Animal Nutrition 18 (2024) 340–355

Contents lists available at ScienceDirect

Animal Nutrition

journal homepage: http://www.keaipublishing.com/en/journals/aninu/

Original Research Article

Dietary supplementation with calcitriol or quercetin improved eggshell and bone quality by modulating calcium metabolism

Yu Fu [a, b], Jianmin Zhou [a], Martine Schroyen [b], Jing Lin [a], Haijun Zhang [a], Shugeng Wu [a], Guanghai Qi [a], Jing Wang [a, *]

[a] Key Laboratory of Feed Biotechnology, Laboratory of Quality & Safety Risk Assessment for Animal Products on Feed Hazards (Beijing), Ministry of Agriculture and Rural Affairs, Institute of Feed Research, Chinese Academy of Agricultural Sciences, Beijing 100081, China
[b] Precision Livestock and Nutrition Laboratory, TERRA Teaching and Research Centre, Gembloux Agro-Bio Tech, University of Liège, B-5030 Gembloux, Belgium

ARTICLE INFO

Article history:
Received 6 November 2023
Received in revised form
3 March 2024
Accepted 16 April 2024
Available online 20 April 2024

Keywords:
Uterine calcium transport
Bone remodeling
Eggshell quality
Bone quality
Calcitriol
Quercetin

ABSTRACT

This study was aimed to investigate the effects of dietary calcitriol or quercetin supplementation on eggshell and bone quality of laying hens. In trial 1, 72 Hy-Line Brown layers (80-week-old) with weak-shelled strength (25 to 30 N) were assigned into 4 dietary treatments with 6 replicates of 3 birds and fed a basal diet (4% calcium level) or basal diets supplemented with 0.5% calcium, 5 μg/kg calcitriol or 500 mg/kg quercetin for 4 weeks. In trial 2, 360 Hy-Line Brown layers (60-week-old) were divided into 3 groups with 8 replicates of 15 birds: control group (basal diet), calcitriol group (basal diet + 5 μg/kg calcitriol), and quercetin group (basal diet + 500 mg/kg quercetin). This trial lasted for 12 weeks. The results showed that dietary calcitriol or quercetin improved eggshell quality in both trials ($P < 0.05$). In trial 2, compared with the control group, both calcitriol and quercetin supplementations improved femoral bone quality, calcium retention of hens and calcium content in uterine fluid at 18.5 h post-oviposition (PO) ($P < 0.05$), along with enhancing uterine morphology. Compared to the control group, supplemental calcitriol or quercetin up-regulated the relative mRNA expression levels of uterine transient receptor potential cation channel, subfamily V, member 6 (*TRPV6*) at 8.5 h PO and plasma membrane calcium-ATPase (*PMCA*), vitamin D receptor (*VDR*), estrogen receptor alpha (*ERα*) at 18.5 h PO ($P < 0.05$), but down-regulated the uterine caspase 3 (*CASP3*) relative mRNA expression level at 8.5 h PO ($P < 0.05$). Meanwhile, the femoral relative mRNA expression levels of tartrate-resistant acid phosphatase (*TRAP*) (up-regulated at 8.5 and 18.5 h PO) and alkaline phosphatase (*ALP*) (up-regulated at 8.5 h PO but down-regulated at 18.5 h PO) were also affected by calcitriol or quercetin supplementation ($P < 0.05$). Compared to the calcitriol, quercetin increased hen-day egg production and femoral medullary bone volume/bone tissue volume but reduced femoral stiffness ($P < 0.05$), which were accompanied by increased relative mRNA expression levels of uterine *TRPV6*, estrogen receptor beta (*ERβ*) at 18.5 h PO ($P < 0.05$). Overall, both dietary calcitriol and quercetin could improve eggshell and bone quality by modulating calcium metabolism of aged layers. Compared to calcitriol, dietary quercetin up-regulated the expression of uterine calcium transporters, without affecting eggshell quality.

1. Introduction

Cracked eggs and brittle bones are two of the most important issues during the late egg production period of laying hens, and they are generally characterized by lower eggshell weight and thinner eggshell thickness (Fu et al., 2022; Gautron et al., 2021; Rodriguez-Navarro et al., 2002) as well as lower bone mass (Bain et al., 2016; Fleming et al., 2006). This suggests that calcium

* Corresponding author.
E-mail address: wangjing@caas.cn (J. Wang).

Peer review under the responsibility of Chinese Association of Animal Science and Veterinary Medicine.

Production and Hosting by Elsevier on behalf of KeAi

https://doi.org/10.1016/j.aninu.2024.04.007

metabolic disorder is a major factor contributing to the reduced eggshell and bone quality during the late laying period of hens. Previous studies have reported that impaired calcium metabolism during the late laying period is associated with a vitamin D_3 metabolism disorder (Bar, 2008) and a decrease of estrogen levels and receptors (Hansen et al., 2003; Liu et al., 2018). Therefore, increasing the circulating level or sensitivity of calcitriol (1, 25-dihydroxyvitamin-D_3, active vitamin D_3) and/or estrogen in hens during the late laying period may be a viable option to mitigate the decline both in eggshell and bone quality.

Dietary addition of calcitriol could increase the blood concentration of calcitriol (Tsang and Grunder, 1993), leading to an increased eggshell weight and weight ratio of layers, and its optimal supplementation level was found to be 5 μg/kg (Soares et al., 1988; Tsang et al., 1990). It is also effective in promoting bone formation and bone mineral retention at oviposition (Frost et al., 1990; Newbrey et al., 1992). Quercetin is a bioactive flavonoid that shares a chemical structure resembling that of estrogen, and its addition in layers' diets increased the circulating estrogen level of laying hens (Liu et al., 2023). A study found evidence of an improvement in eggshell quality with supplementation of 400 to 600 mg/kg of quercetin in the diets (Liu et al., 2013), while another study showed no effect with its inclusion (Liu et al., 2014). To the best of our knowledge, there are few reports on the effects of quercetin on the bone quality of laying hens, however, it has been shown to play a role in preventing bone loss and promoting bone mineral deposition in rats and broilers (Wang et al., 2022; Wong et al., 2020). Thus, further exploration and comparison of the effects of quercetin and calcitriol on eggshell and bone quality are warranted to provide a reference for selecting feed additives during the late laying period.

The regulatory mechanisms of calcitriol and quercetin on eggshell and bone quality are poorly reported. Calcitriol and quercetin have the ability to modulate the development of reproductive organs by activating the vitamin D receptor (VDR) and estrogen receptors (ER), respectively (Amevor et al., 2021; Cheng et al., 2023; Irani and Merhi, 2014). Additionally, the vitamin D responsive element and estrogen responsive element have been identified as present in the promoter region of uterine calcium transporters such as transient receptor potential cation channel, subfamily V, member 6 (TRPV6), calbindin (CALB), plasma membrane calcium-ATPase (PMCA), and sodium-calcium exchanger (NCX) (Bar, 2008, 2009; Jonchère et al., 2012). Thus, supplementing calcitriol or quercetin in diets may improve eggshell quality by stimulating uterine calcium transport. Distinct calcium sources are utilized in the uterine environment during the process of eggshell calcification. Generally, the eggshell calcium is primary derived from the intestinal absorption during the initiation stage of eggshell calcification. However, during the growth stage, it is mainly of skeletal origin (Nys and Le Roy, 2018). Specialized adjustments in uterine calcium transport and bone remodeling could be present to meet the calcium requirements for eggshell calcification. Bone remodeling involves both bone formation and bone resorption, which collectively determine bone quality (Dacke et al., 2015). Exploring the changes in calcium transport and bone remodeling during the initiation and growth stages of eggshell calcification could offer valuable insights into how calcitriol or quercetin affect eggshell and bone quality. Additionally, there is likely a cooperative effect between calcitriol and estrogen in regulating calcium transport, as both have the ability to stimulate the expression of the *VDR* (Inoue et al., 2010) and *ER* (Santos-Martínez et al., 2021). Therefore, conducting a comparative analysis of the effects of calcitriol and quercetin on calcium metabolism could contribute to a more comprehensive understanding of their respective functions and mechanisms.

The first trial of this study investigated the effects of dietary calcium, calcitriol and quercetin supplementation on the eggshell quality of aged laying hens with a low eggshell breaking strength to determine the validity of these additives on improving eggshell quality. The second trial of this study compared the effects of dietary calcitriol and quercetin additions on eggshell and bone quality in a normal commercial flock. The changes in uterine calcium transport and bone remodeling at the initiation and growth stages of eggshell calcification were further explored to reveal the impact of dietary calcitriol and quercetin on eggshell and bone quality of aged laying hens. The findings of this study carry significant implications for selecting more effective strategies to improve eggshell and bone quality in laying hens.

2. Materials and methods

2.1. Animal ethics statement

Animal management and experimental procedures were approved by the Animal Care and Use Committee of Institute of Feed Research, Chinese Academy of Agricultural Sciences (approval No. AEC-CAAS-20200902). All animal experiments were conducted in accordance with the ARRIVE guidelines.

2.2. Experimental design

In the first trial, a total of 1950 Hy-Line Brown laying hens at 79 weeks of age were selected from a commercial farm and caged individually. All eggs were collected from one single day to determine the average breaking strength of this flock, which was 35.68 N. In the following week, 72 hens that laid at least 5 eggs with an average eggshell breaking strength of 25 to 30 N were considered as hens which laid weak eggs. These hens (80 weeks of age) were randomly assigned to 4 dietary treatments with 6 replicates (3 birds each). The basal diet (4% calcium level) was formulated to meet or slightly exceed Chinese Feeding Standard of Chicken (NY/T 33-2004) and National Research Council (NRC, 1994) requirements (Table 1). The other treatments were fed the basal diet supplemented with 0.5% calcium (high calcium), 5 μg/kg calcitriol or 500 mg/kg quercetin. All birds were housed in individual cages, and

Table 1
Ingredient and nutrient levels of the basal diet in trial 1 (air-dried basis, %).

Ingredient	Content	Nutrient level[2]	Content
Corn	59.00	AME, MJ/kg	11.11
Soybean meal	24.53	Crude protein	16.41
Soybean oil	1.80	Calcium	3.89
Limestone	10.60	Methionine	0.41
DL-methionine	0.12	Lysine	0.75
50% choline chloride	0.12	Total phosphorus	0.43
Calcium hydrogen phosphate	0.90	Available phosphorus	0.26
Sodium chloride	0.15	Methionine + Cysteine	0.69
Sodium sulfate	0.20		
Wheat bran	2.40		
Vitamin and mineral premix[1]	0.18		
Total	100.00		

AME = apparent metabolizable energy.
[1] Vitamin and mineral premix provided the following per kilogram of the diet: vitamin A 9500 IU; vitamin D_3 4125 IU; vitamin E 15 IU; vitamin K 2 mg; thiamine 1 mg; riboflavin 8.5 mg; calcium pantothenate 11 mg; niacin 32.5 mg; pyridoxine 8 mg; biotin 0.5 mg; folic acid 1.25 mg; vitamin B_{12} 0.02 mg; Mn 65 mg; I 1 mg; Fe 60 mg; Cu 8 mg; Zn 66 mg; phytase 500 mg.
[2] The numbers of AME and available phosphorus (AP) are calculated based on Feeding Standard of Chicken (NY/T 33-2004), while the others are measured. AME = Corn × AME1 + Soybean meal × AME2 + Soybean oil × AME3 + Methionine × AME4; AP = Corn × AP1 + Soybean meal × AP2 + Calcium hydrogen phosphate × AP3 + Limestone × AP4.

all hens in one replicate were provided with a shared trough feeder for the same diet. The trial consisted of a one-week observation period and a four-week treatment period. All birds were fed the same diet (basal diet) during the observation period.

In the second trial, 360 healthy Hy-Line Brown laying hens (60-week-old) were collected from a commercial farm and then randomly divided into 3 groups that consisted of 8 replicates with 5 adjacent cages each (3 birds per cage). Layers were fed a basal diet or a basal diet supplemented with either 5 μg/kg calcitriol or 500 mg/kg quercetin. The basal diet was based on Chinese Feeding Standard of Chicken (NY/T 33-2004) and National Research Council (NRC, 1994) requirements as shown in Table 2. All hens in one replicate were provided with a shared trough feeder for the same diet. The feeding trial lasted 12 weeks (from 61 to 72 weeks of age) following a one-week adaption period.

Diet samples (approximately 300 g each) were collected using a quartering division method and stored at −20 °C for testing nutrient composition. The samples were dried, milled, and sifted through a 40-mesh sieve (0.425 mm particle size) prior to analysis. Crude protein content was determined through the Kjeldahl method (Kjeltec 8420, Foss Co., Ltd., Beijing, China; method 984.13, AOAC, 2006). Calcium content was analyzed using a flame atomic absorption spectrophotometry (Z2000, Hitachi Co., Ltd., Tokyo, Japan; method 968.08, AOAC, 2006), and total phosphorus content was determined by a colorimetric procedure (UV2700, Shimadzu Co., Ltd., Kyoto, Japan; method 965.17, AOAC, 2006). The amino acid contents were determined using an automatic amino acid analyzer (L8800, Hitachi Co., Ltd., Tokyo, Japan) after hydrolyzing the samples with 6 mol/L HCl at 100 °C for 22 h according to a previous study (Jin et al., 2019). The analyzed values of nutrients are listed in Tables 1 and 2. Additionally, the values of apparent metabolizable energy and available phosphorus listed in Tables 1 and 2 are calculated based on Chinese Feeding Standard of Chicken (NY/T 33-2004).

The temperatures were set to 18 to 22 °C with 60% to 65% humidity throughout the whole trial. All birds were kept under controlled photoperiod (16 h light/8 h dark) and received feed and water ad libitum.

Table 2
Ingredient and nutrient levels of the basal diets in trial 2 (air-dried basis, %).

Ingredient	Content	Nutrient level[2]	Content
Corn	61.00	AME, MJ/kg	11.10
Soybean meal	23.86	Crude protein	16.53
Soybean oil	1.20	Calcium	3.53
Limestone	9.50	Methionine	0.37
DL-methionine	0.12	Lysine	0.80
50% choline chloride	0.12	Total phosphorus	0.45
Calcium hydrogen phosphate	0.90	Available phosphorus	0.27
Sodium chloride	0.15	Methionine + Cysteine	0.65
Sodium sulfate	0.20		
Wheat bran	2.75		
Vitamin and mineral premix[1]	0.20		
Total	100.00		

AME = apparent metabolizable energy.
[1] Vitamin and mineral premix provided the following per kilogram of the diet: vitamin A 9500 IU; vitamin D_3 4125 IU; vitamin E 15 IU; vitamin K 2 mg; thiamine 1 mg; riboflavin 8.5 mg; calcium pantothenate 11 mg; niacin 32.5 mg; pyridoxine 8 mg; biotin 0.5 mg; folic acid 1.25 mg; vitamin B_{12} 0.02 mg; Mn 65 mg; I 1 mg; Fe 60 mg; Cu 8 mg; Zn 66 mg; phytase 500 mg.
[2] The numbers of AME and available phosphorus (AP) are calculated based on Feeding Standard of Chicken (NY/T 33-2004), while the others are measured. AME = Corn × AME1 + Soybean meal × AME2 + Soybean oil × AME3 + Methionine × AME4; AP = Corn × AP1 + Soybean meal × AP2 + Calcium hydrogen phosphate × AP3 + Limestone × AP4.

2.3. Sample collection

In the first trial, 12 eggs per replicate were randomly selected each week to determine the eggshell quality during the observation and treatment periods.

In the second trial, a total of 18 eggs (on three consecutive days) from each replicate were randomly collected at the end of 60, 64, 68 and 72 weeks of age to detect eggshell quality. At the end of trial, 6 eggshells per replicate were randomly selected to determine eggshell ultrastructure, and an additional 6 eggshells per replicate, weighing close to the average eggshell weight, were collected to further measure eggshell components.

After the feeding trial, 2 birds per replicate were randomly selected and maintained in the individual cages to monitor their oviposition period according to the method reported by a previous study (Feng et al., 2023). One of these two birds (per replicate) was sampled at 8.5 h post-oviposition (PO, the initiation stage of eggshell calcification), while the other was sampled at 18.5 h PO (the growth stages of eggshell calcification). The blood was collected by wing vein bleed, and the serum was rapidly separated by centrifugation at 1300 × *g* for 15 min and stored at −20 °C. Then, the birds were euthanized by cervical dislocation for immediate tissue sampling.

All birds had eggs in their uterus, and the characteristics of these eggs met their corresponding eggshell calcification stages. The uterine fluid was carefully aspirated as the egg was removed at the growth stage of eggshell calcification. However, the uterine fluid was too little to collect for further testing at the initiation stage of eggshell calcification. Then, an approximately 1 cm ring segment was removed from the middle part of the uterus. One piece of 1 cm^2 uterine tissue was cut from this segment and fixed in the 4% paraformaldehyde solution for uterine histomorphology. All uterine mucosae were collected from the remaining portion of this segment and stored in liquid nitrogen for further RNA isolation. All uterine tissue collection was performed by one person to ensure consistency.

The humerus and femur on both sides were removed and carefully cleaned of excess tissue. The right bones were stored in liquid nitrogen for further RNA isolation. The left bones collected at 8.5 h PO were used to measure the mineral measurements, mechanical properties and components, and the left bones collected at 18.5 h PO were used to measure geometrical characteristics and histological parameters. The left bones collected at 8.5 h PO were covered with a gauze moistened with 0.9% saline solution and stored at −20 °C, then thawed and rehydrated in saline before testing. The left bones sampled at 18.5 h PO were firstly used to rapidly measure part of the geometrical characteristics, then truncated at the middle. The proximal portion was immersed in formalin to fix, and the other part was used to measure the remaining geometrical characteristics.

2.4. Laying performance

In the first trial, the normal and broken egg number were recorded daily. Eggs were collected and weighed daily during the observation and treatment periods. Hen-day egg production (HDEP), breakage rate and qualified egg rate were calculated weekly with following formula:

HDEP (%) = [(normal egg number + broken egg number)/hen number] × 100;

Breakage rate (%) = (broken egg number/hen number) × 100;

Qualified egg rate (%) = (normal egg number/hen number) × 100.

Only total feed intake for the whole treatment period was recorded.

In the second trial, egg number and egg weight were recorded daily, and total feed intake was recorded weekly. The HDEP, average egg weight, average daily feed intake and feed conversion ratio were calculated every 4 weeks. Feed conversion ratio was expressed as grams of feed intake per gram of egg weight.

2.5. Eggshell physical and mechanical properties, ultrastructure, and components

Eggshell quality determination was carried out as described before (Fu et al., 2021b). The egg weight was determined first. Then, the thickness from 3 points (equator and both poles) for each egg was taken using an Egg Shell Thickness Gauge (Israel Orka Food Technology Ltd., Ramat Hasharon, Israel) and the values averaged to obtain eggshell thickness. The eggshell breaking strength was measured using the Egg Force Reader (Israel Orka Food Technology Ltd., Ramat Hasharon, Israel). The eggshell weight was recorded after removing contents and drying at room temperature until constant weight. The eggshell weight ratio was calculated as eggshell weight/egg weight × 100.

Two pieces (0.5 cm^2 each) from the equator of each eggshell were carefully removed and fixed on the sample stage with conductive adhesive. Their vertical profiles were photographed at 180× magnification under a scanning electronic microscopy (SU8000, Hitachi Co., Ltd., Tokyo, Japan). Three images (field dimensions 675 μm × 470 μm) per piece were taken randomly. Then, the eggshell ultrastructure was measured as described in a previous study (Dunn et al., 2012). The effective layer thickness was assessed by measuring the length from the top of the cuticle to the bottom of the palisade layer. The mammillary layer thickness was measured as the length from the bottom of the palisade layer to the top of the membrane. The calcified layer referred to the combined effective and mammillary layers. The thickness ratio was calculated as the percentage of each layer relative to the calcified layer. The mammillary knob width was calculated as the length of the mammillary knobs/the number of the mammillary knobs.

Six eggshells per replicate were cleaned and eggshell membranes were manually removed by the same individual for consistency. These six eggshells were then air-dried, weighed, ground and mixed into one sample for the detection of eggshell calcium and phosphorus content as described in a previous report (Fu et al., 2021a). Briefly, 0.5 g of eggshell powder from each sample (produced by 6 eggshells per replicate) was digested with a microwave digestion system (MDS-10, Shanghai Xinyi Instrument Technology Co., Ltd, Shanghai, China) after completely dissolving in the hydrogen peroxide and nitric acid. The digestion solution was adjusted to 50 mL with deionized water, then a flame atomic absorption spectrophotometry (Z2000, Hitachi Co., Ltd., Tokyo, Japan) was used to assay calcium content, and a spectrophotometer (UV2700, Shimadzu Co., Ltd., Kyoto, Japan) was employed to assay phosphorus content. Total calcium and phosphorus contents per egg were calculated as the multiple of average eggshell weight ($n = 6$) and calcium or phosphorus contents.

2.6. Bone geometrical, mineral, mechanical and compositional characteristics

The left humeri and femurs sampled at 18.5 h PO were weighed first and placed in a measuring cylinder containing water to determine bone volume, from which density was calculated. A string and a digital caliper were used to measure their length and midpoint circumference. The humeri and femurs were then truncated at the mid-point to measure the external (H) and internal (h) cortical bone diameters in the medial–lateral plane as well as the external (B) and internal (b) cortical bone diameters in the anterior–posterior plane. The mean relative wall thickness, cortical cross-sectional area, cross-sectional moment of inertia, radius of gyration and mean cortical index were calculated as the equations listed in Table S1 according to a previous report (Brzóska et al., 2005).

The left humeri and femurs sampled at 8.5 h PO were used to detect bone mineral content (BMC) and bone mineral density (BMD) according to a previous study (Fu et al., 2022). Briefly, segments of 1 cm length from the skeletal proximal, middle and distal regions were selected to test the BMC and BMD with dual energy X-ray absorptiometry (DTX-200, Osteometer MediTech Inc., Hawthorne, USA). The air was used to calibrate the measurements.

The three-point bending method was used to determine the bone mechanical properties following mineral measurements. A TMS-Pro texture analyzer (Food Technology Corp., Sterling, Virginia, USA) was used to record the load–deformation curve. According to the previous reports (Brzóska et al., 2005), the minimum load to fracture was defined as bone strength, the slope of the maximum elastic load–displacement curve was considered as bone stiffness. The work to fracture indicates the total energy absorbed by bone during its deformation and fracture, which was calculated by the area under the load–displacement.

The fractured humeri and femurs were used to determine the components. All bone fragments from each bone were carefully collected at all steps to ensure that all measurements were conducted on the whole bone. The fractured humeri and femurs were defatted for a period of 3 days with petroleum ether, with daily solution changed. They were then dehydrated in 95% ethanol for 1 day. Afterwards, the bones were dried at 105 °C until reaching a constant weight, and the fat-free dry bone weight was determined. The fat-free dry bone was crushed and collected into a crucible. The crucible containing the bone fragments was carbonized on an electrothermal plate at 200 to 350 °C for 2 h and then ashed in a muffle furnace at 600 °C for 8 h. The ash content was calculated and expressed as a percentage of the fat-free dry bone. The ash was then used to determine the calcium and phosphorus contents in bones based on the method mentioned above (2.5).

2.7. Uterus and bone histomorphometry

Uterine tissues were fixed in the 4% paraformaldehyde solution overnight at 4 °C. The fixed tissue was dehydrated by 75% ethanol for 4 h, 85% ethanol for 2 h, 90% ethanol for 2 h, 95% ethanol for 1 h, absolute ethanol for 30 min twice, ethanol-dimethylbenzene for 10 min, and dimethylbenzene for 10 min in a dehydrator (Diapath S.p.A., Martinengo, Italy). Then, the tissue was embedded in molten paraffin with an embedding machine (Wuhan Junjie Electronics Co., Ltd., Wuhan, China). The wax block was cooled at −20 °C and cut into 4 μm sections with a tissue spreader (Zhejiang Kehua Instrument Co., Ltd., Zhejiang, China). The paraffin sections were picked up by the glass slides and baked in an oven at 60 °C until dewaxed. Histopathological observation was performed with a Pannoramic scanner (3DHISTECH Ltd., Budapest, Hungary) after staining with hematoxylin and eosin (HE). The villus length, the width of uterine mucosal folds, the quantity score of uterine mucosal folds and the ratio of edema or dissolution of tubular glands (EDTG) were defined as previously reported (de Moraes et al., 2021; Feng et al., 2023). The length of mucosal folds was determined from the top of the villus to its root (the junction with lamina propria), and the width of the uterine mucosal folds was defined as the width of the mucosal fold base. The score of uterine folds was stratified in four groups based on the Ishak Semi-Quantitative Score (Ishak et al., 1995): score 1 (primary folds only), score 2 (primary and some secondary

folds), score 3 (primary, secondary and some tertiary folds), score 4 (numerous tertiary folds). Histological evaluation was done by the same histologist in a blinded manner. Two samples (3 images each sample) per replicate were taken and averaged for statistical analysis.

The humerus and femur samples were fixed overnight in 10% formalin at room temperature, and were then decalcified in 14% ethylenediaminetetraacetic acid (EDTA). The decalcification solution was replaced every 2 days until the needle could easily penetrate the cortical bone. The decalcified bone samples were dehydrated, embedded, sectioned and dewaxed in the same way as uterine tissue section preparation. Once dewaxed, a Goldner's Trichrome stain was performed using a commercial kit (Servicebio technology Co. Ltd., Wuhan, China) according to the manufacturer's protocol. Bone histology images were taken within the central or inner region of the medullary cavity with a Pannoramic scanner (3DHISTECH Ltd., Budapest, Hungary). The blinded histological analysis was performed according to our previous study (Fu et al., 2022). Bone tissue area (T.Ar), inner medullary bone perimeter (Mb.Pm) and area (Mb.Ar) were analyzed using Image-Pro Plus 6.0 software (Media Cybernetics, MD, USA). Medullary bone volume/bone tissue volume (BV/TV), number (Mb.N), thickness (Mb.Th) and separation (Mb.Sp) were calculated by the following equations:

$$BV/TV\ (\%) = (Mb.Ar/T.Ar) \times 100;$$

$$Mb.N\ (n/mm) = (1.199/2) \times (Mb.Pm/T.Ar);$$

$$Mb.Th\ (\mu m) = (2000/1.199) \times (Mb.Ar/Mb.Pm);$$

$$Mb.Sp\ (\mu m) = (2000/1.199) \times [(T.Ar - Mb.Ar)/Mb.Pm].$$

2.8. RNA extraction and quantitative real-time PCR (qRT-PCR)

The tissues were ground manually with a mortar and pestle in liquid nitrogen. Total RNA of the uterus and bones were extracted with TRNzol reagent (Tiangen Biotech Co. Ltd., Beijing, China) and EASYspin Plus Bone Tissue RNA kit (Aidlab Biotechnologies Co. Ltd., Beijing, China) according to the instructions. RNA integrity was confirmed by an agarose gel electrophoresis. The purity and concentration of RNA were assessed with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). An Easy Script First-Strand cDNA Synthesis SuperMix kit (TransGen Biotech Co. Ltd., Beijing, China) was used to synthesize cDNA with 1.5 μg total RNA. qRT-PCR was conducted using a Light Cycler 480 system (Roche, Basel, Switzerland) with SuperReal PreMix kit (Tiangen Biotech Co. Ltd., Beijing, China). Primer sequences, target amplicon size, melting temperature (Tm) and primer efficiencies can be found in Table 3. The relative mRNA expression levels were calculated by $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) with the reference gene of avian β-actin, where ΔCt = Ct(target gene) − Ct(β-actin), ΔΔCt = ΔCt(treatment group) − ΔCt(control group), $2^{-\Delta\Delta Ct}$ = relative expression level.

2.9. Calcium retention of hens, as well as the calcium contents in the serum and uterine fluid

Calcium retention of hens was determined as Macelline et al. (2022) described. Briefly, 1 cage with 3 birds were selected from each replicate at the end of experiment for the further test. Before the test, the hens were subjected to a 24-h fasting period. During the 3-day collection period, total excreta were collected and weighed after removing the feed and feathers in the excreta, and the total feed intake was also recorded. The excreta samples were dried and ground to pass through a 0.5-mm sieve. The contents and total calcium excretion were determined as mentioned above (2.5). Calcium retention of hens was counted as following equation and then presented as the average value of 3 hens per replicate in statistical analysis.

$$\text{Calcium retention}\ (\%) = [(\text{feed intake} \times \text{calcium}_{\text{diet}}) - (\text{excreta output} \times \text{calcium}_{\text{excreta}})]/(\text{feed intake} \times \text{calcium}_{\text{diet}}) \times 100.$$

The calcium contents (1 bird per replicate at each time point) of serum and uterine fluid were measured using a microplate reader with a calcium assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) after completely thawing at 4 °C.

2.10. Statistical analysis

All analysis were performed with SAS 9.4 Software (SAS Inc.). The normality of the data and the homogeneity of variances were tested first. The qRT-PCR data were $\log_{10}$ transformed prior to statistical analysis to ensure normality. One-way ANOVA was conducted within each age, followed by Duncan's Multiple Range Test for the comparisons among groups. Differences were considered significant when the *P*-value was below 0.05.

3. Results

3.1. Laying performance and eggshell quality in trial 1

The results of laying performance of the first trial are presented in Fig. 1. There were no significant differences in HDEP, breakage rate and qualified egg rate of laying hens among groups during the observation period (80 weeks of age) ($P > 0.05$). During 82, 83, 84 and 81 to 84 weeks of age, the hens in the high calcium group had a lower HDEP compared with those in other groups ($P < 0.05$). During 82 weeks of age, the birds supplemented with calcitriol or quercetin had higher HDEP than the control group ($P < 0.05$). During 83 and 84 weeks of age, no significant differences were observed among the control, calcitriol, and quercetin groups ($P > 0.05$). From 81 to 84 weeks of age, the HDEP of the quercetin-supplemented birds was higher than that of the control birds ($P < 0.05$), with comparable HDEP to the birds supplemented with calcitriol ($P > 0.05$). The high calcium group had the highest breakage rate among the groups during 81, 82 and 81 to 84 weeks of age, with no significant differences between the control, calcitriol and quercetin groups ($P > 0.05$). During 83 weeks of age, dietary addition with calcitriol or quercetin declined breakage rate compared with dietary calcium addition ($P < 0.05$), while they did not differ significantly compared to the control diet ($P > 0.05$). Compared with other groups, dietary calcium supplementation significantly decreased the qualified egg rate during 81, 82, 83, 84 and 81 to 84 weeks of age ($P < 0.05$). The qualified egg rate of the quercetin group was higher than that of the control and calcitriol groups during 82 and 83 weeks of age ($P < 0.05$), while there was no significant difference during 81 and 84 weeks of age ($P > 0.05$). Compared with the control, supplementation with calcitriol or quercetin significantly increased the qualified egg rate of 81 to 84 weeks of age ($P < 0.05$). From 81 to 84 weeks of age, the average daily feed intakes did not differ among all groups ($P > 0.05$).

The results of eggshell quality are presented in Table 4. Compared with the control and high calcium groups, dietary calcitriol supplementation significantly enhanced the eggshell breaking strength at the end of 82, 83 and 84 weeks of age ($P < 0.05$), and quercetin supplementation only increased that at the end of 82 and 84 weeks of age ($P < 0.05$). At the end of 83 and 84 weeks of age, dietary calcitriol supplementation significantly increased eggshell thickness compared to the control and high

Table 3
The primers used for qRT-PCR assays.

Gene name	Primer sequence (5′ to 3′)	Accession no.	Tm, °C	Target amplicon size, bp	Efficiency, %
β-actin	F: TATGTGCAAGGCCGGTTTC R: TGTCTTTCTGGCCCATACCAA	NM_205518.2	55.89	110	97.22
CASP3	F: ACTCTGGAATTCTGCCTGATGACA R: CATCTGCATCCGTGCCTGA	NM_204725.2	57.72	129	102.40
CASP8	F: CCCTGAAGACAGTGCCATTT R: GGGTCGGCTGGTCATTTTAT	NM_204592.4	55.28	106	92.90
CASP9	F: TGGAGAAGCGTTTCAGGTCC R: ATGGGAGAGGATGACCACGA	XM_046931415.1	57.59	143	92.29
Bax	F: GTGATGGCATGGGACATAGCTC R: TGGCGTAGACCTTGCGGATAA	XM_422067.4	58.31	90	102.38
Bcl	F: TGTTTCTCAAACCAGACACCAA R: CAGTAGGCACCTGTGAGATCG	NM_205339.3	54.06	205	96.76
TRPV6	F: TAAGACATTTGCCTGCCACA R: TTCAGCCCAGGAGTCAATCT	XM_040661661.2	54.28	241	94.05
NCX	F: GGATTGTGGAGGTTTGGGAAGG R: CTGTTTGCCAGCTCGGTATTTC	NM_001398209.1	58.61	131	108.19
PMCA	F: TTACTGGTCTGACGTGCATTG R: AATCTTTGCCCTCCAAACAC	NM_001397870.1	55.04	204	106.33
CALB	F: TGTTATGGAGTGCAGGATGG R: TAGAGCGAACAAGCAGGTGA	NM_205513.1	59.54	148	99.32
VDR	F: CCGGATTCAGGGATCTGACG R: AAGTCATTGCTTCCGCAGGT	NM_205098.2	58.19	133	101.56
ERα	F: TGAGCTGGAGACTCTGAGCA R: CCGTACACTGGAGCGGTAGT	NM_205183.2	58.09	147	101.56
ERβ	F: ATGACTTGCTGCTGGAGA R: CAGACCTGGAAATGTGAAAC	NM_204794.3	53.43	103	109.58
ALP	F: GGAGAAGGACCCCGAATACTG R: TTGACGCCGCAGAGGTAAG	NM_205360.2	57.51	300	101.83
Runx2	F: CACGCTGCTAAACCCAAACT R: GACTCATCCATCCTGCCACT	NM_204128.2	55.93	108	91.91
OCN	F: GAAGAGGCAGAAGAGGTTCG R: AGATAGTCACAGGGAGGGTAGC	NM_001201386.2	55.58	108	107.86
OPN	F: TAGGAGTTGCTGCTGGGATT R: CCTGGTGGTACCTGTGTGTG	NM_204535.5	56.07	218	102.12
TRAP	F: CTGGCTTTGGGCGATAACT R: TCGGAGTGTCGGCTGTATG	XM_040693093.2	55.40	185	92.71
CTSK	F: ACGTCCCGGAGGTTGATTTG R: CCACCTCCTCGCTGGTCATA	NM_204971.3	57.91	130	95.16

F = forward primer; R = reverse primer; Tm = melting temperature; *CASP3* = caspase 3; *CASP8* = caspase 8; *CASP9* = caspase 9; *Bax* = BCl-2-associated X protein; *Bcl* = B-cell lymphoma; *TRPV6* = transient receptor potential cation channel, subfamily V, member 6; *NCX* = sodium-calcium exchanger; *PMCA* = plasma membrane calcium-ATPase; *CALB* = calbindin; *VDR* = vitamin D receptor; *ERα* = estrogen receptor alpha; *ERβ* = estrogen receptor beta; *ALP* = alkaline phosphatase; *RUNX2* = runt-related transcription factor 2; *OCN* = osteocalcin; *OPN* = osteopontin; *TRAP* = tartrate-resistant acid phosphatase; *CTSK* = cathepsin K.

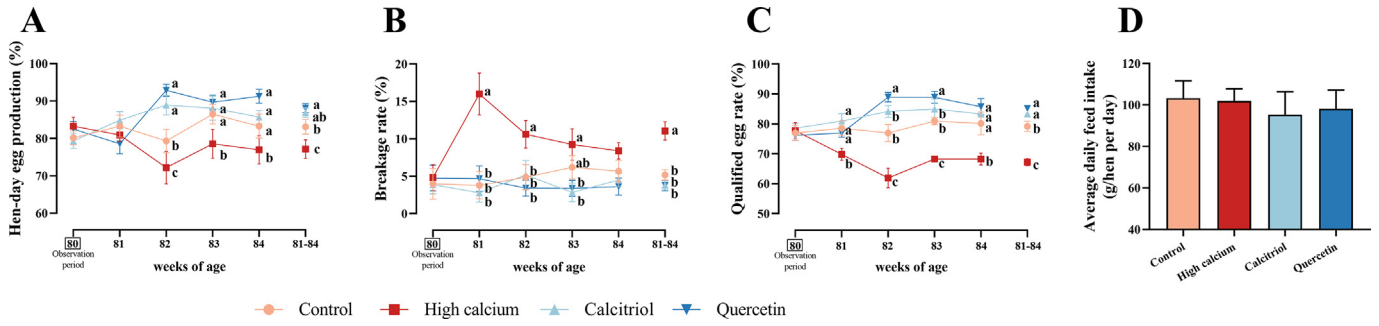

Fig. 1. Effects of dietary supplementation with calcitriol or quercetin on laying performance of aged laying hens with weak-shelled eggs (25 N < average eggshell breaking strength < 30 N). (A) Hen-day egg production. (B) Breakage rate. (C) Qualified egg rate. (D) Average daily feed intake from 81 to 84 weeks of age. All hens were fed the control diet during the observation period (80 weeks of age) and then fed the treatment diets from 81 weeks of age. Control = basal diet; High calcium = basal diet supplemented with 0.5% calcium; Calcitriol = basal diet supplemented with 5 μg/kg calcitriol; Quercetin = basal diet supplemented with 500 mg/kg quercetin. $^{a-c}$Values in the same week with no common letters differ significantly ($P < 0.05$). Values are presented as mean with standard error, $n = 6$.

calcium diets ($P < 0.05$). The birds fed with calcitriol or quercetin had higher eggshell weight than those fed a high calcium diet at the end of 82 weeks of age ($P = 0.009$). There was no significant distinction observed between the calcitriol and quercetin groups, and likewise, no significant differences were found between the control and high calcium groups ($P > 0.05$).

3.2. Laying performance

As shown in Table 5, dietary quercetin supplementation improved HDEP during 69 to 72 weeks of age and 61 to 72 weeks of age compared to both control and calcitriol groups ($P < 0.05$). However, the HDEP of birds in the control group and the calcitriol

Table 4
Effects of dietary supplementation with calcium, calcitriol or quercetin on eggshell quality of aged laying hens (80 to 84 weeks of age) with weak-shelled eggs.[1]

Weeks of age	Group[2]				SEM	*P*-value
	Control	High calcium	Calcitriol	Quercetin		
Eggshell breaking strength, N						
80	28.40	28.51	27.08	27.79	0.851	0.935
81	28.43	26.94	31.00	30.06	0.820	0.318
82	26.19^{b}	25.31^{b}	29.65^{a}	30.46^{a}	0.699	0.007
83	26.80^{b}	27.47^{b}	31.79^{a}	29.10^{ab}	0.568	0.004
84	28.27^{b}	28.70^{b}	32.73^{a}	32.42^{a}	0.651	0.010
Eggshell thickness, ×0.01 mm						
80	42.82	41.39	41.92	40.94	0.347	0.240
81	43.58	41.07	44.75	41.90	0.452	0.058
82	42.14	41.99	43.13	42.36	0.370	0.075
83	41.26^{b}	40.50^{b}	43.46^{a}	42.02^{ab}	0.446	0.039
84	41.82^{b}	41.59^{b}	43.61^{a}	42.22^{ab}	0.336	0.034
Eggshell weight, g						
80	5.84	5.61	5.74	5.44	0.089	0.444
81	5.83	5.51	5.96	5.62	0.104	0.411
82	5.60^{ab}	5.26^{b}	5.85^{a}	5.88^{a}	0.079	0.009
83	5.76	5.47	6.01	6.01	0.084	0.054
84	5.78	5.53	6.00	5.89	0.086	0.076
Eggshell weight ratio, %						
80	9.22	8.91	8.92	8.66	0.107	0.337
81	9.33	8.82	9.31	8.83	0.124	0.269
82	9.13	8.57	9.16	9.03	0.130	0.377
83	9.13	8.61	9.26	9.26	0.104	0.075
84	9.02	8.95	9.21	9.00	0.099	0.816

[a, b]Within a row, values with no common letter superscripts differ significantly ($P < 0.05$).
[1] Weak-shelled eggs were defined as the average eggshell breaking strength from 25 to 30 N. All hens were fed the control diet during the observation period (80 weeks of age) and then fed the treatment diets from 81 weeks of age. Values are presented as mean and standard error of the mean (SEM), $n = 6$.
[2] Control = basal diet; High calcium = basal diet supplemented with 0.5% calcium; Calcitriol = basal diet supplemented with 5 μg/kg calcitriol; Quercetin = basal diet supplemented with 500 mg/kg quercetin.

Table 5
Effects of dietary supplementation with calcitriol or quercetin on laying performance of aged laying hens (61 to 72 weeks of age).[1]

Weeks of age	Group[2]			SEM	*P*-value
	Control	Calcitriol	Quercetin		
Hen-day egg production, %					
61 to 64	86.81	86.71	88.05	0.627	0.683
65 to 68	85.62	86.51	88.19	0.523	0.060
69 to 72	85.98^{b}	85.12^{b}	88.49^{a}	0.510	0.022
61 to 72	86.13^{b}	86.11^{b}	88.24^{a}	0.356	0.020
Average egg weight, g					
61 to 64	61.11	61.22	60.72	0.214	0.671
65 to 68	60.53	60.99	60.47	0.163	0.422
69 to 72	61.13	61.37	60.62	0.158	0.190
61 to 72	60.92	61.19	60.60	0.142	0.265
Average daily feed intake, g/hen per day					
61 to 64	106.49	105.21	109.13	0.809	0.206
65 to 68	102.44	102.83	105.41	0.772	0.264
69 to 72	105.34	105.95	106.95	0.373	0.211
61 to 72	104.76	104.66	106.93	0.534	0.174
Feed conversion ratio					
61 to 64	2.01	1.98	2.02	0.018	0.750
65 to 68	2.05	2.02	2.05	0.018	0.762
69 to 72	1.96	1.96	1.92	0.020	0.709
61 to 72	2.00	1.99	2.00	0.011	0.908

[a, b]Within a row, values with no common letter superscripts differ significantly ($P < 0.05$).
[1] Values are presented as mean and standard error of the mean (SEM), $n = 8$.
[2] Control = basal diet; Calcitriol = basal diet supplemented with 5 μg/kg calcitriol; Quercetin = basal diet supplemented with 500 mg/kg quercetin.

group did not differ ($P > 0.05$). Additionally, dietary supplementation with calcitriol or quercetin did not affect average egg weight, average daily feed intake or feed conversion ratio ($P > 0.05$).

3.3. Eggshell physical and mechanical properties, ultrastructure, and components

The results for the eggshell physical and mechanical properties are presented in Table 6. At the end of 68 and 72 weeks of age, dietary calcitriol and quercetin addition significantly raised eggshell breaking strength compared with the control ($P < 0.05$). In comparison with the control, the eggshell thickness was significantly increased with calcitriol or quercetin addition at the end of 72 weeks of age ($P = 0.040$). The eggshell weight of the end of 72 weeks of age was higher in the calcitriol group than in the control group ($P = 0.026$).

Compared with the control, dietary addition with calcitriol or quercetin significantly increased the thickness and ratio of the effective layer as well as the thickness of the calcified layer ($P < 0.05$), but decreased the thickness and ratio of the mammillary layer (Fig. 2, $P < 0.05$).

The eggshell component results are summarized in Table 7. The eggshell had higher phosphorus content in the calcitriol group than the other groups ($P = 0.040$), with no significant differences between the control and quercetin groups ($P > 0.05$). Compared with the control, the total calcium content per eggshell and total phosphorus content per eggshell were significantly increased in the hens fed with calcitriol or quercetin ($P < 0.05$). The eggshell physical and mechanical properties (Table 6), ultrastructure (Fig. 2), and components (except for phosphorus content, Table 7) did not show significant differences between calcitriol and quercetin supplementations ($P > 0.05$).

Table 6
Effects of dietary supplementation with calcitriol or quercetin on eggshell quality of aged laying hens (60 to 72 weeks of age).[1]

Weeks of age	Group[2]			SEM	*P*-value
	Control	Calcitriol	Quercetin		
Eggshell breaking strength, N					
60	39.70	38.84	38.11	0.772	0.713
64	35.41	37.41	34.84	0.610	0.252
68	32.53^{b}	35.82^{a}	34.87^{a}	0.490	0.006
72	34.71^{b}	39.91^{a}	38.05^{a}	0.863	0.029
Eggshell thickness, × 0.01 mm					
60	44.75	44.22	44.29	0.339	0.798
64	43.29	44.21	43.43	0.214	0.191
68	43.76	44.71	44.59	0.200	0.083
72	43.83^{b}	45.78^{a}	45.69^{a}	0.377	0.040
Eggshell weight, g					
60	6.02	5.87	5.78	0.062	0.255
64	5.71	5.87	5.70	0.043	0.231
68	5.66	5.85	5.84	0.053	0.258
72	5.86^{b}	6.16^{a}	5.96^{ab}	0.048	0.026
Eggshell weight ratio, %					
60	9.66	9.29	9.62	0.100	0.293
64	9.51	9.66	9.41	0.060	0.307
68	9.56	9.75	9.79	0.096	0.579
72	9.73	10.08	9.87	0.090	0.291

[a, b]Within a row, values with no common letter superscripts differ significantly ($P < 0.05$).
[1] All hens were fed the control diet at 60 weeks of age and then fed the treatment diets from 61 weeks of age. Values are presented as mean and standard error of the mean (SEM), $n = 8$.
[2] Control = basal diet; Calcitriol = basal diet supplemented with 5 μg/kg calcitriol; Quercetin = basal diet supplemented with 500 mg/kg quercetin.

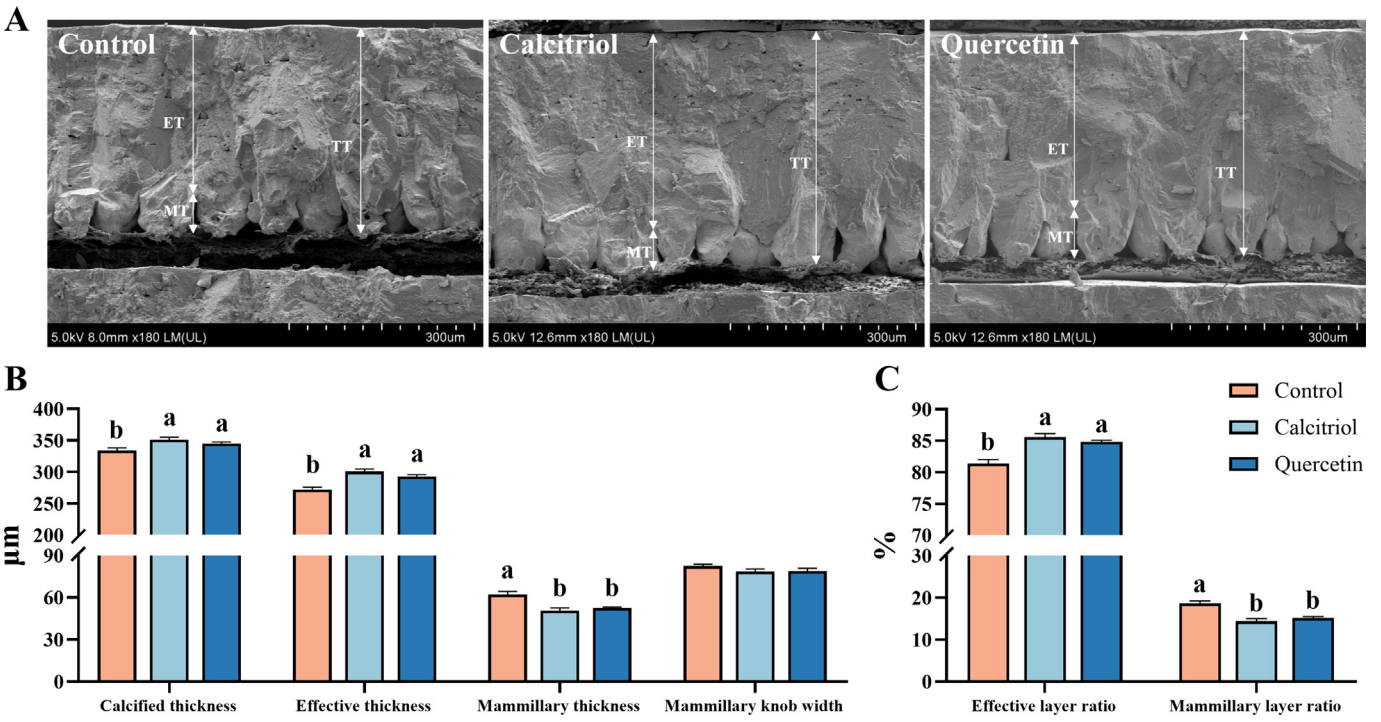

Fig. 2. Effects of dietary supplementation with calcitriol or quercetin on eggshell ultrastructure of aged laying hens (72 weeks of age). (A) Vertical profiles. (B) Thickness. (C) Thickness ratio. Control = basal diet; Calcitriol = basal diet supplemented with 5 μg/kg calcitriol; Quercetin = basal diet supplemented with 500 mg/kg quercetin; TT = total thickness (calcified layer thickness); ET = effective layer thickness; MT = mammillary layer thickness. All hens were fed treatment diets from 61 weeks of age. [a, b]Values in the same index with no common letters differ significantly ($P < 0.05$). Values are presented as mean with standard error, $n = 8$.

Table 7
Effects of dietary supplementation with calcitriol or quercetin on eggshell components of aged laying hens (72 weeks of age).[1]

Item	Group[2]			SEM	*P*-value
	Control	Calcitriol	Quercetin		
Calcium content, mg/g	369.31	366.65	372.74	1.125	0.077
Total calcium content per eggshell, g	2.10[b]	2.24[a]	2.23[a]	0.026	0.046
Phosphorus content, mg/g	1.17[b]	1.30[a]	1.19[b]	0.024	0.040
Total phosphorus content per eggshell, mg	6.64[b]	7.95[a]	7.12[a]	0.180	0.003

[a, b]Within a row, values with no common letter superscripts differ significantly ($P < 0.05$).
[1] All hens were fed treatment diets for 12 weeks. Values are presented as mean and standard error of the mean (SEM), $n = 8$.
[2] Control = basal diet; Calcitriol = basal diet supplemented with 5 μg/kg calcitriol; Quercetin = basal diet supplemented with 500 mg/kg quercetin.

3.4. Bone geometrical, mechanical, compositional, mineral, and histological characteristics

As shown in Table 8, dietary calcitriol or quercetin addition did not affect the humeral and femoral bone geometric characteristics, including length, weight, volume, density, midpoint circumference, mean relative wall thickness, cortical cross-sectional area, cross-sectional moment of inertia, radius of gyration and mean cortical index ($P > 0.05$).

Table 9 describes the results of mineral measurements. No significant differences were found in the humerus ($P > 0.05$). However, dietary supplementation of calcitriol or quercetin significantly increased distal BMD of the femur compared to the control ($P = 0.012$). The femoral proximal BMC of hens in the quercetin group was higher than that in the calcitriol group ($P = 0.044$), while both groups showed no significant differences compared to the control group ($P > 0.05$).

The results of bone mechanical characteristics are listed in Table 10. Compared to the control, the bone stiffness of the femur was increased in the hens supplemented with calcitriol or quercetin ($P = 0.001$), with the calcitriol supplementation showing a more significant effect. However, no significant differences in femoral bone strength, work to fracture or humeral mechanical properties (bone strength, bone stiffness and work to fracture) were observed among all treatments ($P > 0.05$).

Table 11 summarizes the changes in bone components. Dietary calcitriol or quercetin addition did not influence the humerus components ($P > 0.05$). However, when compared to the control, the fat-free dry weight of the femur was increased ($P = 0.027$), and calcium content in ash was significantly decreased with calcitriol supplementation ($P = 0.020$). Additionally, the ash, ash content, total calcium content per bone and total phosphorus content per bone of the femur were significantly raised in both calcitriol- and quercetin-supplemented groups ($P < 0.05$). There was no significant difference in the bone components between the calcitriol and quercetin groups ($P > 0.05$).

Figure 3 demonstrates the results of the femur histomorphometry. Dietary supplementation with quercetin significantly increased BV/TV compared with the other groups ($P < 0.05$), while there was no significant difference observed between the

Table 8
Effects of dietary supplementation with calcitriol or quercetin on bone geometric characteristics of aged laying hens (72 weeks of age).[1]

Item	Groups[2]			SEM	*P*-value
	Control	Calcitriol	Quercetin		
Humerus					
Length, cm	7.96	7.97	8.06	0.042	0.601
Weight, g	4.29	4.81	4.09	0.311	0.654
Volume, cm^3	5.35	5.32	5.75	0.135	0.368
Density, g/cm^3	0.81	0.89	0.72	0.057	0.489
Midpoint circumference, cm	2.25	2.28	2.39	0.031	0.131
Mean relative wall thickness	0.18	0.21	0.20	0.008	0.348
Cortical cross-sectional area, mm^2	10.22	10.48	9.49	0.404	0.609
Cross-sectional moment of inertia, mm^4	37.84	35.40	33.86	2.008	0.742
Radius of gyration, mm	1.92	1.83	1.87	0.028	0.452
Mean cortical index	0.15	0.17	0.16	0.006	0.381
Femur					
Length, cm	8.59	8.73	8.81	0.047	0.171
Weight, g	9.36	9.48	9.37	0.161	0.944
Volume, cm^3	7.35	7.21	7.44	0.121	0.751
Density, g/cm^3	1.28	1.32	1.26	0.015	0.260
Midpoint circumference, cm	2.82	2.71	2.76	0.020	0.119
Mean relative wall thickness	0.18	0.22	0.21	0.010	0.297
Cortical cross-sectional area, mm^2	14.07	15.62	15.59	0.578	0.482
Cross-sectional moment of inertia, mm^4	94.01	89.37	98.23	5.078	0.796
Radius of gyration, mm	2.56	2.40	2.51	0.036	0.179
Mean cortical index	0.15	0.18	0.17	0.007	0.274

[1] All hens were fed treatment diets for 12 weeks. Values are presented as mean and standard error of the mean (SEM), $n = 8$.
[2] Control = basal diet; Calcitriol = basal diet supplemented with 5 μg/kg calcitriol; Quercetin = basal diet supplemented with 500 mg/kg quercetin.

Table 9
Effects of dietary supplementation with calcitriol or quercetin on bone mineral measurements of aged laying hens (72 weeks of age).[1]

Item	Groups[2]			SEM	*P*-value
	Control	Calcitriol	Quercetin		
Humerus					
Distal BMD, g/cm^2	2.52	2.50	2.62	0.046	0.567
Midshaft BMD, g/cm^2	2.70	2.72	2.78	0.055	0.837
Proximal BMD, g/cm^2	2.55	2.52	2.69	0.039	0.221
Average BMD, g/cm^2	2.59	2.58	2.70	0.026	0.127
Distal BMC, g	2.59	2.58	2.72	0.044	0.383
Midshaft BMC, g	1.74	1.72	1.86	0.039	0.269
Proximal BMC, g	2.52	2.51	2.70	0.043	0.160
Average BMC, g	2.28	2.27	2.42	0.030	0.051
Femur					
Distal BMD, g/cm^2	2.49[b]	2.67[a]	2.68[a]	0.032	0.012
Midshaft BMD, g/cm^2	2.41	2.68	2.55	0.056	0.139
Proximal BMD, g/cm^2	2.68	2.61	2.76	0.030	0.114
Average BMD, g/cm^2	2.53	2.66	2.67	0.030	0.104
Distal BMC, g	2.77	2.89	2.95	0.034	0.067
Midshaft BMC, g	2.18	2.33	2.27	0.048	0.460
Proximal BMC, g	2.81[ab]	2.77[b]	2.99[a]	0.041	0.044
Average BMC, g	2.59	2.66	2.74	0.032	0.145

BMD = bone mineral density; BMC = bone mineral content.
[a, b]Within a row, values with no common letter superscripts differ significantly ($P < 0.05$).
[1] All hens were fed treatment diets for 12 weeks. Values are presented as mean and standard error of the mean (SEM), $n = 8$.
[2] Control = basal diet; Calcitriol = basal diet supplemented with 5 μg/kg calcitriol; Quercetin = basal diet supplemented with 500 mg/kg quercetin.

Table 10
Effects of dietary supplementation with calcitriol or quercetin on bone mechanical properties of aged laying hens (72 weeks of age).[1]

Item	Groups[2]			SEM	*P*-value
	Control	Calcitriol	Quercetin		
Humerus					
Strength, N	160.53	138.85	135.58	7.671	0.443
Stiffness, N/mm	75.08	68.76	66.04	3.950	0.701
Work to fracture, mJ	234.23	186.75	185.89	15.123	0.418
Femur					
Strength, N	168.45	224.83	217.77	14.724	0.246
Stiffness, N/mm	83.29[c]	140.68[a]	109.96[b]	7.295	0.001
Work to fracture, mJ	235.67	293.08	270.14	14.499	0.281

[a-c]Within a row, values with no common superscripts differ significantly ($P < 0.05$).
[1] All hens were fed treatment diets for 12 weeks. Values are presented as mean and standard error of the mean (SEM), $n = 8$.
[2] Control = basal diet; Calcitriol = basal diet supplemented with 5 μg/kg calcitriol; Quercetin = basal diet supplemented with 500 mg/kg quercetin.

control group and the calcitriol group ($P > 0.05$). The femur of hens fed with quercetin had thicker medullary bone thickness than that in the control group ($P < 0.05$). However, the calcitriol group showed no significant differences compared to the other groups ($P > 0.05$).

3.5. Calcium retention of hens and calcium content in serum

The results of calcium retention and serum calcium content are depicted in Table 12. Dietary supplementation of calcitriol or quercetin significantly raised the calcium retention of hens compared to the control ($P = 0.003$), with no significant differences between the calcitriol and quercetin supplementations ($P > 0.05$). However, no significant changes were found in the serum calcium content among the groups ($P > 0.05$).

3.6. Quantification of apoptosis-related mRNA and morphology in the uterus

When compared to the control, dietary calcitriol or quercetin supplementation down-regulated the relative mRNA expression level of *CASP3* at the initiation stage of eggshell calcification (Fig. 4A, $P < 0.05$). The HE staining (Fig. 4B) demonstrated that the uterine tissues in all groups had an intact epithelial structure, with tightly arranged epithelial cells. Compared with the control, the supplementation of calcitriol or quercetin significantly declined the ratio of EDTG (Fig. 4F, $P < 0.05$) but failed to significantly affect the villus length, width of mucosal folds, and quantity score of mucosal folds (Fig. 4C to E, $P > 0.05$). However, no significant difference in the uterine morphology and apoptosis-related relative mRNA

Table 11
Effects of dietary supplementation with calcitriol or quercetin on bone components of aged laying hens (72 weeks of age).[1]

Item	Groups[2]			SEM	*P*-value
	Control	Calcitriol	Quercetin		
Humerus					
Fat-free dry weight, g	2.89	3.23	2.76	0.180	0.570
Ash, g	1.60	1.84	1.61	0.089	0.498
Ash content, %	55.57	57.50	58.80	0.694	0.161
Calcium content in ash, mg/g	376.99	377.12	382.58	2.042	0.470
Total calcium content per bone, g	0.60	0.62	0.69	0.032	0.512
Phosphorus content in ash, mg/g	171.26	172.20	174.73	0.930	0.305
Total phosphorus content per bone, g	0.27	0.28	0.32	0.015	0.508
Femur					
Fat-free dry weight, g	5.08^{b}	6.26^{a}	5.80^{ab}	0.190	0.027
Ash, g	2.65^{b}	3.54^{a}	3.24^{a}	0.132	0.010
Ash content, %	51.94^{b}	56.41^{a}	55.79^{a}	0.758	0.022
Calcium content in ash, mg/g	388.23^{a}	376.10^{b}	381.35^{ab}	1.891	0.020
Total calcium content per bone, g	1.03^{b}	1.33^{a}	1.24^{a}	0.049	0.025
Phosphorus content in ash, mg/g	178.73	174.35	175.48	0.814	0.064
Total phosphorus content per bone, g	0.47^{b}	0.62^{a}	0.57^{a}	0.023	0.018

$^{a, b}$Within a row, values with no common letter superscripts differ significantly ($P < 0.05$).
[1] All hens were fed treatment diets for 12 weeks. Values are presented as mean and standard error of the mean (SEM), $n = 8$.
[2] Control = basal diet; Calcitriol = basal diet supplemented with 5 μg/kg calcitriol; Quercetin = basal diet supplemented with 500 mg/kg quercetin.

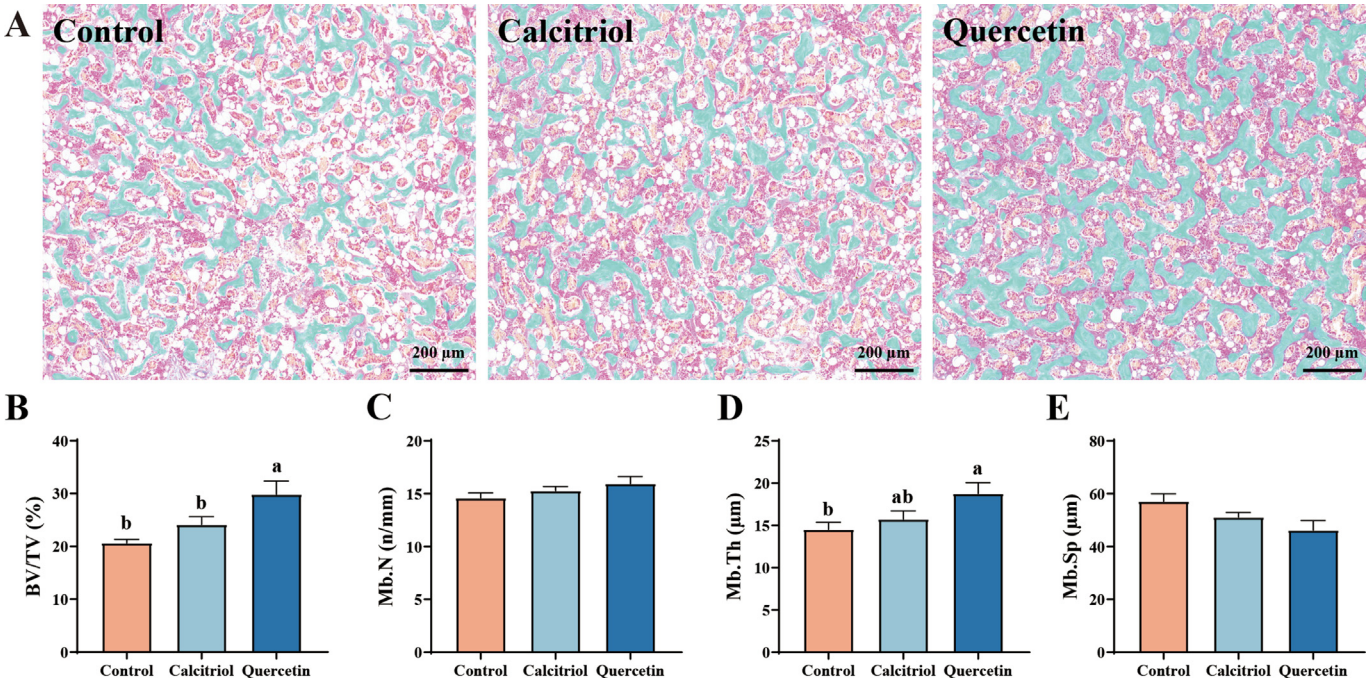

Fig. 3. Effects of dietary supplementation with calcitriol or quercetin on femur histomorphometry of aged laying hens (72 weeks of age). (A) The medullary bone (green) stained with Goldner's trichrome. (B) Medullary bone volume/bone tissue volume (BV/TV). (C) Medullary bone number (Mb.N). (D) Medullary bone thickness (Mb.Th). (E) Medullary bone separation (Mb.Sp). Control = basal diet; Calcitriol = basal diet supplemented with 5 μg/kg calcitriol; Quercetin = basal diet supplemented with 500 mg/kg quercetin. All hens were fed treatment diets from 61 weeks of age. $^{a, b}$Values in the same index with no common letters differ significantly ($P < 0.05$). Values are presented as mean with standard error, $n = 8$.

expression was observed between the calcitriol and quercetin groups (Fig. 4, $P > 0.05$).

3.7. Quantification of calcium transport-related mRNA in the uterus and the calcium content in uterine fluid

The relative mRNA expression levels of calcium transporters in the uterus are exhibited in Fig. 5A and B. At the initiation stage of eggshell calcification, dietary supplementation with calcitriol or quercetin significantly up-regulated the relative mRNA expression level of *TRPV6* and down-regulated the relative mRNA expression level of *PMCA* in the uterus when compared to the control ($P < 0.05$), with no significant differences observed between the calcitriol and quercetin supplementations ($P > 0.05$). The relative mRNA expression level of uterine *CALB* in the calcitriol group was not significant changed when compared to the quercetin group ($P > 0.05$) but significantly increased compared to the control ($P < 0.05$). However, the relative mRNA expression level of *CALB* in the uterus of hens fed with the quercetin supplementation diet and the control diet did not differ ($P > 0.05$). No significant differences were found in the relative mRNA expression levels of uterine *NCX*, *VDR*, *ERα*, and *ERβ* ($P > 0.05$). At the growth stage of eggshell

Table 12
Effects of dietary supplementation with calcitriol or quercetin on calcium retention and calcium content in serum of aged laying hens (72 weeks of age).[1]

Item	Groups[2]			SEM	*P*-value
	Control	Calcitriol	Quercetin		
Calcium retention of hens, %	48.85[b]	57.43[a]	55.64[a]	1.178	0.003
Calcium content in serum, mmol/L[3]					
Initiation stage	2.52	2.53	2.66	0.048	0.421
Growth stage	1.52	1.52	1.56	0.024	0.721

[a, b]Within a row, values with no common letter superscripts differ significantly ($P < 0.05$).
[1] All hens were fed treatment diets for 12 weeks. Values are presented as mean and standard error of the mean (SEM), $n = 8$.
[2] Control = basal diet; Calcitriol = basal diet supplemented with 5 μg/kg calcitriol; Quercetin = basal diet supplemented with 500 mg/kg quercetin.
[3] Initiation stage = 8.5 h post-oviposition; growth stage = 18.5 h post-oviposition.

calcification, the relative mRNA expression levels of uterine *TRPV6* and *ERβ* were higher in the quercetin group than in the control and the calcitriol groups ($P < 0.05$). Compared to the control, dietary supplementation with calcitriol or quercetin raised the relative mRNA expression levels of *PMCA*, *VDR*, and *ERα* in the uterus ($P < 0.05$). Dietary addition with quercetin up-regulated the relative mRNA expression level of uterine *CALB* compared with the control ($P < 0.05$). However, no significant difference was observed in the relative mRNA expression levels of *TRPV6*, *CALB* and *ERβ* between the calcitriol supplementation and the control ($P > 0.05$), and similarly for the relative mRNA expression levels of *PMCA*, *CALB*, *VDR* and *ERα* between the calcitriol and the quercetin supplementations ($P > 0.05$).

Figure 5C displays the results of calcium content in uterine fluid at the growth stage of eggshell calcification. Compared to the control, dietary calcitriol or quercetin supplementation significantly increased the calcium content in the uterine fluid ($P < 0.05$), while no significant difference was observed between calcitriol and quercetin supplementations ($P > 0.05$).

3.8. Quantification of bone remodeling-related mRNA in humerus and femur

Figure 6 presents the differences in relative mRNA expression levels of bone remodeling-related genes in the humerus and femur. In the humerus, at the growth stage of eggshell calcification, addition of dietary quercetin significantly up-regulated the relative mRNA expression levels of *ALP* and *CTSK* compared to the control and calcitriol supplementations ($P < 0.05$). However, there was no significant difference observed between the calcitriol group and the control group ($P > 0.05$). At the growth stage of eggshell calcification, the relative mRNA expression level of humeral *TRAP* was higher in the calcitriol group than in the quercetin group ($P < 0.05$), which was higher than in the control group for both groups ($P < 0.05$). At the initiation stage of eggshell calcification, dietary calcitriol or quercetin addition significantly increased the relative mRNA expression levels of *ALP*, *OPN*, and *TRAP* in the femur when compared to the control ($P < 0.05$), while no significant difference was found between the calcitriol and quercetin supplementations ($P > 0.05$). The relative mRNA expression level of femoral *ERα* did not show significant differences between the control and calcitriol groups ($P > 0.05$), however both groups showed an increase in expression when compared to the quercetin group ($P < 0.05$). Dietary calcitriol addition significantly increased the relative mRNA expression level of femoral *VDR* compared with the control and quercetin addition ($P < 0.05$). However, no significant difference was found in the relative mRNA expression level of femoral *VDR* between the control and quercetin groups ($P > 0.05$). At the growth stage of eggshell calcification, dietary supplementation with calcitriol or quercetin down-regulated the relative mRNA expression level of *ALP* in the femur compared to the control ($P < 0.05$), however, no significant difference was found between the calcitriol and quercetin additions ($P > 0.05$). Dietary calcitriol or quercetin addition increased the relative mRNA expression level of the femoral *TRAP* when compared to the control ($P < 0.05$), with quercetin showing a more obvious difference ($P < 0.05$).

4. Discussion

Layers that laid eggs with low eggshell breaking strength should be given more attention since the laid eggs were more prone to breakage and cracks during egg collection and transportation (Alfonso-Carrillo et al., 2021). Increasing calcium deposition in eggshells is an effective strategy for improving eggshell quality. In the current study, dietary extra calcium supplementation did not improve eggshell quality; instead, it increased the breakage rate and reduced HDEP and the rate of qualified eggs. A possible explanation is that a high dietary calcium level (4.2% to 4.5%) reduced the relative mRNA expression of intestinal *CALB*, leading to decreased calcium absorption and retention (Wang et al., 2021). This ultimately resulted in lower eggshell quality and an increased egg breakage rate (Atteh and Leeson, 1985; Wang et al., 2021). Calcitriol plays a major role in regulating calcium metabolism. The supplementation of quercetin increased the circulating levels of estrogen in aged laying hens (Liu et al., 2023), which could also modulate calcium metabolism. Our results demonstrated that the hens supplemented with calcitriol or quercetin improved eggshell quality, which is in line with previous reports (Frost et al., 1990; Liu et al., 2013; Soares et al., 1988). Additionally, the elevated estrogen level caused by dietary quercetin supplementation could be a significant factor in the increase of HDEP, as estrogen stimulates oviduct development and folliculogenesis (Song et al., 2011). In the current study, the positive effects of calcitriol and quercetin on eggshell quality were also observed in general commercial aged laying hens, although the significant improvements were not evident until at least 8 weeks. It can be explained that the calcitriol and quercetin took effect more quickly on the hens with weak eggshells. Overall, the supplementation of calcitriol or quercetin could improve the eggshell quality in aged laying hens, and the quercetin group showed an additional increase in HDEP.

The supplementation of either 5 μg/kg calcitriol or 500 mg/kg quercetin resulted in similar improvements on eggshell quality, which were associated with comparable changes in ultrastructure and components. Both the calcitriol and quercetin groups showed the increases in the thickness and ratio of the effective layer that contributes to the resistance against the initiation and propagation of cracks caused by an external force (Zhang et al., 2017). This enhancement may be linked to the increase in total calcium per eggshell in the current study. These results suggest that adding dietary calcitriol or quercetin could alleviate the age-related decrease in eggshell calcium deposition (Park and Sohn, 2018), thereby improving eggshell ultrastructure and mechanical characteristics.

Improved eggshell quality generally accompanies an intense medullary bone resorption and a high risk of osteoporosis, as approximately 20% to 40% of eggshell calcium is derived from bone stock (Alfonso-Carrillo et al., 2021). Previous studies have reported positive effects of calcitriol and quercetin on bone quality (Newbrey et al., 1992; Wang et al., 2022; Wong et al., 2020). Consistently, dietary supplementation of calcitriol or quercetin simultaneously improved eggshell quality along with bone quality in the current study. Additionally, calcitriol or quercetin supplementation was shown to act primarily on the skeleton with medullary bone since

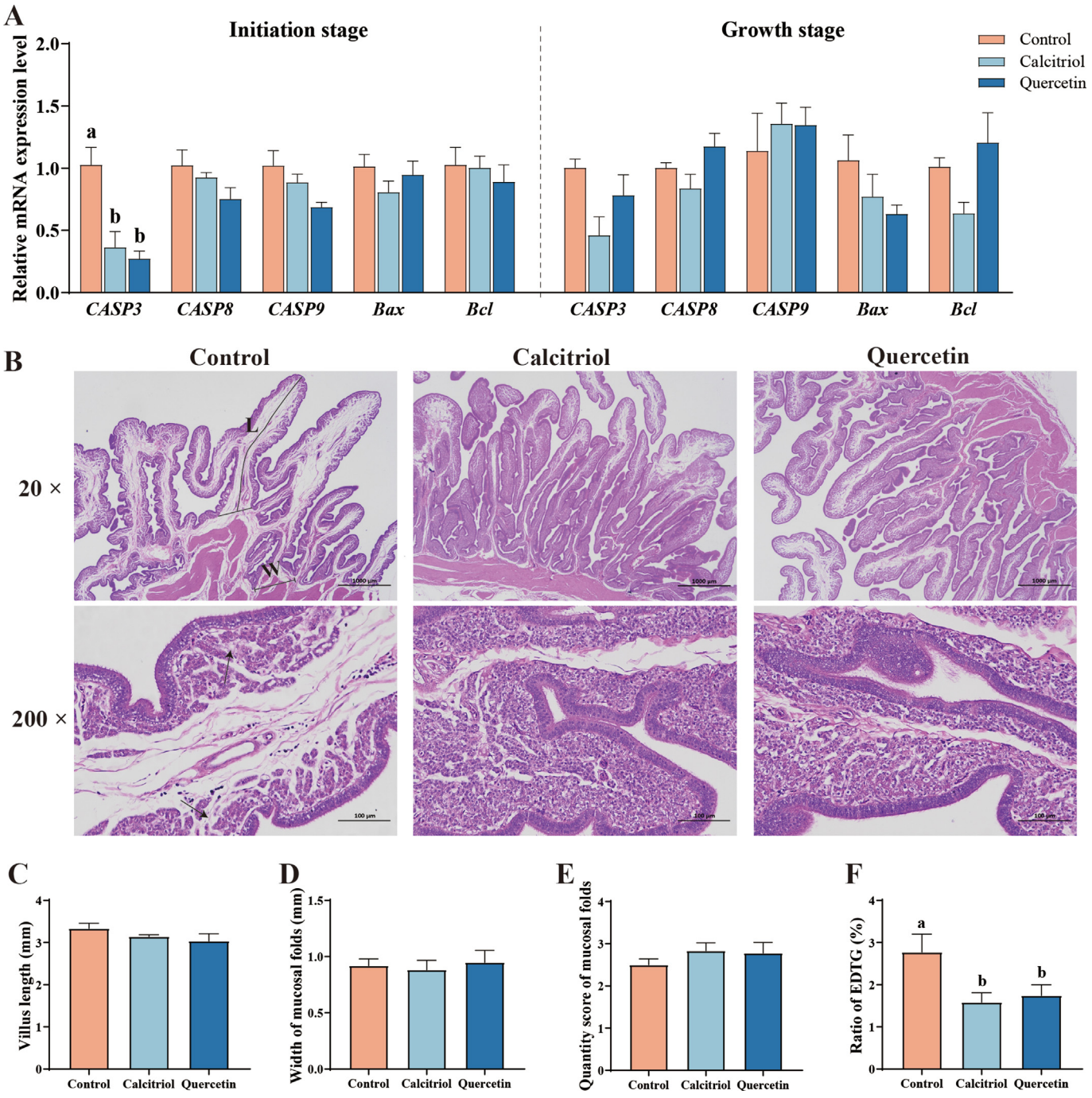

Fig. 4. Effects of dietary supplementation with calcitriol or quercetin on the uterine morphology and the relative mRNA expression levels of apoptotic genes in age laying hens (72 weeks of age). (A) The mRNA expression levels of apoptotic genes at the initiation (8.5 h post-oviposition) and growth (18.5 h post-oviposition) stages of eggshell calcification. (B) Hematoxylin and eosin (HE) stain of uterine tissues. (C) Villus length. (D) Width of mucosal folds. (E) Quantity score of mucosal folds. (F) Ratio of edema or dissolution of tubular glands (EDTG). Black arrow indicates EDTG. *CASP3* = caspase 3; *CASP8* = caspase 8; *CASP9* = caspase 9; *Bax* = BCl-2-associated X protein; *Bcl* = B-cell lymphoma; L = villus length; W = width of mucosal folds; Control = basal diet; Calcitriol = basal diet supplemented with 5 μg/kg calcitriol; Quercetin = basal diet supplemented with 500 mg/kg quercetin. All hens were fed treatment diets from 61 weeks of age. [a, b]Values in the same index with no common letters differ significantly ($P < 0.05$). Values are presented as mean with standard error, $n = 8$.

the improvements to the bone quality were mainly manifested in the femur rather than in the humerus. The calcitriol and quercetin supplementations increased femoral ash, total calcium/phosphorus per bone, distal BMD and stiffness, indicating a better bone in these two groups. The enhanced femoral stiffness in the calcitriol and quercetin groups could lead to a better resistance to deformation (Seeman and Pierre, 2006) and is expected to result in a lower probability of fracture (Kobayashi et al., 1996). Skeletal mechanical properties are determined by the geometrical, compositional, and structural characteristics. Dietary supplementation with calcitriol or quercetin did not affect bone geometrical characteristics but increased femoral ash, ash content, total calcium and phosphorus per bone. Furthermore, dietary quercetin also enhanced bone microarchitecture by increasing medullary bone thickness and BV/

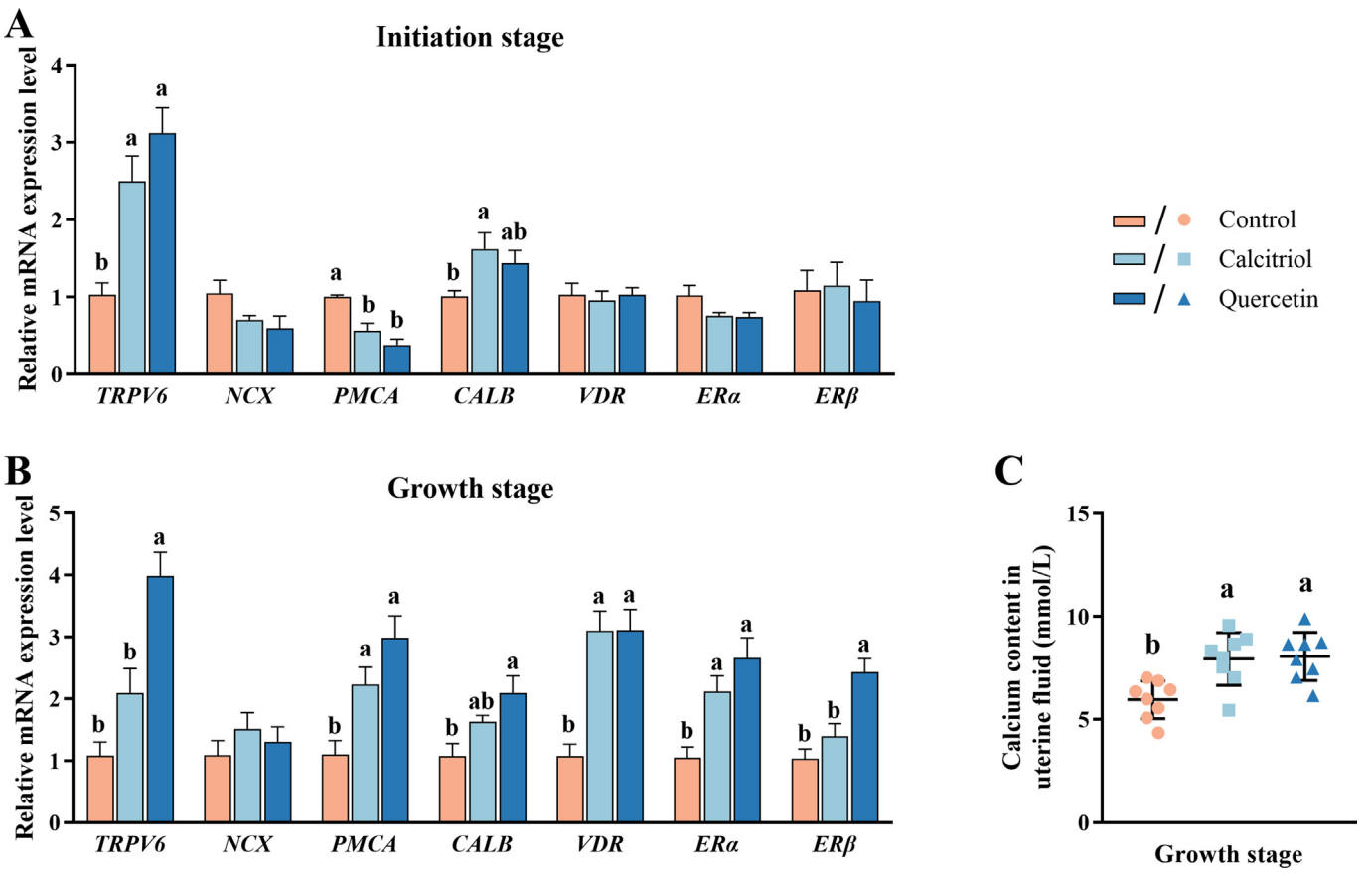

Fig. 5. Effects of dietary supplementation with calcitriol or quercetin on uterine calcium transport of aged laying hens (72 weeks of age). The relative mRNA expression levels of genes related to calcium transport at the initiation (A, 8.5 h post-oviposition) and growth (B, 18.5 h post-oviposition) stages of eggshell calcification; (C) Calcium content in the uterine fluid at the growth stage of eggshell calcification. *TRPV6* = transient receptor potential cation channel, subfamily V, member 6; *NCX* = sodium-calcium exchanger; *PMCA* = plasma membrane calcium-ATPase; *CALB* = calbindin; *VDR* = vitamin D receptor; *ERα* = estrogen receptor alpha; *ERβ* = estrogen receptor beta; Control = basal diet; Calcitriol = basal diet supplemented with 5 μg/kg calcitriol; Quercetin = basal diet supplemented with 500 mg/kg quercetin. All hens were fed treatment diets from 61 weeks of age. [a, b]Values in the same stage of eggshell calcification with no common letters differ significantly ($P < 0.05$). Values are presented as mean with standard error, $n = 8$.

TV. These findings suggest that both calcitriol and quercetin could promote mineral retention in the femur, potentially contributing to increased bone stiffness (Luo and Yang, 2019). Furthermore, the increase in medullary bone was significantly greater in the quercetin group compared to the calcitriol group. This difference may be attributed to estrogenic effect of quercetin, which facilitated the transition of bone turnover from cortical bone to medullary bone (Whitehead and Fleming, 2000). Additionally, increased medullary bone in the femur may account for a lower bone stiffness, since medullary bone has a lower structural strength than cortical bone (Fleming et al., 2006). Thus, dietary supplementation with calcitriol or quercetin improved bone stiffness by increasing mineral retention, in which the quercetin supplementation tended to form more medullary bone, leading to a lower bone stiffness compared to the calcitriol supplementation.

The improved eggshell and bone quality observed with calcitriol or quercetin supplementation was associated with increases in eggshell calcium deposition and skeletal calcium retention. As expected, the calcium retention of the layers increased with the addition of calcitriol or quercetin. An intact and healthy uterine tissue, along with highly expressed calcium transporters are the prerequisites for promoting eggshell calcification. CASP3 is the terminal caspase involved in apoptosis signaling. Its activation induces cell death in the apoptosis response, ultimately leading to tissue damage (McIlwain et al., 2013). In the current study, dietary calcitriol or quercetin supplementation down-regulated the relative mRNA expression level of *CASP3*. This finding was consistent with previous studies that reported a decrease in the apoptosis of granulosa cells due to calcitriol and quercetin, thereby promoting follicular development (Cheng et al., 2023; Yang et al., 2018), which indicates their positive effect on the development of poultry reproductive organs. This was also demonstrated by the improvements in uterine morphology showing a lower ratio of EDTG in the calcitriol and quercetin groups. The improvements of uterine morphology could promote eggshell calcium deposition and elevate eggshell quality while damaged uterine tissue could hinder calcium transport and the synthesis of matrix proteins (Feng et al., 2023).

There may be a complex relationship among calcitriol, estrogen, and their receptors. Earlier studies pointed out that exogenous estrogen could activate VDR in the duodenal mucosa and the uterus of rats (Liel et al., 1999). In this study, the relative mRNA expression level of *VDR* was up-regulated in the quercetin group during the growth stage of eggshell calcification, which was consistent with a previous study (Inoue et al., 2010). These observations indicate that quercetin might exert an estrogen-like effect, activating VDR, and potentially increasing the uterine sensitivity to vitamin D_3. Meanwhile, the *ERα* transcript was up-regulated with calcitriol supplementation during the growth stage of eggshell calcification, suggesting that calcitriol potentially activated the ERα-related pathway. In vitro and in vivo studies showed that calcitriol could activate the ERα transcription either directly via activating the

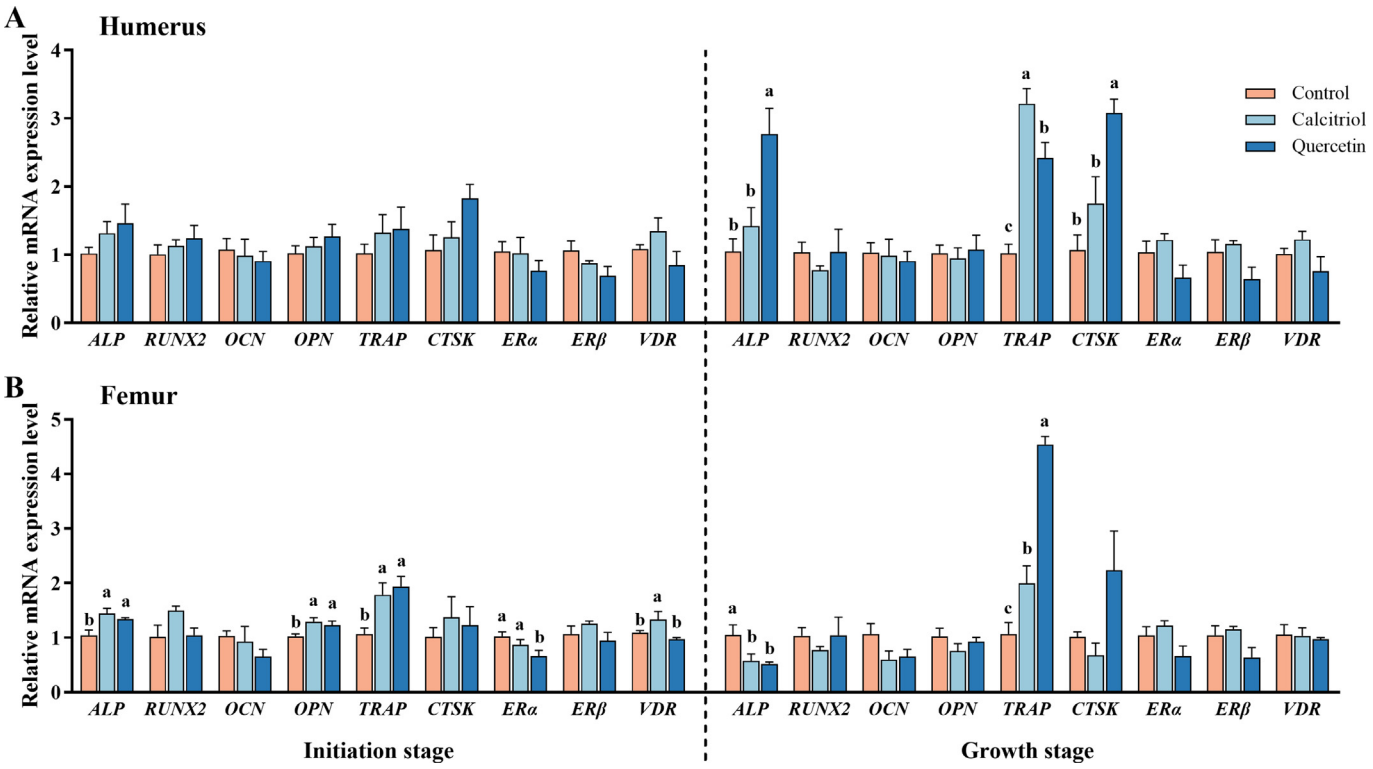

Fig. 6. Effects of dietary supplementation with calcitriol or quercetin on the relative mRNA expression levels of genes related to bone remodeling in the humerus (A) and femur (B) of aged laying hens (72 weeks of age). Initiation stage, at 8.5 h post-oviposition; Growth stage, at 18.5 h post-oviposition. *ALP* = alkaline phosphatase; *RUNX2* = runt-related transcription factor 2; *OCN* = osteocalcin; *OPN* = osteopontin; *TRAP* = tartrate-resistant acid phosphatase; *CTSK* = cathepsin K; *ERα* = estrogen receptor alpha; *ERβ* = estrogen receptor beta; *VDR* = vitamin D receptor; Control = basal diet; Calcitriol = basal diet supplemented with 5 μg/kg calcitriol; Quercetin = basal diet supplemented with 500 mg/kg quercetin. All hens were fed treatment diets from 61 weeks of age. $^{a-c}$Values in the same stage of eggshell calcification with no common letters differ significantly ($P < 0.05$). Values are presented as mean with standard error, $n = 8$.

vitamin D response element in the promoter region of ERα (Santos-Martínez et al., 2014, 2021) or, indirectly, via regulating the estrogen metabolism (Tsang and Grunder, 1984). Thus, there may be similarities in the regulatory effects of calcitriol and quercetin on uterine calcium transporters during the growth stage of eggshell calcification. This is notably achieved through the activation of both VDR- and ER-related pathways. The PMCA extrudes calcium out of cells against an electrochemical gradient (Bar, 2008). The upregulation of *PMCA* in the calcitriol and quercetin groups may promote uterine calcium transport (Brionne et al., 2014). Unlike in the kidney and intestine, the activity of PMCA in the uterus was not affected (Nys and de Laage, 1984) or only slightly influenced (Grunder et al., 1990) by calcitriol. Upon estrogen stimulation, the activity and concentration of PMCA were also essentially independent of calcitriol, however depended on the activation of the ER pathway (Corradino et al., 1993; Nys and de Laage, 1984). Taken together, the increases in the relative mRNA expression level of *PMCA* in the calcitriol and quercetin groups may be the result of an increased ERα during the growth stage of eggshell calcification.

In the current study, supplementation with quercetin, rather than with calcitriol, significantly up-regulated the uterine *CALB* and *TRPV6* transcripts during the growth stage of eggshell calcification, which coincides with previous studies demonstrating that estrogen stimulated the uterine *CALB* and *TRPV6* mRNA expression independently of vitamin D_3 (Navickis et al., 1979; van Abel et al., 2005). Additionally, the relative mRNA expression alteration of *ERβ* exhibited a similar tendency to that of *CALB* and *TRPV6*. However, the biological function of ERβ in the uterus of laying hens has received little attention and needs further investigation. It is worthwhile mentioning that during the initiation stage of eggshell calcification, dietary supplementation of calcitriol or quercetin altered uterine relative mRNA expression levels of *TRPV6*, *CALB* and *PMCA*. However, unlike in the growth stage, there was no impact on the relative mRNA expression levels of *VDR*, *ERα* and *ERβ*, suggesting that these regulatory effects were not associated with alterations in receptor expression. One possible reason is that calcitriol or quercetin supplementation changed the circulating levels of other hormones such as parathyroid hormone, progesterone, and calcitonin, which could further affect uterine calcium transporter relative mRNA expression (Bar, 2009; Cheng et al., 2023; Wang et al., 2022; Yang et al., 2018). While the relative mRNA expression patterns differed between hens supplemented with calcitriol and quercetin, both groups exhibited similar improvements in calcium transport, as evidenced by the comparable calcium concentration in uterine fluid. This may explain the reason for a comparable eggshell quality.

The physiological coupling of bone formation and bone resorption determines the deposition and release of skeletal calcium and, ultimately, bone quality. *ALP* gene expression induces hydroxyapatite growth, an early manifestation of bone mineralization (Nizet et al., 2020). TRAP and CTSK are specific markers of osteoclasts, which are respectively responsible for mineral dissolution and organic matter degradation (Dacke et al., 2015). A synchronous increase of humeral *ALP*, *TRAP* and *CTSK* relative mRNA expression levels in the quercetin group during the growth stage of eggshell calcification indicated a high bone turnover state. This suggested a rapid calcium mobilization and timely recovery of bone mass, ensuring the fulfillment of calcium requirements for both eggshell calcification and bone remodeling processes. In the calcitriol group, only *TRAP* relative mRNA expression level was increased

at the growth stage of eggshell calcification, suggesting that the calcitriol group may be subjected to a risk of bone mineral loss in the humerus. However, no phenomenon associated with bone loss was identified in the current study. To conclude, in the humerus, dietary supplementation with quercetin enhanced the expression of genes related to both bone formation and resorption. This effect may support a rapid export of skeletal calcium without compromising overall mineral retention.

Calcitriol and quercetin supplementation down-regulated the relative mRNA expression level of *ALP* at the growth stage of eggshell calcification, but up-regulated *TRAP* relative mRNA expression level at the growth and initiation stages. These data suggested that calcitriol and quercetin could facilitate the entry of skeletal calcium into circulation by decreasing bone formation (Nizet et al., 2020) and enhancing bone resorption (Hayman, 2008). The reason might be that skeletal calcium was mobilized to meet the higher calcium requirement for eggshell calcification in the calcitriol and quercetin groups. Less bone formation and more bone resorption would yield bone loss. Contrary to preconception, we found that the bone components were increased in the calcitriol and quercetin groups, which could be attributed to the upregulation of bone formation-associated genes such as *ALP* and *OPN* at the initiation stage of eggshell calcification (McKee et al., 1992; Nizet et al., 2020; Ren et al., 2023). In the current study, the relative mRNA expression levels of *VDR* and *ERs* were not altered in the humerus at both stages of eggshell calcification and in the femur at the growth stage, which was similar to the results in the uterus at the initiation stage of eggshell calcification. This might be because dietary calcitriol or quercetin could affect osteoblastic as well as osteoclastic activities by altering the levels of systemic hormones (e.g., parathyroid hormone, progesterone, and calcitonin) (Cheng et al., 2023; Dacke et al., 2015; Wang et al., 2022; Yang et al., 2018), rather than by activating *ERs* and *VDR* relative mRNA expression. Taken together, in the femur, dietary calcitriol or quercetin addition might promote the calcium transport from femur to the uterus and facilitate the recovery of bone mass and quality.

5. Conclusion

In summary, dietary supplementation with calcitriol or quercetin could improve eggshell and bone quality by affecting calcium metabolism. Specifically, dietary calcitriol or quercetin addition exhibited a better uterine morphology and an improved calcium transport-related relative mRNA expression pattern, which promoted eggshell calcification and enhanced eggshell ultrastructure, and eventually increased eggshell quality. Dietary supplementation with calcitriol or quercetin up-regulated genes associated with bone resorption to facilitate the delivery of skeletal calcium into circulation and up-regulated genes related to femoral bone formation to promote the recovery of bone mass. Compared to calcitriol, dietary quercetin addition exhibited a higher HDEP and enhanced uterine *TRPV6* and *CALB* relative mRNA expression levels, while it failed to affect eggshell quality. In the femur, hens fed with quercetin tended to form more medullary bone and showed a lower bone stiffness compared to hens fed with calcitriol. In addition to the classical VDR and ERs pathway, dietary calcitriol or quercetin addition may modulate eggshell and bone quality by mediating other hormones, which deserves further investigation.

Author contributions

Yu Fu: Conceptualization, Investigation, Data curation, Formal analysis, Writing - Original draft. **Jianmin Zhou:** Visualization, Writing - Review and Editing. **Martine Schroyen:** Conceptualization, Writing - Review and Editing. **Jing Lin:** Validation. **Haijun Zhang:** Methodology, Software. **Shugeng Wu:** Resources. **Guanghai Qi:** Conceptualization, Supervision, Writing - Review and Editing. **Jing Wang:** Conceptualization, Supervision, Writing - Review and Editing, Funding acquisition.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

Acknowledgments

This study was financially supported by the National Natural Science Foundation of China (32172743), the earmarked fund for China Agriculture Research Systems (CARS-40) and the Agricultural Science and Technology Innovation Program (ASTIP) of CAAS. The authors acknowledge the support from Chinese Scholarship Council.

Appendix supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2024.04.007.

References

Alfonso-Carrillo C, Benavides-Reyes C, de los Mozos J, Dominguez-Gasca N, Sanchez-Rodríguez E, Garcia-Ruiz AI, et al. Relationship between bone quality, egg production and eggshell quality in laying hens at the end of an extended production cycle (105 weeks). Animals (Basel) 2021;11:623.

Amevor FK, Cui Z, Du X, Ning Z, Shu G, Jin N, et al. Combination of quercetin and vitamin E supplementation promotes yolk precursor synthesis and follicle development in aging breeder hens via liver-blood-ovary signal axis. Animals (Basel) 2021;11:1915.

AOAC. Official methods of analysis. 18th ed. Gaithersburg, MD, USA: AOAC International; 2006.

Atteh JO, Leeson S. Effects of dietary fat level on laying hens fed various concentrations of calcium. Poult Sci 1985;64:2090–7.

Bain MM, Nys Y, Dunn IC. Increasing persistency in lay and stabilising egg quality in longer laying cycles. What are the challenges? Br Poult Sci 2016;57:330–8.

Bar A. Calcium homeostasis and vitamin D metabolism and expression in strongly calcifying laying birds. Comp Biochem Physiol A Mol Integr Physiol 2008;151:477–90.

Bar A. Calcium transport in strongly calcifying laying birds: mechanisms and regulation. Comp Biochem Physiol A Mol Integr Physiol 2009;152:447–69.

Brionne A, Nys Y, Hennequet-Antier C, Gautron J. Hen uterine gene expression profiling during eggshell formation reveals putative proteins involved in the supply of minerals or in the shell mineralization process. BMC Genomics 2014;15:220.

Brzóska MM, Majewska K, Moniuszko-Jakoniuk J. Mechanical properties of femoral diaphysis and femoral neck of female rats chronically exposed to various levels of cadmium. Calcif Tissue Int 2005;76:287–98.

Cheng M, Song Z, Guo Y, Luo X, Li X, Wu X, et al. 1α,25-dihydroxyvitamin D_3 improves follicular development and steroid hormone biosynthesis by regulating vitamin D receptor in the layers model. Curr Issues Mol Biol 2023;45:4017–34.

Corradino RA, Smith CA, Krook LP, Fullmer CS. Tissue-specific regulation of shell gland calbindin D28K biosynthesis by estradiol in precociously matured, vitamin D-depleted chicks. Endocrinology 1993;132:193–8.

Dacke CG, Sugiyama T, Gay CV. The role of hormones in the regulation of bone turnover and eggshell calcification. In: Scanes CG, editor. Sturkie's avian physiology. San Diego: Academic Press; 2015. p. 549–75.

de Moraes LR, Delicato MEA, da Silva Cruz A, Pereira da Silva H, de Vasconcelos Alves CVB, Campos DB, et al. Methionine supplementing effects on intestine, liver and uterus morphology, and on positivity and expression of Calbindin-D28k and TRPV6 epithelial calcium carriers in laying quail in thermoneutral conditions and under thermal stress. PLoS One 2021;16:e0245615.

Dunn IC, Rodríguez-Navarro AB, Mcdade K, Schmutz M, Preisinger R, Waddington D, et al. Genetic variation in eggshell crystal size and orientation is large and these traits are correlated with shell thickness and are associated with eggshell matrix protein markers. Anim Genet 2012;43:410–8.

Feng J, Lu M, Ma L, Zhang H, Wu S, Qiu K, et al. Uterine inflammation status modulates eggshell mineralization via calcium transport and matrix protein synthesis in laying hens. Anim Nutr 2023;13:411–25.
Fleming RH, McCormack HA, McTeir L, Whitehead CC. Relationships between genetic, environmental and nutritional factors influencing osteoporosis in laying hens. Br Poult Sci 2006;47:742–55.
Frost TJ, Roland Sr DA, Untawale GG. Influence of vitamin D_3, 1α-hydroxyvitamin D_3, and 1,25-dihydroxyvitamin D_3 on eggshell quality, tibia strength, and various production parameters in commercial laying hens. Poult Sci 1990;69:2008–16.
Fu Y, Wang J, Schroyen M, Chen G, Zhang HJ, Wu SG, et al. Effects of rearing systems on the eggshell quality, bone parameters and expression of genes related to bone remodeling in aged laying hens. Front Physiol 2022;13:962330.
Fu Y, Wang J, Zhang HJ, Wu SG, Zhou JM, Qi GH. The partial replacement of sodium chloride with sodium bicarbonate or sodium sulfate in laying hen diets improved laying performance, and eggshell quality and ultrastructure. Poult Sci 2021a;100:101102.
Fu Y, Zhang HJ, Wu SG, Zhou JM, Qi GH, Wang J. Dietary supplementation with sodium bicarbonate or sodium sulfate affects eggshell quality by altering ultrastructure and components in laying hens. Animal 2021b;15:100163.
Gautron J, Réhault-Godbert S, Van de Braak TGH, Dunn IC. Review: what are the challenges facing the table egg industry in the next decades and what can be done to address them? Animal 2021;15(Suppl 1):100282.
Grunder AA, Tsang CP, Narbaitz R. Effects of vitamin D_3 metabolites on physiological traits of white leghorn hens. Poult Sci 1990;69:1204–8.
Hansen KK, Kittok RJ, Sarath G, Toombs CF, Caceres N, Beck MM. Estrogen receptor-alpha populations change with age in commercial laying hens. Poult Sci 2003;82:1624–9.
Hayman AR. Tartrate-resistant acid phosphatase (TRAP) and the osteoclast/immune cell dichotomy. Autoimmunity 2008;41:218–23.
Inoue J, Choi JM, Yoshidomi T, Yashiro T, Sato R. Quercetin enhances VDR activity, leading to stimulation of its target gene expression in Caco-2 cells. J Nutr Sci Vitaminol (Tokyo) 2010;56:326–30.
Irani M, Merhi Z. Role of vitamin D in ovarian physiology and its implication in reproduction: a systematic review. Fertil Steril 2014;102:460–8.
Ishak K, Baptista A, Bianchi L, Callea F, De Groote J, Gudat F, et al. Histological grading and staging of chronic hepatitis. J Hepatol 1995;22:696–9.
Jin CL, Ye JL, Yang J, Gao CQ, Yan HC, Li HC, Wang XQ. mTORC1 mediates lysine-induced satellite cell activation to promote skeletal muscle growth. Cells 2019;8:1549.
Jonchère V, Brionne A, Gautron J, Nys Y. Identification of uterine ion transporters for mineralisation precursors of the avian eggshell. BMC Physiol 2012;12:10.
Kobayashi S, Inoue S, Hosoi T, Ouchi Y, Shiraki M, Orimo H. Association of bone mineral density with polymorphism of the estrogen receptor gene. J Bone Miner Res 1996;11:306–11.
Liel Y, Shany S, Smirnoff P, Schwartz B. Estrogen increases 1,25-dihydroxyvitamin D receptors expression and bioresponse in the rat duodenal mucosa. Endocrinology 1999;140:280–5.
Liu HN, Liu Y, Hu LL, Suo YL, Zhang L, Jin F, et al. Effects of dietary supplementation of quercetin on performance, egg quality, cecal microflora populations, and antioxidant status in laying hens. Poult Sci 2014;93:347–53.
Liu J, Fu Y, Zhou S, Zhao P, Zhao J, Yang Q, et al. Comparison of the effect of quercetin and daidzein on production performance, anti-oxidation, hormones, and cecal microflora in laying hens during the late laying period. Poult Sci 2023;102:102674.
Liu XT, Lin X, Mi YL, Zeng WD, Zhang CQ. Age-related changes of yolk precursor formation in the liver of laying hens. J Zhejiang Univ Sci B 2018;19:390–9.
Liu Y, Li Y, Liu HN, Suo YL, Hu LL, Feng XA, et al. Effect of quercetin on performance and egg quality during the late laying period of hens. Br Poult Sci 2013;54:510–4.
Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods 2001;25:402–8.
Luo Y, Yang H. Comparison of femur stiffness measured from DXA and QCT for assessment of hip fracture risk. J Bone Miner Metab 2019;37:342–50.
Macelline SP, Chrystal PV, Mcquade LR, Mclnerney BV, Kim Y, Bao Y, et al. Graded methionine dietary inclusions influence growth performance and apparent ileal amino acid digestibility coefficients and disappearance rates in broiler chickens. Anim Nutr 2022;8:160–8.
McIlwain DR, Berger T, Mak TW. Caspase functions in cell death and disease. Cold Spring Harb Perspect Biol 2013;5:a008656.
McKee MD, Glimcher MJ, Nanci A. High-resolution immunolocalization of osteopontin and osteocalcin in bone and cartilage during endochondral ossification in the chicken tibia. Anat Rec 1992;234:479–92.
Ministry of Agriculture of the People's Republic of China. China National Feeding Standard of Chicken (NY/T 33-2004). Beijing, China: China Agriculture Press; 2004.
Navickis RJ, Katzenellenbogen BS, Nalbandov AV. Effects of the sex steroid hormones and vitamin D_3 on calcium-binding proteins in the chick shell gland. Biol Reprod 1979;21:1153–62.
Newbrey JW, Truitt ST, Roland DA, Frost TJ, Untawale GG. Bone histomorphometry in $1,25(OH)_2D_3$- and vitamin D_3-treated aged laying hens. Avian Dis 1992;36:700–6.
Nizet A, Cavalier E, Stenvinkel P, Haarhaus M, Magnusson P. Bone alkaline phosphatase: an important biomarker in chronic kidney disease - mineral and bone disorder. Clin Chim Acta 2020;501:198–206.
NRC (National Research Council). Nutrient Requirements of Poultry. 9th ed. Washington (DC): National Academic Press; 1994. p. 19–25.
Nys Y, de Laage X. Effects of suppression of eggshell calcification and of $1,25(OH)_2D_3$ on Mg^{2+}, Ca^{2+} and $Mg^{2+}HCO_3^-$ ATPase, alkaline phosphatase, carbonic anhydrase and CaBP levels—II. The laying hen intestine. Comp Biochem Physiol A Comp Physiol 1984;78:839–44.
Nys Y, Le Roy N. Calcium homeostasis and eggshell biomineralization in female chicken. In: Feldman D, editor. Vitamin d. San Diego: Academic Press; 2018. p. 361–82.
Park JA, Sohn SH. The influence of hen aging on eggshell ultrastructure and shell mineral components. Korean J Food Sci Anim Resour 2018;38:1080–91.
Ren Y, Zhao T, Zhang K, Zhu Z, Li L, Li Y, et al. Effect of replacing dicalcium phosphate with mono-dicalcium phosphate to supplement phosphorus on laying performance, phosphorus-calcium metabolism and bone metabolism of aged laying hens. Front Vet Sci 2023;10:1196334.
Rodriguez-Navarro A, Kalin O, Nys Y, Garcia-Ruiz JM. Influence of the microstructure on the shell strength of eggs laid by hens of different ages. Br Poult Sci 2002;43:395–403.
Santos-Martínez N, Díaz L, Ordaz-Rosado D, García-Quiroz J, Barrera D, Avila E, et al. Calcitriol restores antiestrogen responsiveness in estrogen receptor negative breast cancer cells: a potential new therapeutic approach. BMC Cancer 2014;14:230.
Santos-Martínez N, Díaz L, Ortiz-Ortega VM, Ordaz-Rosado D, Prado-Garcia H, Avila E, et al. Calcitriol induces estrogen receptor α expression through direct transcriptional regulation and epigenetic modifications in estrogen receptor-negative breast cancer cells. Am J Cancer Res 2021;11:5951–64.
Seeman ED, Pierre D. Bone quality - the material and structural basis of bone strength and fragility. N Engl J Med 2006;354:2250–61.
Soares JH, Ottinger MA, Buss EG. Potential role of 1,25 dihydroxycholecalciferol in egg shell calcification. Poult Sci 1988;67:1322–8.
Song G, Seo HW, Choi JW, Rengaraj D, Kim TM, Lee BR, et al. Discovery of candidate genes and pathways regulating oviduct development in chickens. Biol Reprod 2011;85:306–14.
Tsang CP, Grunder AA. Effect of dietary contents of cholecalciferol, 1α,25-dihydroxycholecalciferol and 24,25-dihydroxycholecalciferol on blood concentrations of 25-hydroxycholecalciferol, 1α,25-dihydroxycholecalciferol, total calcium and eggshell quality. Br Poult Sci 1993;34:1021–7.
Tsang CP, Grunder AA, Narbaitz R. Optimal dietary level of 1α,25-dihydroxycholecalciferol for eggshell quality in laying hens. Poult Sci 1990;69:1702–12.
Tsang CPW, Grunder AA. Effects of vitamin D_3 deficiency on estradiol-17β metabolism in the laying hen. Endocrinology 1984;115:2170–5.
van Abel M, Hoenderop JG, Bindels RJ. The epithelial calcium channels TRPV5 and TRPV6: regulation and implications for disease. Naunyn Schmiedebergs Arch Pharmacol 2005;371:295–306.
Wang B, Wang S, Ding M, Lu H, Wu H, Li Y. Quercetin regulates calcium and phosphorus metabolism through the Wnt signaling pathway in broilers. Front Vet Sci 2022;8:786519.
Wang J, Wang WW, Qi GH, Cui CF, Wu SG, Zhang HJ, et al. Effects of dietary *Bacillus subtilis* supplementation and calcium levels on performance and eggshell quality of laying hens in the late phase of production. Poult Sci 2021;100:100970.
Whitehead CC, Fleming RH. Osteoporosis in cage layers. Poult Sci 2000;79:1033–41.
Wong SK, Chin KY, Ima-Nirwana S. Quercetin as an agent for protecting the bone: a review of the current evidence. Int J Mol Sci 2020;21:6448.
Yang JX, Chaudhry MT, Yao JY, Wang SN, Zhou B, Wang M, et al. Effects of phytoestrogen quercetin on productive performance, hormones, reproductive organs and apoptotic genes in laying hens. J Anim Physiol Anim Nutr (Berl) 2018;102:505–13.
Zhang YN, Zhang HJ, Wu SG, Wang J, Qi GH. Dietary manganese supplementation modulated mechanical and ultrastructural changes during eggshell formation in laying hens. Poult Sci 2017;96:2699–707.