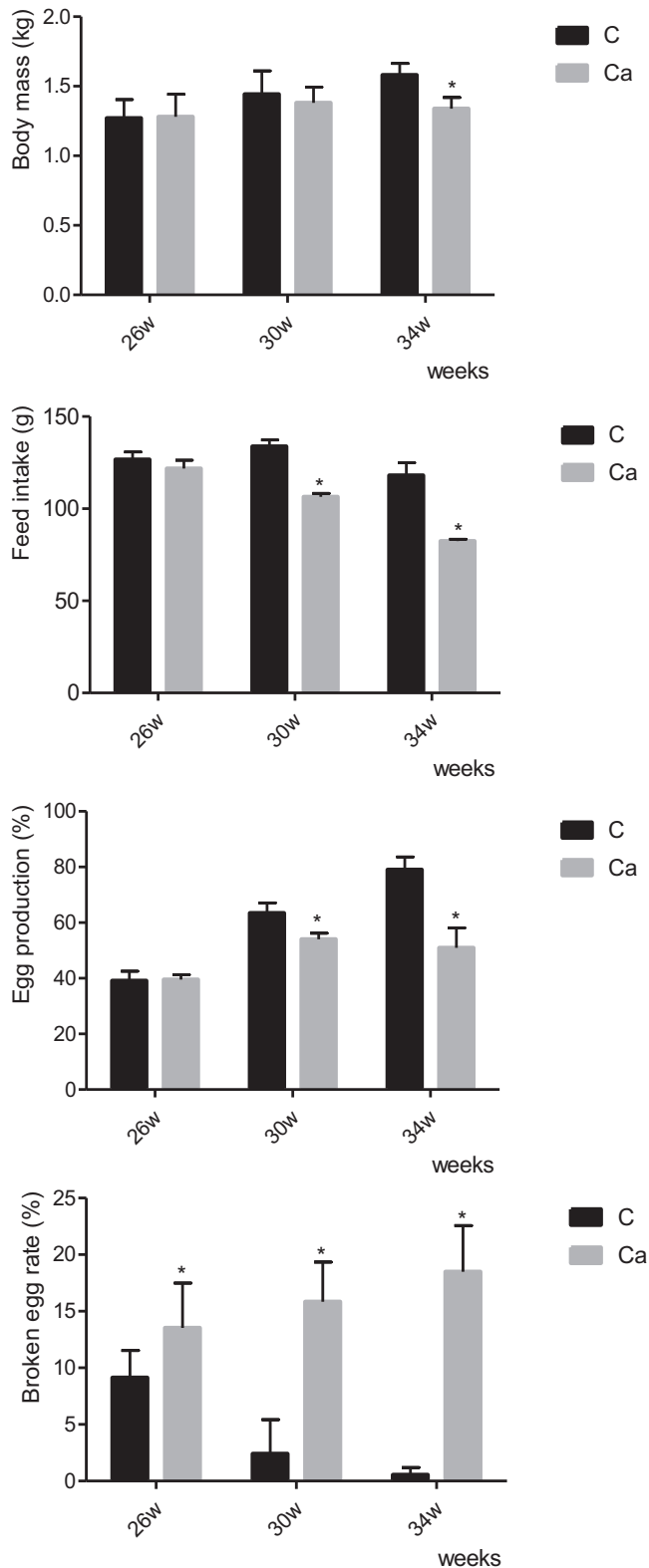
**Figure 1.** The productive properties in treated group (Ca) and control group (C). *Difference in 2 groups (*P* < 0.05).

## Biochemical Indicators

Calcium and phosphorus concentrations in serum were measured using an automatic biochemistry analyzer (BS-300; Mindray, Shenzhen, China) using the accompanying commercial calcium and phosphorus kits.
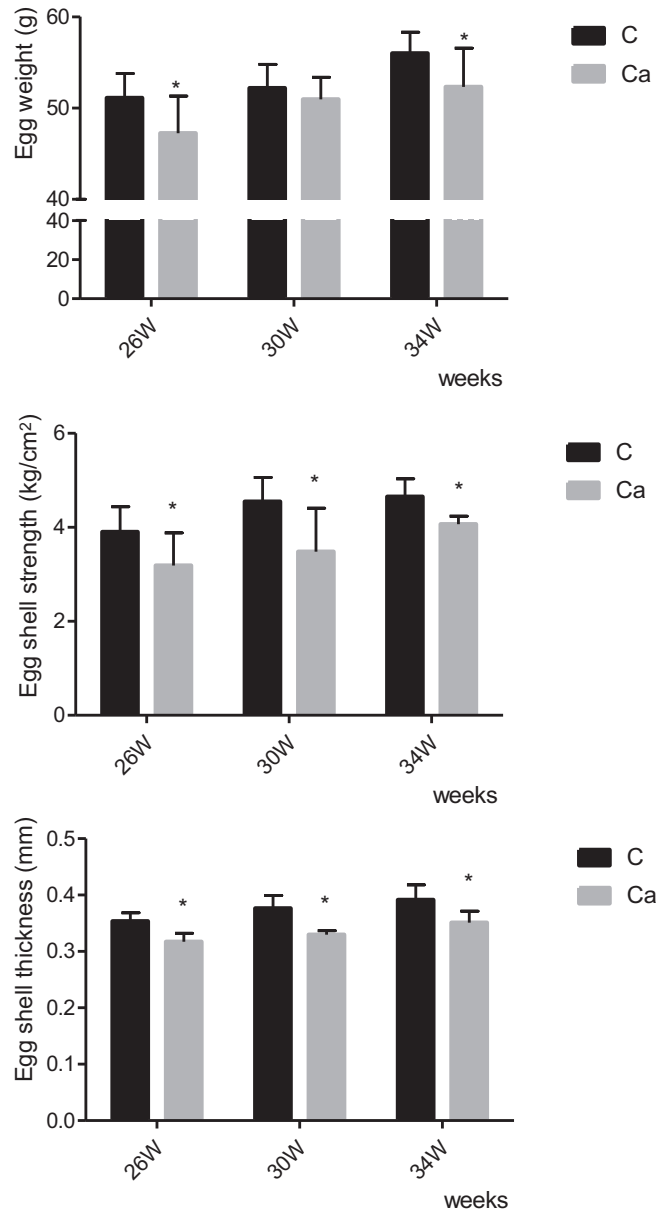


**Figure 2.** Eggshell traits in treated group (Ca) and control group (C). *Difference in 2 groups (*P* < 0.05).

## Statistical Analysis

Date were analyzed by using ANOVA two-way test with subsequent post hoc tests. A significant difference (*P* < 0.05) was denoted with an asterisk (*) and analyzed with GraphPad 5.01 software (GraphPad Software Inc., San Diego, CA).

## RESULTS

During the experiment period, the body mass of hens and egg production in control group were increasing with days. There was obviously a decrease in feed consumption and egg production in 30 wk and 34 wk in the treated group compared with the control group (Figure 1). Furthermore, the broken egg rate increased (*P* < 0.05) in the treated group. Two uteruses in 12 treated birds became degenerated and necrosed in
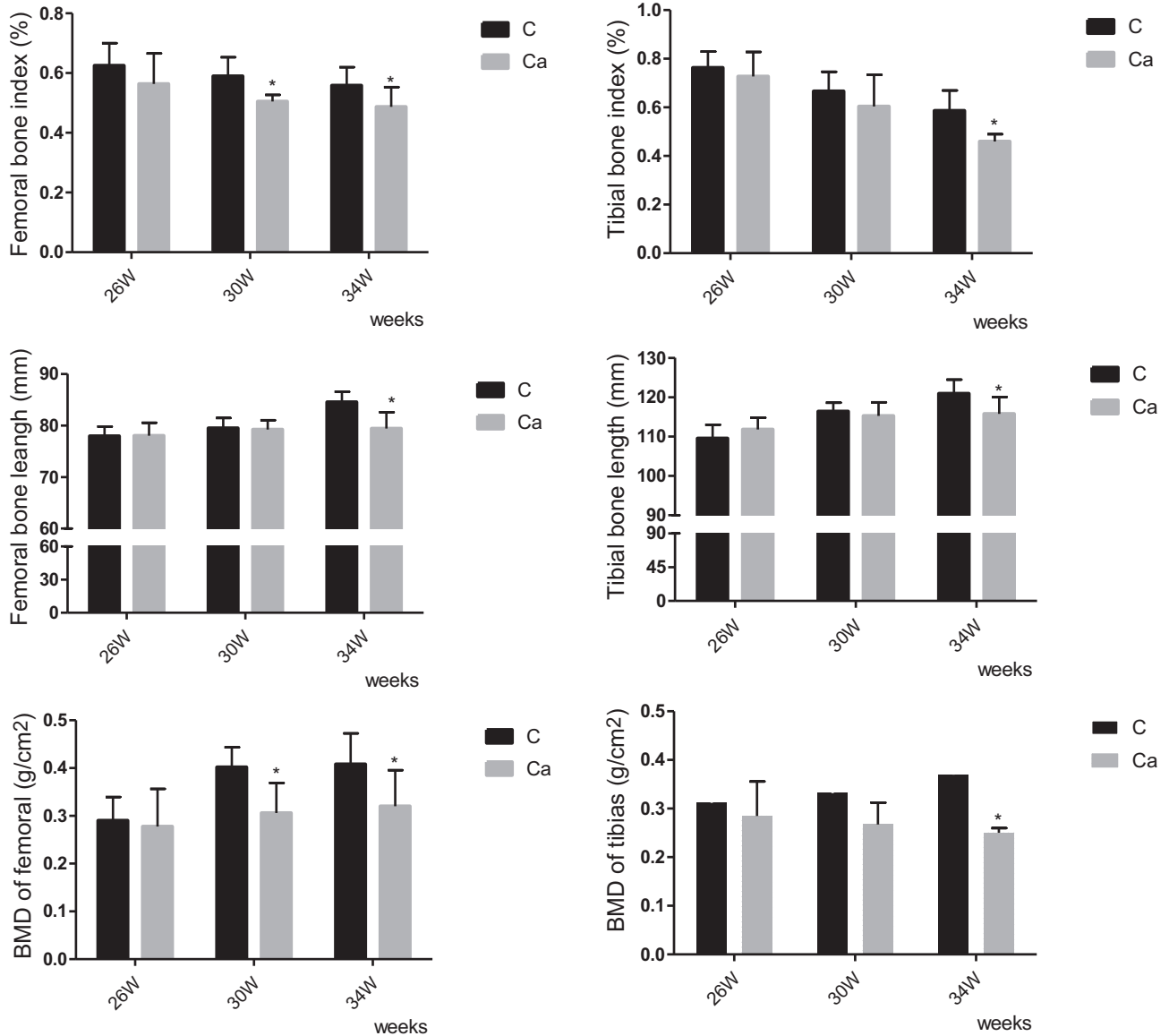
**Figure 3.** The bone properties and bone mineral density (BMD) of femurs and tibias in treated group (Ca) and control group (C). *Difference between 2 groups ($P < 0.05$).

34 wk. The uterine wall became thinner, and some eggs in the 2 uteruses had partly calcified or became smelly.

Eggshell traits are shown in Figure 2. There were no obvious differences in egg weight between 2 groups. However, the eggshell strength and eggshell thickness in the treated group were lower ($P < 0.05$) than those in the control group in 26, 30, and 34 wk.

Femoral bone index and BMD were significantly lower ($P < 0.05$) at 30 wk and 34 wk in treated than in control hens (Figure 3). However, tibial bone index and BMD were lower ($P < 0.05$) only at 34 wk (Figure 3). The bone length grew longer with days in control hens; however, the femoral and tibial bone length were shorter ($P < 0.05$) in treated hens than those in control hens at 34 wk.

The results of measurement in biomechanical properties of femurs and tibias were given in Figure 4. The values of stiffness, Young's modulus, and breaking strength of femurs in treated birds were lower

($P < 0.05$) than those in control birds at 26 wk and 34 wk. However, the values of stiffness, Young's modulus, and breaking strength of tibias in treated birds were lower ($P < 0.05$) than those of tibias in control birds at 34 wk.

As shown in Figure 5, the cortical thicknesses of femurs and tibias were thinner ($P < 0.05$) in the treated group than those in the control group at the same time. Furthermore, the cortical thicknesses of femurs and tibias in control birds were increasing with consuming feed from 26 wk to 34 wk.

There were no differences between the calcium and phosphorus concentrations in serum (Figure 6), but there were increasing trends with the extension of feeding time in both groups.

## Histopathology of Femurs and Tibias

Representative hematoxylin and eosin images of the distal diaphysis of femurs and tibias are displayed in
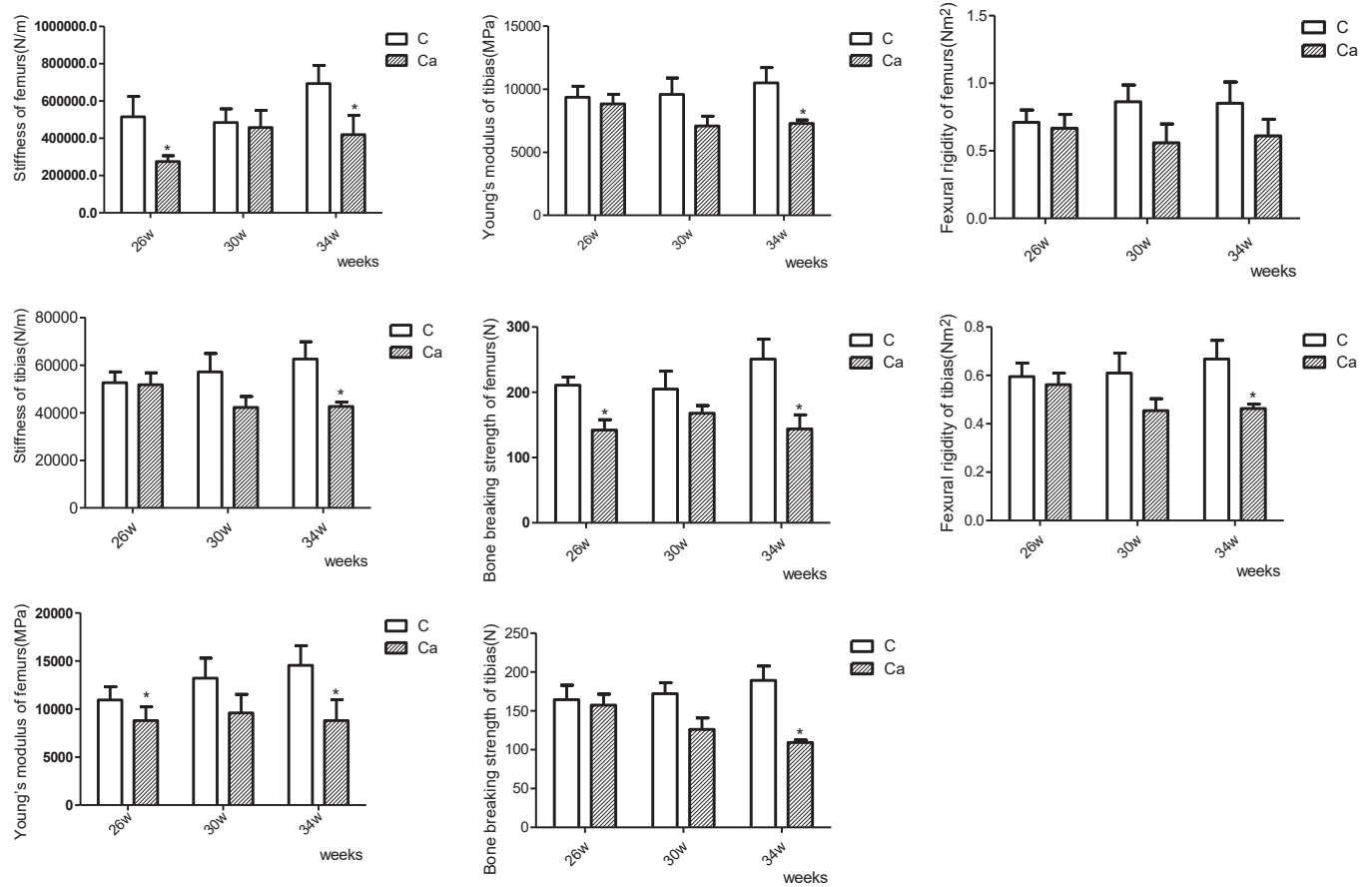
**Figure 4.** Biomechanical properties of femurs and tibias in treated group (Ca) and control group (C). *Difference between 2 groups ($P < 0.05$).

Figure 7 in treated group (Ca) and control group (C) at 26 wk, 30 wk, and 34 wk. In general, the cortical bone of femurs from low-calcium birds was thinner than that from control birds. On the other hand, bone from low-calcium birds show more large resorption cavities at the junction of cortical bone and cancellous bone in which the cortical bone was partially replaced by osteoid bone (Figures 7A, 7C and 7E). In contrast, the cortical bone from control birds was thicker and generally does not show resorption centers (Figures 7B, 7D, and 7F). The trabeculae network was generally fragmented, and the trabecular bone became less cohesive and more vacuolated from femurs in the treated group. The photomicrographs of tibial bone had similar changes with femoral bone, such as more cavities in cortex bone and more vacuolations in cancellous bone in 34 wk. Furthermore, there appeared more osteoid in cortical bone and cancellous bone in tibias at 30 wk and 34 wk.

## DISCUSSION

Modern laying hens have a high susceptibility to bone fracture. In a survey of end-of-lay hens arriving at processing plants, Gregory and Wilkins (1989) reported that broken bones were found in about 30% of hens before slaughter and that the proportion had risen to 90% by the time that the carcasses had reached the end of the processing line. Osteoporosis is the major cause of skeletal problems in laying hens, especially during peak production, and is commonly caused by calcium deficiency in the feed (Whitehead, 2002; Riczu et al., 2004). Wilson et al. (1992) has confirmed that the bone loss is caused mainly by the progressive development of osteoporosis. Osteoporosis may result, in part, from prolonged periods of high egg production during which time structural bone is mobilized without an opportunity for regeneration (Whitehead and Wilson, 1992; Knowles and Wilkins, 1998). In correspondence with the statement of Roland (1986) that laying hens fed calcium-deficient diets will reduce egg production, the low-calcium (1.5%) diet obviously had an effect on body mass, feed consumption, and egg production in the present study. The main reason perhaps is reduction of digestive and absorptive abilities of birds fed with low-calcium diets for a long period. Furthermore, it is an important factor that uterine hypofunction owing to deficient calcium verified by the present study brought out reduction of egg production. Low dietary calcium had no adverse influence on egg weight (Roland et al., 1996) that was consistent with this research. In the present study, the broken eggs rate increased and the eggshell strength and eggshell thickness in the treated group were lower than those in the control group. Hence, our results suggested that a low-calcium diet resulted in decrease of egg production and eggshell quality.
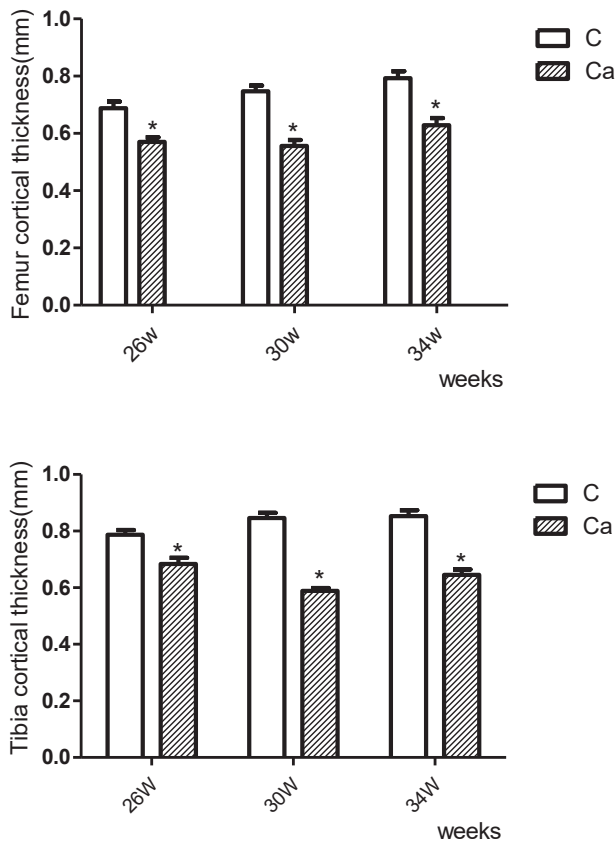
**Figure 5.** The femoral and tibial cortical thickness in treated group (Ca) and control group (C). *Difference between 2 groups ($P < 0.05$).



**Figure 6.** The calcium and phosphorus concentrations in serum in treated group (Ca) and control group (C).

Whitehead and Fleming (2000) proposed that the major causes of osteoporosis are a switch in osteoblastic bone formation from structural to medullary bone and continued osteoclastic resorption of structural bone at the onset of hen sexual maturity. Deficient calcium in diets usually may be more prone to generate osteoporosis. A laying hen has 3 bone types: cortical, cancellous (or trabecular), and medullary bone (Riczu et al., 2004). The 2 former types provide most of the structural integrity to the bone, whereas the latter type acts as a source of available calcium for eggshell formation (Dacke et al., 1993). Although mobilization of medullary bone to increase calcium availability (Whitehead and Fleming, 2000; Whitehead, 2004) also results in resorption of exposed structural bone during the period of eggshell construction, the net effect of cortical and trabecular bone resorption without subsequent reconstruction is structural bone loss and skeletal weakening in the course of the production cycle. On the histopathology of femurs and tibias in this study, thickness of cortical bone was decreased, and there was a less cohesive system and less well-connected trabeculae. Actually, cortical and trabecular structural bone formation is ceased in favor of woven, medullary bone deposition at the onset of sexual maturity (Wilson et al., 1992; Hudson et al., 1993; Whitehead and Fleming, 2000). The decrease of tibial bone strength between 25 and 50 wk implies considerable loss of structural bone (Whitehead and Fleming, 2000). In accordance with the histomorphology
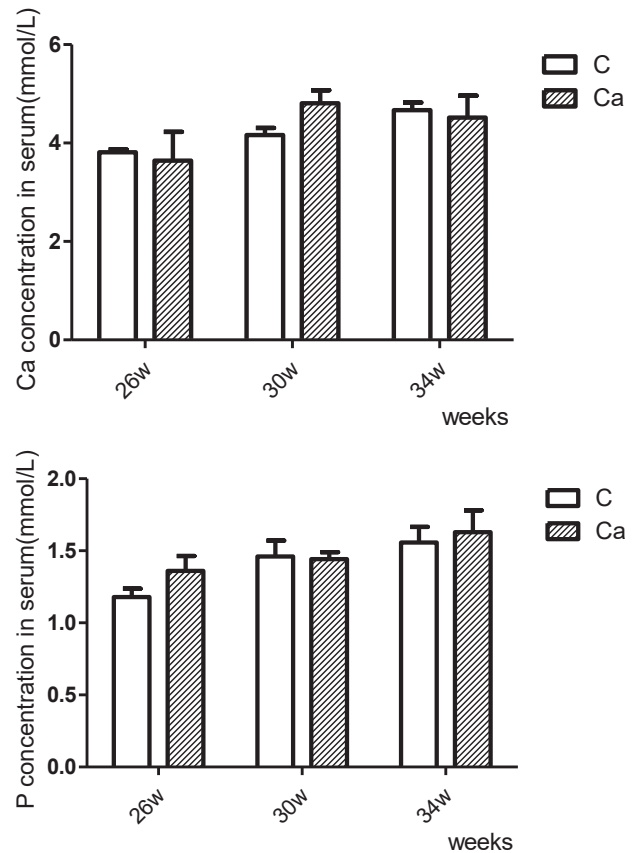
of femurs and tibias in this study, the cortical bone and trabeculae became thinner, and more osteoid were present in low-calcium birds because of widespread loss of structural bone.

The breaking strength of a hen's bone is closely related to morphometric measurement and radiographic density of its structural components (Fleming et al., 1994). Whitehead and Wilson (1992) recorded a loss of bone strength throughout the laying period of normal birds. Fleming et al. (1998) stated that tibial breaking strength decreased between 25 and 50 wk, which implies considerable loss of structural bone. In this study, the biomechanical properties and BMD of femurs and tibias were increasing from 26 to 34 wk in control birds; however, both of them in femurs and tibias were obviously decreased in low dietary calcium birds. The results indicated that low-calcium diets facilitated development of osteoporosis and change of bone structure over time.

Various measurements of bone-breaking strength were good indicators in any study related to bone minerals. Furthermore, the thickness of cortical bone was the most important structural parameter contributing to bone-breaking strength, and cortical bone had the greatest contribution to global bone mechanical properties (Rodriguez-Navarro et al., 2018). The cortical thicknesses of femur and tibia from low-calcium hens were obviously thinner than those of control hens. On the other hand, the cortical thickness and bone strength in control birds increased with consuming feed, which was consistent
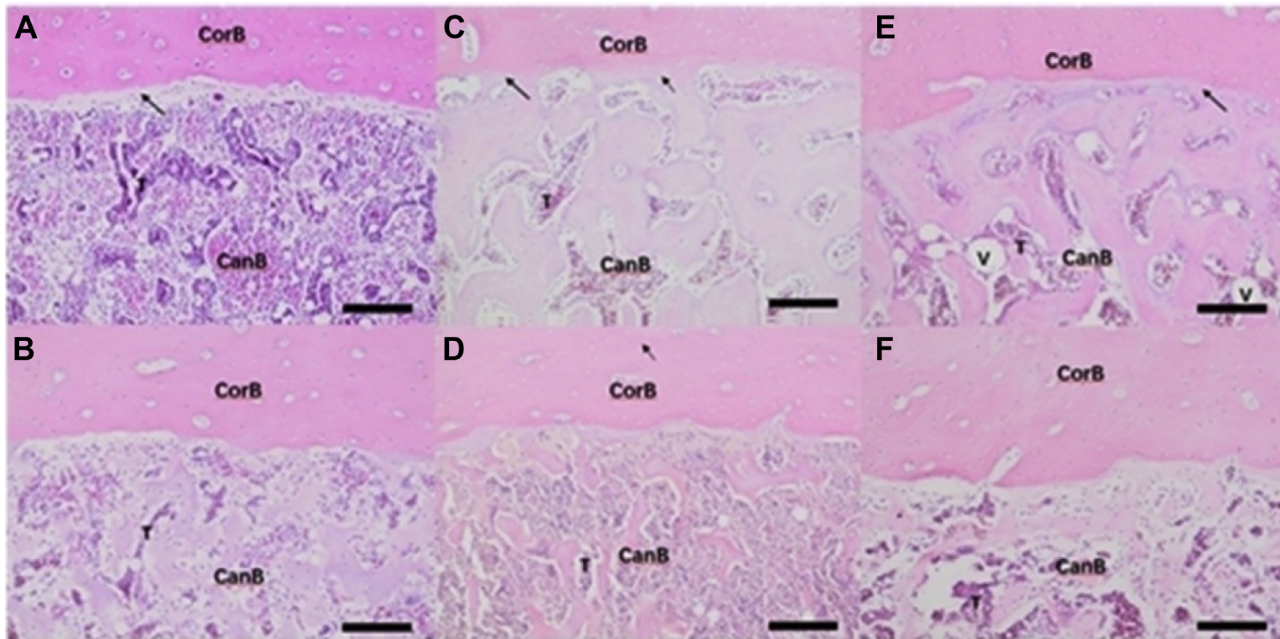
**Figure 7.** Distal diaphysis of femur and tibia in 26 wk, 30 wk, and 34 wk. (A) Femur in low-Ca group in 26 wk; (B) femur in control group in 26 wk; (C) tibia in low-Ca group in 30 wk; (D) tibia in control group in 30 wk; (E) femur in low-Ca group in 34 wk; (F) femur in control group in 34 wk. The cortex turned thinner, cancellous bone (CanB) immerse cortex bone (CorB) in part, and the boundary between cortex bone and cancellous bone was dim (see arrow in A, C, and E). The trabeculae bone (T) network was fewer, less cohesive, generally fragmented, and more laminar in low-calcium birds than in control birds. Some cell nucleus in cancellous bone disappeared and became vacuolation (V) and less well-connected trabeculae in cancellous bone in 34 wk. The osteoid (↑) appeared between cortex bone and cancellous bone in 30 wk and 34 wk. There were more cavities in cancellous bone in low-Ca in 34 wk.

with the report of Mc Coy and Reilly (1996) that the density and breaking strength of the bones from non-cage layer osteoporosis hens were consistently greater than those of the CLF hens. Jiang et al. (2013) also stated that hens fed control calcium (3.7%) and high-calcium diets (4.4%) had higher bone strength and bone density than those fed low-calcium diets (2.62%). Both femurs and tibias are weight-bearing bones and have similar structural characteristics as per the assessment of CLF. Hence, researchers prefer one of them as a bone-related index to measure. Furthermore, the cortical thicknesses of femurs and tibias showed similar changes with both biomechanical properties and bone properties in the present study, suggesting low-calcium diets decreased the bone quality and strength in hens.

Approximately 99% of total body calcium is located in the skeleton, and most of the remaining calcium is intracellular, with less than 0.1% of total calcium mass being present in the extracellular fluid. In the present study, there were no differences in concentrations of calcium and phosphorus in serum in 2 groups, although other indexes of bone showed distinguished differences. Because of the importance of calcium in physiologic processes, blood calcium levels are preserved in a normal level until bone reserves are severely depleted. Therefore, hypocalcemia may not be measurable in serum although the skeletal problems had occurred.

In conclusion, our results demonstrate that low-calcium diets can facilitate development of osteoporosis characterized by an increase of osteoid and loss of structural bone in histomorphology and a decrease in the values of bone quality and strength in femurs and tibias, accompanied with a decrease in egg production and egg qualities in hens. This study may elucidate the developing mechanism of CLF.

## ACKNOWLEDGMENTS

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