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Calcium transport in uterus and bone in relationship to eggshell and bone qualities in aged laying hens

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Abstract

Cracked eggs and brittle bones are two major issues affecting the economic benefits of laying hens at the late laying period. Eggshell and bone quality is regulated by calcium transport in the uterus and bones of the hens. There may be an interaction between bone remodeling and eggshell calcification since bone resorption is an important calcium source for eggshell calcification. This dissertation explored the alterations of uterine calcium transport and bone remodeling as well as their impact on the eggshell and bone quality of aged hens. The relationship between eggshell and bone quality, alongside the possible mechanisms contributing to the reduction of their quality, were also examined in this thesis.

The first study investigated the effects of rearing systems on eggshell quality and bone parameters as well as the expression of genes related to bone remodeling and uterine calcium transport in aged laying hens. Layers were housed in the conventional caging system (CCS) or aviary system (AVS) from 55 days of age to 95 weeks of age. The eggshell quality decreased with age. However, at 95 weeks of age, the AVS had higher eggshell mechanical properties and components compared with the CCS, which may be attributed to the upregulated uterine calcium transporter (transient receptor potential cation channel, subfamily V, member 6 (TRPV6)) in the AVS. The AVS increased the mechanical properties, total calcium per bone, and mineral measurements of humeri, which may be associated with increased expression levels of hormone receptors (vitamin D receptor (VDR), estrogen receptor alpha (ERα), and fibroblast growth factor 23 (FGF 23)). The simultaneous upregulations of bone formation (alkaline phosphatase (ALP) and osteocalcin (OCN)) and resorption (tartrate-resistant acid phosphatase (TRAP)) related genes may contribute to the unchanged femur quality of the AVS relative to the CCS. Overall, compared with the CCS, the AVS simultaneously alleviated the deterioration of eggshell and bone qualities of aged laying hens, which may be related to the changes in the expression of genes associated with bone remodeling and uterine calcium transport. Subsequent experiments were carried out within the CCS due to the more significant decline observed in eggshell and bone quality of aged hens.

The second study compared the differences in eggshell quality, bone parameters and their correlations of aged hens laid eggs with high (HBS, 44.83 ± 1.31 N) or low (LBS, 24.43 ± 0.57 N) eggshell breaking strength. Their uterine physiological characteristics and bone remodeling processes were further explored to reveal the mechanism of eggshell and bone quality reduction and their interaction. Eggshells in the LBS showed poor quality, ultrastructural deterioration, and total calcium reduction. Bone quality was negatively correlated with eggshell quality, marked with enhanced structures and increased components in the LBS. In the LBS, the mammillary knobs and effective layer grew slowly. At the initiation stage of eggshell calcification, transcriptional profiling revealed the differentially expressed genes (DEGs) were relevant to apoptosis due to the cellular calcium overload. The level or activity of apoptosis-related proteins (p62, Bax, Bcl-2, and caspase-8), TUNEL assay, and hematoxylin-eosin staining results showed increased apoptosis and tissue damage in

the uterus of the LBS. Similar damages were also observed in the uterus collected at the growth stage, although few DEGs were identified at this stage. The expressions of runt-related transcription factor 2 (RUNX 2) and OCN were upregulated in the humeri of the LBS. An enlarged diameter and more structural damages of endocortical bones and decreased ash were observed in the femurs of the HBS. The lower eggshell breaking strength may be attributed to a reduced calcium transport due to uterine tissue damage, which could affect eggshell calcification and lead to a weak ultrastructure. Impaired uterine calcium transport may result in reduced femoral bone resorption and increased humeral bone formation to maintain a higher mineral and bone quality in the LBS. The improvements in calcium metabolism of aged laying hens (such as by supplementing calcitriol or quercetin) have a potential to increase eggshell and bone quality.

The third study compared the effects of dietary calcitriol or quercetin addition on eggshell and bone quality of aged laying hens. Dietary calcitriol or quercetin supplementation improved eggshell quality (breaking strength, ultrastructure, and total calcium) and femoral bone quality (stiffness, components). Both of them increased calcium retention of hens and calcium concentration in uterine fluid at the growth stage of eggshell calcification. Additionally, dietary supplementation with calcitriol or quercetin downregulated apoptosis-related gene expression (caspase 3) at the initiation stage of eggshell calcification and improved uterine morphology. They also increased the transcripts of uterine calcium transporters (initiation stage: TRPV6; growth stage: PMCA) and hormone receptors (growth stage: VDR, ERα). These changes may be beneficial for the uterine calcium transport. Moreover, dietary addition of calcitriol or quercetin declined the femoral transcript of ALP but increased that of TRAP at the growth stage of eggshell calcification, which may contribute to the skeletal calcium delivery to the uterus. However, the concurrent upregulations in bone formation (ALP and osteopontin (OPN)) and resorption (TRAP) related genes at the initiation stage promoted the recovery of femoral bone mass. By contrast, hens fed with quercetin had higher egg production and femurs with more medullary bone and lower stiffness. Overall, dietary supplementation with calcitriol or quercetin could simultaneously improve eggshell and bone quality by modulating the calcium metabolism of aged layers.

In conclusion, eggshell quality was closely associated with bone remodeling but the relationship performs differentially due to the inconsistent physiological status of laying hens under different conditions. Compared with the CCS, the AVS that provides more space for movement would simultaneously improve bone remodeling and uterine calcium transport thus alleviating the decline of bone and eggshell qualities in aged laying hens. Under the same environment, diet, and age, the lower eggshell breaking strength was related to impaired uterine calcium transport, which may result in reduced femoral bone resorption and increased humeral bone formation to maintain a higher mineral and bone quality. Dietary calcitriol or quercetin addition could simultaneously improve eggshell and bone quality by modulating calcium metabolism in aged hens. A better-quality shell was accompanied by increased uterine calcium transport and active bone resorption. The various impacts of different

conditions on bone formation may be the main reason for diverse correlations between eggshell and bone quality. Additionally, in aged laying hens, the reduced eggshell quality may be related to the less bone resorption, the uterine tissue damages and the lower calcium metabolic capacity of hens, while the deteriorated bone quality may be associated with rearing systems (the opportunities of movement), the high intensity eggshell calcification and the reduced calcium metabolic capacity of hens.

Keywords: eggshell quality, bone quality, eggshell calcification, uterine calcium transport, bone remodeling, humerus, femur, laying hen

Résumé

Les œufs fissurés et les os fragiles représentent deux problématiques majeures affectant la rentabilité économique des poules pondeuses en fin de période de ponte. La qualité de la coquille d'œuf et des os est régulée par le transport du calcium dans l'utérus et les os des poules. Une interaction potentielle entre le remodelage osseux et la calcification de la coquille d'œuf a été envisagée, car la résorption osseuse constitue une source cruciale de calcium pour la calcification de la coquille. Cette thèse explore les modifications du transport utérin du calcium et du remodelage osseux, ainsi que leur impact sur la qualité de la coquille d'œuf et des os chez les poules vieillissantes. La relation entre la qualité de la coquille d'œuf et des os, ainsi que les mécanismes possibles contribuant à la réduction de leur qualité, a également été examinée.

La première étude a exploré les effets des systèmes d'élevage sur la qualité de la coquille d'œuf, les paramètres osseux, ainsi que l'expression des gènes liés au remodelage osseux et au transport utérin du calcium chez les poules pondeuses vieillissantes. Les sujets ont été logés dans le système de cages conventionnelles (CCS) ou le système de volières (AVS) de 55 jours à 95 semaines d'âge. La qualité de la coquille d'œuf a décliné avec l'âge. Toutefois, à 95 semaines, les œufs produits dans le système AVS présentaient des propriétés mécaniques et des composants de la coquille supérieurs aux œufs produits en CCS, ce qui pourrait être lié à une surexpression du transporteur de calcium utérin (canal cationique à potentiel transitoire, sous-famille V, membre 6 (TRPV6)) dans le sytème AVS. Le logement en AVS a amélioré les propriétés mécaniques, le calcium total par os et les mesures minérales des humérus, ce qui pourrait être associé à une surexpression des récepteurs hormonaux (récepteur de la vitamine D (VDR), récepteur des œstrogènes alpha (ERα) et facteur de croissance des fibroblastes 23 (FGF 23)). Les régulations positives (activation) simultanées des gènes liés à la formation osseuse (phosphatase alcaline (ALP) et ostéocalcine (OCN)) et à la résorption osseuse (phosphatase acide résistante au tartrate (TRAP)) pourraient contribuer à la qualité inchangée du fémur dans le système l'AVS par rapport au CCS. Globalement, comparé au CCS, l'AVS a simultanément atténué la détérioration de la qualité de la coquille d'œuf et des os chez les poules pondeuses vieillissantes, possiblement liée aux changements dans l'expression des gènes associés au remodelage osseux et au transport utérin du calcium. Des expériences ultérieures ont été menées dans le CCS en raison de son déclin plus marqué observé dans la qualité de la coquille d'œuf et des os des poules vieillissantes.

La deuxième étude a comparé les différences de qualité de la coquille d'œuf, les paramètres osseux et leurs corrélations chez les poules âgées pondant des œufs avec une résistance à la rupture élevée (HBS, 44,83 ± 1,31 N) ou faible (LBS, 24,43 ± 0,57 N). Les caractéristiques physiologiques de leur utérus et les processus de remodelage osseux ont été approfondis pour élucider le mécanisme de réduction de la qualité de la coquille d'œuf et des os, ainsi que leur interaction. Les coquilles d'œufs du LBS présentaient une qualité médiocre, une détérioration ultrastructurale et une réduction du calcium total. La qualité osseuse était négativement corrélée à la qualité de la coquille d'œuf, marquée par de meilleures structures et le calcium total par os accrus

dans le LBS. Dans le LBS, les mamelons et la couche effective présentaient une croissance plus lente. Au stade d'initiation de la calcification de la coquille d'œuf, l'analyse transcriptionnelle a révélé que les gènes exprimés de manière différentielle (DEG) étaient liés à l'apoptose en raison de la surcharge calcique cellulaire. Les résultats de l'activité ou du niveau des protéines liées à l'apoptose (p62, Bax, Bcl-2 et caspase-8), de l'analyse TUNEL et de la coloration à l'hématoxyline-éosine ont montré une apoptose accrue et des dommages tissulaires dans l'utérus du LBS. Bien que peu de DEG aient été identifiés au stade de croissance, des dommages tissulaires utérins similaires ont également été observés dans le LBS. Les expressions du facteur de transcription 2 lié à la course (RUNX 2) et de l'OCN ont été surexprimées dans les humérus du LBS. Un diamètre agrandi, davantage de dommages structurels des os endocorticaux et une diminution des cendres ont été observés dans les fémurs du HBS. La résistance à la rupture plus faible de la coquille d'œuf dans le LBS pourrait être attribuée à un transport réduit du calcium en raison des dommages tissulaires utérins, ce qui pourrait affecter la calcification de la coquille d'œuf et entraîner une faible ultrastructure. Un transport utérin défectueux du calcium peut entraîner une réduction de la résorption osseuse fémorale et une augmentation de la formation osseuse humérale pour maintenir une qualité minérale et osseuse supérieure dans le LBS. Les améliorations du métabolisme du calcium chez les poules pondeuses vieillissantes (telles que la supplémentation en calcitriol ou en quercétine) ont le potentiel d'augmenter la qualité de la coquille d'œuf et des os.

La troisième étude a comparé les effets de l'ajout alimentaire de calcitriol ou de quercétine sur la qualité de la coquille d'œuf et des os chez les poules pondeuses vieillissantes. La supplémentation alimentaire en calcitriol ou en quercétine a amélioré la qualité de la coquille d'œuf (résistance à la rupture, ultrastructure et calcium total) et la qualité osseuse fémorale (rigidité, composants). Les deux ont augmenté la rétention de calcium chez les poules et la concentration de calcium dans le liquide utérin au stade de croissance de la calcification de la coquille d'œuf. De plus, la supplémentation alimentaire en calcitriol ou en quercétine a régulé à la baisse l'expression des gènes liés à l'apoptose (caspase 3) au stade d'initiation de la calcification de la coquille d'œuf et a amélioré la morphologie utérine. Elle a également augmenté les transcriptions des transporteurs de calcium utérins (stade d'initiation : TRPV6 ; stade de croissance : PMCA) et des récepteurs hormonaux (stade de croissance : VDR, ERα). Ces changements peuvent être bénéfiques pour le transport utérin du calcium. De plus, l'ajout alimentaire de calcitriol ou de quercétine a diminué la transcription fémorale de l'ALP mais a augmenté celle de la TRAP au stade de croissance de la calcification de la coquille d'œuf, ce qui peut contribuer à la fourniture de calcium squelettique à l'utérus. Cependant, les uprégulations simultanées des gènes liés à la formation osseuse (ALP et ostéopontine (OPN)) et à la résorption osseuse (TRAP) au stade d'initiation ont favorisé la récupération de la masse osseuse fémorale. En revanche, les poules nourries avec de la quercétine ont eu une production d'œufs plus élevée et des fémurs avec plus d'os médullaire et une rigidité inférieure. Dans l'ensemble, la supplémentation alimentaire en calcitriol ou en

quercétine pourrait simultanément améliorer la qualité de la coquille d'œuf et des os en modulant le métabolisme du calcium chez les poules vieillissantes.

En conclusion, la qualité de la coquille d'œuf était étroitement associée au remodelage osseux, mais la nature de cette relation varie en fonction du statut physiologique des poules pondeuses dans des conditions diverses. Comparé au CCS, l'AVS, qui offre plus d'espace pour le mouvement, améliorerait simultanément le remodelage osseux et le transport utérin du calcium, atténuant ainsi le déclin de la qualité des os et de la coquille d'œuf chez les poules pondeuses vieillissantes. Dans le même environnement, avec la même alimentation et le même âge, la résistance plus faible de la coquille d'œuf était liée à un transport utérin altéré du calcium, entraînant une réduction de la résorption osseuse fémorale et une augmentation de la formation osseuse humérale pour maintenir une qualité minérale et osseuse supérieure. L'ajout alimentaire de calcitriol ou de quercétine pourrait simultanément améliorer la qualité de la coquille d'œuf et des os en modulant le métabolisme du calcium chez les poules vieillissantes. Une coquille de meilleure qualité était accompagnée d'un transport utérin du calcium accru et d'une résorption osseuse active. Les divers impacts des différentes conditions sur la formation osseuse pourraient être la principale raison des corrélations diverses entre la qualité de la coquille d'œuf et des os. De plus, chez les poules pondeuses vieillissantes, la réduction de la qualité de la coquille d'œuf pourrait être liée à une moindre résorption osseuse, des dommages tissulaires utérins et une capacité métabolique du calcium inférieure, tandis que la détérioration de la qualité osseuse pourrait être associée aux systèmes d'élevage (opportunités de mouvement), à l'intensité élevée de la calcification de la coquille.

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List of abbreviations

$1,25\text{-}(OH)_2\text{-}D_3$: 1α,25-dihydroxyvitamin D_3

$25\text{-}OH\text{-}D_3$: 25-hydroxyvitamin D_3

ALP: Alkaline phosphatase

AVS: Aviary system

BALP: Bone-specific alkaline phosphatase

BMC: Bone mineral content

BMD: Bone mineral density

BSP: Bone sialoprotein

BV/TV: Trabecular bone volume/tissue volume

Ca: Calcium

CALB: Calbindin

CASP3: Caspase 3

CASP8: Caspase 8

CASP9: Caspase 9

CCS: Conventional caging system

COL I: Collagen type I

Cts K: Cathepsin K

DEGs: Differentially expressed genes

E_2: Estrogen

ERα: Estrogen receptor alpha

ERβ: Estrogen receptor beta

ET: Effective layer thickness

FGF 23: Fibroblast growth factor 23

HBS: High eggshell breaking strength group

HDEP: Hen-day egg production

HE: Hematoxylin-eosin

LBS: Low eggshell breaking strength group

M-CSF: Macrophage colony-stimulating factor

MT: Mammillary layer thickness

NCX: Sodium-calcium (Na^{+}/Ca^{2+}) exchange

OCN: Osteocalcin

ON: Osteonectin

OPG: Osteoprotegerin

OPN: Osteopontin

P: Phosphorus

PMCA: Plasma membrane calcium-ATPase

PO: Post-oviposition

PTH: Parathyroid hormone

RIA: Radioimmunoassay

RANK: Receptor activator of nuclear factor-kappa B

RANKL: Receptor activator of nuclear factor-kappa B ligand

RUNX 2: Runt-related transcription factor 2

SD: Standard deviation

SEM: Standard error of the mean

Tb.N: Trabecular number

Tb.Sp: Trabecular separation

Tb.Th: Trabecular thickness

TRAP: Tartrate-resistant acid phosphatase

TRPV6: Transient receptor potential cation channel, subfamily V, member 6

TT: Total thickness

VDR: Vitamin D receptor.

Chapter 1

General introduction

Increased broken eggs and the weakening of bones in the late laying period pose considerable risks to the economic benefit and animal welfare of the poultry industry (Dunn et al., 2009; Ren et al., 2018). During the laying period, the average eggshell breakage rate increases from 6% to 20% with age (Bell, 2002; Hanlon et al., 2022; Lolli et al., 2013). Therein, the percentage of broken eggs during the whole production chains (from laying to retail) was higher in the conventional cages (17% - 44.63%) than in the aviary (2% - 5.47%) (Hamilton et al., 2021; Mertens et al., 2006). The incidence of bone fracture in laying hens is up to 30% in the conventional cages at the late laying period (Clark et al., 2008; Gregory et al., 1989; Huang et al., 2020a), while the incidence of broken bones occurred with a lower frequency in the aviary (1.3% - 4.6%) (Leyendecker et al., 2005). Eggshells and bones, as the main calcified products and structures, may have some relationships in their formation process and qualities.

1. Uterine physiological status and the calcium transporters affecting eggshell calcification in laying hens

1.1. Eggshell ultrastructure formation and its effects on mechanical properties

During ovulation, the yolk is released from the largest follicle into the oviduct, after which it gets encapsulated with albumen proteins secreted by the magnum. At around 3.5 - 4 h post-ovulation, the yolk wrapped with albumen enters the isthmus and two eggshell membrane formation begins. Shortly after that (1 - 1.5 h), the egg descends to the uterus (eggshell gland) and the eggshell formation is initiated. As shown in Figure 1-1, three main stages are involved in the eggshell temporal and spatial deposition, determining a multilayered ultrastructure of the eggshell. The first one is the initiation stage of eggshell calcification (from 5 to 10 h post-ovulation). The deposition of the first calcite crystal on the organic nucleation sites of the outer eggshell membrane marks the onset of eggshell calcification, subsequently calcite crystals grow radially within these sites to form the mammillary knobs. As calcite crystals continue to pile up, adjacent mammillary knobs fuse in competition for available space (Rodriguez-Navarro et al., 2000). This first stage is a “slow” phase, since only 0.1 g of eggshell is deposited per hour during this phase. The second one is the growth stage of eggshell calcification (from 10 to 22 h post-ovulation) with a rapid deposition of calcite (0.33 g per hour) (Eastin et al., 1978a). During this stage, the fusion of adjacent mammillary knobs builds a base of the eggshell palisade layer, in which calcite crystals are rapidly growing perpendicular to the eggshell surface, developing a palisade layer and a thin vertical layer. The last one, the termination stage of eggshell calcification, is characterized by the arrest of calcification and the formation of the cuticle. At this point, the eggshell ultrastructure is established, including an inner eggshell membrane (~20 μm), an outer eggshell membrane (~60 μm), a mammillary layer (~100 μm), an effective layer (palisade and vertical layers; ~200 μm) and a cuticle (~8 μm).

The calcified layer, consisting of a mammillary layer and an effective layer, dominates the mechanical properties of eggshells (Bain, 1992; Zhang et al., 2017). Abnormalities in the mammillary layer may directly decrease the tight junctions between the outer membrane and mammillary knobs, leading to worse eggshell mechanical properties (Macleod et al., 2006). A reduction in the depth of the inter-mammillary crevices contributes to preventing micro-cracking under external forces (Macleod et al., 2006). Abnormalities of the mammillary layer could even affect the physical properties of subsequent structures, such as a declined effective layer thickness or an increased eggshell porosity (Dunn et al., 2012; Radwan, 2016). The thickness of the effective layer forms two-thirds of the total eggshell thickness, and this is crucial for the eggshell breaking strength (Fathi et al., 2007; Radwan, 2016). An average force of 35 N has been noted to be required to break a 0.33 mm thick eggshell (Nys et al., 2022). The cuticle mainly exerts an antibacterial function (Wellman-Labadie et al., 2010), while its effects on the total mechanical properties of eggshells are only poorly reported.

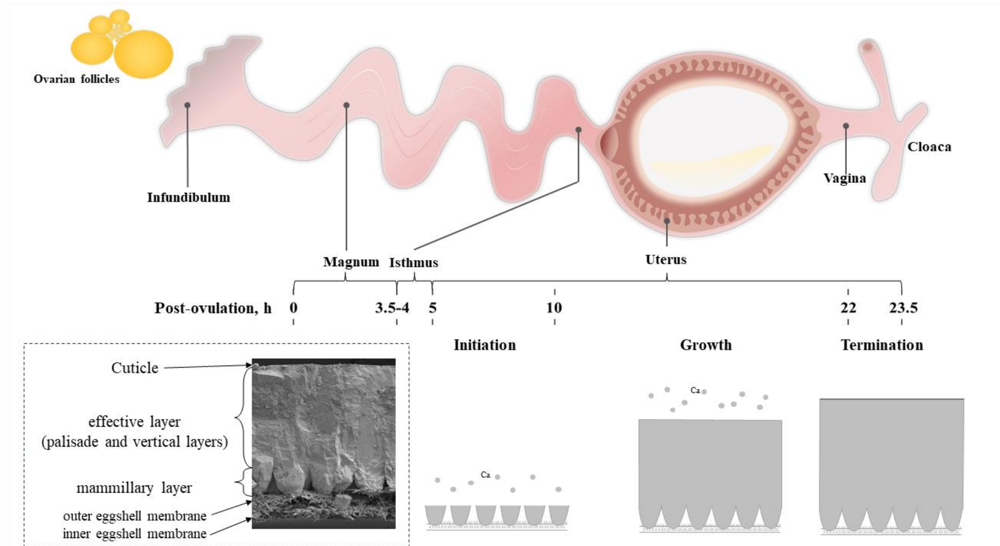

Figure 1-1. Eggshell calcification stages and eggshell ultrastructure formation.

1.2. Uterine physiological status affecting eggshell calcification

The uterus is the main organ responsible for eggshell formation. The uterus is a multilayered structure and is composed of, from inner to outer, a mucosa layer (mucous epithelium and lamina propria), a sub-mucosa layer (vascular system and inner connective tissue), a muscle layer (circular muscle, outer connective tissue, and longitudinal muscle) and a serosa layer (Figure 1-2, Nys et al. (2011), Mohammadpour et al. (2012)). The mucosa layer has numerous mucosal folds appearing leaf-shaped, partially with some secondary or tertiary folding on the surface (Mohammadpour et al., 2012). Numerous tubular glands are presented in the lamina propria and secrete most of the eggshell constituents (Hrabia, 2022). The abundant

secondary and tertiary folds imply a larger area for the production of calcium carbonate which is the main component of the eggshell (de Moraes et al., 2021). Higher mucosal folds can increase the absorption and transport of ions to accelerate eggshell calcification (Ma et al., 2020). The multilayered structure of the sub-mucosa layer and muscle layer controls the translocation and rotation of the egg in the oviduct (Hrabia, 2022). Thus, the physiological status of the uterus may affect the formation of eggshells.

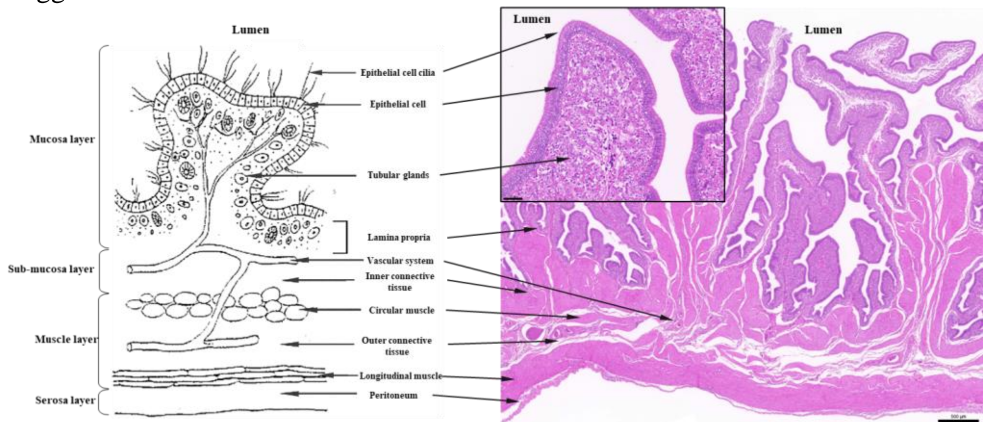

Figure 1-2. Structure diagram of uterus (mode diagram from Gilbert (1979)). Bar = low magnification, 500 μm; high magnification (inset), 50 μm.

A multitude of studies have observed the associations between uterine morphology and eggshell formation. The density of uterine tubular glands was seen to follow a downward trend from 21 to 49 weeks of age, remaining constant thereafter (to 70 weeks of age) (Wistedt et al., 2019). After high-intensity laying cycles, injuries such as deformation, fibrosis, and atrophy in the endometrial villi, as well as the elimination of micro-villi and the edema of tubular glands presented in the uterus of aged laying hens, affecting the biosynthesis of organic matrix and ion transport in the uterus (Feng et al., 2023; Park et al., 2017; Park et al., 2018), which may be a possible reason for age-related decreases in eggshell quality. Furthermore, age-related damages in the endometrial tissue inhibited the processes of ion transport and the crystallization of eggshell formation, leading to an abnormal formation of mammillary knobs (Park et al., 2018). Likewise, the uterus has been found to also exhibit apoptosis in the mucosal epithelial layer, edema or dissolution in tubular glands, as well as atrophy, edema, and fracture in the villi in the laying hens exposed to lipopolysaccharide or Newcastle disease virus, thus affecting eggshell mineralization and eggshell color deposition (Feng et al., 2023; Igwe et al., 2018). Additionally, dietary supplementation with methionine, complex trace elements (zinc, copper, and manganese), or Chinese herbal medicine enhanced the development and maintenance of the uterus mucosa to improve eggshell quality (de Moraes et al., 2021; Jiang et al., 2021; Yu et al., 2023). The previous studies reported that Chinese herbal medicine could regulate the activities of antioxidant enzymes and the expression of inflammatory-related

cytokines to modulate the physiological inflammation occurring in the uterus during eggshell formation, which may improve the uterine morphology and the shell matrix protein biogenesis in oviduct of laying hens (Liu et al., 2023b; Xiao et al., 2019). Thus, the morphological changes in the uterus may influence the biosynthesis of the organic matrix and the efficiency of ion translocation in the process of eggshell formation.

1.3. Calcium transporters involved in eggshell calcification

The chemical composition of eggshells mainly comprises 95% calcium carbonate in the form of calcite and 3.5% organic matrices. Calcium required for eggshell formation is not stored in the uterus before calcification but comes from the blood during calcification. The uterine secretion of calcium is discontinuous and periodic, and the uterus exhibits a higher transport capacity for ions during eggshell calcification especially at the growth stage to satisfy the need for rapid deposition of calcite (Eastin et al., 1978a; Nys et al., 2011). As shown in Figure 1-3, calcium transport involves at least 3 pathways in the uterus of laying hens, including transmembrane transport, extracellular vesicle transport, and paracellular transport. Many proteins related to calcium transport were identified (Table 1-1), which play vital roles in eggshell calcification.

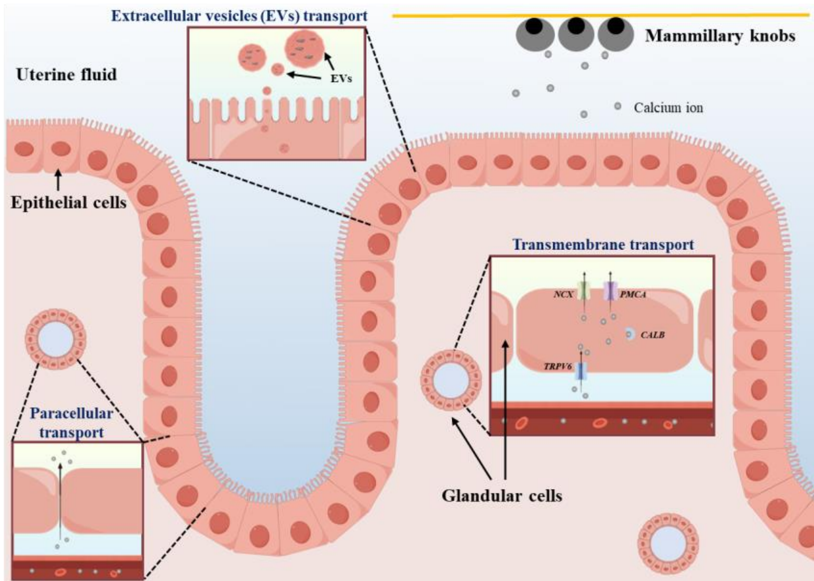

Figure 1-3. Calcium transport pattern diagram in the uterus of laying hens, including the transmembrane transport, extracellular vesicle transport, and paracellular transport. TRPV6, transient receptor potential vanilloid 6; CALB, calbindin; PMCA, plasma membrane Ca-ATPases; NCX, sodium/calcium exchanger.

The localization of abundant calcium transporters in glandular cells highlights the significance of transmembrane transport (Bar, 2009b; Jonchère et al., 2012). Three steps and at least four calcium transporters are considered to be involved in the transmembrane transport of glandular cells. Firstly, due to a much higher calcium

concentration in the plasma (1.2 mM) relative to the uterine cell interior (< 0.0002 mM) (Sauveur et al., 1971), calcium passively enters the uterine cells by passing through voltage-dependent calcium channels. In the kidney, intestine, or plasma, transient receptor potential vanilloid 5 (TRPV5) and TRPV6 are principal epithelial channels for calcium entry into cells (Hoenderop et al., 2005; Yang et al., 2011). However, only TRPV6 was identified in the uterus of laying hens (Jonchère et al., 2012; Yang et al., 2013). Compared with the magnum, TRPV6 is significantly overexpressed in the active uterus, indicating that it may play an important role in uterine calcium transport during eggshell calcification (Jonchère et al., 2012).

Secondly, calbindin proteins and some endoplasmic reticulum proteins (e.g. inositol trisphosphate receptors type 1, 2, and 3 (ITPR1, 2, 3) and ATP-dependent calcium pumps (ATP2A2 and 3)) contribute to the intracellular transport of calcium and the maintenance of low intracellular free calcium concentration (Brionne et al., 2014; Sah et al., 2018). Calbindin proteins are highly expressed in the cytoplasm of mammals, including calbindin 9 kDa (Calbindin 9k) and 28 kDa (Calbindin 28k) (Christakos et al., 2003), but in birds only the 28 kDa variant (encoded by CALB1) is expressed (Jande et al., 1981; Lippiello et al., 1975). Calbindin 28k has been widely employed as a marker for calcium secretion since its mucosal concentration reflects the capacity to transfer calcium in the intestine and uterus (Bar, 2008, 2009a, 2009b; Bouillon et al., 2003; Christakos et al., 2020; Nys et al., 2018). The expression level of CALB1 is highly expressed in the uterus during eggshell calcification compared with in the magnum or in the egg-free uterus (Bar et al., 1990; Jonchère et al., 2012; Nys et al., 1989), after which its expression rapidly decreases at the moment of egg expulsion (Nys et al., 1992). This indicates a distinct relationship between the expression of CALB1 and the eggshell calcification. The expression of CALB1 may be beneficial to transport intracellular calcium from the basal membrane to the apical membrane of the glandular cells (Jonchère et al., 2012; Nys et al., 2022). In addition to calcium transport, calbindins and other regulators for intracellular calcium homeostasis serve as calcium buffers that are essential for shielding cells from calcium stress and the apoptotic cellular degradation caused by elevated intracellular calcium levels (Christakos et al., 2007; Elíes et al., 2020).

Thirdly, the calcium is extruded through plasma membrane Ca-ATPases (PMCA) and the sodium/calcium exchanger (NCX) at the apical membrane of the glandular cells (Brionne et al., 2014; Sah et al., 2018). This is an active transport process of calcium against the concentration gradient because of a higher calcium concentration in the uterine fluid (6 - 10 mM) to adapt eggshell calcification (Jonchère et al., 2012; Nys et al., 1991). Early evidence identified the presence of energy-dependent calcium transporters in the uterus (Schraer et al., 1970), where PMCA is localized primarily at the apical membrane of the glandular cells (Wistedt et al., 2019; Yamamoto et al., 1985). Another mechanism of calcium transfer from uterine cells into the uterine fluid involves NCX, by which calcium is transported accompanied by a sodium influx, thereby creating a voltage potential gradient (Lavelin et al., 2001). Although NCX is active in the uterus during eggshell calcification, the major contributor to excrete calcium is believed to be PMCA rather than NCX (Parker et al., 2008) since only a

slight change in calcium excretion has been observed when sodium was removed from the perfusate (with 125 mM/L sodium before) in *vivo* perfusion experiments (Eastin et al., 1978b). Recent publications pointed out that some of the uterine intracellular calcium extrusion into the uterine fluid is secreted by extracellular vesicles containing amorphous calcium carbonate in the epithelial cells (Stapane et al., 2019; Stapane et al., 2020).

These findings provided a reasonable hypothesis of different mechanisms involved in calcium transport. However, the respective contributions of these candidates are difficult to establish since they are qualitative rather than quantitative. Additionally, the contribution of the paracellular pathway is probably minor in the calcium transport, while it favors the maintenance of a stable osmolarity via the secretion of sodium, potassium, chloride, and water (Bar, 2009b; Nys et al., 1999; Nys et al., 2018).

During eggshell calcification, many proteins responsible for transporting other ions (HCO_3^-, Na^+, K^+, Cl^-, H^+) were also identified, and they contribute to the maintenance of cellular ionic homeostasis and the secretion of calcium (Table 1-1, Nys et al. (2022)). Calcium deposition is a complex biological process regulated by multiple proteins. The recent development of high-throughput technologies has facilitated the identification of many proteins in the uterus and eggshells (Brionne et al., 2014; Marie et al., 2015). Among these proteins, major functions have been assigned such as calcium binding, amorphous calcium carbonate (ACC) stabilization, calcite crystal deposition, matrix organization and antimicrobial function (Table 1-1, Marie et al. (2015), Stapane et al. (2019)).

Table 1-1. Major proteins involved in eggshell calcification[1]

Gene symbol	Name	Description
Calcium ion (Ca^{2+}) transport		
Transmembrane transport		
TRPV2, 4, 6	Transient receptor potential cation channel subfamily V member 2, 4, 6	Ca^{2+} channel
CALB1	Calbindin 28 K	Intracellular transport
Otop2	Otopetrin	Ca^{2+} intracellular transporter?
ATP2A1, 2, 3	Endoplasmic reticulum calcium ATPase 1, 2, 3	Ca^{2+} ATPase
ITPR1, 2, 3	IP3 receptor1, 2, 3	Ca^{2+} channel
RYR1	Ryanodine receptor 1	Ca^{2+} channel
ATP2B1, 2, 4 (PMCA1, 2, 4)	Plasma membrane calcium transporting ATPase 1, 2, 4	Ca^{2+}/H^+ exchanger
SLC8A1, 3	Sodium/calcium exchanger 1, 3	Na^+/Ca^{2+} exchanger
TRPM7	Transient receptor potential cation channel subfamily M member 7	Ca^{2+} channel
TRPC1	Transient receptor potential cation channel subfamily C member 1	Ca^{2+} channel

CACNA 1D, 1E, 1H	Voltage-dependent L-type calcium channel subunit alpha1D, -1E, -1H	Ca^{2+} channel
Extracellular vesicle transport		
Annexin-1, 2, 8	Vesicular calcium channels	Ca^{2+} entry in vesicles
EZR	Ezrin	Membrane fusion (plasma membrane cytoskeleton linker)
MFGE8	Milk fat globule-EGF factor 8 protein	Calcium-binding
EDIL3	EGF-like repeats discoidin I-like domains 3	Calcium-binding; targeting of ACC in vesicles
Paracellular transport		
CLDN2	Claudin 2, 10, 12	Paracellular cation channel Tight junction permeability
JAM	Junctional adhesion molecule	Integral membrane protein localizing at intercellular junctions of endothelial and epithelial cells
TJP 1, 2, 3 (ZO 1, 2, 3)	Tight junction proteins 1, 2, 3 (Zonula occludens 1, 2, 3)	Connect transmembrane proteins with the actin cytoskeleton
OCLN	Occludin	Integral membrane protein localizing at tight junctions
Other ions transport		
CA2, 4, 7, 9	Carbonic anhydrase 2, 4, 7, 9	Catalyze HCO_3^- formation
SLC26A9	Solute carrier family 26 member 9	HCO_3^-/Cl^- exchanger
SLC4A8, 9	Solute carrier family 4 member 8, 9	HCO_3^-/Cl^- exchangers
CFTR	Cystic fibrosis transmembrane conductance regulator	Cl^- channel
CLCN2	Chloride voltage-gated channel 2	Cl^- channel
CLCN5	Chloride voltage-gated channel 5	Cl^-/H^+ exchanger
SLC4A4, 5, 7, 9, 10	Solute carrier family 4 member 4, 5, 7, 9, 10	Na^+/HCO_3^- cotransporters
SCNN1A, B, G	Amiloride-sensitive sodium channel subunit α, β, γ	Na^+ channel
ATP1A1, B1	Sodium/potassium-transporting ATPase subunit α-1, β-1	Na^+/K^+ exchangers
ATP6V1B2, C2	Vacuolar H ATPase B subunit osteoclast isozyme	H^+ pump
KCNJ2, 15, 16	Inward rectifier potassium channel 2, 15, 16	Inward rectifiers K^+ channels
KCNMA1	Calcium-activated potassium channel subunit α-1	K^+ channel

Calcium-binding, amorphous calcium carbonate (ACC) stabilization, calcite crystal deposition and eggshell matrix		
SPARC	Secreted protein acidic and cysteine rich	Calcium-binding
NUCB2	Nucleobindin 2	Calcium-binding
OVAL	Ovalbumin	ACC stabilization
LYZ	Lysozyme	ACC stabilization; antimicrobial
TF	Ovotransferrin	Calcite crystal morphology; antimicrobial
OC17	Ovocleidin 17	ACC/calcium carbonate deposition
OPN/SPP1	Osteopontin	Mineralization modifier
OC116/MEPE	Ovocleidin 116	Mineralization modifier
OCX36	Ovocalyxin 36	Proinflammatory mediator; antimicrobial
OCX32	Ovocalyxin 32	Protease inhibitor; antimicrobial
SPINK7	Ovomucoid	Serine protease inhibitor, Kazal like domains
PPIB	Peptidylprolyl isomerase B	Accelerates protein folding
GPC4	Glypican 4	Heparan sulfate proteoglycan
LOC 428,451	Prostatic acid phosphatase like	Protein phosphatase

[1] Data from Marie et al. (2015), Stapane et al. (2019), Gloux et al. (2020a), Le Roy et al. (2021) and Nys et al. (2022).

2. Bone remodeling-related genes and hormones regulating bone quality

The skeleton is a complex tissue containing matrices and cells such as osteoblasts, osteocytes, and osteoclasts, which are responsible for bone quality by regulating bone remodeling (Siddiqui et al., 2016). A high-quality bone is vital for maintaining the health, welfare, and economic sustainability of laying hens (Whitehead, 2004).

2.1. Special bones in laying hens

In the avian species, the skeleton has undergone a multitude of structural changes and functional adaptations to adapt a bird's flight habits and egg-laying behavior (Canoville et al., 2019; Novitskaya et al., 2017). Bone consists of 65% - 67% inorganic minerals (mainly calcium hydroxyapatite) and organic collagen fibrils (mainly type I collagen). Collagen constitutes the primary scaffold of bone, providing oriented support for the inorganic minerals. Bones are classified into woven bone or lamellar bone based on the orientation of collagen. The woven bone is an immature bone exhibiting loosely organized collagen fibers, whereas the lamellar bone (Figure

1-4) such as cortical (compact) or cancellous (trabecular, spongy) bone is a mature bone with oriented and ordered collagen fibrils. These bones have similar material content but vary in their shape and material structures (cortical or cancellous). The majority of bones exhibit cortical bone on their outer surfaces, while the cancellous bone is mostly presented in the flat bones (keel sternum, skull) or the metaphyseal region of many long bones (humerus, tibia, and femur). Compared with the cortical bone, the cancellous bone possesses a high bone turnover (Clarke, 2008; Florencio-Silva et al., 2015) and a low modulus of elasticity and compressive strength (Sheikh et al., 2015). During bone development, the spaces among spongy bone are filled with bone matrix to form the compact bone (Logan, 1942).

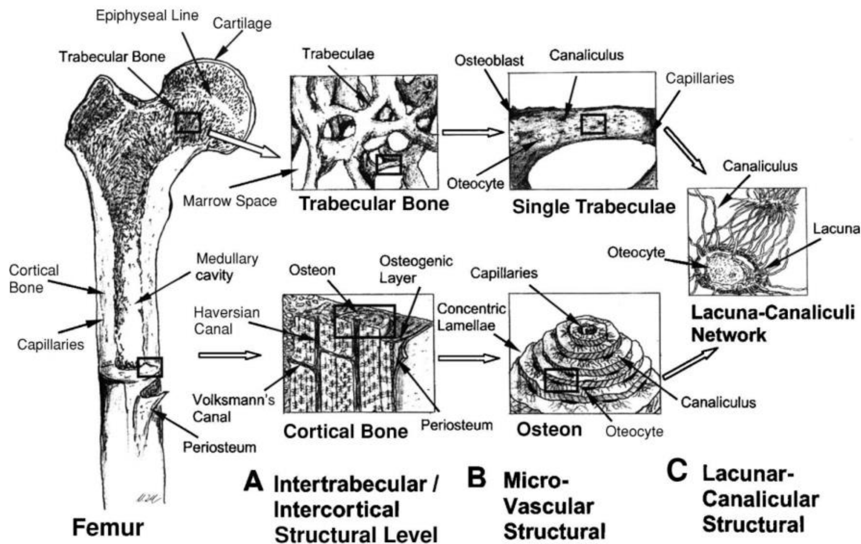

Figure 1-4. Bone possesses a hierarchical structure, with trabecular and cortical bone exhibiting differences at both the macro and microscopic levels. However, they share fundamental similarities in terms of their lamellar structure (Liebschner et al., 2005).

Unlike cortical and cancellous bones, medullary bone is characterized by a high vascularity and randomly oriented hydroxyapatite crystals, which exists within the marrow cavities of the bone midshaft (mid-diaphysis) in birds (Figure 1-5, Canoville et al. (2019); Squire et al. (2017); van de Velde et al. (1985)). When the hens reach sexual maturity, the medullary bone begins to form in response to hormones (mainly estrogen) (Alfonso-Carrillo et al., 2021; Dacke et al., 1993; Fisher et al., 1982). Medullary bone, as a major calcium reservoir, is absorbed during eggshell calcification due to increased osteoclast activity driven by hormones (mainly PTH, vitamin D_3 and calcitriol) (Canoville et al., 2019; van de Velde et al., 1984; Whitehead, 2004). However, medullary bone re-mineralizes during periods without eggshell calcification (Kerschnitzki et al., 2014). The amount of medullary bone rapidly increases, followed by a slow accumulation in the subsequent egg-laying period (Whitehead, 2004). During the intensive laying period, it is formed at the expense of cortical bone mass, which results in the development of osteoporosis and the high

incidence of bone fractures, notably in calcium-deficient diets or during the late laying period (Cransberg et al., 2001; Eusemann et al., 2018a; Fleming et al., 2006). Medullary bones are found in the femur and tibia of almost all female birds, and the humerus of most birds (over 60%) are firm and light without medullary bones (Figure 1-6, Canoville et al. (2019); Whitehead (2004)).

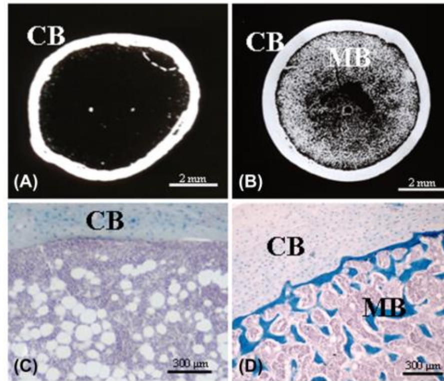

Figure 1-5. Micrographs (A and B) and light microscopy (C and D, stained with alcian blue staining) of cross-sections of femurs from male chickens (A and C) and egg-laying hens (B and D). CB, Cortical bone; MB, medullary bone. Medullary bone is developed within the marrow cavities of long bones in the egg-laying hen (Dacke et al., 2015).

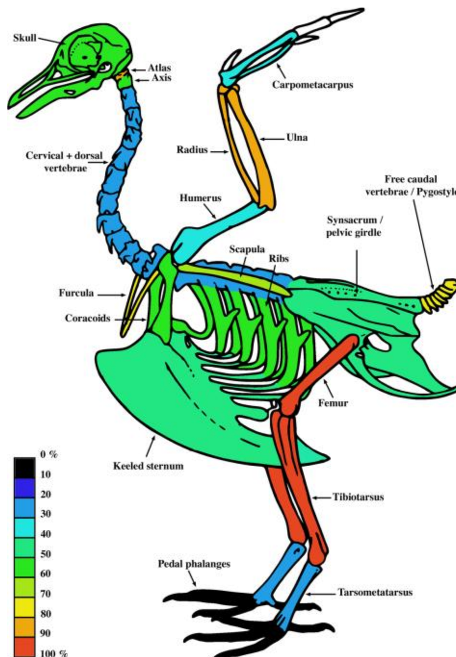

Figure 1-6. Overall distribution and prevalence of medullary bone in the different regions of the avian skeleton. The color-coding refers to the proportion of individuals in 40 female birds

(38 species) that presented medullary bone in a given skeletal element (Canoville et al., 2019).

2.2. Bone quality

Bone quality has been defined as a comprehensive description encompassing a range of bone tissue characteristics. In birds, the deterioration of bone quality contributes to the risks of osteoporosis and fracture (Julian, 2005) due to intensive egg production and eggshell formation (Kim et al., 2005; Kim et al., 2012). Bone mechanical properties such as strength, stiffness, and work to fracture are described in Table 1-2, and they are key factors in assessing fracture risk (Bishop et al., 2000; Currey, 1979). They are dependent on the material composition and structure of the bones (Jerome et al., 1999; Rodriguez-Navarro et al., 2018). The inorganic minerals increase the stiffness of the fabric and the organic collagen fibrils provide flexibility (Wahl et al., 2006). The amount of bone ash and its composition are directly reflecting the quantity and content of inorganic minerals, and have been used to indicate the bone status of chickens (Kim et al., 2012). Numerous studies indicate that bone strength is highly positively correlated with bone ash and fat-free dry matter (Shim et al., 2012; Świątkiewicz et al., 2018; Zhang et al., 1997).

Table 1-2. Definitions of the mechanical parameters in bones

Mechanical property	Definition	Methods[1]
Bone strength	The overall resistance of the entire bone to fracture	Three-point bending test, the minimum load required to fracture the bone
Bone stiffness	The bone's resistance to deformation under load	Three-point bending test, the slope of the maximum elastic load-displacement curve
Work to fracture	The total work done by the applied load to deform and fracture the bone, or the total energy absorbed by the bone until fracture occurs	Three-point bending test, the area under the load-displacement curve up to the point of fracture

[1] The methods are reported by Brzóska et al. (2005) and Cui et al. (2019).

Bone is woven at submicroscopic, microscopic, and macroscopic levels into a structure to limit the propagation of cracks (Seeman, 2006). Histological analyses of bone provide visual representations of the bone architecture. Although micro-computed tomography (micro-CT) scanners can visualize and quantify bone microstructure on a three-dimensional scale, their application is limited due to their high cost (Kim et al., 2012). In contrast, the staining of mineralized bone (e. g. Goldner's or von Kossa) is an alternative method for bone microstructure observation. The structural characteristic is determined by material composition and geometry (Turner et al., 1993). The geometric characteristics of bone are not only indicative of volume and size but also of the spatial distribution of minerals that provide bone

strength, which enable it to function as a structural support organ (Ferretti et al., 2001). The introduction of geometric properties of bones can improve the accuracy of predicting bone strength based on material properties (Zhang et al., 1997). Additionally, the application of multiple scanning and imaging techniques, including digitized fluoroscopy, dual-energy X-ray absorptiometry (DEXA), and peripheral quantitative computed tomography (pQCT), describe the bone mineral content (BMC) and bone mineral density (BMD). The BMC describes the mineral quantity within the measured area, while the BMD provides a 2- or 3-dimensional distribution of bone mineral. Some non-X-ray techniques to the bone such as quantitative ultrasound (QUS), magnetic resonance imaging (MRI), spatially offset Raman spectroscopy (SORS) and microwave tomography (MWT) also offer new insights into the measurements of bone mass, density, mechanical properties, architecture, composition and mineral crystallinity (Amin et al., 2020; Florkow et al., 2022; Gautam et al., 2023; Li et al., 2016). These are complementary measures to evaluate bone health and improve fracture risk prediction (Bouxsein, 2003; Marshall et al., 1996; Surowiec et al., 2024).

The measurements of bone parameters enable researchers to accurately assess the bone status of layers and the availability of additives (Cheng et al., 1990; Cui et al., 2019). The mechanical property is the ability of skeletons to resist external loads and strains (Hernandez et al., 2006), which is a direct index to evaluate the risk of bone fracture. Normally, the mechanical property is evaluated by directly simulating skeletal deformation and fracture patterns, which has certain adverse effects to bones, even being potentially destructive. The measurement of bone structure has helped identify the specific structure alterations taken place in the different physiological status. It can be performed by the non-destructive imaging of living tissues. The clinical estimation of fracture risk is based on the bone mineral measurement, and DEXA is a non-invasive, simple, cost-effective and commonly used diagnostic screening tool (Cummings et al., 2002). It is sensitive to bone loss, while it can only account for approximately 60% of the variation in bone strength (Bailey et al., 2018). It is necessary to conduct a comprehensive examination of these parameters in certain cases. For example, BMD (by DEXA) or bone ash may not effectively assess bone quality when the medullary bone is present. On the one hand, both medullary bone and structural bone exhibit radiodensity (Whitehead, 2004). On the other hand, although bone loss occurs in the structural bone during the laying period, the accumulation of medullary bone means that the total bone mass may be kept constant over the laying period (Whitehead, 2004). At this time, the confirmation of structural and mechanical properties thus becomes especially crucial. These parameters can only reveal the results of bone modeling, but we cannot specifically figure out whether the results are caused by altered bone formation or bone resorption. For instance, decreased mineral loss due to reduced osteoclast and/or increased mineral retention due to enhanced osteogenesis may both result in a higher mineral content. Thus, the measurements of bone parameters are more suitable to evaluate the results caused by a physiological process change over a period of time, rather than to explore its specific change mechanism.

2.3. Biomarkers involved in bone remodeling

Biomarkers in the bone remodeling process may favor the identification of adaptability and contribution of bone in certain physiological states. Bone tissues comprise osteocytes, osteoblasts, and osteoclasts. The osteocytes are transformed from the osteoblasts under the action of various growth factors such as WNT, BMP, and TGF-β (Bonewald, 2011; Florencio-Silva et al., 2015). The osteocytes do not make bone, they govern the bone remodeling through their interaction with osteoblasts and osteoclasts and regulate bone mass (Bonewald et al., 2008; Turner et al., 2009).

The osteoblasts are responsible for bone formation, while the osteoclasts are in charge of bone resorption (Figure 1-7). The osteoblasts differentiate under the regulation of a transcription factor runt-related transcription factor 2 (RNUX 2), then synthesize and secrete type I collagen (COL I), alkaline phosphatase (ALP), osteocalcin (OCN), osteopontin (OPN) and other proteins that regulate bone formation (Clarke, 2008; Moreira et al., 2019; Murshed, 2018). COL I elevates the osteogenic differentiation potential of mesenchymal stem cells and facilitates extensive remodeling of the collagenous matrix, resulting in a bone-like structure with calcification (Leisten et al., 2012; Schneider et al., 2010). A lower expression of COL I may impair bone accretion, accounting for the osteoporotic syndrome in aged laying hens (Gloux et al., 2020b). ALP and OCN are acknowledged as markers for the early and late osteogenic stages, respectively (Farley et al., 1994; Xu et al., 2019). ALP elevates local inorganic phosphate rates, promoting mineralization, while concurrently reducing the concentration of extracellular pyrophosphate which is an inhibitor of mineral formation (Vimalraj, 2020). Nizet et al. (2020) indicated that the expression of ALP promotes hydroxyapatite growth, marking the initiation of early skeletal mineralization. OCN is the major non-collagenous protein of bone and has been used as an osteoblast activity biomarker to evaluate bone remodeling in humans and rodents (Christenson, 1997; Seibel, 2005; Wang et al., 2021b). It plays an important role in the biosynthesis of the bone organic matrix (Xu et al., 2022) and the anchoring of calcium and phosphate (Mishra et al., 2015). In chickens, age-associated decrease in the serum concentration of OCN may be a reason for the increased incidence of osteoporotic fractures with age (Webster, 2004; Whitehead, 2004), which may be related to the decreased OCN expression level in the osteoblasts (Sabir et al., 2023). OPN plays a crucial role in regulating the mineralization processes of the extracellular matrix (McKee et al., 1992), and its upregulation results in a wider hypertrophic zone within chicken tibial growth plates (Ren et al., 2023).

Osteoclasts, a kind of multinuclear giant cells, are responsible for bone degradation (Burgess et al., 1999). They have a ruffle border that interacts with the bone surface, producing hydrogen and chloride ions as well as catalytic enzymes such as tartrate-resistant acid phosphatase (TRAP) and cathepsin K (CTSK) to dissolve and resorb bone matrices (Clarke, 2008). TRAP is a common biomarker identifying osteoclasts (Burstone, 1959; Yin et al., 2019). Although the deletion of TRAP did not influence osteoclast differentiation and the formation of resorption pits, it impaired the bone

resorption capacity (Hayman et al., 1996; Hayman, 2008). CTSK is responsible for the degradation of bone matrix protein, and inhibiting it leads to a reduction in the bone resorption activity of osteoclasts, alleviating osteoporosis-like symptoms (Costa et al., 2011; Duong le et al., 2016). The osteoclastogenesis is regulated by the RANK/RANKL/OPG pathway (Hiyama et al., 2019). RANK is expressed in osteoclast precursors, and its interaction with RANKL triggers the activation of the NF-κB signaling pathway in osteoclast precursors, leading to osteoclast maturation (Khosla, 2001). However, the decoy receptor OPG, secreted by osteoblasts, seems to prevent this process by competing with RANK for binding to RANKL (Boyce et al., 2007). The RANKL/OPG ratio is regarded as a pivotal determinant for osteoclast maturation and activation (Dacke et al., 2015).

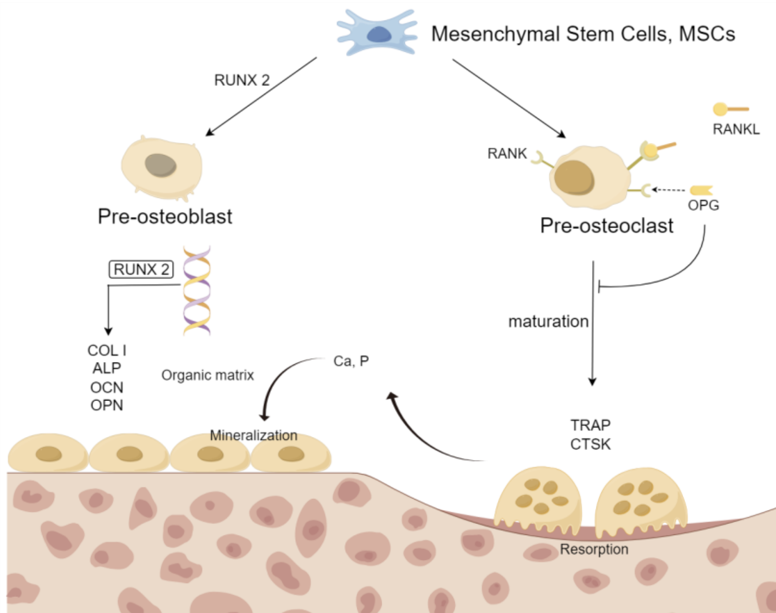

Figure 1-7. Bone remodeling and related biomarkers. MSCs, mesenchymal stem cells; RUNX 2, runt-related transcription factor 2; COL I, Collagen type I; OCN, osteocalcin; ALP, alkaline phosphatase; OPN, osteopontin; OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor-kappa B ligand; RANK, receptor activator of nuclear factor-kappa B; TRAP, tartrate-resistant acid phosphatase; CTSK, cathepsin K.

The imbalance between the activities of osteoblasts and osteoclasts results in changes in bone mass. The increase in osteoclast bone resorbing activity induces osteoporosis in laying hens (Jendral et al., 2008; Whitehead, 2004). Thus, the detection of key regulators in bone remodeling contributes to revealing the process of bone remodeling.

2.4. Hormones involved in bone remodeling

Bone remodeling is controlled by various systemic hormones and local signaling factors. Osteoblasts function on bone surfaces and are subject to regulation by

numerous hormones including parathyroid hormone (PTH), vitamin D_3, calcitonin, estrogen, growth hormone, and thyroid hormone, many of which promote their proliferation and matrix production (Florencio-Silva et al., 2015; Rath et al., 2000; Rutkovskiy et al., 2016). Some of these hormones such as PTH, vitamin D_3, calcitonin, estrogen, glucocorticoid, and prostaglandins are also involved in the regulation of osteoclast activities (Okamoto et al., 2019; Ono et al., 2018).

Chicken PTH is a polypeptide of 88 amino acids (Khosla et al., 1988; Lim et al., 1991), and it shows distinct homology with the biologically active 1 - 34 region of mammalian PTH (Russell et al., 1989). In laying hens, an elevation in the PTH stimulates bone resorption in the medullary bone (Taylor et al., 1954). Previous studies found that, after treating Japanese quails with PTH during the inactive phase of eggshell calcification, medullary bone exhibited bone resorption characterized by the formation of ruffled borders in osteoclasts with clear areas rich in filamentous fibers at the borders (Miller, 1977; Miller et al., 1985). Similar results were observed in another report (Sugiyama et al., 1994). The influence of PTH on other bones is contingent upon the periodicity of the PTH signal (Silva et al., 2015), and intermittent spikes in serum PTH levels can lead to an anabolic effect on rat metatarsal bones (Dobnig et al., 1997). Additionally, PTH stimulates the expression of RUNX 2 and induces the RUNX 2-dependent transcription that benefits the production of bone biomarkers linked with bone formation (Krishnan et al., 2003). Parathyroid hormone-related peptide may have similar physiological functions since it also shares the active conserved region (1 - 34) with the PTH (Dacke et al., 2015).

It is well established that vitamin D_3 is sequentially hydroxylated in the liver and kidney by the enzymes 25-hydroxylase (CYP2R1) and 1α-hydroxylase (CYP27B1) to form 25-hydroxyvitamin D_3 and 1,25-dihydroxyvitamin D_3 (calcitriol). Of these, only calcitriol is active. Calcitriol trigger the activation of the vitamin D receptor (VDR), facilitating the absorption of calcium and, consequently, regulating blood calcium levels (Holick, 1994). When serum calcium levels are sufficient, calcitriol promotes calcium deposition into bones (Harahap et al., 2022; Lips, 2012). A low dosage of calcitriol (10 nmol/L) alleviated the toxicity and apoptosis of White Leghorn chickens bone marrow stromal cells (BMSCs) exposed to cadmium and enhanced osteogenic differentiation, including increased ALP activity, and elevated protein expression of COL I and RUNX 2 (Tong et al., 2023). Additionally, some studies reported that vitamin D_3 influenced bone mineralization by stimulating osteoblast activity and proliferation (van Driel et al., 2017; Zofkova, 2018). Therefore, vitamin D_3 and its metabolites may play crucial roles in the differentiation of osteoblasts and bone marrow stromal cells and stimulate the synthesis of bone formation biomarkers, contributing to bone mineralization. However, high-dose calcitriol (100 nmol/L) may inhibit osteoblastic mineralization by suppressing the activation of NF-κB and Smad pathways in primary osteoblast precursors or MC3T3 pre-osteoblastic cells (Yamaguchi et al., 2012).

In mammals, estrogen has been observed to not only stimulate osteoblast proliferation and the expressions of matrix proteins, hormone receptors, and ALP (Majeska et al., 1994; Robinson et al., 1997) but also inhibit osteoclast formation and

promote osteoclast apoptosis (Kanatani et al., 1998; Shevde et al., 2000). Likewise, in chicken, estrogen significantly enhanced osteoblast proliferation and ALP activity, accelerating cell cycle and cellular DNA synthesis *in vitro* (Chen et al., 2010). Serum ALP and TRAP levels were reduced in parallel to the decreases in the level and receptor expression level of estrogen upon estrogen deprivation with letrozole, indicating a lower bone turnover, and this eventually resulted in a reduced X-ray density in the tibia of pullets (Deng et al., 2010).

Osteoclasts undergo cell death in response to calcitonin stimuli, followed by the termination of bone resorption (Chen et al., 2018; Ono et al., 2018). Calcitonin exerts calcium-lowering effects by suppressing osteoclastic bone resorption in mammals (Granholm et al., 2007), while similar results were not observed in avian osteoclasts (Dempster et al., 1987; Ito et al., 1985; Miyaura et al., 1981; Nicholson et al., 1986). Osteoclasts from calcium-deficient chicks responded to calcitonin *in vitro* with a 58% reduction in cell spreading area (Pandalai et al., 1990) and an inhibition of bone resorption activity (de Vernejoul et al., 1988). Thus, calcitonin may reduce bone resorption by inhibiting osteoclast activity.

3. Possible relationships between eggshell and bone

3.1. The supply of calcium and the roles of bone remodeling during eggshell calcification

The calcium supply for eggshell calcification may undergo distinct adaptations due to the limitation of the photoperiod on feed access (Figure 1-8). Generally, the initiation stage of eggshell calcification in most hens occurs during the light hours that expose hens to the feed, the eggshell calcium being predominantly from intestinal calcium absorption (Bar, 2009b). However, a considerable time of the growth stage of eggshell calcification happens in the nocturnal fasting period (Nys et al., 2018). During this phase, the medullary bone resorption takes on a prominent role in providing calcium for eggshell calcification as the residual calcium in the intestine is gradually depleted (Bar, 2009b; Kerschnitzki et al., 2014; Nys et al., 2018). There is evidence that the medullary bone accumulates new bone before eggshell calcification (before 6 h post-ovulation), then bone resorption may occur in the cortical bone and medullary bone during 12 - 18 h post-ovulation, and the cortical bone exhibits bone loss during 18 - 24 h post-ovulation (Clunies et al., 1993). Kerschnitzki et al. (2014) also found that the mineral content of medullary bone together with the trabecular thickness and bone volume ratio gradually reduced after the onset of eggshell calcification. Additionally, the blood calcium level decreases while a concurrent rise in phosphorus is observed with eggshell formation (Kerschnitzki et al., 2014). There has been a hundredfold difference in the phosphorus content of eggshell and bone (ash basis) with a similar calcium content (Luck et al., 1979a; Wang et al., 2021a). This provides a hypothesis that the calcium from bone resorption may be used for eggshell calcification and eliminated from the blood during eggshell calcification, while redundancy phosphorus produced by bone resorption may remain in the blood (Figure 1-8). Thus, available evidence suggests that bone resorption occurs with eggshell

calcification, and it may contribute approximately to 20% - 40% of the eggshell calcium (Clunies et al., 1993; Comar et al., 1949). However, increased plasma phosphorus caused by bone resorption was observed to induce the secretion of FGF 23 from osteocytes and osteoblasts. FGF 23 is a phosphorus metabolism regulator that actives the negative feedback of bone resorption (Erben et al., 2017) and inhibits the production of calcitriol in the kidney (Ren et al., 2017), which may reduce the skeletal calcium transportation to the uterus and the calcium absorption in the intestine. Some studies indicated that the elevation in plasma phosphorus following bone resorption disturbed eggshell calcium deposition in the uterus (Miles et al., 1982) and eventually reduced eggshell weight (Sauveur et al., 1983). Thus, bone resorption is a major provider of calcium supply for eggshell calcification. Further studies are needed to explore bone remodeling changes in the key period of eggshell calcification and the possible effects on the quality of eggshells and bones.

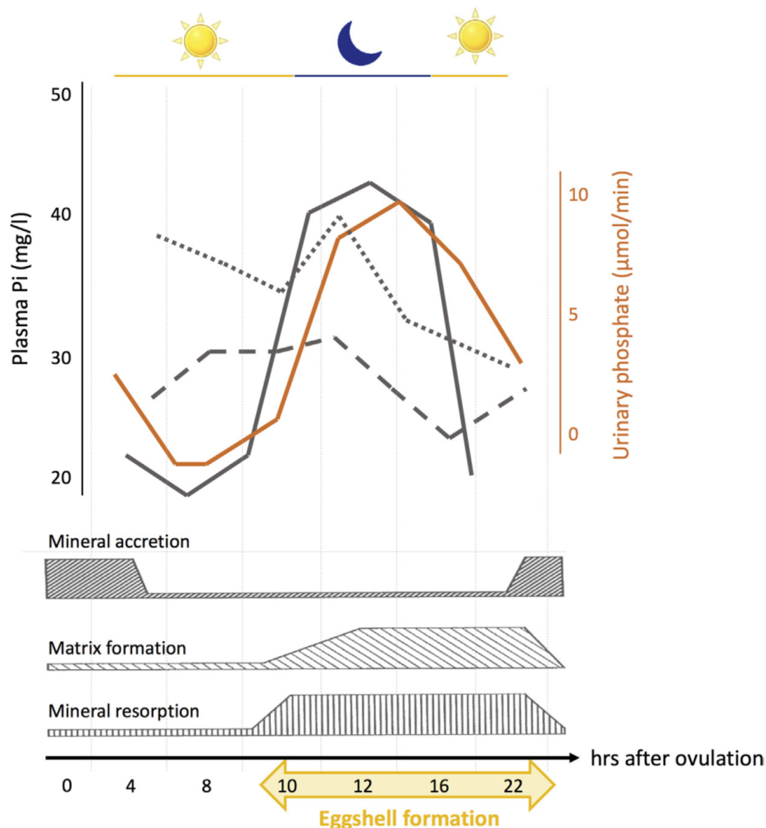

Figure 1-8. Plasma levels of inorganic phosphorus and urinary phosphorus excretion excretion during the laying cycle. Eggshell formation maily occurred 10 - 22 hours after ovulation within the oviduct. Laying hens were fed 3.5% dietary calcium in the form of flour (continuous line) or larger particle sizes (dotted line). Urinary calcium levels were assessed specifically in hens fed with flour calcium at the 3.5% level (orange line). Dashes indicate the plasma inorganic phosphorus levels of cockerels. During the nocturnal fasting period, the

residual calcium in the intestine is rapidly depleted, especially when calcium flour is provided, thereby increasing bone resorption (Nys et al., 2018).

3.2. Possible relationships between eggshell and bone quality

As mentioned previously, bone resorption occurs with eggshell formation, which may change the balance of bone remodeling and finally affect bone quality (Feng et al., 2011). Some studies indicated that hens with low egg production exhibited a lower bone fracture incidence compared to those with high egg production (Cransberg et al., 2001; Eusemann et al., 2018b; Hocking et al., 2003; Rufener et al., 2019). This appears to indicate that the more eggshells are produced, the poorer the hen's bone quality is. Kim et al. (2005) reported that low-quality bones were correlated with high-quality eggshells since rapid bone metabolism was synchronous with more eggshell calcium deposition. However, the link between bone quality and egg production/eggshell quality is controversial. When the hens experience different rearing systems leading to skeletal differences, bone quality was not associated with egg production other than a weak correlation in humeral cortical density (Jendral et al., 2008). Gebhardt-Henrich et al. (2015) suggested that, although the hens suffered a greater number of bone fractures when their laying rate came to its peak, there was no significant correlation between bone quality and total egg number. Additionally, some scholars pointed out that there is no exact relationship between bone and egg production/eggshell quality based on the results from the single bone (humerus) or single bone structure (trabecular bone) (Alfonso-Carrillo et al., 2021; Rennie et al., 1997). The comparative results in multiple lines also endorsed this perspective, suggesting that improvements in both eggshell and bone quality can be achieved simultaneously in the breeding program (Clark et al., 2008; Jansen et al., 2020a). It is essential to conduct a systematic exploration of bone and eggshell quality in different situations, which contributes to revealing the role of bones in eggshell formation and provides possible targets for improving both eggshell and bone quality.

4. Factors affecting eggshell and bone quality

It is estimated that up to 40% of skeletal variations are caused by genetic or hereditary factors (Bishop et al., 2000). Despite this, in addition to genetics, the rearing environment and diet also contribute to effective eggshell and bone quality improvements (Bouvarel et al., 2011; Sokołowicz et al., 2018; Whitehead et al., 2000).

4.1. Genetic background

The breeding objectives within the laying hen's industry primarily focus on increasing the laying rate and the overall cumulative egg number (Bain et al., 2016; van Sambeek, 2011). The modern commercial lines (Lohmann LSL-lite, Shaver White Leghorn, and some commercial lines of unknown breed origins) have smaller bones compared to the traditional lines (Smoky Joe White Leghorn, Araucana, Brown Leghorn, Barnvelder, Buff Orpington, Cornish Game, Friesian Fowl, Ixworth, Jersey Giant, J-Line, Light Sussex, Maran, White Dorking, and White Sussex) (Hanlon et al., 2022; Hocking et al., 2003). This suggests that although breeding criteria are primarily targeting egg production, selection results also lead to a decrease in long bone

dimensions and an overall smaller skeletal structure. Compared to the traditional line (Smoky Joe White Leghorn), the commercial lines (Lohmann LSL-lite and Shaver White Leghorns) exhibit higher egg production and high-quality eggshells, even if their eggs are larger (Hanlon et al., 2022). This is a significant outcome for the genetic improvement of egg-type poultry. However, as expected, the selection is also responsible for a reduced overall BMD of medullary bones in the aged hens of the commercial lines (Lohmann LSL lite and Shaver White Leghorns) (Hanlon et al., 2022). Furthermore, the white-egg flock generally shows a greater eggshell quality and a poorer bone quality compared to the brown-egg one at similar laying rates (Akbari Moghaddam Kakhki et al., 2020b; Hocking et al., 2003; Jansen et al., 2020a). Bone calcium reserves of the medullary bone can buffer temporary calcium fluctuations, while structural bones undergo demineralization during prolonged calcium depletion, resulting in bone structure impairment and osteoporosis (Dacke et al., 1993; Zallone et al., 1969). A low-calcium diet and the overconsumption of medullary bone may reduce egg production and eggshell quality (0.5% - 1.7% calcium, Bar et al. (1984); Gilbert et al. (1975); Jansen et al. (2020b)), even resulting in the cessation of egg laying (0.03% - 0.05% calcium, Gilbert et al. (1975); Luck et al. (1979b)). Hens laying brown eggs appear to be more tolerant to calcium depletion, while hens with white eggs are more sensitive, as the white-egg layers challenged with a calcium-deficient diet (1.09% calcium) exhibited a greater drop in egg production and more severe defects in eggshells (Jansen et al., 2020b). Bones in white-egg lines deteriorate more rapidly with age than those in the brown-egg ones (Akbari Moghaddam Kakhki et al., 2020b), which may be partly attributed to their high need for calcium mobilization related to better shell quality. However, the high-laying lines exhibited higher eggshell weight and total eggshell production as well as decreased bone quality regardless of laying brown or white eggs (Jansen et al., 2020b). Thus, the genetic variations affect both eggshell and bone quality, and the relative changes of both traits showed a negative correlation.

4.2. Age

It is widely acknowledged that eggshell and bone quality decrease with age in high-laying flocks (Klionsky et al., 2016; Whitehead et al., 2000). Hy-line Brown is a common and high-productively commercial line of laying hens. Ma et al. (2021) found that the eggshell breaking strength of Hy-line Brown hens was diminished by 27.41% from 31 to 80 weeks of age, which was consistent with other studies (Kim et al., 2014; Samiullah et al., 2017; Sirri et al., 2018). Other lines (commercial lines: Bovans Brown, Lohmann Selected Leghorn, Lohmann Brown; traditional lines: Fayoumi, Dandarawi) also exhibited significant reductions in eggshell quality during the late laying period (Dijkslag et al., 2023; Fathi et al., 2019; Wistedt et al., 2019). This suggested that most lines are suffering from a deterioration in eggshell quality during the late laying period. In addition, the eggshell breaking strength deteriorated more rapidly after around 46 to 49 weeks of age (Ma et al., 2021; Wistedt et al., 2019). The main skeletal development of layers is completed before or during the early stage of egg laying (Hanlon et al., 2022; Rath et al., 2000). An elevation of the circulating

estrogen level at the onset of sexual maturity induces the formation of medullary bones that are considered the calcium reservoir for eggshell formation (Whitehead et al., 2000). When the hens enter the laying period, the BMC of medullary bones elevates with age and peaks at the end of the laying period (100 weeks of age) (Hanlon et al., 2022). However, Yamada et al. (2021) suggested that aged hens (52 weeks of age) exhibited a slight increase in the BMC of medullary bones, while their width and bone volume fraction declined when compared with those from young hens (25 weeks of age). This demonstrated that the medullary bone structure may deteriorate after prolonged egg production. Notably, changes in water solubility, soft tissue deposition, or other non-mineralized tissue may result in an increase in the BMC (Metscher, 2009). Thus, the increased BMC might not be fully representative of the mineral content changes in the medullary bone due to the presence of non-mineralized tissue in the medullary space. As stressed repeatedly, the medullary bone may be consumed during eggshell calcification (Rath et al., 2022; Whitehead, 2004). Meanwhile, the medullary bone can also be supplemented and remodeled by the cortical bone. Pongmanee et al. (2020) suggested that cortical bone was replaced by medullary bone, followed by a decrease in its thickness. Prolonged high-intensity laying behavior leads to mineral loss and structural damage in the cortical bone (Whitehead et al., 2000). The high-laying hens displayed a declined relative bone weight (relative to total body weight) with age during the laying period, while the low-laying ones remained a constant value during the whole laying period and they even had a higher bone weight, cortical BMC, and thickness at the end of laying period (100 weeks of age) (Hanlon et al., 2022). The weakening of both cortical and medullary bone structures contributes to an increase in the susceptibility of bone fracture with age in the high-laying layers, and the bone strength decreased by 11% from 35 to 55 weeks of age (Rath et al., 2000).

4.3. Rearing system

Although a conventional caging system offers advantages in reducing the risks of feather-pecking, cannibalism, and mortality (Fossum et al., 2009; Kjaer, 2009), it restricts the movement of hens and is not conducive to the welfare of birds in terms of expressing natural behaviors (Bessei, 2019). *In vitro* and *in vivo* studies have elucidated the impact of exercise on bone formation and load-bearing capabilities (Robling et al., 2008; Wan et al., 2013; Zymbal et al., 2019). Compared with the conventional caging system, the enriched colony caging system, the aviary system, the barn system, and the free-range system (Table 1-3) provide more activity space and enrichments (e.g., perches, terraces, and platforms) to increase exercise opportunities (Hamilton et al., 2021), which may alter bone characteristics and make them stronger. Previous studies showed that pullets housed in the system equipped with perches or platforms exhibited a higher bone mineral content, breaking strength, and ash content (Casey-Trott et al., 2017c; Khanal et al., 2020a; Khanal et al., 2021). Moreover, this beneficial effect was sustained until 73 weeks of age (Casey-Trott et al., 2017b). In addition, these systems reduced the prevalence of keel-bone fractures in 30- to 70-week-age hens compared with the conventional caging system (Casey-Trott et al., 2017a). Lolli et al. (2013) suggested that the free-range system enhanced

Table 1-3. Rearing systems in laying hens

System type	Description	Space allowance, cm^2 per hen	Equipment				
			feeders	drinkers	perches	nest boxes	scratch area
Conventional caging system	cages	290 - 688	√	√	×	×	×
Enriched colony caging system	cages	696 - 880	√	√	√	√	√/×
Aviary system	a large cage that has multiple levels	754 - 1,850	√	√	√	√	√
Barn system	a room that allows hens to access to entire barn floor	1,064 - 2,050	√	√	√	√	√
Free-range system (organic)	a room that allows hens to access to entire barn floor and outside	1,852 - 24,321	√	√	√	√	√

Data from Abrahamsson et al. (1998); Ahammed et al. (2014); Casey-Trott et al. (2017a); CRS (2014); EU (1999); Leyendecker et al. (2005); Lolli et al. (2013); Lordelo et al. (2017); NFACC (2017).

humeri bone breaking strength and stiffness of aged laying hens (70 weeks of age), while it did not affect tibia quality. The barn and aviary system improved eggshell breaking strength and thickness and decreased cracked and broken eggs of the peak laying hens (21 - 40 weeks of age) (Ahammed et al., 2013; Ahammed et al., 2014). Hens reared in a free-range system laid eggs with higher eggshell thickness and breaking strength and fewer eggs were cracked over the whole laying period (20 - 68 weeks of age) (Lolli et al., 2013). Nevertheless, there is limited documentation on the influence of rearing systems on eggshell quality in laying hens during the late laying period, especially over 70 weeks of age.

4.4. Dietary factors

Cracked eggs and brittle bones are generally characterized by a lower eggshell weight and a thinner eggshell thickness (Gautron et al., 2021; Rodriguez-Navarro et al., 2002) as well as a lower bone mass (Bain et al., 2016; Fleming et al., 2006). Low-quality eggshells and bones may be attributed to inadequate dietary calcium intake (Classen et al., 1982; Härtel, 1990; Whitehead, 2004) and reduced capacity of calcium metabolism (Abe et al., 1982; Franco-Jimenez et al., 2005; Joyner et al., 1987). Thus, increasing dietary calcium levels, promoting calcium absorption and transport may be effective methods for improving eggshell and bone quality.

4.4.1. Calcium levels

The Nutrient Requirements of Poultry (NRC 1994 and NY/T 33-2004) recommends a dietary calcium level of 3.5% for laying hens (100 - 120 g/hen/d), which is four times higher than that for non-laying hens. Härtel (1990) suggested that the dietary calcium level of laying hens should not be lower than 2.5%. Low calcium levels may reduce the total calcium of medullary bones, egg production, and eggshell weight (Clunies et al., 1992). Calcium deficiency quickly causes bone loss (Elaroussi et al., 1994; Webster, 2004) and the declines in bone strength (Jiang et al., 2013). Zhao et al. (2020) reported that calcium deficiency has been observed to adversely impact the quality of both eggshells and bones. A higher dietary calcium intake was found to promote bone mineral retention to keep bones with higher dry weight and ash weight (Cheng et al., 1990). Similarly, increasing dietary calcium levels can improve eggshell strength, thickness, weight, and weight ratio (An et al., 2016; Chowdhury et al., 2002; Safaa et al., 2008), while it fails to affect eggshell deformation and egg specific gravity (Keshavarz et al., 1993; Leeson et al., 1993). However, Wang et al. (2021a) suggested a dietary calcium level of a maximum of 4% to improve eggshell quality, as there was no benefit when increasing to 4.5%, and some eggshells even demonstrated abnormal vaterite particles on the egg surface.

4.4.2. Limestone particles

Limestone particles are mainly composed of calcium carbonate (>35% calcium) and serve as the primary dietary calcium source for laying hens. The desynchrony between intestinal calcium absorption and eggshell calcium deposition leads to enhanced bone resorption to meet the calcium requirement for eggshell formation during the dark period (Kim et al., 2012). Compared with fine limestone particles, coarse limestone particles retained longer in the gizzard to extend the duration of the calcium release at

night (Hervo et al., 2022; Rao et al., 1990). Coarse limestone particles may improve eggshell quality by alleviating the eggshell calcification limitation during the nocturnal fasting period (Hervo et al., 2022; Saki et al., 2019). A meta-analysis concluded that specific gravity, eggshell thickness, and breaking strength were linearly raised with limestone particle size, and specifically, their increments were 0.08%, 1.1%, and 3.0% when the limestone particle size increased from 0.15 to 1.5 mm (Hervo et al., 2022). Lukić et al. (2009) suggested that coarse limestone particles had a more visible effect on reducing eggshell defects in hens fed with a deficient calcium diet (2.5%) compared to those fed with normal calcium (3.8%). This suggested that coarse limestone particles could properly alleviate the adverse effects of calcium deficiency in laying hens. A rise in bone resorption also impacts cortical bone, thereby increasing the incidence of osteoporosis (Whitehead, 2004). Hens fed coarse limestone exhibited a prolonged soluble calcium diffusion after the lights were off, leading to a reduction in bone calcium mobilization and bone loss (Fleming et al., 2006; Fleming, 2008; Gloux et al., 2020a). This leads to an increase in the bone strength of the hens subjected to coarse limestone particles (Hervo et al., 2022). Additionally, de Witt et al. (2009) found that coarse limestone particles enhanced the bone strength of the tibia without affecting the humerus. This may hint that coarse limestone particles mainly alleviate medullary osteolysis to improve bone quality. Furthermore, Xavier et al. (2015) showed that the tibial breaking strength linearly increased as the proportion of coarse limestone increased. However, some studies included a quadratic effect with an optimum of around 60% - 70% (de Oliveira et al., 2013; Molnár et al., 2018).

4.4.3. Calcitriol or flavonoids

A previous study suggested that increasing calcium levels is sometimes not effective in solving the reduction in eggshell and bone quality in aged laying hens (Wang et al., 2021a). Low eggshell and bone quality in aged laying hens may be primarily related to abnormalities in calcium metabolism (Park et al., 2018). Several studies indicated that the impaired calcium metabolism in aged laying hens was associated with a disorder in vitamin D_3 metabolism (Abe et al., 1982; Bar, 2008) and a decrease in estrogen levels and receptors (Hansen et al., 2003; Liu et al., 2018). Thus, enhancing the circulating level or sensitivity of calcitriol (active vitamin D_3) and estrogen by dietary strategies could potentially serve as an option to mitigate the declines in eggshell and bone quality in hens during the late laying period.

Dietary calcitriol supplementation could elevate the blood concentration of calcitriol (Tsang et al., 1993). The uterus is one of the significant targets for calcitriol (Yoshimura et al., 1997). Castaldo et al. (1990) also reported that hens with a high level of calcitriol in the serum had a strong eggshell. Previous study reported that dietary calcitriol supplementation could increase eggshell quality (Soares et al., 1988; Tsang et al., 1990) or slow down the progressive reduction in eggshell quality (Bar et al., 1988). In mammals, calcitriol on the one hand accelerated bone formation and promoted bone maturation (Liu et al., 2015) while on the other hand mitigated or even reversed the loss of bone minerals in susceptible populations (Peppone et al., 2010). Likewise, the addition of calcitriol in layer diets was also effective in promoting bone

formation and retaining bone mineral at oviposition (Frost et al., 1990; Newbrey et al., 1992).

Some flavonoids exhibit a chemical structure that is similar to estrogen, and their additions increased the levels of circulating estrogen and estrogen receptors in laying hens (Huang et al., 2022b; Liu et al., 2023a; Ni et al., 2012; Zhang et al., 2021), which may exert an estrogenic property to regulate eggshell and bone quality. The positive effects of flavonoids on eggshell quality during the late laying period have been widely demonstrated (Cai et al., 2013; Gu et al., 2013; Huang et al., 2022a; Liu et al., 2013; Xiao et al., 2019). However, their impact on eggshell quality at the peak period of laying remains controversial (Liu et al., 2014). Additionally, the increase in dietary total flavonoids promoted bone mineral retention to increase bone ash, calcium content, and BMD (Gu et al., 2013; Huang et al., 2020b).

Quercetin is one of the most well-known flavonoids and presents abundantly in a variety of fruits and vegetables. Dietary quercetin supplementation could increase the levels of estrogen and calcitriol in the blood (Yang et al., 2018). It plays positive roles in preventing oxidative injury and cell death, which may contribute to the maintenance of a healthy tissue (Yang et al., 2018). Additionally, quercetin is also involved in the regulation of calcium and phosphorus metabolism, which prevents bone loss and promotes bone mineral deposition in rats and broilers (Wang et al., 2022; Wong et al., 2020). Thus, dietary supplementation with calcitriol or quercetin may have the potential to improve both eggshell and bone quality in laying hens by improving calcium metabolism.

Chapter 2

Objectives and thesis outline

1. Objectives and research overview

The eggshell and bone quality dramatically reduced during the late laying period, resulting in substantial economic losses in the laying hens' industry. There may be a certain association between eggshell and bone qualities since approximately 20% - 40% of eggshell calcium is derived from bone resorption. Thus, the aim of this dissertation was to explore the relationship between the calcium transport in the uterus and bone and the quality of eggshell and bone in aged laying hens. This thesis explored this relationship in three conditions:

Experiment 1. Effects of rearing systems on the eggshell quality, bone parameters and expression of genes related to bone remodeling in aged laying hens. The rearing system is one of the major factors that affect the bone quality of layers. The hens housed in an aviary system had better bones than those in a conventional caging system. Thus, this first experiment compared the bone and eggshell quality as well as calcium transport in the bones and uterus of hens reared in the conventional caging system or aviary system. Subsequent experiments have been conducted in conventional caging systems because of the more significant reduction in eggshell and bone quality of aged hens in this system.

Experiment 2. Decreased eggshell strength caused by impairment of uterine calcium transport coincide with higher bone minerals and quality in aged laying hens. The hens with hard- or weak-shelled eggs were selected from a commercial flock to compare the quality of eggshell and bone as well as the calcium transport in the uterus and bones. Their interaction mechanism was further analyzed.

Experiment 3. Dietary supplementation with calcitriol or quercetin improved eggshell and bone quality by modulating calcium metabolism. The first and second experiments indicated that calcitriol (1,25-dihydroxyvitamin D_3) and estrogen may play vital roles in the regulation of eggshell calcification and bone remodeling. Thus, this third experiment explored the effects of dietary supplementation with calcitriol or quercetin on eggshell and bone quality as well as the calcium transport of the uterus and bones.

The research overview is represented in Figure 2-1. Our researches have provided some new insight for understanding the interaction and the regulation strategies of eggshell and bone quality in aged laying hens.

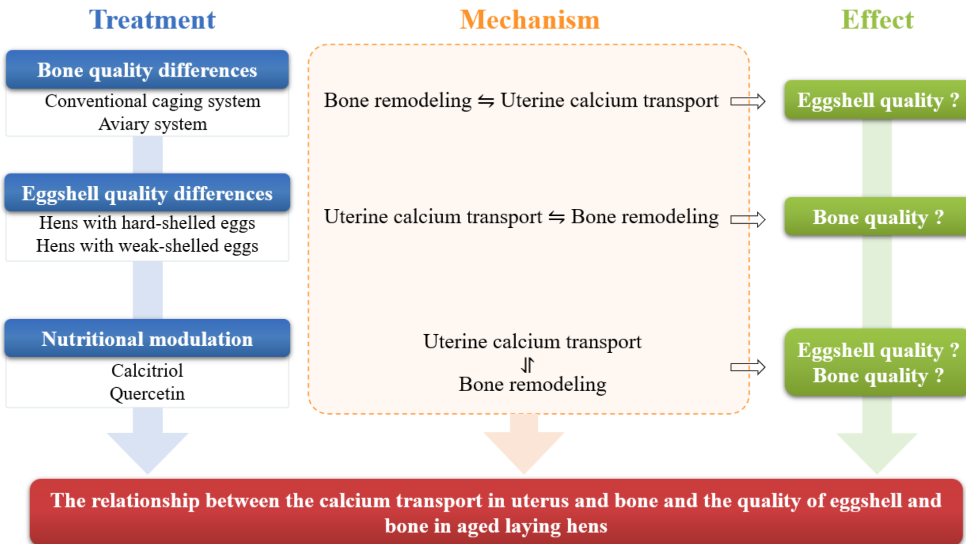

Figure 2-1. A research overview of this thesis

2. Outline of the thesis

Chapter 1 is a general introduction and a review of relevant literature.

Chapter 2 describes the objectives and the outline of the thesis.

Chapter 3 compares the differences between the conventional caging system and aviary system on eggshell quality, bone parameters, and relative expression levels of genes related to uterine calcium transport and bone remodeling in aged laying hens. The aviary system is a cage-free system, and layers housed in this system have bones with higher mineralization and strength compared to the conventional caging system. The comparison results indicated that, compared with the conventional caging system, the aviary system simultaneously alleviated the deterioration of bone and eggshell qualities of aged laying hens. The aviary system upregulated the expression of genes associated with bone formation in the femur and humerus to improve bone quality and increased the expression of a bone resorption gene in the femur to promote the delivery of femoral calcium into the circulating, which may be beneficial in calcium acquisition of the uterus. Additionally, the aviary system upregulated the gene expression level of a calcium transporter to promote uterine calcium transport and improve eggshell quality.

Chapter 4 compares the differences in eggshell quality, bone parameters, uterine calcium transport, and the bone remodeling of hens laying eggs of high or low eggshell breaking strength to explore the mechanism of eggshell and bone quality reduction and their interaction. The hens laid eggs with average breaking strength above 40 N or below 29 N were selected from a commercial flock with an average breaking strength of 34.55 N, which respectively served as a high or low breaking strength group. The results indicated that bone quality was negatively correlated with

eggshell quality. The lower eggshell breaking strength may be attributed to a declined calcium transport due to uterine tissue damage, which could affect eggshell calcification and lead to a weak ultrastructure. Impaired uterine calcium transport may result in reduced femoral bone resorption and increased humeral bone formation to maintain a higher mineral and bone quality in hens laying eggs with low breaking strength.

Chapter 5 investigated the effects and mechanism of dietary calcitriol or quercetin addition on eggshell and bone quality. Calcitriol and estrogen play vital roles in the regulation of uterine calcium transport and bone remodeling. Quercetin could exert an estrogenic property in laying hens. The results showed that dietary calcitriol or quercetin supplementation increased eggshell quality and femoral bone quality. The flocks supplemented with calcitriol or quercetin exhibited a better uterine morphology and an improved calcium transport-related gene expression pattern, which promotes uterine calcium transport and enhances eggshell quality. Additionally, dietary supplementation with calcitriol or quercetin upregulated the genes associated with bone resorption to facilitate the delivery of skeletal calcium into the circulating and upregulated the expression of femoral bone formation genes to promote the recovery of bone mass, which may benefit eggshell calcification and bone mineral retention.

Chapter 6 is the general discussion, followed by perspectives and conclusion.

Chapter 3

Effects of rearing systems on the eggshell quality, bone parameters and expression of genes related to bone remodeling in aged laying hens

Chapter 3. Effects of rearing systems on the eggshell quality, bone parameters and expression of genes related to bone remodeling in aged laying hens

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1. Abstract

Public concerns regarding animal welfare are changing the selection of rearing systems in laying hens. This study investigated the effects of rearing systems on eggshell quality, bone parameters and relative expression levels of genes related to bone remodeling in aged laying hens. A total of 2,952 55-day-old Jing Tint 6 pullets were randomly assigned to place in the conventional caging system (CCS) or aviary system (AVS) and kept until 95 weeks of age. In the AVS group, a delayed decrease of eggshell quality and alleviated symptoms of osteoporosis in the humerus rather than in the femur were observed. Eggshell breaking strength, thickness, weight, weight ratio, stiffness and fracture toughness were decreased linearly with age (from 55 to 95 weeks of age, $P < 0.05$). The AVS group had higher eggshell breaking strength, stiffness and fracture toughness than the CCS group ($P < 0.05$). Higher total calcium and phosphorus per egg were presented in the AVS group at 95 weeks of age ($P < 0.05$). At 95 weeks of age, layers in the AVS group had a humerus with higher weight, volume, length, midpoint perimeter, cortical index, fat-free dry weight, ash content, total calcium per bone, total phosphorus per bone, average bone mineral density, strength, stiffness and work to fracture compared to layers in the CCS group ($P < 0.05$). Such differences did not appear in the femur. The relative expression levels of alkaline phosphatase (ALP) and osteocalcin (OCN) genes in the femur and hormone receptors (vitamin D receptor (VDR), estrogen receptor alpha (ERα) and fibroblast growth factor 23 (FGF 23)) genes in the humerus were significantly upregulated ($P < 0.05$) in the AVS group. The level of tartrate-resistant acid phosphatase (TRAP) transcripts was also increased ($P < 0.05$) in the femur of the AVS group. Overall, compared with the CCS, the AVS alleviated the deterioration of eggshell and bone qualities of aged laying hens, which may be related to the changes in the expression of genes associated with bone remodeling.

Keywords: bone quality, eggshell quality, aviary system, conventional caging system, laying hen

2. Introduction

Although the caging system has an edge in reducing the risk for feather-pecking, cannibalism and mortality (Fossum et al., 2009; Kjaer, 2009), the conventional caging system (CCS) limits the movement of hens and not welfare friendly to birds in aspects of the expression of natural behaviors (Bessei, 2019). In recent decades, the CCS has been prohibited in some regions (e.g. the European Union, Switzerland, Argentina, Australia, Brazil and New Zealand) through legislations and mandatory measures, but it still remains widespread in some countries such as China, Canada and the United States (Bessei, 2019). The transition from the CCS to the alternative or cage-free systems is an inevitable trend under multiple pressures from animal welfare and the international market scrutiny.

Aviary system (AVS), a cage-free system, is a promising alternation, which provides more activity space and enrichments (e.g., perches, terraces and platforms) to increase opportunities of locomotion, develop skills to navigate and reduce the risk

of abnormal behavioral development (Campbell et al., 2016b; Hester, 2014; Janczak et al., 2015). It not only improves animal welfare, but also has a positive impact on bone strengthening (Regmi et al., 2015). An early investigation in the causes of bone loss noted CCS had a higher incidence of osteoporosis (OP) (King, 1965) that is associated with fracture and premature mortality (Whitehead, 2004). Pullets raised in AVS has been evidenced to have bones with higher mineralization and strength (Casey-Trott et al., 2017c; Regmi et al., 2015), and such benefits for bones could persist into the late period of laying (until the 14th month of laying) (Leyendecker et al., 2005). Extended laying period (from 80 weeks of age to 100 weeks of age) is a commonly mentioned target in recent years (Pottgüter, 2016), which presents greater challenges for bone quality of laying hens, since the long-term mobilization of calcium related to eggshell formation may lead to the hen's skeletal calcium to be absorbed into its uterus during eggshell formation, resulting in bone mineral loss and inducing OP (Cransberg et al., 2001; Wilson et al., 1998). Additionally, the changes in bone quality in return could affect eggshell quality, as 20% - 40% of the eggshell calcium content are derived from bone resorption (Clunies et al., 1993; Comar et al., 1949). However, less attention has been given to the effects of rearing systems on eggshell quality. Our current study speculates that hens kept in AVS for a long time may alleviate the deterioration of bone and eggshell qualities compared to those kept in the CCS, which benefits the achievement of extended laying period. Thus, it is necessary to further compare the effects of CCS and AVS on eggshell and bone qualities of aged laying hens (over 80 weeks of age).

Given the complicated interactions between bone and eggshell, the mechanism of rearing systems on avian skeleton quality has been poorly understood. Two special types of skeletons remained during evolution in birds, one of which is a light but firm skeleton (without medullary bone) such as the humerus in charge of the ability to fly, and the other is a long bone containing medullary bone such as the femur (Dacke et al., 2015). The supply of calcium in eggshell formation depends on the rapid mobilization of medullary bone, while the humerus (mainly cortical bone) contributes only a little (Dacke et al., 1993; Dacke et al., 2015). Thus, the humerus and the femur may undergo bone remodeling in different manners. Their joint analysis may contribute to revealing the effects and molecular mechanisms of rearing systems on bone remodeling and eggshell formation.

This study tracked the eggshell quality of hens kept in the CCS and AVS at the late laying period, followed by analyzing their bone quality at the end of laying period. It then focused on the levels of bone remodeling-related transcripts and uterine calcium transport-related transcripts to explore the molecular mechanisms of rearing systems on bone remodeling and eggshell formation. This study may provide references for the selection of rearing systems in laying hens.

3. Materials and methods

3.1. Birds and experimental design

The animal experiment was conducted under the management of the Animal Care and Use Committee of Institute of Feed Research, Chinese Academy of Agricultural Sciences (approval No. AEC-CAAS-20191027). A total of 2,952 55-day-old Jing Tint 6 pullets with similar body weight were purchased from Beijing Huadu Yukou Poultry Industry Co., Ltd. of China and randomly assigned to CCS and AVS. Jing Tint 6 is a commercial line in China, and it was based on Rhode Island Red and White Leghorn and featured with pink eggs (Figure 3-1). It is widely farmed in China due to its good production performance and product quality. Breeder recommended, to 80 weeks, total egg number is around 380, the average egg weight is about 55.8 g, the egg production of the peak laying period is over 95%, the liveability is more than 95%, and the average daily feed intake is around 106 g during laying period, and average body weight at 80 weeks is around 1,800 g. The CCS was a four-tier battery system, whereas the AVS was a four-tier aviary system. Three hundred and sixty of hens were placed in 30 cages (12 hens per cage) of CCS with a space allowance of 450 cm^2/ hen; total cage area = 5,400 cm^2. Another 2,592 hens were placed in 6 units of AVS with floor space allowance of 311 cm^2/ hen, and floor + 3 layers suspended platforms space allowance of 917 cm^2/ hen (floor area 134,400 cm^2, suspended platforms area 261,744 cm^2, total system area 396,144 cm^2). Each system was equally divided into 6 replicates, CCS had 5 cages per replicate and AVS had 1 unit per replicate. The dimension (length × width × height) of CCS cages and AVS units were 60 cm × 90 cm × 45 cm and 480 cm × 280 cm × 330 cm. As shown in Figure 3-2, the AVS had three levels of platforms above the floor, and each level contained two equally sized platforms on either side of the cage interior. The perches of the same length as the platform were mounted on the upper platforms to increase opportunities for locomotion (Hester et al., 2014). Its nesting area was also installed in the platforms and separated by curtains. Each floor is equipped with the manure belt and egg collection trough. In the AVS, the floor and each level platform were equipped with feeders on both sides of the cage, and waterlines with nipple waterers were suspended from the ceilings per level. The feeders and waterers of the CCS were fixed in the similar positions to the AVS.

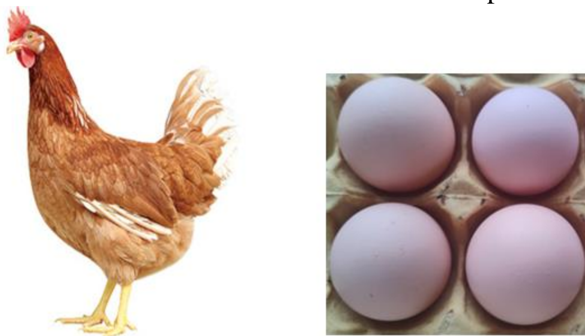

Figure 3-1. Jing Tint 6 laying hen and its eggs

The CCS and AVS were placed in one room of a research farm of China Agricultural University. This study was not terminated until the hens were 95 weeks of age. According to the nutrient requirements of the National Research Council (1994) and Chinese Feeding Standard of Chicken (Ministry of Agriculture of China, 2004), all pullets were fed a grower diet (CP = 15.5%, calcium = 0.8% and non-phytate P = 0.35%) until 18 weeks of age and changed to laying hen diets (19 weeks of age - the beginning of laying: CP = 17%, calcium = 2% and non-phytate P = 0.32%; the beginning of laying – 55 weeks of age: CP:16.5%, calcium = 3.5% and non-phytate P = 0.32%; 55 weeks of age – 95 weeks of age: CP = 15.5%, calcium = 3.8% and non-phytate P = 0.32%) later. All diets were purchased from a specialty feed mill. The detailed composition and nutrient levels of the diets were presented in the Table 3-1. The analytical values of calcium and CP in diets from 55 to 95 weeks of age were 3.92% and 16.1%, respectively. All chickens were given feed and water ad libitum during the whole experiment. Two systems were given the same photoperiod (8-18 weeks of age: 9 h light/15 h dark, 15 lux; 18-30 weeks of age: increased stepwise to 16 h light/8 h dark, 30 lux; 30-95 weeks of age: 16 h light/8 h dark, 30 lux) and room temperature (15 ~ 23℃). The average daily egg production of the CCS and AVS for the whole laying period were 85.76% and 89.48%, respectively. And the average body weight at the end of trial was 1.894 kg in CCS and 1.752 kg in AVS.

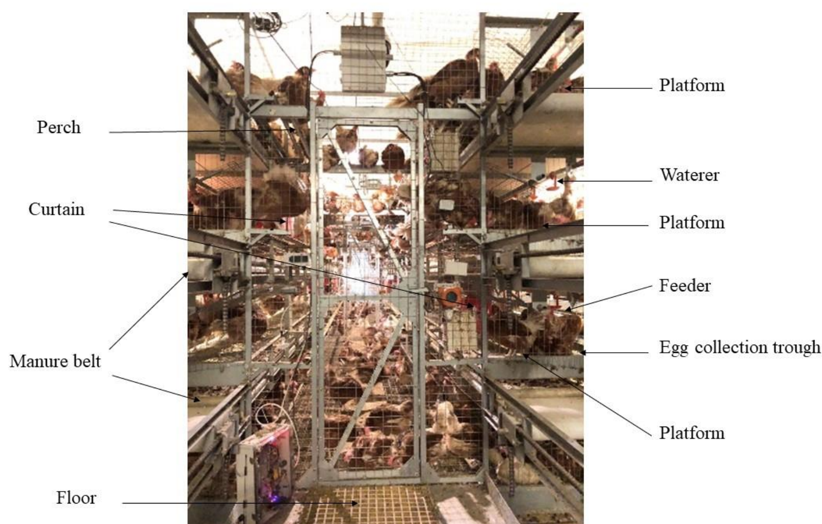

Figure 3-2. Image of aviary system employed in this study. The dimension (length × width × height) of the unit was 480 cm × 280 cm × 330 cm. Image depicts the main equipment inside the system (floor area 134,400 cm^2, total system area 396,144 cm^2), including feeders, waterers, perches, manure belts, litter area, nesting area and suspended platforms (261,744 cm^2).

Table 3-1. The composition and nutrient levels of the diets (air-dry basis)

	9 – 18 weeks	19 weeks - lay	lay – 55 weeks	55 – 95 weeks
Ingredient (%)				
Corn	68.00	63.00	58.40	63.00
Soybean meal	22.20	26.60	26.50	23.20
Limestone	1.25	4.70	9.00	9.90
Dicalcium Phosphate	1.40	1.20	1.20	1.20
Salt	0.36	0.36	0.36	0.36
Soybean oil	0.80	0.80	2.00	-
Wheat bran	3.75	1.10	0.30	0.10
D, L-Methionine	0.12	0.12	0.12	0.12
Choline chloride	0.12	0.12	0.12	0.12
Premix	2.00[1]	2.00[1]	2.00[2]	2.00[2]
Total	100.00	100.00	100.00	100.00
Nutrient levels (calculated value)[3]				
AME (MJ/kg)	11.75	11.50	11.29	10.89
Crude protein (%)	15.53	16.99	16.54	15.53
Methionine (%)	0.37	0.39	0.38	0.37
Lysine (%)	0.76	0.86	0.85	0.77
Methionine + Cysteine (%)	0.65	0.69	0.67	0.64
Calcium (%)	0.82	2.00	3.50	3.81
Phosphorous (%)	0.55	0.53	0.52	0.51
Available phosphorous (%)	0.35	0.32	0.32	0.32

[1] Premix provided the following per kg of the diet: vitamin A 6,000 IU; vitamin D_3 2,000 IU; vitamin E 15 IU; vitamin K 1.5 mg; thiamine 1.5 mg; riboflavin 6 mg; calcium pantothenate 25 mg; niacin 16 mg; pyridoxine 8 mg; biotin 0.5 mg; folic acid 1.25 mg; vitamin B_{12} 0.02mg; Mn 65 mg; I 1 mg; Fe 60 mg; Cu 8 mg; Zn 66 mg; montmorillonite 0.5 g; yeast culture 10 g.

[2] Premix provided the following per kg of the diet: vitamin A 10,000 IU; vitamin D_3 4,125 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} 0.02mg; Mn 65 mg; I 1 mg; Fe 60 mg; Cu 8 mg; Zn 66 mg; montmorillonite 1 g; yeast culture 10 g.

[3] AME is calculated by the AME values of ingredients that were obtained by metabolic tests, and the other nutrients are calculated based on absolute values.

3.2. Sample collection

Each replicate (5 cages for CCS, 1 unit for AVS) was a sampling unit and an experimental unit. On the final day of 55, 65, 75, 85 and 95 weeks of age, all eggs from each replicate were collected individually, and 10 eggs of them were randomly selected to determine eggshell quality. During each time point, 60 eggs were examined

for each system. At the end of the trial, two individuals were chosen randomly from each replicate to be sacrificed. The mucosa samples of uterus and duodenum were immediately removed and snap-frozen in liquid nitrogen. Moreover, humeri and femurs on both lateral sides were dissected out and removed from meat and fat. The left bones were stored at -20ºC for compositional, geometrical, morphological, or mechanical analyses. The right bones were truncated from their center. The proximal portion was immersed in formalin to fix. For the rest of the bone, a segment was cut in close proximity to the incision and snap-frozen in liquid nitrogen. Following collection, all samples frozen in liquid nitrogen were transferred to a freezer at -80ºC until further analysis.

3.3. Egg size and eggshell quality

Sixty eggs from each group were selected for testing at each time point (6 replicates with 10 eggs per replicate). Egg length and width were measured using caliper for calculation of shape index (egg shape index = egg length/egg width). The determination of eggshell quality referred to earlier described methods (Fu et al., 2021a; Mabe et al., 2003). Eggshell thickness was measured with Egg Shell Thickness Gauge (Ramat Hasharon, Israel Orka Food Technology Ltd., Ramat Hasharon, Israel). Eggshell breaking strength was defined as the minimum force required to fracture each egg at a longitudinal compression speed of 5 mm/min. It was measured with Egg Force Reader (Ramat Hasharon, Israel Orka Food Technology Ltd., Ramat Hasharon, Israel) at room temperature with the following parameters: the speed of the cross head, 5 mm/min; rated load capacity, 50 N; overload tolerance, 100 N; accuracy, 0.001 kgf. Eggshell weight was weighted as w_1 after cleaning and drying in room temperature. The sum of dry weights of eggshells from each replicate was W_1. The weight ratio of eggshell was calculated as a percentage score for the weight of each eggshell to the weight of each egg. For eggshell stiffness, a Texture Lab Pro (TMS-Pro, Food Technology Ltd., SV, Sterling, VA, USA) was used with the parameters as follow: the load of sensor, 25 N; the applied load, 10 N; the speed of the cross head, 1 mm/min. According to a previous report (Mabe et al., 2003), eggshell elastic modulus and fracture toughness were calculated as follow: the elastic modulus (N/mm^2) = $[(0.153 \times I^3) - (0.907 \times I^2) + (1.866 \times I) - 0.666] / 0.444 \times (0.408 + 3.026 \times 2 \times T / w) \times (S_d \times w / 2 / T^2)$, the fracture toughness ($N/mm^{3/2}$) = $0.777 \times (2.388 + 2.9934 \times 12 / w) \times F / T^{3/2}$, where I = egg shape index, T = eggshell thickness (mm), w = egg width (mm), Sd = eggshell stiffness (N/mm).

All eggshells from each replicate were mixed and crushed as one sample to analyze calcium and phosphorus contents according to the reported method (Fu et al., 2021b). Briefly, approximately 0.5 g of eggshell powder was taken and mixed with 3 mL nitric acid and 3 mL H_2O_2 in a burning cup and stood for 2 h. Then, the burning cup with eggshell was digested using a microwave digestion instrument (MDS-10, Shanghai Xinyi Instrument Technology co., Ltd, Shanghai, China). After digesting, the solutions were transferred to 50 mL volumetric flasks and adjusted to 50 mL by rinsing 3 to 4 times deionized water. Flame atomic absorption spectrophotometry (Z2000, Hitachi Co., Ltd., Tokyo, Japan) was used to analyze the calcium content as

C_1. The content of phosphorus was measured as C_2 with a spectrophotometer (UV-2700, Shimadzu, Japan). Total calcium and phosphorus per egg were calculated as follow: total calcium per egg = $W_1 \times C_1$, total phosphorus per egg = $W_1 \times C_2$.

3.4. Bone geometric characteristics

Each group consisted of 6 replicates with 2 birds per replicate. The left bones were thawed at 4°C overnight before weighing. The volume was defined as the amount of water displaced when the bone was placed in a measuring cylinder. The bone density was the weight divided by the volume. A vernier caliper and a string were used to measure the length and the midpoint perimeter of the bone. The mean relative wall thickness, the cortical cross-sectional area and the mean cortical index were measured using the proximal portion of the right bone (fixed with formalin) and calculated according to published methods (Brzóska et al., 2005; Cui et al., 2019). Briefly, the horizontal external and internal cortical bone diameters of the mid-diaphyseal cross section were measured as H and h using a digital caliper, and the vertical external and internal cortical bone diameter of that were measured as B and b. The mean relative wall thickness, the cortical cross-sectional area and the mean cortical index were calculated according to the following formula:

Mean relative wall thickness = $[(B - b)/b + (H - h)/h]/2$

Cortical cross-sectional area = $\pi \cdot (H \cdot B - h \cdot b)/4$

Mean cortical index = $[(B - b)/B + (H - h)/H]/2$

3.5. Bone mineral measurements

Following the analyses of geometric characteristics, three regions of each left bone (6 replicates per group, each replicate based on 2 birds) were used for analyzing mineral density (BMD) and bone mineral content (BMC) using a dual energy X-ray absorptiometry system (DTX-200, Osteometer MediTech, Hawthorne, CA, USA). The bone was divided into three aliquots and marked with lead needles. The point located near from the body was considered as proximal, and the other was distal. Similarly, the midpoint of the bone was also marked with a lead needle. Bone segments 0.5 cm above and below the lead marker were used to measure BMD and BMC.

3.6. Bone mechanical properties

After scanning, TMS-Pro was used to assess bone mechanical properties (strength, stiffness and work to fracture) using the three-point bending method reported by a previous study (Brzóska et al., 2005). Six replicates were used for each group and each replicate contains 2 birds. The span of two support bars was 4 cm. The load of sensor was 1,000 N. The bone was vertically loaded at a displacement rate of 2 mm/min until bone fracture.

3.7. Bone components

Each group had 6 replicates and each replicate had 2 birds. The fracture bones were defatted completely and oven-dried at 65ºC. The fat-free dry bone was weighted as W_2 and then burned in a muffle furnace until they were fully burned to obtain bone

ashes. The bone ash content was determined through calculating the percentage of the ash weight versus the fat-free dry weight. The calcium and phosphorus contents in ash were analyzed following the same method described above (Eggshell Quality). Total calcium and phosphorus in bones were calculated according to the following formulas: total calcium/phosphorus per bone = the calcium/phosphorus content in ash / the ash content × W_2.

3.8. Bone histomorphometry

Histomorphological analysis was performed in a blinded manner. The method of Goldner's Trichrome stain was adapted from a previous study (Farr et al., 2017). Femurs and humeri (6 replicates per group with 2 birds per replicate) were decalcified in EDTA until adequately softened. The softened bones were dehydrated, wax dipped, embedded and cut into 4-μm sections. The sections were deparaffinized and stained with Goldner staining solution suit (Servicebio technology Co. Ltd., Wuhan, China). Images were scanned with a panoramic slide scanner (3DHISTECH Ltd., Budapest, Hungary), and suitable areas of interest (AOI) in the proximal and middle parts of the bone were photographed with CaseViewer2.2 software (3DHISTECH Ltd., Budapest, Hungary). The suitable areas of interest (AOI) in proximal part of bone were defined as the entire epiphysis and metaphysis, and the AOI in the middle part of the bone was the mid-diaphyseal region (near the incision). Images of the epiphyseal region should contain the entire epiphyseal structure (including the outer cortical bone). Each image of metaphyseal and diaphyseal regions includes cortical bone on both sides. The regions were consistent across all bone samples. And, the same part was photographed at the same magnification.

The analysis of trabecular bone microarchitecture referred to a previous study (Lei et al., 2017) and was conducted with Image-Pro Plus 6.0 (Media Cybemetics, MD, USA) software. Tissue area (T.Ar), trabecular area (Tb.Ar) and trabecular perimeter (Tb.Pm) were measured. Trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp) and trabecular bone volume/tissue volume (BV/TV) were calculated using the following equations:

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

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

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

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

3.9. Quantification of calcium metabolism-related mRNA

The tissue samples (including humerus, femur, uterus and duodenum; 6 replicates per group with 2 birds per replicate) were ground in liquid nitrogen first. EASYspin Plus Bone Tissue RNA Kit (Aidlab Biotechnologies Co. Ltd., Beijing, China) was used to extract total RNA in the humerus and femur. The total RNA in uterine and duodenal tissues were extracted using TRNzol reagent (Tiangen Biotech Co. Ltd., Beijing, China). The integrity, purity and concentration of RNA were determined by agarose gel electrophoresis and a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA). The cDNA synthesis was done on 1.5 μg total RNA with

Easy Script First-Strand cDNA Synthesis SuperMix kit (TransGen Biotech Co. Ltd., Beijing, China). The resulting cDNA was used for subsequent quantification.

Quantitative real-time PCR (qPCR) was carried out by Light Cycler 480 system (Roche, Basel, Switzerland) with SuperReal PreMix kit (Tiangen Biotech Co. Ltd., Beijing, China). The results were processed using $2^{-\Delta\Delta CT}$ method (Livak et al., 2001). The sequences of primers were listed in Table S2, the housekeeping gene used was avian β-actin.

3.10. Statistical analysis

The replicate was the experimental unit. All analyses were conducted with SAS 9.4 (SAS Inc.). The homogeneity of variances and the normality of the data were tested first. The Proc GLM procedure was used to examine the effects of rearing system, age and the interaction between these two factors on the data of eggshell quality. The linear and quadratic orthogonal polynomial contrasts were further conducted for age response. Eggshell components, bone qualities and qPCR mean values of two rearing systems were compared using T-test procedure. Differences were considered statistically significant at $P < 0.05$.

4. Results

4.1. Egg size and eggshell quality

The results of egg size and eggshell quality are presented in Table 3-2. Egg length and egg shape index were linearly and quadratically increased with age ($P < 0.05$). The eggs in the AVS group had longer length and width compared to those in the CCS group ($P < 0.05$). Eggshell breaking strength, thickness, weight, weight ratio, stiffness and fracture toughness were decreased linearly with age ($P < 0.05$). Additionally, quadratic effects of age were detected on the egg weight, egg shape index, eggshell breaking strength, thickness, weight, weight ratio, stiffness and elastic modulus ($P < 0.05$). The AVS group had higher eggshell breaking strength, stiffness and fracture toughness than the CCS group ($P < 0.05$).

In Figure 3-3, the results of eggshell component show that both total calcium and total phosphorus per egg in the AVS group were significantly higher than those in the CCS group ($P < 0.05$). In addition, eggshell calcium and phosphorus content were numerically higher in the AVS group compared to those in the CCS group, but the differences were not significant (calcium content, $P = 0.071$; phosphorus content, $P = 0.063$).

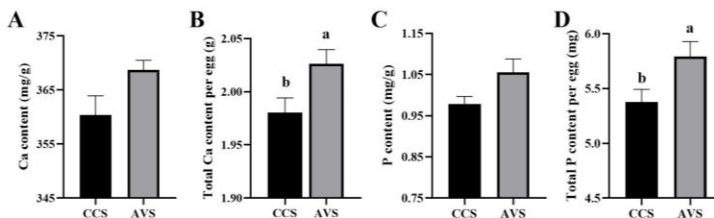

Figure 3-3. Effect of rearing systems on eggshell component of aged laying hens (95 weeks of age) (n = 6). (A) Calcium (Ca) content, (B) Total Ca per egg, (C) Phosphorus (P) content,

(D) Total P per egg. CCS, conventional caging system; AVS, aviary system. a, b Bars with different letters differ significantly at *P* < 0.05.

4.2. Bone geometric characteristics, and components

Figure 3-4 presents the differences in the geometric characteristics and components of the humerus and femur obtained from the AVS and CCS groups. Humeral weight, volume, length, midpoint perimeter and cortical index of the AVS group were obviously higher than those of the CCS group (*P* < 0.05). Compared to the CCS group, the AVS group had a humerus with higher fat-free dry weight, ash content, total calcium per bone and total phosphorus per bone (*P* < 0.05). There were no significant differences in the geometric characteristics and components of femur between the two groups (*P* > 0.05).

4.3. Bone mineral measurements

Table 3-3 shows the comparative results of bone mineral measurements of laying hens. Compared to the CCS group, the AVS group had higher average BMD of the humerus (*P* < 0.05). The AVS group significantly increased distal BMD of the femur in comparison with the CCS group (*P* < 0.05).

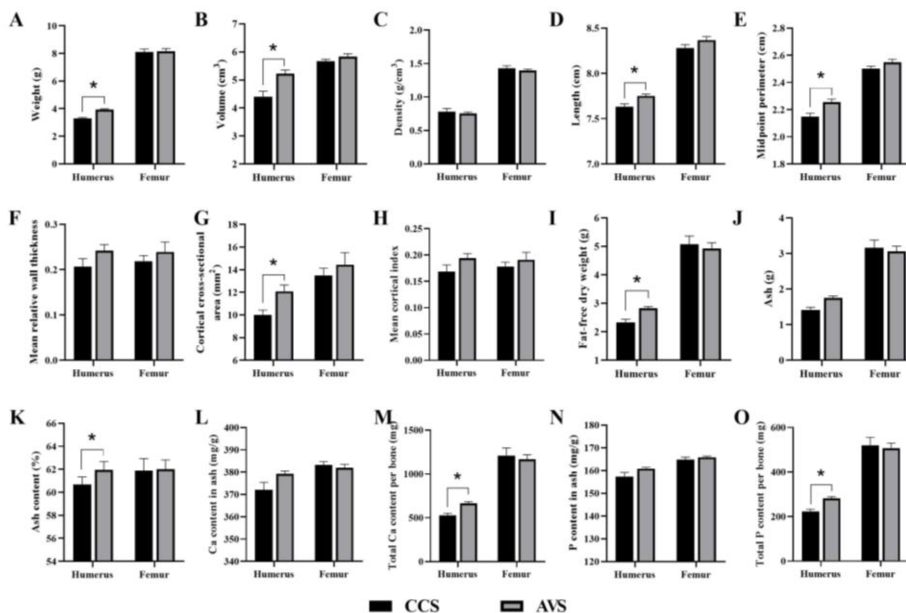

Figure 3-4. Effect of rearing systems on bone geometric characteristics (A-H) and component (I-O) in the aged laying hens (95 weeks of age) (n = 6). (A) Bone weight, (B) Volume, (C) Density, (D) Length, (E) Midpoint perimeter, (F) Mean relative wall thickness, (G) Cortical cross-sectional area, (H) mean cortical index, (I) fat-free dry weight, (J) ash, (K) ash content, (L) calcium (Ca) content in ash, (M) total Ca per bone, (N) phosphorus (P) content in ash, (O) total P per bone. CCS, conventional caging system; AVS, aviary system. Data represent means with standard error. **P* < 0.05.

Table 3-2. Effect of rearing systems on eggshell quality of laying hens (55-95 weeks of age)[1]

Item	Egg size			Eggshell quality						
	Length (mm)	Width (mm)	Shape index (mm/mm)	Breaking strength (N)	Thickness (*0.01 mm)	Weight (g)	Weight ratio (%)	Stiffness (N/mm)	Elastic modulus (N/mm2)	Fracture toughness (N/mm3/2)
System										
CCS	5.84[a]	4.30[a]	1.36	34.95[b]	43.42	5.60	9.16	66.74[b]	4689.23	305.05[b]
AVS	5.79[b]	4.28[b]	1.35	36.57[a]	43.84	5.54	9.24	67.66[a]	4643.77	316.65[a]
SEM	0.10	0.04	0.02	0.39	0.21	0.02	0.04	0.56	29.41	2.42
Age, wk										
55	5.69	4.29	1.33	37.57	44.30	5.62	9.49	72.83	4898.44	317.54
65	5.75	4.28	1.34	38.61	45.28	5.67	9.47	70.98	4566.53	317.52
75	5.87	4.29	1.37	35.20	43.64	5.54	9.07	65.67	4552.50	306.25
85	5.85	4.28	1.37	35.23	43.09	5.53	9.10	64.10	4571.99	313.60
95	5.91	4.31	1.37	32.17	41.82	5.50	8.86	62.42	4743.05	299.33
SEM	0.10	0.04	0.02	0.39	0.21	0.02	0.04	0.56	29.41	2.42
P-value										
System	<0.001	0.028	0.424	0.002	0.145	0.150	0.166	0.029	0.337	0.011
Age										
Linear	<0.001	0.347	<0.001	<0.001	<0.001	0.007	<0.001	<0.001	0.144	0.017
Quadratic	<0.001	0.293	<0.001	<0.001	<0.001	0.027	<0.001	<0.001	<0.001	0.053
System × Age	0.785	0.319	0.476	0.477	0.270	0.871	0.430	0.796	0.089	0.385

[1] Data represent means with standard error. There were 6 replicates per age in each group. CCS, conventional caging system; AVS, aviary system.

[a, b] Values within a column with no common superscripts differ significantly ($P < 0.05$).

Table 3-3. Effect of rearing systems on mineral measurements of bones in aged laying hens[1]

Items	CCS	AVS	*P*-value
Humerus			
Distal BMD (g/cm^2)	2.77±0.04	2.82±0.04	0.344
Midshaft BMD (g/cm^2)	2.81±0.09	2.96±0.04	0.141
Proximal BMD (g/cm^2)	2.73±0.03	2.80±0.06	0.257
Average BMD (g/cm^2)	2.77±0.03[b]	2.86±0.03[a]	0.042
Distal BMC (g)	2.50±0.04	2.59±0.06	0.268
Midshaft BMC (g)	1.62±0.08	1.71±0.04	0.296
Proximal BMC (g)	2.37±0.03	2.49±0.06	0.137
Average BMC (g)	2.16±0.04	2.27±0.05	0.134
Femur			
Distal BMD (g/cm^2)	2.72±0.03[b]	2.82±0.02[a]	0.046
Midshaft BMD (g/cm^2)	2.72±0.09	2.87±0.05	0.148
Proximal BMD (g/cm^2)	2.77±0.04	2.89±0.07	0.154
Average BMD (g/cm^2)	2.73±0.04	2.84±0.04	0.112
Distal BMC (g)	2.64±0.04	2.71±0.05	0.331
Midshaft BMC (g)	2.10±0.06	2.23±0.06	0.130
Proximal BMC (g)	2.62±0.04	2.73±0.08	0.238
Average BMC (g)	2.45±0.04	2.56±0.05	0.124

[1] Data represent means with standard error (n = 6). CCS, conventional caging system; AVS, aviary system; BMD, bone mineral density; BMC, bone mineral content.

[a, b] Values within a column with no common superscripts mean significant difference ($P < 0.05$).

4.4. Bone mechanical properties

As seen in the results summarized in Table 3-4, the strength, stiffness and work to fracture of the humerus in the AVS group were significantly higher than those in the CCS group ($P < 0.05$). However, these two groups did not differ significantly in femoral mechanical properties ($P > 0.05$).

Table 3-4. Effect of rearing systems on bone mechanical properties of aged laying hens[1]

Items	CCS	AVS	*P*-value
Humerus			
strength (N)	130.15±6.09[b]	182.44±9.50[a]	0.001
stiffness (N/mm)	67.67±4.58[b]	92.01±6.45[a]	0.012
work to fracture (mJ)	139.20±11.47[b]	217.19±14.88[a]	0.002
Femur			
strength (N)	270.43±20.18	263.27±16.06	0.787
stiffness (N/mm)	188.33±23.99	152.59±6.96	0.183
work to fracture (mJ)	241.41±22.76	287.82±25.15	0.201

[1] Data represent means with standard error (n = 6). CCS, conventional caging system; AVS, aviary system.

[a, b] Values within a column with no common superscripts mean significant difference ($P < 0.05$).

4.5. Bone Histomorphometry

The representative sections of the humeri and femurs stained with Goldner Trichrome are shown in Figure 3-5 (A-D). The lateral margin of cortical bone was clear, while its medial edge lacked clear boundary with the adjacent trabecular bones. All humerus samples presented large cavities inside. In the humerus, most of trabecular bone and osteoid were distributed among the epiphysis, and a little in the metaphysis and diaphysis. The humerus of the AVS group had more trabecular bone and osteoid than that of the CCS group. The trabecular bone and osteoid of the femur were distributed throughout the bone cavity, accompanied by a few lacunae. Larger lacunae were observed in the femur of the AVS group compared to the CCS group. Figure 3-4 (E-L) shows the results of the blinded analysis of BV/TV, Tb.Th, Tb.N and Tb.Sp. There was a trend towards increased BV/TV ($P = 0.085$) in the humerus of the AVS group.

4.6. Quantification of bone remodeling-related mRNA in bone

The results of the expression levels of key genes involved in bone remodeling are shown in Figure 3-6. Compared to the CCS group, the AVS group significantly increased the relative mRNA expression levels of fibroblast growth factor 23 (FGF 23), vitamin D receptor (VDR) and Estrogen receptor alpha (ERα) genes, but significantly decreased that of receptor activator of nuclear factor-kappa B (RANK) gene in humeri ($P < 0.05$). The relative mRNA expression levels of osteocalcin (OCN), alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP) genes in femurs were significantly upregulated in the AVS group compared to the CCS group ($P < 0.05$).

4.7. Quantification of calcium transport-related mRNA in duodenum and uterus

As seen in the results showed in Figure 3-7, no significant difference was observed in relative expression level of calbindin (CALB) in the mucosa of duodenum ($P > 0.05$). Birds in the AVS group significantly increased the transient receptor potential cation channel, subfamily V, member 6 (TRPV6) gene relative expression level in the mucosa of uterus compared to birds in the CCS group ($P < 0.05$), while no significant effects were observed on the relative expression levels of CALB, Na^+/Ca^{2+} exchange (NCX) and Ca^{2+} ATPase (PMCA) ($P > 0.05$).

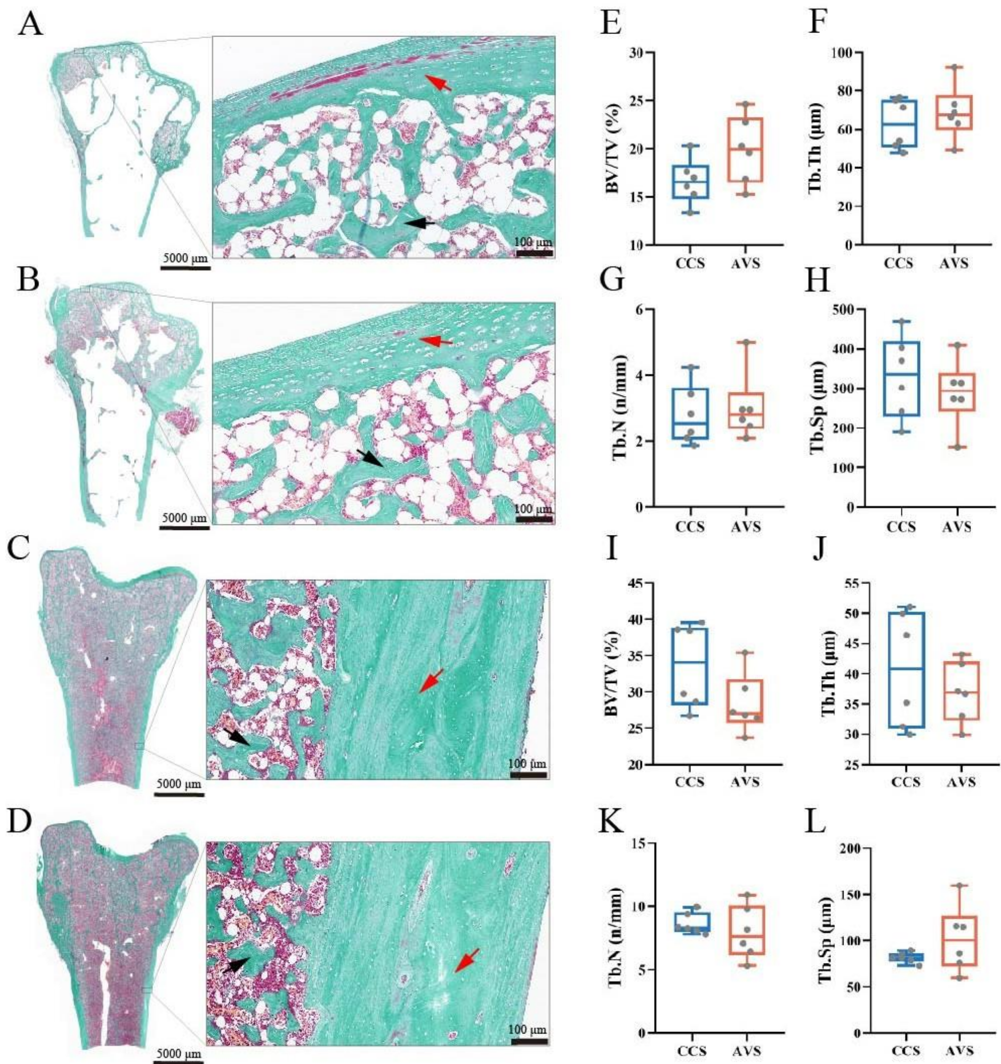

Figure 3-5. Effect of rearing systems on bone histomorphometry of aged laying hens (humerus, (A, B, E-H); femur, (C, D, I-L)). Humeri and femurs were sectioned and stained with Goldner's trichrome ((A, C), the CCS group; (B, D), the AVS group; red arrow, cortical bone; black arrow, trabecular bone). (E, I) Trabecular bone volume/tissue volume (BV/TV), (F, J) Trabecular thickness (Tb.Th), (G, K) Trabecular number (Tb.N), (H, L) Trabecular separation (Tb.Sp). BV/TV, Tb.Th, Tb.N and Tb.Sp were counted under blinded analysis (n = 6). CCS, conventional caging system; AVS, aviary system.

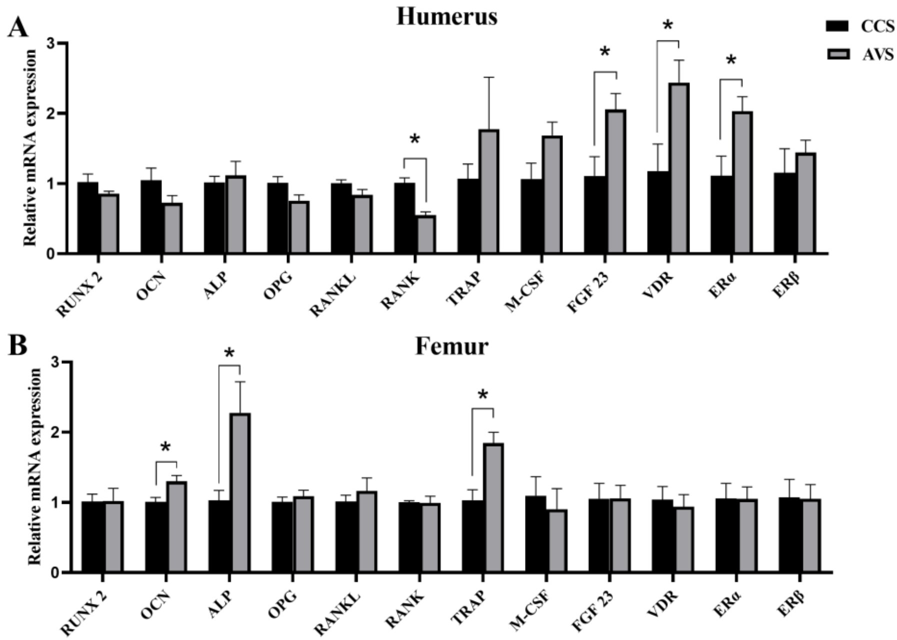

Figure 3-6. Effect of rearing systems on quantification of bone remodeling-related mRNA in bone of aged laying hens (n = 6). (A) in the humerus, (B) in the femur. CCS, conventional caging system; AVS, aviary system. RUNX 2, runt-related transcription factor 2; OCN, osteocalcin; ALP, alkaline phosphatase; OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor-kappa B ligand; RANK, receptor activator of nuclear factor-kappa B; TRAP, tartrate-resistant acid phosphatase; M-CSF, macrophage colony-stimulating factor; FGF 23, fibroblast growth factor 23; VDR, vitamin D receptor; ERα, estrogen receptor alpha; ERβ, estrogen receptor beta. Data represent means with standard error. *$P < 0.05$.

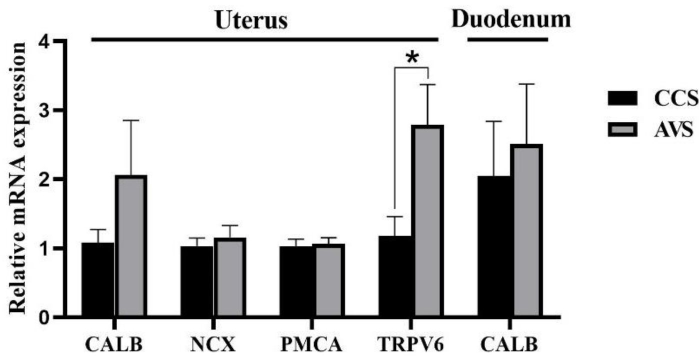

Figure 3-7. Effect of rearing systems on quantification of calcium transport-related mRNA in duodenum and uterus of aged laying hens (n = 6). CCS, conventional caging system; AVS, aviary system. CALB, calbindin; NCX, Na^+/Ca^{2+} exchange; PMCA, Ca^{2+} ATPase; TRPV6, transient receptor potential cation channel, subfamily V, member 6. Data represent means with standard error. *$P < 0.05$.

5. Discussion

Decreased eggshell quality in the late phase of laying poses a considerable threat to the economic benefit of poultry industry. Here, this study traced the eggshell quality of hens from the age of 55 weeks to 95 weeks. In this stage, the egg size gradually increased and elongated as the age advanced, while eggshell breaking strength, thickness, weight, weight ratio, stiffness and fracture toughness exhibited a gradually decreasing trend, which well agreed with previous studies (Gu et al., 2021; Sirri et al., 2018). Notably, the AVS group seemed to delay these changes. Compared to CCS group, the AVS group had higher eggshell breaking strength, stiffness and fracture toughness. Previous report demonstrated that the AVS had eggshell with thicker thickness than CCS (Ahammed et al., 2014; Leyendecker et al., 2005). Thus, the hens that housed in AVS could produce high-quality eggshells. Calcium carbonate makes up about 96% of the eggshell and is a predominant contributor to its mechanical properties (Hincke et al., 2012). We found that total calcium and phosphorus per egg of the AVS group higher than those of the CCS group. This demonstrated that calcium involved in eggshell formation was more in AVS group, and the increase of calcium supply may be a reason of the enhancement in eggshell mechanical properties (Fu et al., 2021a).

During bone remodeling, more bone formation and less bone loss increased bone strength and reduced the incidence of bone fracture (Whitehead, 2004). The AVS group exhibited higher weight, volume, length, midpoint perimeter, cortical index, fat-free dry weight, ash content, total calcium per bone and total phosphorus per bone in the humerus, indicating the AVS group had benefits in bone development and remodeling compared to the CCS group. The ultrastructure and mineral measurements of bones are the primary means of diagnosing OP (Brandi, 2009; Rachner et al., 2011). The average BMD of humerus and the distal BMD of femur were significantly increased in the AVS group compared to the CCS group. Similarly, there was a trend (P = 0.085) towards increased BV/TV in the humerus. Although no objective diagnostic parameters of OP existed in laying hens, the humerus of the CCS group tilted towards OP symptoms (Brandi, 2009) compared to that of the AVS group. The most severe consequence of OP is fracture, which is the leading cause of osteoporosis-related mortality. A three-point bending test was performed in our current study to simulate the process by which a bone was damaged by force. The strength, stiffness and work to fracture of the humerus were lower in the CCS group, indicating the hens held in this group may be susceptible to fracture in routine activities or accidental injuries. The increase of mechanical properties in the AVS group may be attributed to the enhancements of bone ultrastructure and bone mineral measurements, since the increase of trabecular bone connectivity and mineral deposition offered higher bone fracture resistance (Chen et al., 2020a). Previous studies confirmed that exercise played roles in enhancing bone mineral density and ultrastructure (Zymbal et al., 2019), and preventing OP in elder people (Feskanich et al., 2002). These benefits were also confirmed in pullets housed in AVS, which were equipped with perches and platforms to increase opportunities for locomotion (Casey-Trott et al., 2017c; Regmi

et al., 2015). Our study demonstrated that such effects would carry over to the end of laying phase, but only in the humerus and not in the femur, since similar differences were not found in the femur. Similar results were also observed in a previous study, wherein hens housed in the CCS and AVS had no significant differences on the ash and Young's Modulus of the tibia (Regmi et al., 2016). The marrow cavity of the femur contains medullary bone, which is a source of calcium for eggshell formation (Nys et al., 2018). The AVS group with high-quality eggshells had a higher demand for Ca, and its bone resorption was more active, which may be susceptible to more bone loss (Dacke et al., 2015). We speculated that the AVS group was beneficial for both humerus and femur development compared to the CCS group, however, such differences may be weakened in the femur due to the differences of eggshell calcium supply.

Eggshell formation occurs primarily in the uterus. At least four calcium transporters (CALB, TRPV6, PMCA, NCX) have already been identified to be involved in the transcellular transport of calcium in the uterus (Bar, 2009b). TRPV6 plays important roles in epithelial cellular entry for Ca, and there appeared to be a positive correlation between TRPV6 expression levels and calcium transport capacity (Yang et al., 2013). TRPV6 mRNA increased in the AVS group, which may indicate an enhanced calcium transport capacity, resulting in more calcium to enter the uterus. This could be an explanation for the increased content of eggshell calcium. The calcium transport in the intestinal tract primarily occurs in the anterior section of the small intestine and is considered to be dominated by transmembrane activities associated with CALB (Sugiyama et al., 2007). Herein, no differences were found at the mRNA expression level of CALB in the duodenum, hinting no significant differences in intestinal calcium absorption between the CCS and AVS groups.

Bone homeostasis requires the dynamic balance of bone formation and bone resorption (Feng et al., 2011). Bone resorption could lead to bone mineral loss and damage to bone structure when bone homeostasis is out of the balance (Whitehead, 2004). However, this loss is required in some special physiological states, such as eggshell formation, whenever intestinal calcium absorption is insufficient. Bone resorption contributes 20% - 40% of the eggshell calcium content and is one of the major means to supply eggshell calcium (Clunies et al., 1993; Comar et al., 1949). TRAP has been recognized as a histochemical marker for osteoclasts more than several decades (Burstone, 1959). Although TRAP deletion did not affect osteoclast differentiation and resorption pits formation, it impaired the capacity of bone resorption (Hayman et al., 1996). The relative expression value of femur TRAP mRNA in the AVS group was significantly higher compared with the CCS group, which may indicate a higher bone resorption capacity for the femur in the AVS group. ALP and OCN are considered as markers for the early and late osteogenic stages, respectively (Farley et al., 1994; Xu et al., 2019). ALP plays a role in bone mineralization by promoting the hydroxyapatite growth (Nizet et al., 2020). OCN is involved in regulating the process of bone mineralization, and its upregulation promotes terminal osteogenic differentiation (Xu et al., 2019). The relative expression levels of femur ALP and OCN in the AVS group were also higher than those in the

CCS group, suggesting the bone formation of the AVS group might be increased compared to the CCS group. Therefore, the increased expressions of TRAP, ALP and OCN indicated a higher bone remodeling of the femur in the AVS group, which may affect the calcium supply of eggshells, since bone resorption contributes 20% - 40% of the eggshell calcium content and is one of the major means to supply eggshell calcium (Clunies et al., 1993; Comar et al., 1949).

The RANK/RANKL/OPG pathway plays a dominant role in osteoclastogenesis. RANK is expressed on the osteoclast precursors, and its binding to RANKL activates NF-κB signaling in osteoclast precursors and induces osteoclasts maturation (Khosla, 2001). However, the decoy receptor OPG released by osteoblasts competes with RANK for binding to RANKL to block this process (Boyce et al., 2007). The ratio of RANKL/OPG is considered as a critical factor for osteoclast maturation and activation (Dacke et al., 2015; Khosla, 2001). Although the relative expression level of RANK in the CCS group was higher than in the AVS group, no significant differences were observed in the relative expression levels and rate of RANKL and OPG. Thus, there might be no differences between the CCS and AVS on the bone resorption of the humerus.

However, the humerus in the CCS group showed less bone formation. Active vitamin D and estrogen are involved in osteogenesis and bone formation after binding with the target receptor (mainly VDR and ER, respectively) (Chen et al., 2021; Hayashi et al., 2019). The deficiency of either of them accelerated bone loss and led to an OP phenotype (Chokalingam et al., 2012; Yang et al., 2020). Both VDR and ERα in the AVS group were higher expressed compared to those in the CCS group. In the AVS group, the increased expression of VDR and ERα could prevent bone loss (Lam et al., 2014; Peacock et al., 2002). On the contrary, the CCS group exhibited lower osteogenic ability and bone mass and was more prone to OP, which was consistent with the mechanical and compositional results. Similarly, the relative expression of FGF 23 was also higher in the AVS group, and this result may be related to the change of VDR, since FGF 23 is a phosphate regulator and involves a negative feedback regulation of vitamin D (Masuyama et al., 2006). Furthermore, the increased expression value of FGF 23 may also be related to the increased calcium supply of the uterus in the AVS group. It has been reported that the constant stimulation of parathyroid hormone (PTH) secretion induced by the daily eggshell formation in the uterus could cause a chronic overexpression of FGF 23 (Gloux et al., 2020b). In our current study, the regulation of rearing systems on bone formation of humerus may be mainly through hormone-dependent pathways. calcium is precisely transmitted among the organs (i.e., the intestine, the kidney, the uterus and bone) by a complex network mediated by endocrine hormones, which mainly involves 1,25-dihydroxyvitamin D, PTH, and estrogen (Dacke et al., 2015). A previous study demonstrated that the effect of in-cage facilities on bone formation in laying hens was independent of PTH-related pathways (Dale et al., 2015). We found that the effect of caging systems on the humerus may be related to 1,25-dihydroxyvitamin D and estrogen. Further studies will be necessary to determine the regulatory mechanism of

the rearing systems on the secretion of 1,25-dihydroxyvitamin D and estrogen. It is also worth investigating whether the regulations of 1,25-dihydroxyvitamin D and estrogen can increase bone formation of the layers housed in the CCS.

6. Conclusion

In conclusion, compared with the CCS, the AVS alleviated the deterioration of eggshell and bone qualities of aged laying hens. The AVS upregulated the expression of genes associated with bone formation in the femur (ALP, OCN) and humerus (VDR, ERα and FGF 23), which may be partly responsible for the improvement in bone quality. The AVS increased the expression of TRAP gene in the femur, hinting a higher bone resorption in the AVS, which may partly account for the improvement of eggshell quality.

Chapter 4

Decreased eggshell strength caused by impairment of uterine calcium transport coincide with higher bone minerals and quality in aged laying hens

Chapter 4. Decreased eggshell strength caused by impairment of uterine calcium transport coincide with higher bone minerals and quality in aged laying hens

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1. Abstract

Deteriorations in eggshell and bone quality are major challenges in aged laying hens. This study compared the differences of eggshell quality, bone parameters and their correlations as well as uterine physiological characteristics and the bone remodeling processes of hens laying eggs of different eggshell breaking strength to explore the mechanism of eggshell and bone quality reduction and their interaction. A total of 240 74-week Hy-line Brown laying hens were selected and allocated to a high (HBS, 44.83 ± 1.31 N) or low (LBS, 24.43 ± 0.57 N) eggshell breaking strength group. A decreased thickness, weight and weight ratio of eggshells were observed in the LBS, accompanied with ultrastructural deterioration and total calcium reduction. Bone quality was negatively correlated with eggshell quality, marked with enhanced structures and increased components in the LBS. In the LBS, the mammillary knobs and effective layer grew slowly. At the initiation stage of eggshell calcification, a total of 130 differentially expressed genes (DEGs, 122 upregulated and 8 downregulated) were identified in the uterus of hens in the LBS relative to those in the HBS. These DEGs were relevant to apoptosis due to the cellular calcium overload. Higher values of p62 protein level, caspase-8 activity, Bax protein expression and lower values of Bcl protein expression and Bcl/Bax ratio were seen in the LBS. TUNEL assay and hematoxylin-eosin staining showed a significant increase in TUNEL-positive cells and tissue damages in the uterus of the LBS. Although few DEGs were identified at the growth stage, similar uterine tissue damages were also observed in the LBS. The expressions of runt-related transcription factor and osteocalcin were upregulated in humeri of the LBS. Enlarged diameter and more structural damages of endocortical bones and decreased ash were observed in femurs of the HBS. The lower eggshell breaking strength may be attributed to a declined calcium transport due to uterine tissue damages, which could affect eggshell calcification and lead to a weak ultrastructure. Impaired uterine calcium transport may result in reduced femoral bone resorption and increased humeral bone formation to maintain a higher mineral and bone quality in the LBS.

Keywords: bone parameter, calcium transport, eggshell quality, laying hen, tissue damage

2. Introduction

An increase in cracked eggs in the late phase of the laying period heavily reduces the economic benefits of egg farmers and hinders the implementation of an extending laying period (Bain et al., 2016; Gautron et al., 2021). Eggshell breaking strength is the ability of an eggshell to resist damage under external forces, and each decrement of 1 N breaking strength is associated with a 1.33% breakage in a flock with a high egg production (> 70%) (Alfonso-Carrillo et al., 2021). As the age increased (30 to 80 weeks), the eggshell breaking strength declined from around 48 N to 35 N and its coefficients of variation increased from 11.4 to 17.2, resulting an increase in the proportion of individuals with low eggshell breaking strength in the Hy-line Brown laying hens (Ma et al., 2021; Sirri et al., 2018). These hens laying weak-shelled eggs

deserve special attention since they are responsible for a high breakage rate of eggs at the late laying period. Nutritional modulation strategies have been widely recognized as positive means of improving egg, especially eggshell, quality (Eltahan et al., 2023; Saleh et al., 2021a; Saleh et al., 2021b; Zaki et al., 2023). Comparison of eggshells and physiological characteristics of hens with high and low eggshell breaking strength could reveal the mechanism of increased egg breakage rate in aged laying hens, which is beneficial to develop more suitable products or measures for eggshell quality improvement.

Bone quality is receiving the same attention as eggshell quality in aged laying hens, and there may be a certain association between them since bone resorption provided approximately 20% - 40% calcium for eggshell calcification (Clunies et al., 1993; Comar et al., 1949). Medullary bone is considered as a calcium reservoir for eggshell calcification, which mainly exists within the marrow cavities of hind limbs (femur and tibia) in laying hens (Canoville et al., 2019; Whitehead, 2004). Hens that did not lay eggs had more highly mineralized bones with significant amounts of medullary bone (Eusemann et al., 2018a; Kim et al., 2004), while high-laying hens underwent an increase in the bone fracture incidence due to calcium depletion during eggshell calcification (Eusemann et al., 2018a). Kim et al. (Kim et al., 2005) suggested that having poor-quality bones was linked to laying high-quality eggshells as high deposition of eggshell calcium was accompanied by high bone calcium transfer. Thus, the high calcium requirements of eggshell calcification may be a trigger for weakened bones, attributing to intense medullary bone resorption (Riber et al., 2018; Whitehead, 2004). However, Alfonso-Carrillo et al. (Alfonso-Carrillo et al., 2021) concluded that the bone characteristics and eggshell properties were independent because they found no significant correlations between the eggshell (breaking strength, thickness and weight ratio) and bone (geometric and mechanical characteristics) quality of the hens at the end of laying period. Most previous studies were mainly concluded from limited skeletal indicators, presenting difficulties in determining the detailed correlations between bone and eggshell qualities as well as in analyzing the mechanisms underlying the effect of eggshell calcification on bone quality. The bone quality was determined by the bone remodeling, and more bone resorption and/or less bone formation contribute to a reduced bone mass and an increased incidence of bone fracture (Siddiqui et al., 2016). During bone formation, mesenchymal stem cells differentiate osteoblasts, which synthesize and secrete the organic matrix such as collagen type I (COL I), osteocalcin (OCN), osteopontin (OPN), bone sialoprotein (BSP) and osteonectin (ON), then deposit hydroxyapatite in the extracellular matrix and form mineralized bone, ultimately increasing bone mass (Mishra et al., 2015; Termine et al., 1981). As for the process of bone resorption, bone minerals are dissolved and expose organic matrix to cathepsin K (Cts K) that degrades them, resulting in bone loss and structure damage (Cohen Jr., 2006; Xiong et al., 2015). In the current study, we compared the bone quality and bone remodeling variation between hens with high and low eggshell breaking strength under the same feed and environment, which may contribute to a comprehensive understanding of the interaction mechanism of eggshell calcification and bone remodeling.

Eggshell formation takes about 18 hours, and ultimately forms eggshell ultrastructure (including eggshell membrane, mammillary layer, effective layer (palisade layer and vertical crystal layer) and cuticle) to resist external forces. The mammillary layer and effective layer are major structures that determine an eggshell's mechanical properties, which are respectively formed during the initiation (5-10 h post-ovulation) and growth (10-22 h post-ovulation) stages of eggshell calcification (Marie et al., 2015). The eggshell calcium supply may have different adaptations due to the limitation of the photoperiod on feed access (Bar, 2009b). Generally, in the initiation stage of eggshell calcification, the calcium necessary is mainly derived from the intestinal absorption (Bar, 2009b). However, a considerable time of the growth stage of eggshell calcification typically occurs during the nocturnal fasting period (Nys et al., 2018). At this period, medullary bone resorption becomes dominant in the supply for eggshell calcium as the residual calcium in the intestine is gradually consumed (Bar, 2009b). The increase in medullary bone resorption decreases bone minerals, induces osteoporosis, and affects the health of laying hens (Whitehead et al., 2000). Previous research mainly targeted skeletons with medullary bone, such as tibia and femur (Alfonso-Carrillo et al., 2021; Jiang et al., 2014). The tibia is considered as a model bone for the skeletal health examination in poultry species, while the femur is the most labile source of medullary bone calcium (Almeida et al., 2006; Hanlon et al., 2022), thus the femur may be more suitable to explore the function of bone with medullary bone during eggshell calcification. Additionally, birds retained a special type of bones during evolution, that is a hollow bone such as the humerus adapted to fly (Dacke et al., 2015). Humerus and femur may be remodeled in a different manner that affect eggshell calcification due to a differential sensitivity to hormones (Dacke et al., 2015; Fu et al., 2022). Exploring the metabolic characteristics of different bones during eggshell calcification could provide a more comprehensive panorama of the bone remodeling response to eggshell calcification.

This study compared the eggshell quality and bone parameters of hens with high or low eggshell breaking strength to analyze the correlations between eggshell and bones qualities of aged hens. Furthermore, their uterine transcription profiles, histological characteristics and bone remodeling processes during eggshell calcification were explored to reveal the possible mechanism of eggshell and bone quality reduction and the interaction of the uterus and skeletons of aged hens. This study provides some new insights into the crosstalk between bone and uterus, which is conducive to explore the regulation of bone and eggshell quality.

3. Materials and methods

3.1. Birds and experiment design

Animal procedures were approved by the management of the Animal Care and Use Committee of Institute of Feed Research, Chinese Academy of Agricultural Sciences (approval No. AEC-CAAS-20200902). The present study design is depicted in Figure 4-1. A total of 1,950 72-week-old healthy Hy-line Brown laying hens were selected from a commercial farm. Three hens were housed per cage (45 cm × 45 cm × 45 cm)

before 72 weeks of age. Then the hens were caged individually during the trial, and the eggshell breaking strength of each hen was measured daily during a 2-week pre-trial period. The average egg production of this flock was 88.79%. The average eggshell breaking strength of the flock was 34.55 N, in which the hens with average breaking strength above 40 N and below 29 N accounted for 20%, respectively. After the pre-trial, 240 laying hens were selected and divided into high (> 40 N, HBS) and low (< 29 N, LBS) eggshell breaking strength groups according to eggshell breaking strength. The egg production of the selected hens complied with 88.79% ± 5%, and their egg weights were in accordance with 55 to 70 g. The hens that were not selected were still raised in the commercial hen house until culling. The selected hens were transferred to another hen house with the same management, and each group was sub-divided into 12 replicates with 10 hens each. All hens were caged individually and fed with the same basal diet (Table 4-1; calcium level: 3.89%, the ratio of calcium and total P: 9.05:1) and received water ad libitum through the whole trial. All hens underwent an acclimation period of 4 weeks and an observation period of 2 weeks. During the observation period, the oviposition time of each hen was recorded daily using an automatic-monitoring control system (IFR, CAAS, Beijing, China) to determine the calcification periods (Zhang et al., 2017). All hens were subjected to a controlled photoperiod cycle of 16 h light : 8 h dark (light: 5:30-21:30).

Table 4-1. Ingredient and nutrient levels of the experimental diets (air-dried basis)

Ingredient	%	Nutrient level[2]	%
Corn	59.00	AME (MJ/kg)	11.11
Soybean meal	24.53	Crude protein[3]	16.37 (16.41)
Soybean oil	1.80	Calcium[3]	3.99 (3.89)
Limestone	10.60	Methionine	0.37
DL-Methionine	0.12	Lysine	0.80
50% choline chloride	0.12	Total phosphorus[3]	0.46 (0.43)
Calcium hydrogen phosphate	0.90	Available phosphorus	0.26
Sodium chloride	0.15	Methionine +Cysteine	0.65
Sodium sulfate	0.20	Ratio of calcium and total phosphorus	8.67:1 (9.05:1)
Wheat bran	2.40		
Vitamin and mineral premix[1]	0.18		
Total	100.00		

[1] Premix provided the following per kg of the diet: vitamin A, 9 500 IU; vitamin D_3, 4 125 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] Nutrient levels are calculated values. AME is calculated by the AME values of ingredients that were obtained by metabolic tests, and the other nutrients are calculated based on absolute values.

[3] Numbers in parentheses are the analyzed value.

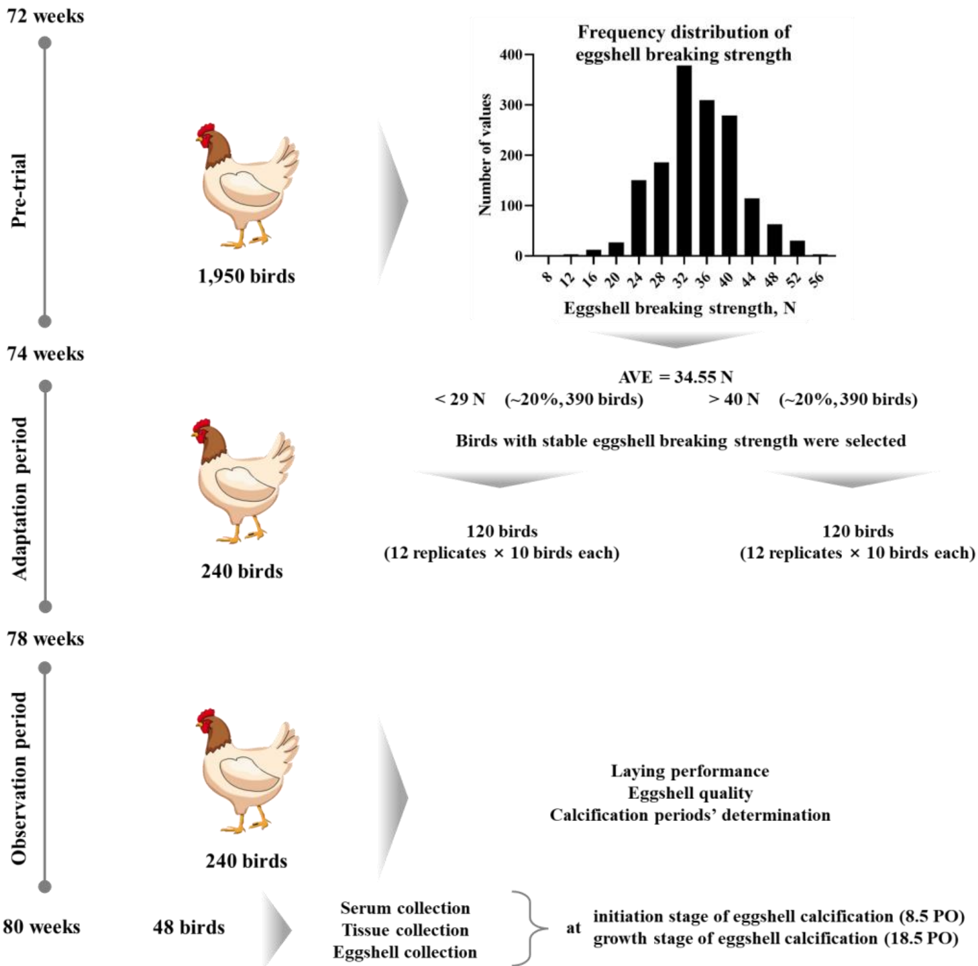

Figure 4-1. Trial design and sampling scheme. PO, post-oviposition.

3.2. Sample collection

A total of 25 eggs from each replicate were collected on 5 consecutive days of the observation period (5 eggs/replicate/d) to detect eggshell measurements. At 80 weeks of age, 12 birds (1 bird per replicate) in each group were selected at both 8.5 h post-oviposition (PO) and 18.5 h PO, corresponding to the initiation and growth stages of eggshell calcification, respectively. The selected hens were collected blood samples first, then subjected to euthanasia and tissue sampling. Serum was immediately separated and stored at -80°C after blood collection. The uterus morphology and the position of the egg were observed before the tissue was removed. Then uterus tissues were quickly removed and placed on ice. The uterine mucosa was removed immediately and stored at -80°C until further analysis. An additional piece of uterine

tissue was fixed in the 4% paraformaldehyde solution for uterine histomorphology. The egg was carefully transferred from the uterus, and the eggshell was slowly isolated from the egg and dried naturally at room temperature. The humerus and femur on both sides of each bird were removed, and residual tissues were cleared. The right bones, after removing the cartilage, were stored at -80°C until analysis. The left bones sampled at 18.5 h PO were truncated in the middle of the bone mid-diaphysis, with the proximal parts fixed in formalin. The remaining left bones were frozen at -20°C for bone geometrical, mineral and compositional analysis.

3.3. Laying performance

During the observation period, egg number and egg weight were recorded daily by replicate, and total feed consumption for each replicate was weighed. The egg production rate, egg mass, average egg weight, average daily feed intake and feed conversion ratio were calculated. Egg production rate was calculated as the ratio of the number of eggs produced per day to the number of birds, expressed as a percentage. Egg mass was represented as the weight of egg production per laying hen per day. Feed conversion ratio was calculated as grams of total feed consumption/total egg weight for each replicate.

3.4. Eggshell quality

3.4.1. Eggshell mechanical properties

Eggshell quality (2 groups, 12 replicates per group, 25 eggs per replicate) was determined according to the methods as described earlier (Fu et al., 2021a). The egg weight was weighed first. Then, the eggshell thickness was tested with the Egg Shell Thickness Gauge (Israel Orka Food Technology Ltd., Ramat Hasharon, Israel) and calculated as the mean of equator and both poles of the egg. Afterwards, eggshell breaking strength was detected by the Egg Force Reader (Israel Orka Food Technology Ltd., Ramat Hasharon, Israel). After removing the egg content, the eggshell was washed, dried and weighed. The eggshell weight ratio was calculated as the percent ratio of the eggshell weight over the total egg weight.

3.4.2. Eggshell ultrastructure

Five eggshells per replicate (collected on the observation period) were collected to assess eggshell ultrastructure (in total 60 eggshells per group). The eggshell was washed with distilled water to remove the dirt and residual albumen. After drying at room temperature, two pieces (0.5 cm^2 each) of each eggshell were taken from the equatorial region, fixed in the specimen stage, and coated with gold powder. The vertical profiles of eggshells were imaged using a scanning electronic microscopy (SU8000, Hitachi Co., Ltd., Tokyo, Japan). The thickness of effective and mammillary layers was measured with the SEM ruler according to a previous report (Zhang et al., 2017). Briefly, the mammillary thickness was assessed by measuring the length from the top of the membrane to the lower edge of the palisade layer. The effective thickness was taken as the length from the top of the cuticle to the bottom of the palisade layer. The calcified layer referred to the combined effective and mammillary layers. The thickness ratio was defined as the percentage of each layer

relative to the calcified layer. Each sample was measured 3 times at random. Two pieces of each eggshell, which were obtained at 8.5 h PO, were fixed at an aluminum plate to observe their vertical and external surfaces. Eggshells (two pieces with 0.5 cm^2 each) collected at 18.5 h PO were similarly fixed to photograph the vertical profile. The thickness of effective, mammillary and calcified layers was determined as described above.

3.4.3. Eggshell components

Five eggshells per replicate, weighing close to the average eggshell weight, were collected on the observation period. The eggshells were first washed, dried and weighed (W_1). Then, equal weights from each eggshell were taken, mixed and crushed into one sample for the determination of eggshell calcium and phosphorus content according to a previously reported method (Fu et al., 2021a). Briefly, 0.5 g of eggshell power was dissolved in 3 mL hydrogen peroxide and 3 mL nitric acid then withheld for 2 h. A microwave digestion system (MDS-10, Shanghai Xinyi Instrument Technology co., Ltd, Shanghai, China) was used to further digest the samples. The calcium and phosphorus contents were determined as C_1 and C_2 by a flame atomic absorption spectrophotometry (Z2000, Hitachi Co., Ltd., Tokyo, Japan) and a spectrophotometer (UV-2700, Shimadzu Corp., Kyoto, Japan), respectively. Total calcium and phosphorus per eggshell were calculated using the following formula: total calcium per eggshell = $W_1/5 \times C_1$, total phosphorus per egg = $W_1/5 \times C_2$.

3.5. *Bone quality*

3.5.1. Bone geometrical characteristics

The left bones (1 bone per replicate) sampled at 8.5 h PO were thawed at 4°C overnight and equilibrated at room temperature for 2 h. The whole bone was weighed (W_2) first, then it was placed in a measuring cylinder with some water and the increased volume was recorded as bone volume (V). The density of bone was calculated according to the following formula: W_2/V. The length and midpoint perimeter of the bone were measured using a string and a digital caliper. A digital caliper was used to determine the diameters of the incision site of the distal left bones collected at 18.5 h PO. The external and internal cortical bone diameters were defined as H and h in the horizontal (medial-lateral plane) and as B and b in the vertical (anterior-posterior plane). The cortical cross-sectional area, mean relative wall thickness and mean cortical index were calculated with equations as previously described (Kwiecień et al., 2014; Tatara et al., 2005): cortical cross-sectional area = $\pi \times [(H \times B) - (h \times b)]/4$; mean relative wall thickness = $[(B - b)/b + (H - h) \times h]/2$; mean cortical index = $[(B - b) \times B + (H - h)/H]/2$.

3.5.2. Bone mineral measurements

After the analysis of geometrical characteristics, the left bones (1 bone per replicate, sampled at 8.5 h PO) without any soft tissues were used to determine bone mineral content (BMC) and bone mineral density (BMD) with a dual energy X-ray absorptiometry (DTX-200, Osteometer MediTech, Hawthorne, CA, USA) according to previous studies (Fu et al., 2022; Shim et al., 2012). The air was used to calibrate

the measurements. Three regions were tested: the proximal, middle and distal, each of which was 1 cm long. The detection regions were kept consistent for all samples.

3.5.3. Bone components

The bone (1 sample per replicate), after measuring BMC and BMD, was used to determine bone components. The bone was broken first, and at each subsequent step, all bone fragments from each sample were carefully collected to ensure that all measurements were conducted on the whole bone. The samples were dehydrated in ethanol and defatted with petroleum ether, followed by drying in the oven overnight at 105 °C. The fat-free dry bone was weighed. Then, the determination of bone ash was carried out using a muffle furnace. The ash content of the bone sample was calculated via division of the ash weight by the fat-free dry weight. The bone calcium and phosphorus contents in ash were measured as described by the method above ("Eggshell quality, ultrastructure and components"), followed by calculating the ratio of calcium and phosphorus. Total calcium/phosphorus per bone was the product of bone calcium/phosphorus content in ash and ash weight.

3.5.4. Bone histomorphometry

The method of Goldner's Trichrome stain was carried out as previously reported (Farr et al., 2017). The formalin-fixed specimens (1 sample per replicate) were decalcified, dehydrated and embedded in paraffin. A microtome was used to slice the paraffin blocks into the sections that were 4 μm thick. After dewaxing, Goldner-Trichrome staining was performed with a commercial kit (Servicebio technology Co. Ltd., Wuhan, China) according to the manufacturer's instructions. Pannoramic scanner system (3DHISTECH Ltd., Budapest, Hungary) was used to scan the images of the bones.

3.6. RNA extraction, library construction, sequencing and data analysis in the uterus

Total RNA of the uterus samples was extracted with TRNzol reagent (Tiangen Biotech Co. Ltd., Beijing, China), and its integrity was verified by the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA). RNA Libraries were prepared using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB Inc., Ipswich, MA, USA). Briefly, the mRNA was enriched using magnetic beads (for eukaryotes) with Oligo (dT) and broken into short fragments of approximately 150 bp in fragmentation buffer. Fragmented mRNA was used as a template, and first-strand cDNA was achieved using random hexamers. The second strand cDNA was subsequently synthesized by adding dNTPs, RNase H, DNA polymerase I and buffer. The double-stranded cDNA was purified with AMPure XP beads, then terminal repair and 3′-end single nucleotide A (adenine) addition were performed for adaptor ligation. Fragment size selection of library was also carried out using AMPure XP beads. The cDNA library was constructed by PCR amplification. AMPure XP system (Beckman Coulter, Beverly, USA) was used for the purification of PCR products, and Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA) was used to assess library quality. Finally, the TruSeq PE Cluster Kit v4-cBot-HS (Illumina, San Diego, CA,

USA) was used to perform the clustering of the index-coded samples on a cBot Cluster Generation System, and the libraries were sequenced on an Illumina platform (Illumina, San Diego, CA, USA). The sequence data have been submitted to the NCBI Sequence Read Archive under the accession numbers: PRJNA1000560.

The raw reads were filtered by removing low-quality reads with ambiguous 'N' base, adapter sequences, rRNA, and short reads (less than 20 nt) with FasTX clipper v0.0.13. The resulting clean reads was mapped using HISAT2 (Kim et al., 2015) with *Gallus gallus GRCg6* as the reference annotation file. The expression levels of mapped genes were normalized as FPKM using StringTie. The differentially expressed genes (DEGs) between the HBS and LBS groups were analyzed separately at 8.5 and 18.5 h PO. The analysis of DEGs was performed using the DESeq2 software, with the following screening threshold: |Fold change| > 1.3 and the false discovery rate (FDR, adjusted with Benjamini and Hochberg's approach) < 0.05. The DEGs were mainly obtained from 8.5 h PO, while there were only a few differences of transcriptional profiles at 18.5 h PO between HBS and LBS. Thus, only DEGs at 8.5 h PO were used for onward analysis. To elaborate on the activated pathways associated with the differences of mammillary knobs' growth at 8.5 h PO, we analyzed for enriched gene ontology (GO) biological processes on DEGs using ClueGO.

3.7. qRT-PCR validation of RNA sequencing results

Twelve genes of each eggshell calcification stage were selected for qRT-PCR validation. The cDNA was synthesized using reverse transcription with Easy Script First-Strand cDNA Synthesis SuperMix kit (TransGen Biotech Co. Ltd., Beijing, China) following manufacturer's instruction. Each reverse transcription included 1.5 μg RNA. Quantitative PCR assays were performed utilizing a CFX96 C1000TM thermal cycler (Bio-Rad, CA, USA). And each assay was done with 3 technical replicates. Primer sequences are listed in Additional file 2. Primer efficiencies ranged from 91.08% to 109.70%.

3.8. Apoptosis-related markers in the uterus

The p62, p53, LC3 and Immunoglobulin A protein contents of the uterus were quantified using an ELISA method. The kits of p62 and p53 were purchased from Shanghai Enzyme-linked Biotechnology Co., Ltd (Shanghai, China), and those of LC3 and Immunoglobulin A were obtained from Jiangsu Meimian Industrial Co., Ltd (Jiangsu, China). The activities of caspase-3 and caspase-8 were assessed using the caspase-3 and caspase-8 activity assay kits (Beyotime, Shanghai, China) according to the manufacturer's protocol. The protein concentrations were measured with a Bradford protein assay kit (Beyotime, Shanghai, China).

Western blot analysis was performed to detect the relative expression of Bcl-2 and Bax proteins. Total proteins were extracted by a commercial kit (Beyotime, Shanghai, China), supplemented with protease/phosphatase inhibitors (Beyotime, Shanghai, China). Lysis buffer contained 20 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100 and a cocktail of protease inhibitors. The protein concentrations were measured with a BCA protein assay kit (Beyotime, Shanghai, China). A total of 20 μg protein

was loaded in each lane. Following electrophoresis, the protein samples were transferred to a PVDF membrane (Bio-Rad, CA, USA). The membrane was blocked with 5% non-fat dry milk (TBS solution with 0.1% Tween) for 45 minutes with agitation at room temperature, followed by overnight incubation with primary antibodies for Bax (Abclonal, #A12009), Bcl-2 (Abclonal, #A11025) and GAPDH (Abcam, #EPR16891), respectively. The membrane was then washed 3 times for 10 minutes each time, and incubated with secondary antibody for 1 h at room temperature. After washing membrane again, the blots were visualized with ECL reagent (Beyotime, Shanghai, China) in a dark room. Image analysis was conducted using Image-Pro Plus 6.0 software.

3.9. Hematoxylin-eosin (HE) and TUNEL staining

Tissue samples of the uterus were fixed in formalin overnight and embedded in paraffin blocks. The blocks were processed for routine microtome and stained by HE for histopathological observation. Another section was used to assay apoptosis with a fluorescein TUNEL assay kit (G1501, Servicebio Technology Co., Ltd., Hubei, China) based on manufacturer's instruction. Firstly, tissue sections were deparaffinized and rehydrated. Proteinase K solution was then added to retrieve the antigen. Subsequently, membranes were disrupted, the TUNEL reaction solution was added, and the nuclei were stained with DAPI solution. Finally, microscopic examination and image collection were conducted using a fluorescence microscope (Nikon Instruments Inc., Tokyo, Japan). Blue color indicates cell nucleus, and green color indicates positive apoptosis cells.

3.10. Quantification of bone turnover-related mRNA in bone

The humerus and femur samples without cartilage were removed from -80°C. The sample was hammered into pieces and further ground manually with a mortar and pestle in the liquid nitrogen. Total RNA was extracted using EASYspin Plus Bone Tissue RNA Kit (Aidlab Biotechnologies Co. Ltd., Beijing, China) according to the kit instructions. Agarose gel electrophoresis was used to confirm RNA integrity, and a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA) was used to determine the purity and concentration of the RNA. The cDNA synthesis and quantification of target gene expression were performed as described in the "qRT-PCR validation of RNA sequencing results". The primers are also supplemented in Additional file 2. Primer efficiencies ranged from 91.91% to 107.86%.

3.11. Calcium, phosphorus, bone remodeling-related enzyme and hormone concentrations in serum

Serum was thawed at 4°C and analyzed for calcium and P concentrations using a microplate reader with calcium and P assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The bone-specific alkaline phosphatase (BALP) level was detected using a commercial kit (Shanghai Meilian biological Technology Co., LTD., Shanghai, China), and the tartrate resistant acid phosphatase (TRAP) activity were

determined using a TRAP activity kit (Shanghai Meilian biological Technology Co., LTD., Shanghai, China). Radioimmunoassay (RIA) kits were purchased from Beijing Sino-UK Institute of Biological Technology (Beijing, China) to determine parathyroid hormone (PTH), estrogen (E_2), 25-hydroxyvitamin D_3 (25-OH-D_3) and 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2$-D_3) concentrations in serum.

3.12. Statistical analysis

Replicates (n = 12) were the experimental units for all analysis. The normal distribution (Shapiro-Wilk test) and homoscedasticity (Levene's test) of all data were firstly checked using SAS 9.4 (SAS Inc., Cary, NC, USA). An unpaired two-tailed Student's t-test was also conducted in SAS 9.4 (SAS Inc., Cary, NC, USA) to analyze the significance of the difference between the HBS and LBS. Data are presented as mean ± SD (standard deviation). Differences were considered significant at a *P* value < 0.05. The correlation between eggshell and bone qualities was analyzed using Origin software. Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method and normalized to avian β-actin as housekeeping gene.

4. Results

4.1. Laying performance and eggshell quality

The results of laying performance as well as eggshell quality and components are shown in Table 4-2. No significant differences were found in the laying performance (egg production rate, egg mass, average egg weight, average daily feed intake and feed conversion ratio) between HBS and LBS ($P > 0.05$). The eggshell breaking strength, thickness, weight and weight ratio were significantly lower in the LBS than those in the HBS ($P < 0.05$). HBS and LBS showed no significant difference in calcium and phosphorus contents of eggshell ($P > 0.05$). However, total calcium per eggshell and total phosphorus per eggshell were significantly reduced in the LBS ($P < 0.05$).

Scanning electron microscopy images in Figure 4-2A show the eggshell ultrastructure of laying hens in HBS and LBS. Compared with the HBS, the LBS had a thinner thickness of calcified layer and effective layer (Figure 4-2B, $P < 0.05$). The LBS significantly decreased the thickness ratio of effective layer while increasing that of mammillary layer (Figure 4-2B, $P < 0.05$).

4.2. Bone quality

The bone geometrical characteristics are presented in Table 4-3. The mean cortical index of both humeri and femurs were significantly higher in the LBS than in the HBS ($P < 0.05$). The mean relative wall thickness of femur in the LBS was thicker than that of the HBS ($P < 0.05$). No significant differences were observed between the two groups on the bone length, weight, volume, density, midpoint perimeter and cortical cross-sectional area ($P > 0.05$).

Table 4-2. Differences in laying performance and eggshell qualities of the hens laying eggs with different eggshell breaking strength[1]

Items	HBS	LBS	*P*-value
Laying performance			
Egg production rate, %	89.35±2.75	86.37±3.88	0.060
Egg mass, g/hen per day	56.36±2.54	55.36±3.76	0.456
Average egg weight, g	63.19±1.69	64.16±2.30	0.250
Average daily feed intake, g/hen per day	107.74±6.31	105.43±5.78	0.360
Feed conversion ratio, g/g	1.92±0.16	1.91±0.15	0.938
Eggshell quality			
Breaking strength, N	46.79±2.56	25.99±2.51	<0.001
Eggshell thickness, mm	0.35±0.02	0.30±0.03	<0.001
Eggshell weight, g	6.59±0.42	5.85±0.41	<0.001
Eggshell weight ratio, %	10.42±0.42	9.13±0.54	<0.001
Eggshell components			
Calcium content, mg/g	374.64±7.32	378.27±7.67	0.229
Total calcium per eggshell, g	2.47±0.10	2.19±0.08	<0.001
Phosphorus content, mg/g	0.96±0.09	0.94±0.11	0.619
Total phosphorus per eggshell, mg	6.29±0.57	5.49±0.73	0.007

[1] Data represent means with standard deviation based on 12 replicates with 25 eggs each. HBS, high eggshell breaking strength group; LBS, low eggshell breaking strength group.

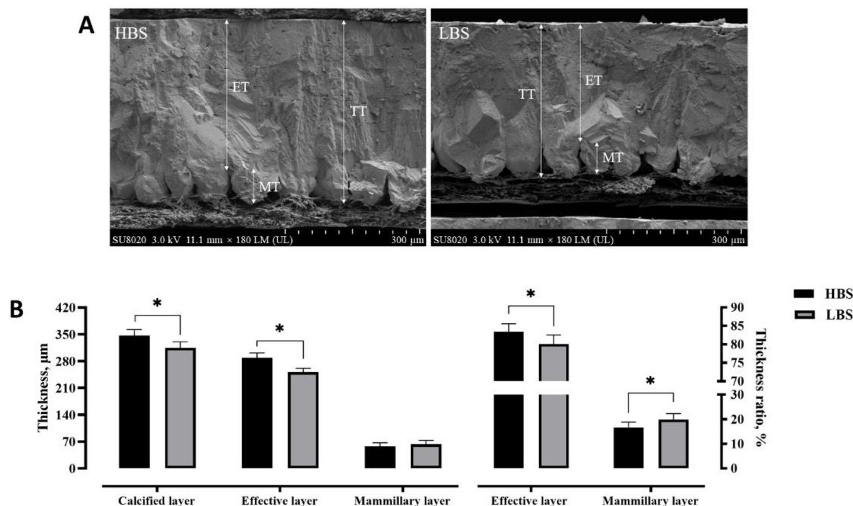

Figure 4-2. Differences in the eggshell ultrastructure of hens laying eggs with different eggshell breaking strength. Images of eggshell vertical profiles (A) in high (HBS) and low (LBS) eggshell breaking strength groups under a scanning electron microscope. Results of the eggshell ultrastructural characteristics (B). TT, total thickness; ET, effective layer thickness; MT, mammillary layer thickness. Data represent means with standard deviation based on 12 replicates with 5 eggshells each. An asterisk (*) indicates a significant difference ($P < 0.05$) between groups.

Table 4-3. Differences in bone geometrical characteristics of the hens laying eggs with different eggshell breaking strength[1]

Items	HBS	LBS	*P*-value
Humerus			
Length, cm	7.96±0.25	7.93±0.14	0.712
Weight, g	3.13±0.09	3.36±0.54	0.234
Volume, cm^3	5.14±0.71	5.21±0.31	0.802
Density, g/cm^3	0.62±0.09	0.64±0.09	0.554
Midpoint perimeter, cm	2.17±0.14	2.08±0.06	0.085
Cortical cross-sectional area, mm^2	9.08±0.80	9.91±1.20	0.079
Mean relative wall thickness	0.18±0.02	0.22±0.07	0.061
Mean cortical index	0.15±0.02	0.18±0.04	0.045
Femur			
Length, cm	8.65±0.09	8.78±0.06	0.204
Weight, g	9.05±0.27	9.11±0.14	0.829
Volume, cm^3	7.16±0.26	7.21±0.12	0.875
Density, g/cm^3	1.27±0.02	1.27±0.02	0.976
Midpoint perimeter, cm	2.61±0.06	2.68±0.03	0.313
Cortical cross-sectional area, mm^2	12.11±2.25	13.99±2.59	0.087
Mean relative wall thickness	0.15±0.03	0.18±0.04	0.042
Mean cortical index	0.13±0.02	0.15±0.02	0.047

[1] Data represent means with standard deviation based on 12 replicates with 1 bird each. HBS, high eggshell breaking strength group; LBS, low eggshell breaking strength group.

Table 4-4. Differences in bone mineral measurements of the hens laying eggs with different eggshell breaking strength[1]

Items	HBS	LBS	*P*-value
Humerus			
Distal BMD, g/cm^2	2.83±0.18	2.96±0.20	0.096
Midshaft BMD, g/cm^2	2.89±0.22	3.14±0.30	0.028
Proximal BMD, g/cm^2	2.68±0.22	2.99±0.22	0.002
Distal BMC, g	2.57±0.14	2.62±0.17	0.436
Midshaft BMC, g	1.66±0.19	1.73±0.19	0.366
Proximal BMC, g	2.35±0.20	2.59±0.18	0.005
Femur			
Distal BMD, g/cm^2	2.75±0.21	2.86±0.19	0.218
Midshaft BMD, g/cm^2	3.04±0.26	3.04±0.35	0.959
Proximal BMD, g/cm^2	2.78±0.26	2.80±0.19	0.876
Distal BMC, g	2.50±0.18	2.55±0.17	0.551
Midshaft BMC, g	1.74±0.14	1.68±0.20	0.354
Proximal BMC, g	2.44±0.22	2.41±0.18	0.732

[1] Data represent means with standard deviation based on 12 replicates with 1 bird each. HBS, high eggshell breaking strength group; LBS, low eggshell breaking strength group; BMD, bone mineral density; BMC, bone mineral content.

Table 4-4 compares bone mineral measurements between HBS and LBS. The humeral midshaft and proximal BMD of the LBS were significantly greater than those of the HBS ($P < 0.05$). The humerus in the LBS had significantly higher proximal BMC compared with that in the HBS ($P < 0.05$). However, there were no significant difference on mineral measurements of the femur between these two groups ($P > 0.05$).

Differences in the bone components between the HBS and LBS are presented in Table 4-5. The LBS had the humeri with higher fat-free dry weight, ash, organic matter, total calcium and phosphorus per bone in comparison with the HBS ($P < 0.05$). The ash and ash content of the femur were significantly higher in the LBS compared with those in the HBS ($P < 0.05$), while calcium and phosphorus contents in ash were lower ($P < 0.05$).

Table 4-5. Differences in bone components of the hens laying eggs with different eggshell breaking strength[1]

Items	HBS	LBS	*P*-value
Humerus			
Fat-free dry weight, g	2.24±0.12	2.53±0.30	0.021
Ash, g	1.39±0.08	1.55±0.17	0.022
Ash content, %	62.07±0.99	61.22±1.66	0.203
Organic matter, g	0.85±0.05	0.98±0.14	0.016
Calcium content in ash, %	36.75±0.35	37.19±0.57	0.137
Total calcium per bone, mg	513.59±17.49	586.49±62.02	0.020
Phosphorus content in ash, %	16.28±0.22	16.47±0.31	0.249
Total phosphorus per bone, mg	227.49±7.77	259.75±28.35	0.023
Ratio of calcium and P	2.25±0.01	2.26±0.03	0.916
Femur			
Fat-free dry weight, g	5.32±0.53	5.70±0.52	0.099
Ash, g	3.00±0.35	3.40±0.43	0.025
Ash content, %	56.33±3.17	59.45±2.64	0.021
Organic matter, g	2.32±0.29	2.30±0.14	0.848
Calcium content in ash, %	37.41±0.37	36.63±0.60	0.022
Total calcium per bone, mg	1113.62±94.55	1235.27±137.7	0.105
Phosphorus content in ash, %	16.48±0.17	16.07±0.29	0.012
Total phosphorus per bone, mg	490.46±40.33	541.34±54.71	0.097
Ratio of calcium and phosphorus	2.27±0.01	2.28±0.03	0.509

[1] Data represent means with standard deviation based on 12 replicates with 1 bird each. HBS, high eggshell breaking strength group; LBS, low eggshell breaking strength group.

Representative images of the humerus and femur stained with Goldner-Trichrome are illustrated in Figure 4-3. The lateral edge of the cortical bone was clear and flat in

both groups, whereas its medial margin in the femur had indistinct borders with adjacent trabecular bone. The cortical thickness of both femur and humerus was thicker in the LBS than in the HBS. The medial edge of the humerus in the LBS was attached by a thick layer of spongy bone, while no similar structure was observed in the HBS. More irregular erosions and demineralized regions were seen in the femoral intracortical region in the HBS. In contrast, the endocortical surface was flatter in the femur of the LBS.

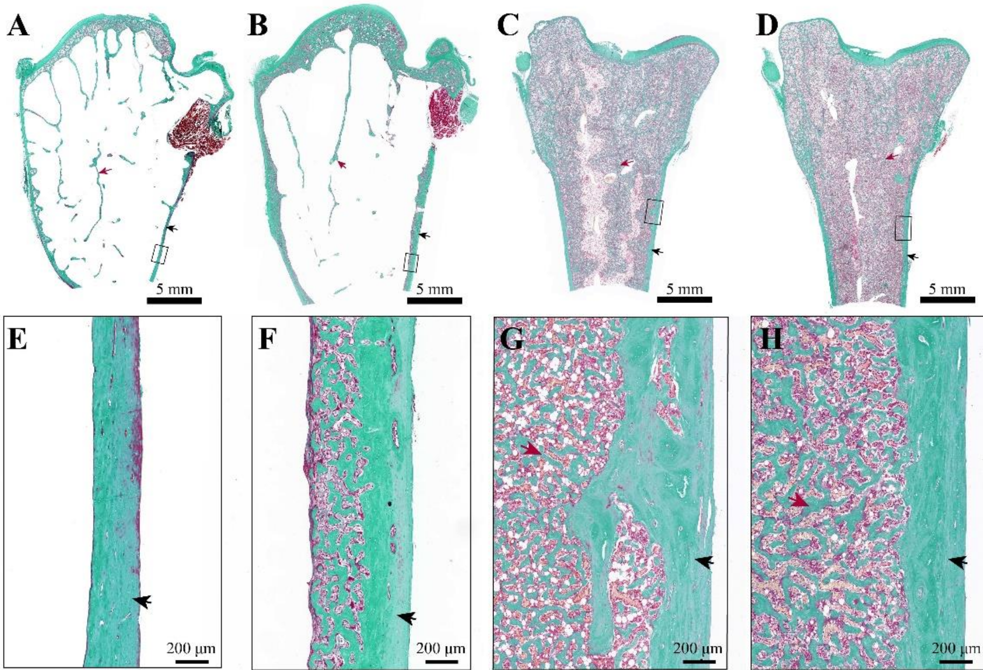

Figure 4-3. Differences in bone histomorphometry of the hens laying eggs with different eggshell breaking strength. The insets (E-H) are the zoomed-in images of the black boxes in (A-D). (A, B, E, F) in the humerus; (C, D, G, H) in the femur; (A, E, C, G) in the HBS; (B, F, D, H) in the LBS. Black arrows, cortical bones; red arrows, trabecular bones. HBS, high eggshell breaking strength group; LBS, low eggshell breaking strength group.

4.3. Correlations between eggshell and bone qualities

Figure 4-4 demonstrates the correlations between eggshell and bone quality. There were negative correlations between eggshell breaking strength and mean cortical index (r = -0.434), midshaft BMD (r = -0.556), proximal BMD (r = -509), proximal BMC (r = -0.465), fat-free dry weight (r = -0.501), ash (r = -0.507), organic matter (r = -0.483), calcium content in ash (r = -0.570), phosphorus content in ash (r = -0.453), total calcium per bone (r = -0.682), total phosphorus per bone (r = -0.670) of the humerus as well as ash content (r = -0.436), total calcium per bone (r = -0.479) and total phosphorus per bone (r = -0.481) of the femur ($P < 0.05$). Eggshell thickness, weight ratio, total calcium per eggshell and effective layer thickness were negatively

correlated with midshaft BMD (r = -0.637, 0.665, -0.555, -0.530), total calcium per bone (r = -0.830, -0.717, -0.623, -0.508), total phosphorus per bone (r = -0.837, -0.710, -0.595, -0.489) of the humerus (P < 0.05). Eggshell weight ratio, total calcium per eggshell, calcified layer thickness and effective layer thickness were negatively correlated with femoral ash content (r = -0.590, -0.508, -0.433, -0.493, P < 0.05). Negative correlations were also observed between: total calcium per eggshell, total phosphorus per eggshell and total calcium per bone (r = -0.518, -0.458), total phosphorus per bone (r = -0.511, -0.467) of the femurs (P < 0.05).

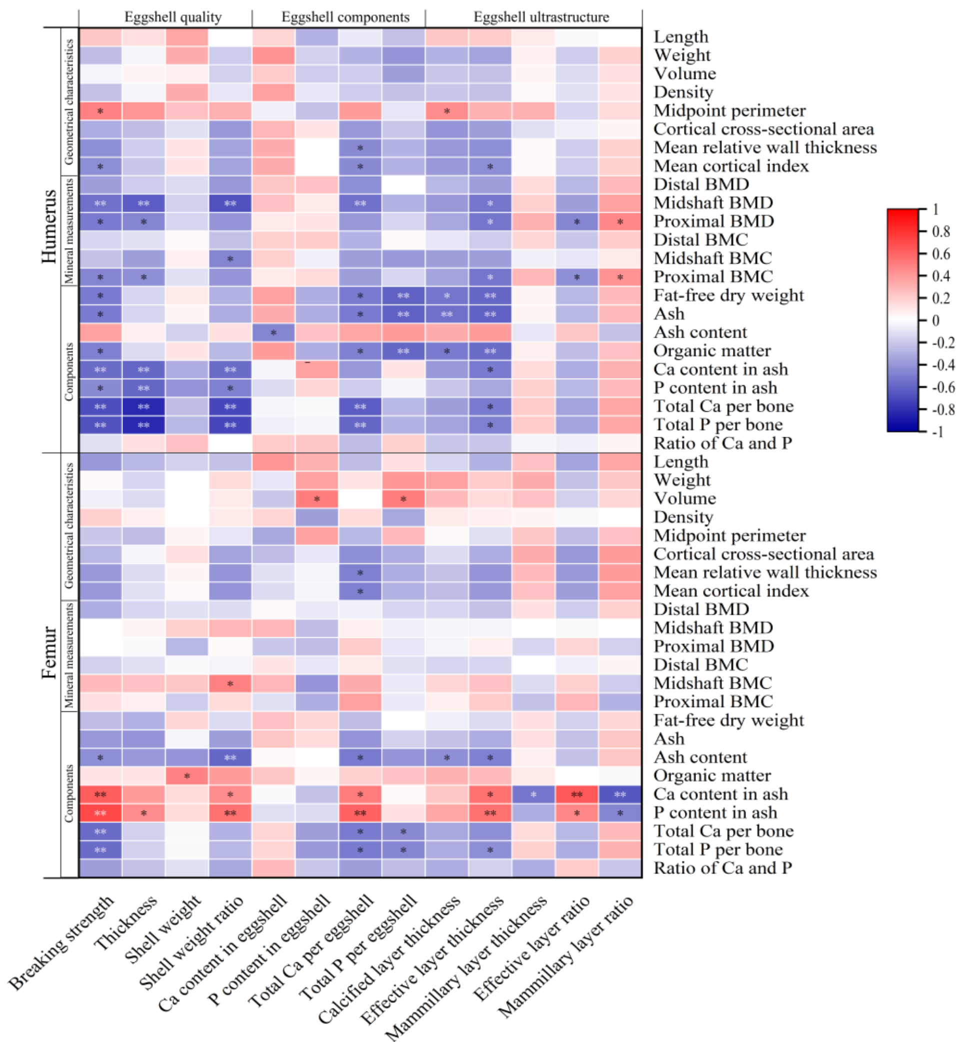

Figure 4-4. Correlation matrix for the eggshell and bone qualities. Item correlations are color graded. Red indicates positive correlations, and blue indicates negative correlations. *P < 0.05, **P < 0.01. Ca, calcium; P, phosphorus; BMD, bone mineral density; BMC, bone mineral content.

4.4. Eggshell ultrastructure at the initiation (8.5 h PO) and growth (18.5 h PO) stages of eggshell calcification

As shown in Figure 4-5D, at 8.5 h PO, images of vertical profiles identified the mammillary knobs were shorter in the LBS than in the HBS, indicating they grew more slowly in the LBS. Meanwhile, external ultrastructural analyses displayed the mammillary knobs were smaller and had less fusion in the LBS compared with the HBS. Additionally, at 18.5 h PO, the thickness of calcified layer and effective layer was thinner in the LBS compared to the HBS ($P < 0.05$). However, no significant difference was observed in the thickness of mammillary layer ($P > 0.05$).

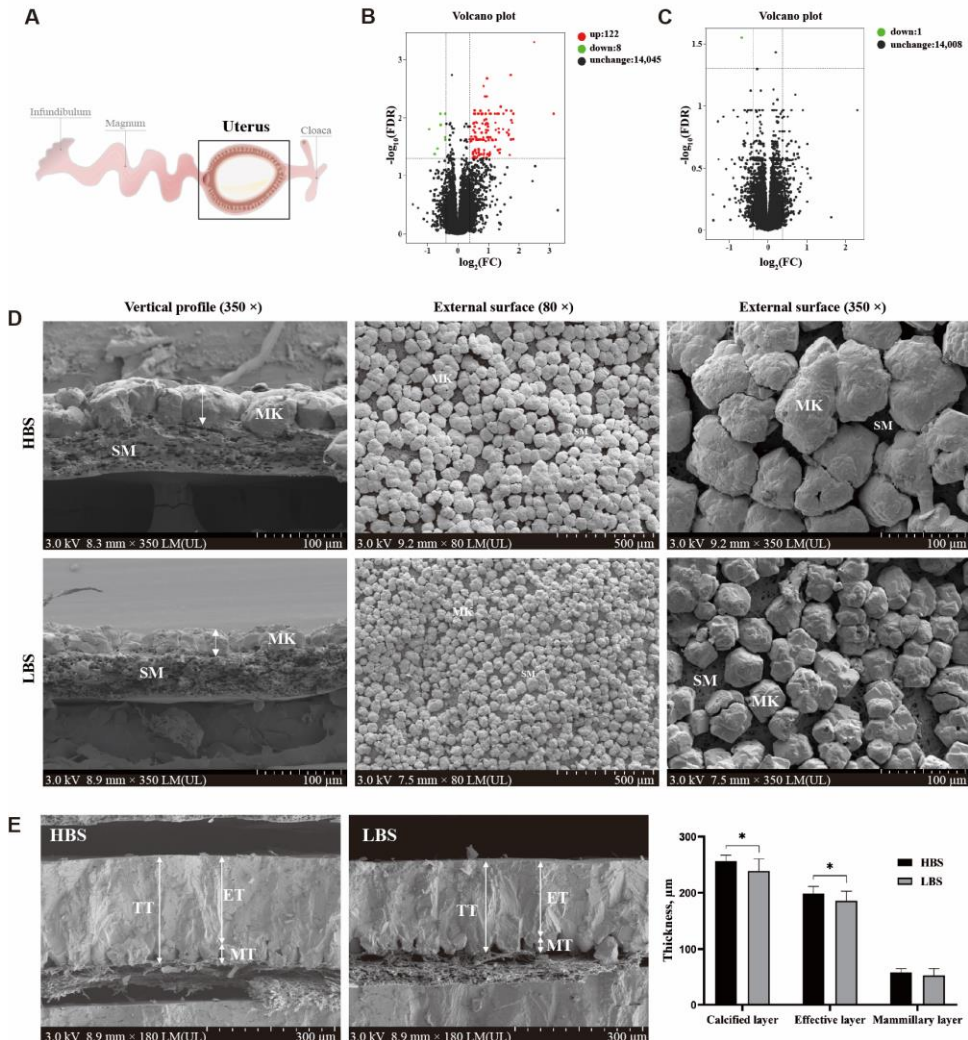

Figure 4-5. Volcano plot of DEGs and eggshell ultrastructure at the initiation (8.5 h PO) and growth (18.5 h PO) stages of eggshell calcification. A shows a mimetic diagram of

sampling, the uterus and eggshell were removed to determine the transcriptome and ultrastructure, respectively. (B, D) at the initiation stage of eggshell calcification; (C, E) at the growth stage of eggshell calcification. SM, shell membrane; MK, mammillary knob; TT, total thickness; ET, effective layer thickness; MT, mammillary layer thickness; PO, post-oviposition; HBS, high eggshell breaking strength group; LBS, low eggshell breaking strength group. An asterisk (*) indicates a significant difference ($P < 0.05$) between groups.

4.5. Differentially expressed genes (DEGs) in uterus at initiation (8.5 h PO) and growth (18.5 h PO) stages of eggshell calcification

A total of 48 uterine samples were obtained in the current study, of which two samples from the LBS at 8.5 h PO were rejected for further analysis, since they did not pass quality inspection. A total of 2,309,035,290 clean reads were sequenced from 46 RNA libraries, over 38.6 million reads for each sample (Additional file 3). Of these reads, 86.77%-89.66% were mapped to a unique position on the reference genome (Additional file 3). The Q30 (%) of all test samples were above 93.66%, and GC contents were stable at 48.43% - 50.27%, indicating the sequence results were accurate and reliable. A total of 130 DEGs were identified at 8.5 h PO between the HBS and LBS (Figure 4-5B & Additional file 4), in which 122 genes were upregulated and 8 genes were downregulated in the LBS relative to the HBS. However, volcano plot showed only 1 DEGs at 18.5 h PO between the two groups (Figure 4-5C). Twelve genes of each calcification stage were selected for qPCR validation, and the gene expression patterns were in accordance with the transcriptome results (Additional file 5).

4.6. Functional enrichment analysis and gene network construction of DEGs at the initiation stage of eggshell calcification (8.5 h PO)

Functional enrichment analysis and gene network construction of DEGs at 8.5 h PO (Figure 4-6) revealed the strong representation of regulation of cell killing, regulation of hemopoiesis, negative regulation of hemopoiesis, regulation of blood coagulation, as well as negative regulation of cell activation. Enrichment of DEGs that are active in positive regulation of immune effector process was also obvious. Twelve upregulated DEGs (BCL2L14, C1QA, C1QB, CARD11, CD3E, HCLS1, IL2RB, NCKAP1L, NFKBIA, PRKCB, SYK, TNFAIP8L1) were related to apoptosis and further annotated in the Additional file 6, which may suggest an active apoptosis in the LBS. Seven upregulated DEGs (EDN2, IKZF1, PRKCB, UBASH3B, LCP1, MGP, SLC24A4, annotated in the Additional file 6) were associated with calcium transport. Of these, EDN2, IKZF1, PRKCB and UBASH3B involved the regulation of cellular calcium ion homeostasis.

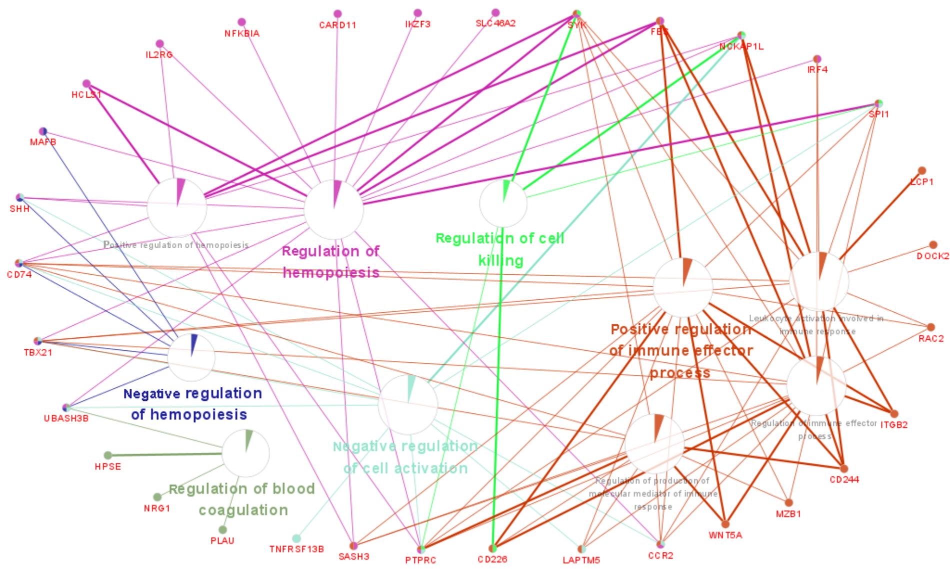

Figure 4-6. Functional enrichment analysis and gene network construction of DEGs at the initiation stage of eggshell calcification (8.5 h post-oviposition). Enrichment of DEGs that are active in regulation of cell killing, regulation of hemopoiesis, negative regulation of hemopoiesis, regulation of blood coagulation, negative regulation of cell activation, as well as positive regulation of immune effector process was noted. GO terms are presented as nodes in functionally grouped networks based on the GO cluster algorithm, where only the most significant term per group is labeled. The pie chart illustrates the percentage distribution of genes shown in the related functional groups**.**

4.7. Identification of apoptosis-related indicators in the uterus

As shown in Figure 4-7, the level of p62 protein was significantly higher in the LBS ($P < 0.05$). The activity of caspase-8 was increased in the LBS ($P < 0.05$). The LBS significantly increased the relative protein expression of Bax, while reducing Bcl-2 expression and the ratio of Bcl-2/Bax ($P < 0.05$). Representative images of TUNEL assay are illustrated in Figure 4-7H. The uterus of the LBS showed a significant increase in TUNEL-positive cells ($P < 0.05$, Figure 4-7G). The HE staining (Figure 4-7H) showed that the uterine tissue from both groups had an intact epithelial structure, with the epithelial cells arranging tightly. However, the uterine tissue in the LBS exhibited multiple interstitial edemas and loose connective tissue with a few inflammatory cell infiltrations.

4.8. Quantification of bone remodeling-related mRNA in bones at initiation (8.5 h PO) and growth (18.5 h PO) stages of eggshell calcification

Figure 4-8A demonstrates the relative expression levels of bone remodeling genes. At 8.5 h PO, a significant increase of runt-related transcription factor 2 (RUNX 2)

expression was shown in the humerus of the LBS compared to that of the HBS (P < 0.05) while no significant differences were observed in the expression levels of other genes ($P > 0.05$). The expressions of humeral RUNX 2 and OCN genes were significantly upregulated in the LBS compared with those in the HBS ($P < 0.05$). No significant differences were observed in the expression of bone remodeling-related genes in femurs (RUNX 2, OCN, OPN, COL I, ALP, TRAP, Cts K, $P > 0.05$).

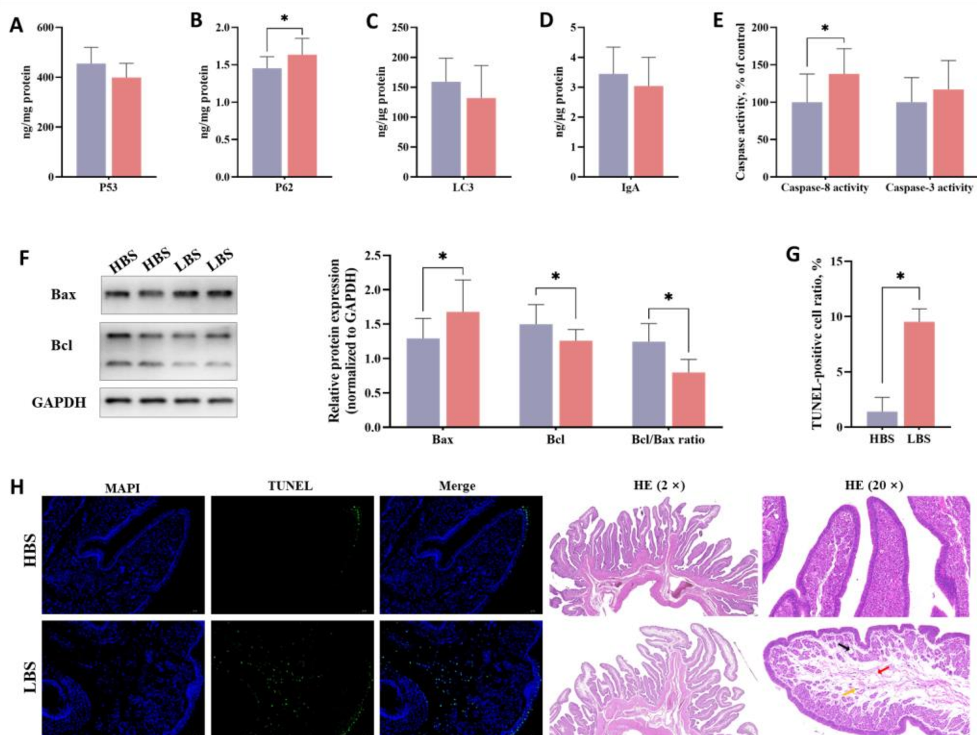

Figure 4-7. Identification of apoptosis-related indicators at the initiation stage of eggshell calcification (8.5 h post-oviposition). The protein contents of p53 (A), p62 (B), LC3 (C), Immunoglobulin A (IgA, D); the activity of caspase-8 and caspase-3 (E); western blot results and quantification of Bax and Bcl-2 (F); TUNEL-positive cells ratio (G); TUNEL staining (20 ×) and HE staining (H), interstitial edemas (black arrow), loose connective tissue (red arrow) and inflammatory cell infiltration (yellow arrow). HBS, high eggshell breaking strength group; LBS, low eggshell breaking strength group. An asterisk (*) indicates a significant difference ($P < 0.05$) between the HBS and LBS.

4.9. Changes of calcium, phosphorus, bone remodeling-related enzymes and hormones concentrations in serum during eggshell calcification

The activity of TRAP and the levels of BALP, Ca, P, PTH, E_2, 1,25-$(OH)_2$-D_3 and 25-OH-D_3 are presented in Figure 4-8B-D. Compared with the HBS, the LBS had a higher serum BALP level at 18.5 h PO ($P < 0.05$) while no difference was observed

at 8.5 h PO ($P > 0.05$). No significant differences appeared in the activity of TRAP, nor in the levels of calcium and P between the HBS and LBS at both calcification periods ($P > 0.05$). The LBS significantly increased the level of serum PTH, but decreased that of 1,25-$(OH)_2$-D_3 and 25-OH-D_3 at 18.5 h PO ($P < 0.05$). At 8.5 h PO, the serum E_2 level was significantly higher in the LBS than in the HBS ($P < 0.05$), while there were no differences in the levels of PTH, 1,25-$(OH)_2$-D_3 and 25-OH-D_3 ($P > 0.05$).

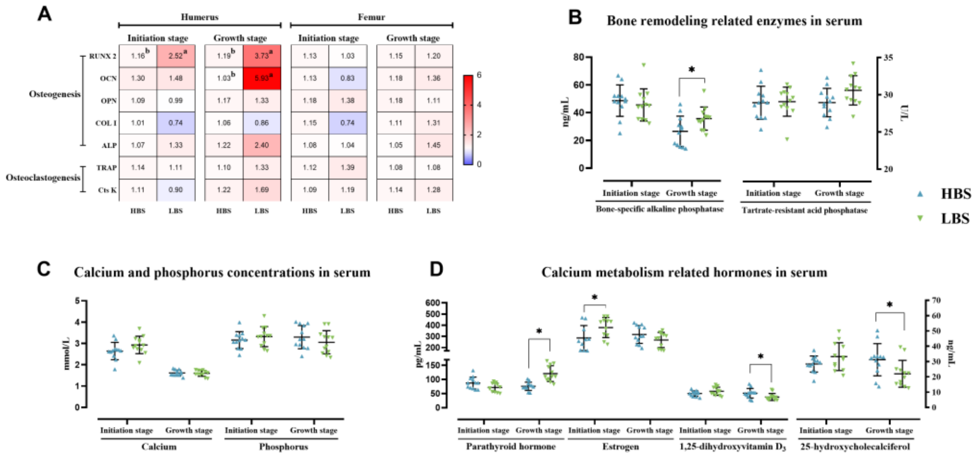

Figure 4-8. Differences in the bone remodeling markers and hormones of the hens laying eggs with different eggshell breaking strength. The quantification of bone remodeling related mRNA in bone (A), bone remodeling related enzymes (B) as well as calcium and phosphorus concentrations (C) and calcium metabolism related hormones (D) in serum. RUNX 2, runt-related transcription factor 2; OCN, osteocalcin; OPN, osteopontin; COL I, collagen 1; ALP, alkaline phosphatase; TRAP, tartrate-resistant acid phosphatase; Cts K, cathepsin K. HBS, high eggshell breaking strength group; LBS, low eggshell breaking strength group. Different superscripts in the adjacent cells indicate significant differences ($P < 0.05$). Both different superscripts ($^{a-b}$) and asterisk (*) denote significant difference ($P < 0.05$) between the HBS and LBS.

5. Discussion

Extended laying period (from first 72-80 weeks of age to 100 weeks of age) is a commonly mentioned target in the laying hen industry (Pottgüter, 2016). A deterioration in eggshell quality is one of the main reasons for currently replacing flocks at or around 72 weeks of age (Bain et al., 2016). The hens used in the current study was selected from a flock at 72 weeks of age. The average eggshell breaking strength of this flock was 34.55 N, and 20% of the hens in this flock laying eggs with eggshell breaking strength lower than 29 N. The eggs laid by these hens may be more prone to break and crack during the collection and transportation (Alfonso-Carrillo et al., 2021). Consistent with a previous study (Zhang et al., 2019), the eggs with low eggshell breaking strength were accompanied by a thinner thickness, which was also reflected by an ultrastructural observation that LBS group showed a thinner effective

layer and subsequently total calcified layer. The eggshell effective layer comprises about two thirds of the total calcified layer and plays crucial roles in resisting the inception and propagation of cracks (Radwan, 2016). The formation of eggshell ultrastructure is largely affected by the deposition of calcium carbonate during its calcification process (Hincke et al., 2012). As predicted, the eggshell of the LBS showed a lower weight, weight ratio and total calcium content. Taken together, eggshell breaking strength reduction in late laying period may be related to the deterioration of ultrastructure (decreased effective layer) caused by the decrease of calcium carbonate deposition.

The correlation between eggshell and bone quality may be different in various conditions since eggshell and bone quality were also influenced by diets and environmental factors (Chen et al., 2020b; Eltahan et al., 2023; Fu et al., 2022; Saleh et al., 2021b; Saleh et al., 2021a; Zaki et al., 2023). Our previous study suggested that eggshell quality exhibited simultaneous improvements with bone quality by changing a conventional caging system to an aviary system (Fu et al., 2022). However, the present study exhibited negative correlations between bone and eggshell quality under the same feed and environmental factors, indicating that the HBS had higher risks for osteoporosis. This is in accordance with previous studies that reported hens with hard-shelled eggs or high production had poor-quality skeletons (Alfonso-Carrillo et al., 2021; Dacke et al., 2015). The negative correlations between eggshell and bone quality were mainly reflected in the components. It suggested that the bone remodeling tended to preserve bone mass in the hens with weak-shelled eggs, while it was prone to lose bone mass to address the calcium needs of eggshell calcification in the hens with hard-shelled eggs. Thus, under the same diet and environment, the negative correlations between eggshell and bone quality may be associated with the competition and delivery of calcium between the uterus and bones.

The uterus transports calcium to meet the calcium demands of eggshell calcium carbonate deposition at different stages of eggshell calcification. During the initiation stage of eggshell calcification, calcite crystals radially grow around the mammillary cones, then the adjacent mammillary knobs gradually come together and fuse at the spatial competition, forming the bases of palisade layer (Nys et al., 2004). During this period, eggshell mammillary knobs grew slowly with few fusions in the LBS, indicating a decreased deposition of calcium carbonate. However, such difference did not affect the frequency of abnormal mammillary knobs (Additional file 7) in the ultrastructural characterization of intact eggshells. During the growth stage of eggshell calcification, the eggshell effective layer thickness was thinner in the LBS, which was attributed to less early fusion of mammillary knobs at the initiation stage of eggshell calcification as well as reduced calcium carbonate deposition at the growth stage of eggshell calcification. Thus, the eggshell ultrastructural deterioration (decreased effective layer thickness) was related to the decreasing of calcium carbonate deposition at both initiation and growth stages of eggshell calcification.

Uterus transports calciumand secretes matrix proteins to regulate the deposition of calcium carbonate during eggshell calcification. The uterine transcriptome analysis showed that the differences of calcium carbonate deposition were mainly caused by

the transcriptional variations at the initiation stage of eggshell calcification, since only a few DEGs were observed at the growth stage, while 130 genes were differentially expressed at the initiation stage. The mass transport of calciumin the uterus is required for the maintenance of eggshell calcification. In this study, 7 DEGs (EDN2, IKZF1, PRKCB, UBASH3B, LCP1, MGP, SLC24A4) were associated with calcium transport, of which EDN2, IKZF1, PRKCB and UBASH3B involved cellular calcium ion homeostasis and positive regulation of cytosolic calcium ion concentration. Their upregulations in the LBS may induce a rise in cytosolic calcium ion concentration, which could lead to apoptosis due to the cellular calcium overload (Boeckel et al., 2018; Murphy, 1999). In agreement with this, the upregulation of a GO cluster relevant for cell killing along with the genes related to apoptosis (BCL2L14, C1QA, C1QB, CARD11, CD3E, CSF1R, E2F8, HCLS1, IL2RB, NCKAP1L, NFKBIA, PRKCB, SPI1, SYK, TNFAIP8L1) suggested an excessive apoptosis, which may aggravate tissue damages in the LBS. MGP is a negative regulator for vascular calcification (Zebboudj et al., 2003), and its upregulation of gene expression may lead to a disorder in chickens via inhibiting Ca-dependent function (Zhang et al., 2008). LCP1 is of great significance in the adipogenesis and lipid metabolism, and the overexpression of LCP1 could suppress lipid catabolism and increase adipogenesis and lipogenesis (Subramani et al., 2021). The lipid accumulation in the aged laying hens would hinder uterine function (Gongruttananun, 2018) and interfere eggshell calcification (Feng et al., 2020). The SLC24A4 family exports calcium out of the cell with the potassium via entry of sodium (Cervetto et al., 1989; Schnetkamp et al., 1989). However, its localization in the uterus and biological function in eggshell calcification were not clear and require further inquiry. Additionally, the enrichment of a GO cluster on hemopoiesis linked with tissue repair may contribute to the repair of tissue damage that result from apoptosis-mediated cell death (Gundry et al., 2017). The upregulation of *NRG1* was concordant with this, which is involved in the development and regeneration of the chicken reproductive tract through mediating E_2 (Jeong et al., 2017). The differences in the immune repones between the hens with different eggshell breaking strength, such as the positive regulation of immune effector process and regulation of blood coagulation, could be due to the phagocytic clearance of apoptotic cells. Overall, the hens with weak-shelled eggs may induce intracellular calcium overload to trigger excessive apoptosis and aggravate uterine tissue damages, and hematopoietic repair may be involved in the subsequent repair of injury. The transcriptional differences may be linked with certain genotypes and environmental factors. Although efforts have been made in this study to control environmental and genotypic variables to the greatest extent possible, some potential differences such as individual behavioral differences are unlikely to be entirely avoided. In the further study, more relevant information is needed to comprehensively decipher the possible mechanisms underlying the reduction in eggshell strength.

Maintenance of tissue integrity is fundamental to transport calcium in the uterus. Although, as a compensatory mechanism, the hematopoiesis system was activated and exerted its repair property, the extents of uterine injury and repair were not understood. Thus, the apoptosis and tissue homeostasis indexes were further analyzed in the

current study to assess the mechanism underlying the decrease in the eggshell calcium deposition. Autophagy, an evolutionarily conserved process among eukaryotes, plays a critical role in homeostasis in cells and tissues by the clearance of detrimental and damaged proteins and dysfunctional organelles (Maiuri et al., 2007). The levels of p62 and LC3 identify the progression of autophagy induction, of which the former is an autophagy substrate, and the latter increases synchronously with increased autophagic flux (Klionsky et al., 2016). An increased level of p62 with a constant LC3 in the LBS hinted a disruption in the downstream steps of autophagy, that was unable to clear autophagosomes and degrade p62 (Wang et al., 2016). On the one hand, this could lead to the cellular dysfunction and aggravate apoptosis (Cao et al., 2021). On the other hand, autophagy defect may facilitate excessive inflammatory responses and create tissue damages (Wang et al., 2021c). Apoptosis acts as an important defense mechanism of host against infection, in which caspase 8, caspase 3, Bcl-2 and Bax play key roles. The increased caspase 8 activity in the LBS indicates the initiation of extrinsic apoptotic pathway, which could enhance the clearance of virus-infected cells (Ravindra et al., 2009). Bcl-2 (anti-apoptotic) and Bax (pro-apoptotic) belong to Bcl-2 family that is a central regulator of apoptosis, the reduction in Bcl-2/Bax ratio of the LBS suggested cell death in the apoptosis response (Luo et al., 2010). Activation of the pro-apoptotic effect in the LBS was further confirmed by an increase of TUNEL-positive cells in its uterus. Additionally, most apoptotic cells were localized at the inner of the uterine fold, which was consistent with the HE staining result that revealed the edema or dissolution in tubular glands. In general, the uterus of the LBS was more sensitive to apoptosis and tissue damages at the initiation stage of eggshell calcification. Similarly, HE staining showed the LBS also had obvious tissue damage at the growth stage of eggshell calcification. Uterine tissue injury is bound to hamper calcium transport, reducing eggshell quality even inducing weak-shelled and soft-shelled eggs (Nii et al., 2014; Park et al., 2018). Thus, the breaking strength reduction and ultrastructural deterioration of eggshells may be attributed to a declined calcium transport due to uterine tissue damages.

The blockage of calcium transport resulting from tissue damage may influence the deposition and release of skeletal calcium. Although the expression levels of genes related to bone remodeling did not differ significantly in the femur, the geometrical and compositional changes suggested increased bone resorption in the femur of HBS. In the current study, femoral geometric metrics showed no difference in the midpoint perimeter, while significant decreases in the mean relative wall thickness and mean cortical index were observed in the HBS, leading to a larger endocortical bone diameter. Such enlarged diameter and more structural damages of endocortical bones, such as irregular erosions in the endocortical surface and more demineralized regions in the intracortical region, are usually representative of endocortical resorption in human beings (Shen et al., 2016; Sheu et al., 2015). Therefore, more resorption could occur in the femur of hens laid hard-shelled eggs to meet the calcium requirement during eggshell calcification, in return decreased bone quality.

Humerus exhibited similar variations as the femur manifested by enlarged endocortical diameter and the lower skeletal minerals and components in the HBS.

However, unlike the femur, such difference in the humerus may be related to more endocortical formation in the LBS rather than more endocortical resorption in the HBS, since the LBS had a thicker layer of woven bone that lined the intracortical portion of the bone. The woven bone, an intermediate form of bone development, represented the initiation of intramembranous ossification in the LBS (Rath et al., 2022). RUNX 2 is involved in the regulation of genes responsible for the biosynthesis of bone-specific protein (Merriman et al., 1995; Rath et al., 2022). At the growth stage of eggshell calcification, increased RUNX 2 in the LBS appeared to upregulate *OCN* expression that would facilitate the biosynthesis of skeletal organic matrix (Xu et al., 2022) and the anchoring of calcium and phosphate (Mishra et al., 2015), which is a prerequisite for the increment of the cortical bone formation. Additionally, BALP is a typical serum marker that reflects the rate of bone formation in bone tissue (Liu et al., 2021), and increased serum BALP level in the LBS indicated a more intense osteogenesis in bones. Thus, the humerus of hens with weak shells had more bone formation at the growth stage of eggshell calcification. The coupling of bone formation and bone resorption maintain bone homeostasis. During eggshell formation, following the bone resorption, bone displays an intense osteoblastic activity that remodels new bone for the next cycle of eggshell calcification (van de Velde et al., 1984). The uterus of the LBS did not require extensive calcium consumption due to its blockage of calcium transport, thus calcium redundancy obtained by bone resorption may be recovered to perform new bone formation. In contrast, in the HBS, bone formation may be inhibited in the humerus due to an increased acquisition of calcium in the uterus.

Blood calcium concentration and its regulatory factors may be the key signaling for the crosstalk between uterus and skeletons due to their highly dependence on the ca. PTH and 1,25-OH)$_2$-D$_3$ are two major calcium-regulating hormones that involved in calcium homeostasis during eggshell calcification by directly and indirectly mediating bone remodeling and intestinal calcium absorption (Bar, 2008). The effect of PTH on bones is contingent on the periodicity of the PTH signal (Silva et al., 2015), and the intermittent increase of the serum PTH level would result in an anabolic effect on rat metatarsal (Dobnig et al., 1997). Thus, increased humerus formation in the LBS may be related to the temporary increase of serum PTH at the growth stage of eggshell calcification. Increased serum PTH may account for the enhancement of RUNX 2-dependent transcription via mediating RUNX 2 protein expression in the LBS (Krishnan et al., 2003), thereby stimulating the synthesis of bone biomarkers associated with formation. The secretion of PTH is subject to a direct regulation of calcium-sensing receptors and a negative feedback regulation by vitamin D receptors (Brown et al., 1995; Ritter et al., 2011). In the LBS, the diminished serum active vitamin D$_3$ may maintain the PTH at a high level by attenuating negative regulation. The changes in vitamin D$_3$ metabolites may be involved in the process of uterine damage on bone remodeling, since inflammation could affect vitamin D$_3$ metabolism by mediating the downregulation of 1α-hydroxylase and the upregulation 24-hydroxylase (Shanmugasundaram et al., 2012). Additionally, the effect of inflammatory cytokines on bone turnover is well accepted (Klein, 2018; Souza et al.,

2013). Thus, the uterine damages may regulate the bone remodeling through other pathways such as inflammatory cytokines, which are warranted for further investigation.

6. Conclusions

In conclusion, in aged laying hens, the lower eggshell breaking strength may be attributed to a declined calcium transport due to uterine tissue damages, which could affect eggshell calcification and lead to a weak ultrastructure (Figure 4-9). Impaired calcium transport in the uterus may result in reduced femoral bone resorption and increased humeral bone formation to maintain a higher minerals and bone quality in the LBS (Figure 4-9). Blood hormones such as PTH, 1,25-$(OH)_2$-D_3 and 25-OH-D_3 may be acting as mediators involved in signaling between bone and uterus.

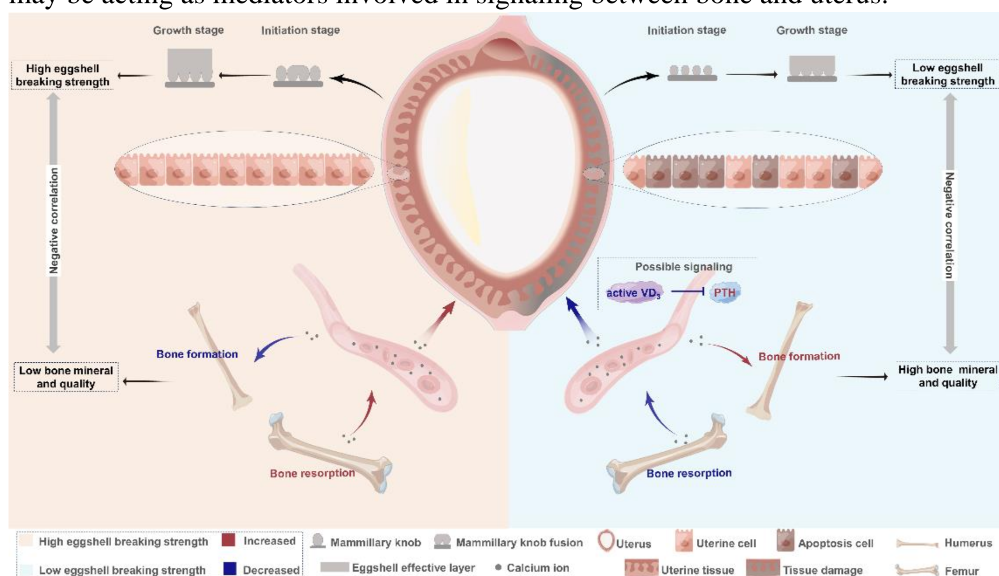

Figure 4-9. A schematic model displaying the differences of calcium transport and bone remodeling between hens laid eggs with low and high eggshell breaking strength.

Chapter 5

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

Chapter 5. Dietary supplementation with calcitriol or quercetin improved eggshell and bone quality by modulating calcium metabolism

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1. Abstract

Two trials were conducted in this study to investigate the effects of dietary calcitriol or quercetin supplementation on eggshell and bone quality of laying hens and sought to explore the regulatory mechanism underlying the effects. 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: a basal diet (4% calcium level) or basal diets supplemented with 0.5% calcium, 5 µg/kg calcitriol or 500 mg/kg quercetin. In trial 2, a total of 360 60-week-old Hy-Line Brown layers were divided into 3 groups: the control, calcitriol (5 µg/kg) supplementation, and quercetin (500 mg/kg) supplementation. Results showed that dietary calcitriol or quercetin supplementation improved eggshell quality in both trial 1 and 2 ($P < 0.05$). In trial 2, both calcitriol and quercetin supplementations improved femoral bone quality, calcium retention and calcium content in uterine fluid at 18.5 h post-oviposition (PO), along with enhancing uterine morphology ($P < 0.05$). Supplemental calcitriol or quercetin up-regulated the gene expression of uterine transient receptor potential cation channel, subfamily V, member 6 (TRPV6) at 8.5 h PO and plasma membrane calcium-ATPase, vitamin D receptor (VDR), estrogen receptor (ER) alpha at 18.5 h PO ($P < 0.05$), but down-regulated the uterine caspase 3 gene expression at 8.5 h PO ($P < 0.05$). Meanwhile, the femoral gene expression of tartrate-resistant acid phosphatase (up-regulated at both 8.5 h 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 treatment, hens received quercetin showed higher hen-day egg production and femoral medullary bone but lower femoral stiffness ($P < 0.05$), which were accompanied by increased expression of uterine TRPV6, ERβ at 18.5 h PO and humeral ALP, cathepsin K at 8.5 h PO ($P < 0.05$). To sum up, both dietary calcitriol and quercetin can improve eggshell and bone quality by modulating calcium metabolism of aged layers. Compared to the calcitriol, dietary quercetin up-regulated the uterine calcium transporters' expression in a VDR-independent manner, but without affecting eggshell quality.

Keywords: eggshell quality; bone quality; calcium transport; bone remodeling; laying hen.

2. 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 a lower eggshell weight and a thinner eggshell thickness (Fu et al., 2022; Gautron et al., 2021; Rodriguez-Navarro et al., 2002) as well as a lower bone mass (Bain et al., 2016; Fleming et al., 2006). This suggested that the calcium 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 an 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 declines in eggshell and bone quality.

Dietary addition of calcitriol could increase the blood concentration of calcitriol (Tsang et al., 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., 2023a). A study found evidence of an improved effect of supplementing 400 to 600 mg/kg of quercetin to the diets on eggshell quality (Liu et al., 2013) while another study showed no effect of 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 (ERs), respectively (Amevor et al., 2021; Cheng et al., 2023; Irani et al., 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, 2009b; 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 et al., 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 a likely cooperation 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 ERs (Santos-Martínez et al., 2021). Therefore, conducting a comparative analysis of the effects of calcitriol and quercetin on calcium metabolism contributes 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 supplementations on eggshell quality of aged laying hens with a low eggshell breaking strength to determine the validity of these additives on 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.

3. Materials and methods

3.1. Birds and experimental design

Animal management and experimental procedures were approved by the Animal Care and Use Committee of Institute of Feed Research, Chinese Academy of Agricultural Sciences. All animal experiments were conducted in accordance with the ARRIVE guidelines.

In the first trial, according to chapter 4 in this thesis, 72 80-week-old Hy-Line Brown laying hens with an average eggshell breaking strength of 25 to 30 N were randomly assigned to 4 dietary treatments with 6 replicates (3 birds each). The control 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 5-1). The other treatments were fed a control diet supplemented with 0.5% calcium (high calcium), 5 μg/kg calcitriol or 500 mg/kg quercetin. All birds were housed in individual cages (45 cm × 45 cm × 45 cm), and 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 (control diet) during the observation period.

In the first trial, dietary high calcium did not improve eggshell quality; instead, it decreased eggshell quality and increased breakage rate. Thus, the effects in dietary high calcium were no longer examined in the second trial. In the second trial, 360 60-week-old healthy Hy-Line Brown laying hens were collected from a commercial farm and then randomly divided into 3 groups that consisted of 8 replicates with 5 adjacent cages each. Three hens were housed per cage (45 cm × 45 cm × 45 cm). Layers were fed a basal diet, a basal diet with 5 μg/kg calcitriol or 500 mg/kg quercetin. The basal diet based on Chinese Feeding Standard of Chicken (NY/T 33-2004) and National Research Council (NRC, 1994) requirements is shown in Table 5-2. 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) after 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 was determined by a colorimetric procedure (UV2700, Shimadzu Co., Ltd., Kyoto, Japan; method 965.17, AOAC 2006). The amino acid levels 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. The analyzed values of nutrients are listed in the Table 5-1 and 5-2.

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. In this study, all the hens used were housed in groups of three in individual cages (45 cm × 45 cm × 45 cm) before the trials.

Table 5-1. Ingredient and nutrient levels of the experimental diets in trial 1 (air-dried basis)

Ingredient	%	Nutrient level[2]	%
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		

[1] Premix provided the following per kg of the diet: vitamin A, 9 500 IU; vitamin D_3, 4 125 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) and Tables of Feed Composition and Nutritive Value in China (2021, 32nd edition), while the others are analyzed. AME = Corn × AME1 + Soybean meal × AME2 + Soybean oil × AME3 + Methionine × AME4. AP = Corn × AP1 + Soybean meal × AP2 + Calcium hydrogen phosphate × AP3 + Limestone × AP4.

Table 5-2. Ingredient and nutrient levels of the experimental diets in trial 2 (air-dried basis)

Ingredient	%	Nutrient level[2]	%
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		

[1] Premix provided the following per kg of the diet: vitamin A, 9 500 IU; vitamin D_3, 4 125 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) and Tables of Feed Composition and Nutritive Value in China (2021, 32nd edition), while the others are analyzed. AME = Corn × AME1 + Soybean meal × AME2 + Soybean oil × AME3 + Methionine × AME4. AP = Corn × AP1 + Soybean meal × AP2 + Calcium hydrogen phosphate × AP3 + Limestone × AP4.

3.2. Sample collection

In the first trial, 12 eggs per replicate were randomly selected weekly 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 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 and store at -20°C. Then, the birds were euthanized by cervical dislocation immediately for tissue sampling. All birds had eggs in their uterus, and the characteristics of these eggs meet 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, 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 mucosa 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 a 0.9% saline solution and stored at -20°C, then thawed and rehydrated in saline before testing. The left bones sampled at 18.5 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.

3.3. 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.

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

Eggshell quality determination was referred to the method described before (Fu et al., 2021b). Briefly, the egg weight was weighed first. Then, the thickness from 3 points (equator and both poles) for each egg were detected with Egg Shell Thickness Gauge (Israel Orka Food Technology Ltd., Ramat Hasharon, Israel) and averaged as 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 weighed after removing contents and drying at room temperature until reaching a constant weight. The eggshell weight ratio was calculated as eggshell weight/egg weight × 100%.

Two pieces (~0.5 cm2 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 according to a previous study (Dunn et al., 2012). Briefly, 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 manually removed the eggshell membranes by the same individual for consistency. Then, these six eggshells were air-dried, weighed, ground and mixed into one sample for the detection of eggshell calcium and phosphorus content according to a previous report (Fu et al., 2021a). Briefly, 0.5 g of eggshell power 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 perform calcium content assay, and a spectrophotometer (UV2700, Shimadzu Co., Ltd., Kyoto, Japan) was employed to detect phosphorus content. Total calcium and phosphorus per egg were calculated as multiplying average eggshell weight ($n = 6$) by calcium or phosphorus content.

3.5. 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, then the density was calculated. A string and a digital caliper were used to measure their length and midpoint circumference. Then, the humeri and femurs were 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 the Table S3 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 a 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 analyzer 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 deform 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 day with petroleum ether, with daily solution changed. They were then dehydrated in 95% ethanol for 1 day. Afterwards, the bones 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. Ash was then used to determine the calcium and phosphorus contents in bones based on the method mentioned above (*Eggshell physical and mechanical properties, ultrastructure, and components*).

3.6. 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 embedding in molten paraffin with the 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 the oven at 60 °C to dewax. 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). Briefly, as shown in Fig. 5B, 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 then decalcified in 14% 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 Cybemetics, 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)×100;

Mb.N (n/mm) = (1.199/2)×(Mb.Pm/T.Ar);

Mb.Th (μm) = (2000/1.199)×(Mb.Ar/Mb.Pm);

Mb.Sp (μm) = (2000/1.199)×[(T.Ar–Mb.Ar)/Mb.Pm].

3.7. RNA extraction and quantitative real-time PCR (qPCR)

The tissues were ground manually with a mortar and pestle in the 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. Quantitative real-time PCR (qPCR) 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, Tm and primer efficiencies can be found in Table S4. The relative gene expression levels were calculated by $2^{-\Delta\Delta Ct}$ method (Livak et al., 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.

3.8. Calcium retention of hens, as well as the calcium concentrations of the serum and uterine fluid

Calcium retention of hens was determined as described in Macelline et al. (2022). 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 sample were dried and ground to pass through a 0.5 mm sieve. The contents and total calcium excretion were determined as mentioned above (*Eggshell physical and mechanical properties, ultrastructure, and components*). Calcium retention coefficient of hens was counted as following equation and then presented as the average value of 3 hens per replicate in statistical analysis.

Calcium retention coefficient (%) = [(feed intake×$calcium_{diet}$)–(excreta output×$calcium_{excreta}$)]/(feed intake×$calcium_{diet}$)×100

The total calcium concentrations (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.

3.9. 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 relative qPCR 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 considered significant when the *P* value below 0.05.

4. Results

4.1. Trial 1

The results of laying performance of the first trial are presented in the Figure 5-1. There were no significant differences in HDEP, breakage rate and qualified egg rate of laying hens among groups during the observation period (from the end of 79 weeks of age to the end of 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 ($P < 0.05$), while these birds had comparable HDEP with the birds supplemented with calcitriol ($P > 0.05$). The hens in 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 among the birds in 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 birds in the quercetin addition group was higher than that of birds in the control and calcitriol addition 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, the 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 daily feed intakes were respectively 103.27 ± 8.37, 101.96 ± 5.80, 95.28 ± 11.14, 98.19 ± 9.03 g per hen in the control, high calcium, calcitriol and quercetin supplemented groups, with no significant difference ($P > 0.05$).

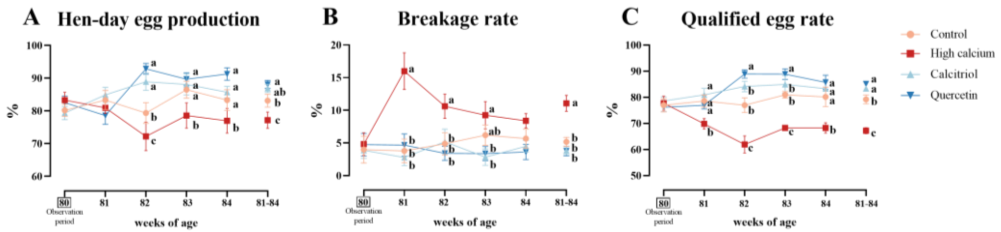

Figure 5-1. Effects of dietary supplementation with calcitriol or quercetin on laying performance of aged laying hens with weak-shelled eggs (25 N < average eggshell breaking strength < 30 N): (A) hen-day egg production, (B) breakage rate, (C) qualified egg rate. All hens were fed the control diet during 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.

The results of eggshell quality are presented in Table 5-3. Compared with the control and calcium-supplemented treatments, dietary calcitriol supplementation significantly enhanced the eggshell breaking strength at the end of 82, 83 and 84 weeks of age, and the 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 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 ($P < 0.05$). There was no significant distinction observed between the calcitriol and quercetin groups, and likewise, no significant differences were found between the control and calcium-supplemented groups ($P > 0.05$).

Table 5-3. 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	Control	High calcium	Calcitriol	Quercetin	SEM	*P*-value
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, *0.01mm						
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

Values are presented as mean and standard error of the mean (SEM), n = 6.

[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. 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, b] Within a row, values with no common superscripts differ significantly ($P < 0.05$).

4.2. Trial 2

4.2.1. Laying performance

As shown in Table 5-4, dietary quercetin supplementation improved HDEP during 69 to 72 and 61 to 72 weeks of age ($P < 0.05$). However, the HDEP of birds in the control group and the calcitriol supplementation group did not differ ($P > 0.05$). Additionally, dietary supplementation with calcitriol or quercetin did not affect

average egg weight, average daily feed intake and feed conversion ratio (g feed/g egg) ($P > 0.05$).

Table 5-4. Effects of dietary supplementation with calcitriol or quercetin on laying performance of aged laying hens (60 to 72 weeks of age) [1]

Weeks of age	Control	Calcitriol	Quercetin	SEM	*P*-value
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, g feed/g egg					
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

Values are presented as mean and standard error of the mean (SEM), n = 8.

[1] Control, a basal diet; Calcitriol, a basal diet supplemented with 5 μg/kg calcitriol; Quercetin, a basal diet supplemented with 500 mg/kg quercetin.

[a, b] Within a row, values with no common superscripts differ significantly ($P < 0.05$).

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

The results of eggshell physical and mechanical properties are presented in the Table 5-5. 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 ($P < 0.05$). The eggshell weight of the end of 72 weeks of age was higher in the calcitriol supplementation group than in the control group ($P < 0.05$).

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, but declined the thickness and ratio of the mammillary layer (Figure 5-2, $P < 0.05$).

The eggshell component results are summarized in the Table 5-6. The eggshell had higher phosphorus content in the calcitriol group than the other groups ($P < 0.05$), with no significant differences between the control and quercetin groups ($P > 0.05$). Compared with the control, the total calcium per eggshell and total phosphorus per eggshell were significantly increased in the hens fed with calcitriol or quercetin ($P < 0.05$). The eggshell physical and mechanical properties, ultrastructure, and components (except for phosphorus content) did not show significant differences between calcitriol and quercetin supplementations ($P > 0.05$).

Table 5-5. 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	Control	Calcitriol	Quercetin	SEM	*P*-value
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, *0.01mm					
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

Values are presented as mean and standard error of the mean (SEM), n = 8.

[1] All hens were fed the control diet at 60 weeks of age and then fed the treatment diets from 61 weeks of age. Control, basal diet; Calcitriol, basal diet supplemented with 5 μg/kg calcitriol; Quercetin, basal diet supplemented with 500 mg/kg quercetin.

[a, b] Within a row, values with no common superscripts differ significantly ($P < 0.05$).

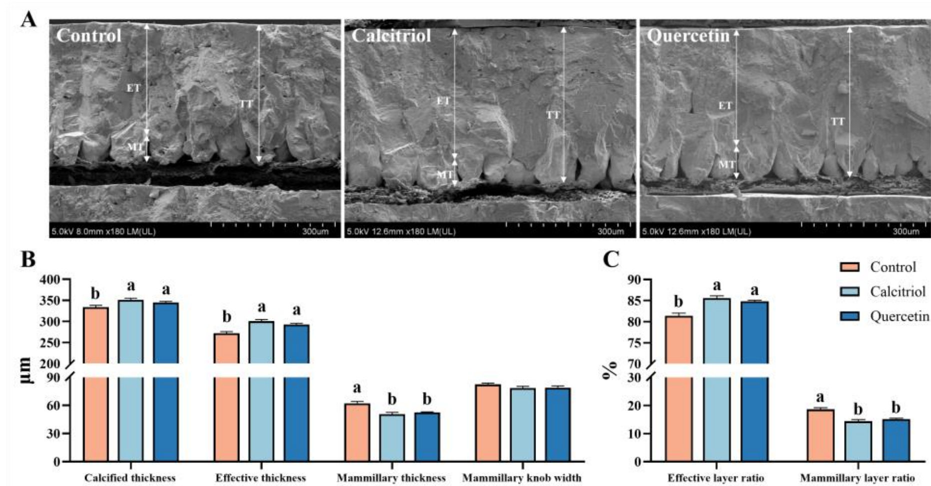

Figure 5-2. Effects of dietary supplementation with calcitriol or quercetin on eggshell ultrastructure of aged laying hens (72 weeks of age): (A) vertical profiles, (B) thickness, (C) thickness ratio. Control, basal diet; Calcitriol, basal diet supplemented with 5 μg/kg calcitriol; Quercetin, basal diet supplemented with 500 mg/kg quercetin. All hens were fed treatment diets from 61 weeks of age. TT, total thickness (calcified layer thickness); ET, effective layer thickness; MT, mammillary layer thickness. [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 5-6. Effects of dietary supplementation with calcitriol or quercetin on eggshell components of aged laying hens (72 weeks of age)[1]

Items	Control	Calcitriol	Quercetin	SEM	*P*-value
Calcium content, mg/g	369.31	366.65	372.74	1.125	0.077
Total calcium 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 per eggshell, mg	6.64^{b}	7.95^{a}	7.12^{a}	0.180	0.003

Values are presented as mean and standard error of the mean (SEM), n = 8.

[1] Control, a basal diet; Calcitriol, a basal diet supplemented with 5 μg/kg calcitriol; Quercetin, a basal diet supplemented with 500 mg/kg quercetin. All hens were fed treatment diets for 12 weeks.

[a, b] Within a row, values with no common superscripts differ significantly ($P < 0.05$).

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

As shown in Table 5-7, 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 5-7. Effects of dietary supplementation with calcitriol or quercetin on bone geometric characteristics of aged laying hens (72 weeks of age)[1]

Items	Control	Calcitriol	Quercetin	SEM	*P*-value
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	7.97	0.20	0.010	0.542
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

Values are presented as mean and standard error of the mean (SEM), n = 8.

[1] Control, a basal diet; Calcitriol, a basal diet supplemented with 5 μg/kg calcitriol; Quercetin, a basal diet supplemented with 500 mg/kg quercetin. All hens were fed treatment diets for 12 weeks.

Table 5-8 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* < 0.05). The femoral proximal BMC of hens in the quercetin addition group was higher than that in the calcitriol supplementation group (*P* < 0.05), while both groups showed no significant differences compared to the control group (*P* > 0.05).

Table 5-8. Effects of dietary supplementation with calcitriol or quercetin on bone mineral measurements of aged laying hens (72 weeks of age)[1]

Items	Control	Calcitriol	Quercetin	SEM	*P*-value
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

Values are presented as mean and standard error of the mean (SEM), n = 8.

[1] Control, a basal diet; Calcitriol, a basal diet supplemented with 5 μg/kg calcitriol; Quercetin, a basal diet supplemented with 500 mg/kg quercetin. All hens were fed treatment diets for 12 weeks. BMD, bone mineral density; BMC, bone mineral content.

The results of bone mechanical characteristics are listed in Table 5-9. Compared to the control, the bone stiffness of the femur was increased in the hens supplemented with calcitriol or quercetin ($P < 0.05$), with the calcitriol supplementation showing a more significant effect ($P < 0.05$). However, no significant differences in the femoral bone strength and work to fracture and the humeral mechanical properties (bone strength, bone stiffness and work to fracture) were observed among all treatments ($P > 0.05$).

Table 5-10 summarizes the changes in bone components. Dietary calcitriol or quercetin addition did not influence the humerus components ($P > 0.05$). However, compared with the control, the fat-free dry weight was increased, and the calcium content in ash was significantly decreased with the calcitriol supplementation ($P < 0.05$). Additionally, the ash, ash content, total calcium per bone and total phosphorus per bone were significantly raised in both calcitriol- and quercetin-supplemented groups ($P < 0.05$). There was no significant difference on the bone components between the calcitriol and quercetin groups ($P > 0.05$).

Table 5-9. Effects of dietary supplementation with calcitriol or quercetin on bone mechanical properties of aged laying hens (72 weeks of age)[1]

Items	Control	Calcitriol	Quercetin	SEM	*P*-value
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

Values are presented as mean and standard error of the mean (SEM), n = 8.

[1] Control, a basal diet; Calcitriol, a basal diet supplemented with 5 μg/kg calcitriol; Quercetin, a basal diet supplemented with 500 mg/kg quercetin. All hens were fed treatment diets for 12 weeks.

[a - c] Within a row, values with no common superscripts differ significantly ($P < 0.05$).

Table 5-10. Effects of dietary supplementation with calcitriol or quercetin on bone components of aged laying hens (72 weeks of age)[1]

Items	Control	Calcitriol	Quercetin	SEM	*P*-value
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 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 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 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 per bone, g	0.47[b]	0.62[a]	0.57[a]	0.023	0.018

Values are presented as mean and standard error of the mean (SEM), n = 8.

[1] Control, a basal diet; Calcitriol, a basal diet supplemented with 5 μg/kg calcitriol; Quercetin, a basal diet supplemented with 500 mg/kg quercetin. All hens were fed treatment diets for 12 weeks.

[a, b] Within a row, values with no common superscripts differ significantly ($P < 0.05$).

Figure 5-3 demonstrates the results of the femur histomorphometry. Dietary supplementation with quercetin significantly increased BV/TV compared with the other treatments ($P < 0.05$), while there was no significant difference observed between the control group and the calcitriol addition 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 addition group showed no significant differences compared to the other groups ($P > 0.05$).

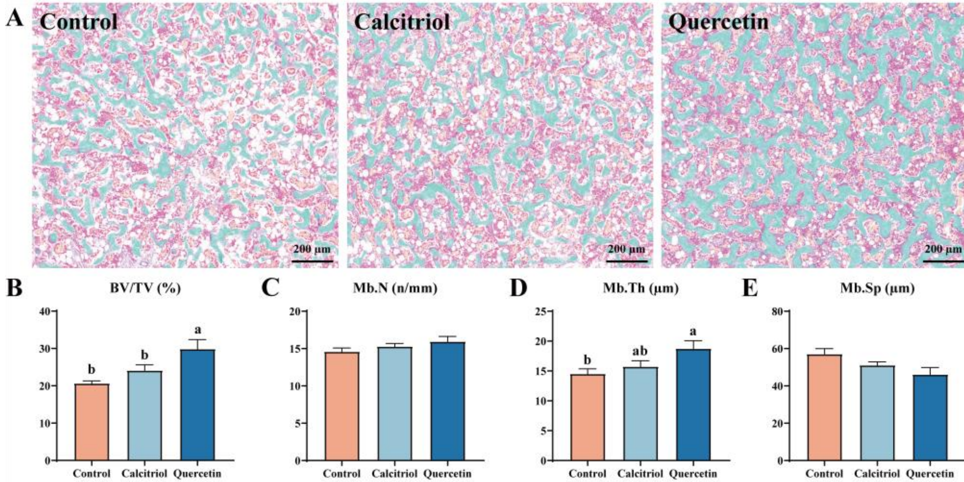

Figure 5-3. Effects of dietary supplementation with calcitriol or quercetin on femur histomorphometry of aged laying hens (72 weeks of age). (A) the medullary bone (green) stained with Goldner's trichrome, (B) medullary bone volume/bone tissue volume (BV/TV), (C) medullary bone number (Mb.N), (D) medullary 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.

4.2.3. Calcium retention coefficient of hens and calcium concentration in serum

The results of calcium retention coefficient and serum total calcium concentration are depicted in Figure 5-4. Dietary supplementation of calcitriol or quercetin significantly raised the calcium retention coefficient of hens compared to the control ($P < 0.05$), with no significant differences between the calcitriol and quercetin supplementations ($P > 0.05$). However, no significant changes were found in the serum total calcium concentration among three groups ($P > 0.05$).

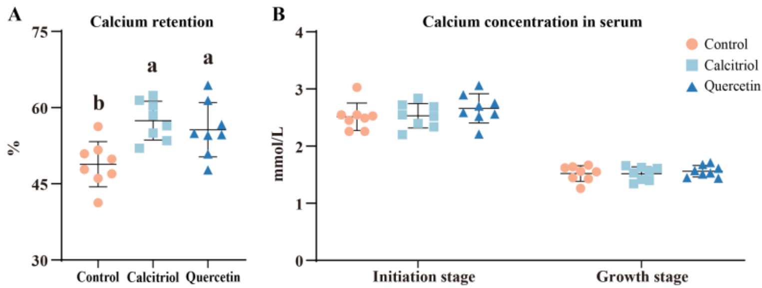

Figure 5-4. Effects of dietary supplementation with calcitriol or quercetin on calcium retention (A) and calcium concentration in serum (B) of aged laying hens (72 weeks of age). Each dot in the calcium retention of hens represents an average value of 3 hens per replicate, while it in calcium concentration in serum represents the value of 1 hen per replicate. 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 ($P < 0.05$). n = 8.

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

Compared to the control, dietary calcitriol or quercetin supplementation down-regulated the gene expression of caspase 3 (CASP3) at the initiation stage of eggshell calcification (Figure 5-5A, $P < 0.05$). The HE staining (Figure 5-5B) 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 (Figure 5-5F, $P < 0.05$) but failed to significantly affect the villus length, width of mucosal folds, and quantity score of mucosal folds (Figure 5-5C-E, $P > 0.05$). However, no significant difference in the uterine morphology and apoptosis-related gene expression was observed between the calcitriol and quercetin groups (Figure 5-5A-B, $P > 0.05$).

4.2.5. Quantification of calcium transport-related mRNA in the uterus and the calcium concentration in uterine fluid

The gene expression levels of calcium transporters in the uterus are exhibited in Figure 5-6A-B. At the initiation stage of eggshell calcification, dietary supplemented with calcitriol or quercetin significantly up-regulated the gene expression of TRPV6 and down-regulated the gene expression of PMCA in the uterus compared to the control ($P < 0.05$), with no significant differences observed between the calcitriol and quercetin supplementations ($P > 0.05$). The gene expression level of uterine CALB in the calcitriol group was not significant changed compared to quercetin group ($P > 0.05$) but significantly increased compared to the control ($P < 0.05$). However, the gene 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 gene expression levels of uterine NCX, VDR, ERα, and ERβ ($P > 0.05$). At the growth stage of eggshell calcification, the gene expression levels of uterine TRPV6 and ERβ were higher in the quercetin supplemented group

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

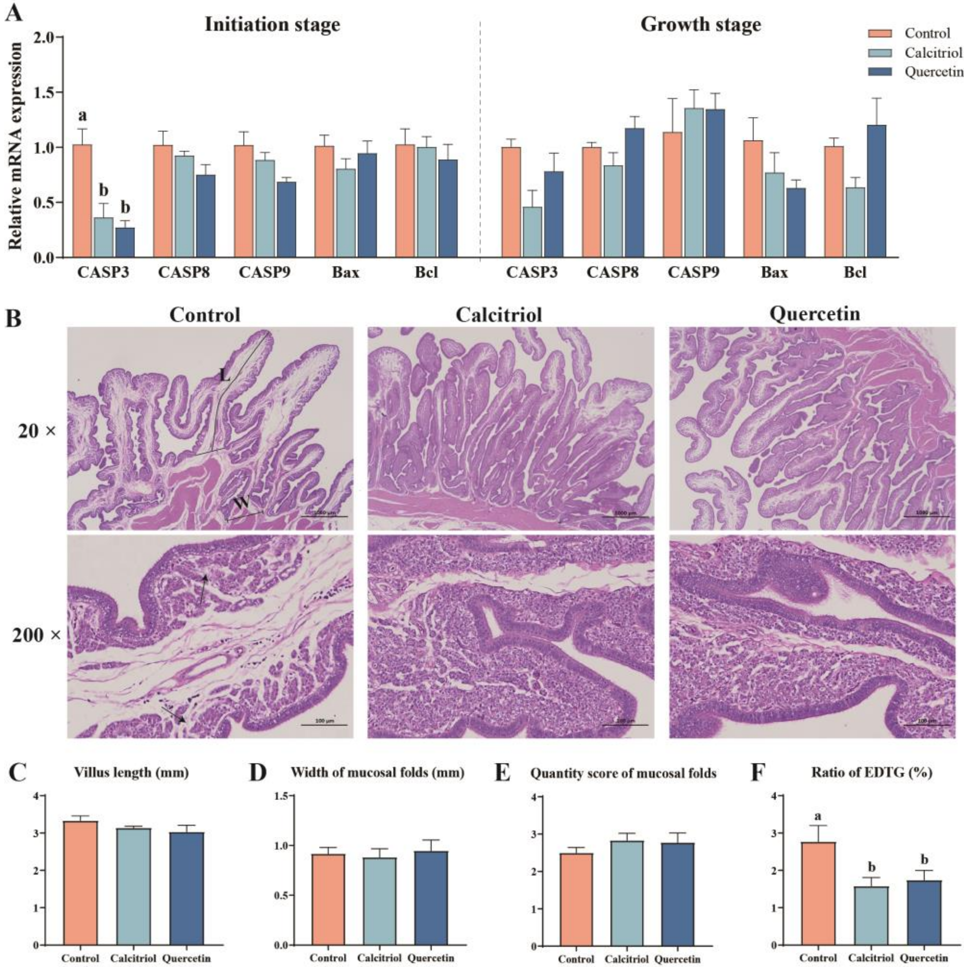

Figure 5-5. Effects of dietary supplementation with calcitriol or quercetin on the uterine morphology and the mRNA expression of apoptotic genes in age laying hens (72 weeks of age). (A) the mRNA expression of apoptotic genes at the initiation (8.5 h post-oviposition) and growth (18.5 h post-oviposition) 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). CASP3, caspase 3; CASP8, caspase 8; CASP9, caspase 9; L, villus length; W, width of mucosal

folds; black arrow, EDTG. Control, basal diet; Calcitriol, basal diet supplemented with 5 μg/kg calcitriol; Quercetin, basal diet supplemented with 500 mg/kg quercetin. [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.

Figure 5-6C displays the results in total calcium concentration of uterine fluid at the growth stage of eggshell calcification. Compared to the control, dietary calcitriol or quercetin supplementation significantly increased the calcium level in the uterine fluid ($P < 0.05$), while no significant difference was observed between calcitriol and quercetin supplementations ($P > 0.05$).

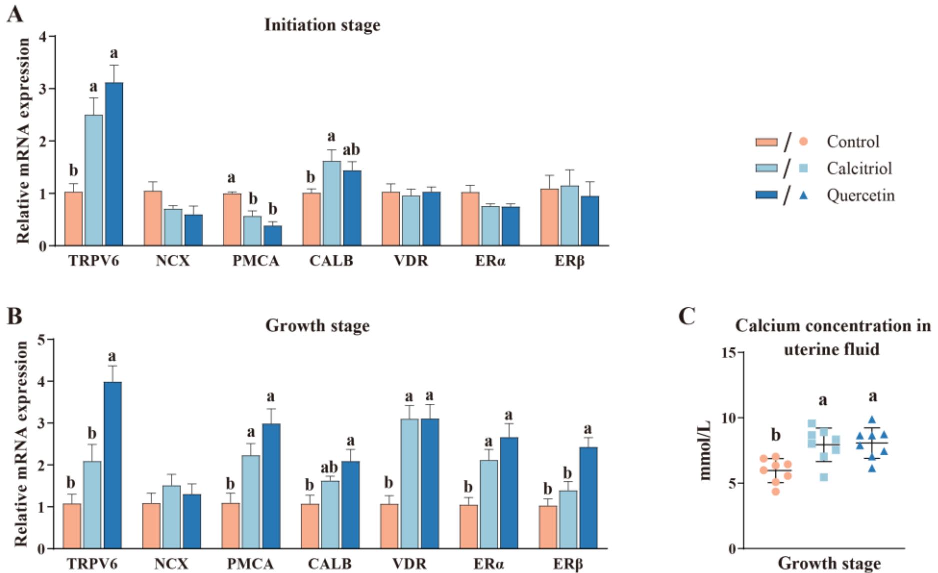

Figure 5-6. Effects of dietary supplementation with calcitriol or quercetin on uterine calcium transport of aged laying hens (72 weeks of age). The mRNA expression of genes related to calcium transport at the initiation (A, 8.5 h post-oviposition) and growth (B, 18.5 h post-oviposition) stages of eggshell calcification, (C) the uterine fluid at the growth stage of eggshell calcification. TRPV6, transient receptor potential cation channel, subfamily V, member 6; NCX, sodium-calcium exchange; 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. [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.

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

Figure 5-7 presents the differences in bone remodeling-related gene expression of the humerus and femur. In the humerus, at the growth stage of eggshell calcification, dietary quercetin addition significantly up-regulated the gene expression levels of ALP and CTSK compared to the control and calcitriol supplementation ($P < 0.05$).

However, there was no significant difference in these levels between the calcitriol supplementation group and the control group ($P > 0.05$). The gene expression level of humeral TRAP was higher in the calcitriol group than in the quercetin group ($P < 0.05$), and that in both groups was higher than in the control group ($P < 0.05$). At the initiation stage of eggshell calcification, dietary calcitriol or quercetin addition significantly increased the gene expression levels of ALP, OPN, and TRAP in the femur compared to the control ($P < 0.05$), while no significant difference was found between the calcitriol and quercetin supplementations ($P > 0.05$). The gene expression level of femoral ERα did not show significant differences between the control and calcitriol supplementation groups ($P > 0.05$), but that in both groups were significantly higher than in the quercetin supplementation group ($P < 0.05$). Dietary calcitriol addition significantly increased the gene expression of femoral VDR compared with the control and quercetin addition ($P < 0.05$). However, no significant difference was found in the gene expression of femoral VDR between the control and quercetin addition groups ($P > 0.05$). At the growth stage of eggshell calcification, dietary supplementation with calcitriol or quercetin down-regulated the gene expression 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 gene expression of the femoral TRAP than the control ($P < 0.05$), among which the quercetin addition was more obvious ($P < 0.05$).

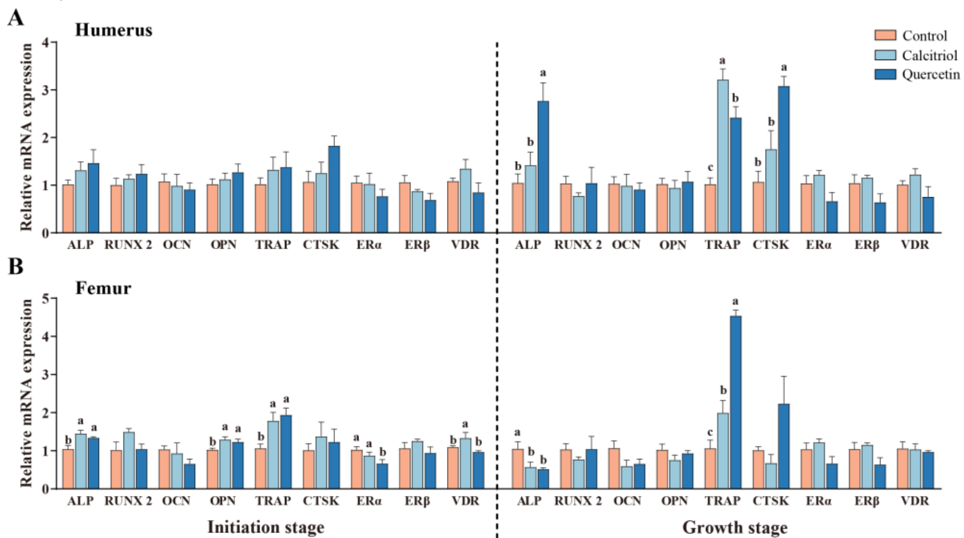

Figure 5-7. Effects of dietary supplementation with calcitriol or quercetin on the mRNA expression of genes related to bone remodeling in the humerus (A) and femur (B) of aged laying hens (72 weeks of age). Initiation stage, at 8.5 h post-oviposition; Growth stage, at 18.5 h post-oviposition; ALP, alkaline phosphatase; RUNX 2, runt-related transcription factor 2; OCN, osteocalcin; OPN, osteopontin; TRAP, tartrate-resistant acid phosphatase; CTSK, cathepsin K; Control, basal diet; Calcitriol, basal diet supplemented with 5 μg/kg calcitriol; Quercetin, basal diet supplemented with 500 mg/kg quercetin. [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.

5. Discussion

The layers that laid eggs with low eggshell breaking strength should be given more attention since the laid eggs were more prone to break and crack 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 trial 1, dietary extra calcium supplementation did not improve eggshell quality of aged hens with weak shells; instead, it increased the breakage rate and reduced HDEP and the rate of qualified eggs. The size of the limestone particles was uniform for all groups, thus the differences between groups may be independent of limestone solubility in the intestine. A possible explanation is that a high dietary calcium level (4.2% to 4.5%) reduced the gene expression of intestinal CALB, leading to decreased calcium absorption and retention. This ultimately resulted in lower eggshell quality and an increased egg breakage rate (Atteh et al., 1985; Wang et al., 2021a). 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., 2023a), 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 trial 2, the positive effects of calcitriol and quercetin on eggshell quality were also observed in general commercial aged laying hens, while the significant improvements were not evident until at least 8 weeks. It can explain that the calcitriol and quercetin took more quickly effect on the hens with weak eggshells since they exerted a positive effect on eggshell quality from the 2nd week in the such flock. 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 in aged hens with weak eggshells.

The supplementation of either 5 μg/kg calcitriol (~0.25 μg kg^{-1} body weight per day) or 500 mg/kg quercetin (~25.65 mg kg^{-1} body weight per day) 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 suggests that adding dietary calcitriol or quercetin could alleviate the age-related decrease in eggshell calcium deposition (Park et al., 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, the calcitriol or quercetin supplementation acted primarily on the skeleton with medullary bone since the improvements of bone 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, 2006) and was 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 bone components. Furthermore, dietary quercetin also enhanced bone microarchitecture by increasing medullary bone thickness and BV/TV. These findings suggest that both calcitriol and quercetin could promote mineral retention in the femur, potentially contributing to increased bone stiffness (Luo et al., 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 an estrogenic effect of quercetin, which facilitated the transition of bone turnover from cortical bone to medullary bone (Whitehead et al., 2000). Compared with the femur of hens fed with calcitriol, when the changes of mineral measurements and components were not significant, more medullary bone in the femur of hens fed with quercetin may account for a lower bone stiffness since the medullary bone has a lower structural strength than cortical bones (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 were 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 gene expression 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. Additionally, a lower ratio of EDTG in the calcitriol and quercetin groups also suggest an improved uterine morphology. The improvements of uterine morphology could promote eggshell calcium deposition and

elevate eggshell quality while a 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). However, the effect of exogenous estrogen on VDR in birds has been rarely reported. In this study, the gene expression 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 vitamin D response element in the promoter region of ERα (Santos-Martínez et al., 2014; Santos-Martínez et al., 2021) or, indirectly, via regulating the estrogen metabolism (Tsang et al., 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 et al., 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 et al., 1984). Taken together, the increases in the gene expression 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 gene expression independently of vitamin D_3 (Navickis et al., 1979; van Abel et al., 2005). Additionally, the gene 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 gene expression of TRPV6, CALB and PMCA. However, unlike in the growth stage, there was no impact on the gene expression 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 that could further affect uterine calcium

transporter gene expression (Bar, 2009b; Cheng et al., 2023; Wang et al., 2022; Yang et al., 2018). While the gene 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 considered 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* gene expression 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 supplemented group, only *TRAP* gene expression was increased at the growth stage of eggshell calcification, suggesting that the calcitriol treatment 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 gene expression of ALP at the growth stage of eggshell calcification, but up-regulated TRAP gene expression 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 in the initiation stage of eggshell calcification (McKee et al., 1992; Nizet et al., 2020; Ren et al., 2023). In the current study, the gene expression 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 gene 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.

China is the main producer of quercetin, and its production was around 98.13 tons in 2020 (source: http://www.chinamrn.com). Quercetin has extensive pharmacological effects, such as anti-oxidant, anti-inflammatory and anti-senescence effects. In recent years, the effectiveness and safety of quercetin on poultry are being explored. This study further explored the effects of quercetin on eggshell and bone quality as well as its mechanisms. The results showed that the quercetin could improve eggshell and bone quality by regulating the calcium metabolism. Future efforts should supplement the results of blood and tissue concentrations of quercetin. The residual contents in the egg, meat, and excreta should also be detected to assess its potential impacts on poultry products and environment. These issues must be resolved before pursuing practical application.

6. Conclusion

Based on all the results, dietary supplementation with calcitriol or quercetin could improve eggshell and bone quality by improving calcium metabolism. Specifically, dietary calcitriol or quercetin addition exhibited a better uterine morphology and an improved calcium transport-related gene expression pattern (Figure 5-8), 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 gene expression in a calcitriol independent manner, however, without affecting eggshell quality more. 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.

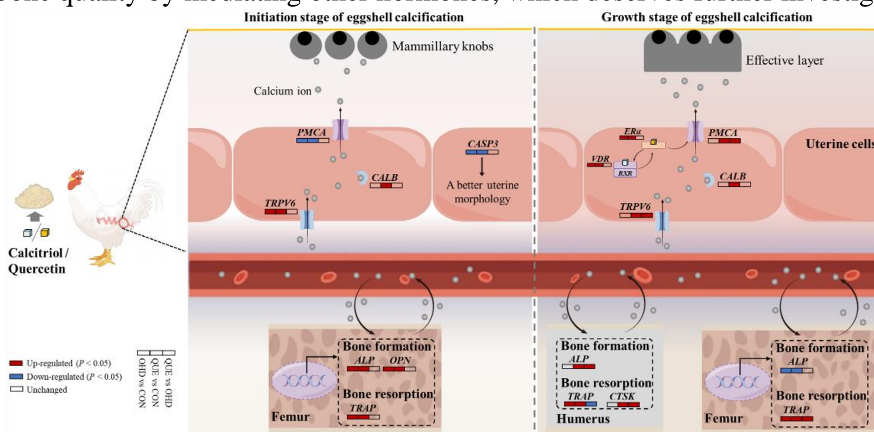

Figure 5-8. A schematic model displaying the mechanism of calcitriol or quercetin addition on uterine calcium transport and bone remodeling.

Chapter 6

General discussion, conclusions and perspective

1. General discussion

The reduction in eggshell and bone quality is a major concern for the late laying period in laying poultry. Eggshell and bone quality is regulated by calcium transport in the uterus and bones. There may be an interaction between bone remodeling and eggshell calcification, as bone resorption is an important calcium source for eggshell formation. Exploring the changes of uterine calcium transport and bone remodeling as well as their effects on eggshell and bone quality contribute to reveal the interaction between the uterus and bones and investigate the possible mechanism of eggshell and bone quality reduction in aged laying hens. In addition to their interaction effects, eggshell and bone quality are also determined by environmental and dietary factors, thus it is necessary to explore the influence of these parameters as well.

This thesis investigated the changes of uterine calcium transport and bone remodeling as well as their effects on eggshell and bone quality of aged hens in three conditions. The relationship between eggshell and bone quality as well as the possible mechanism of their quality reduction were further analyzed.

1.1. Comparison of eggshell and bone relationship among three conditions

Previous studies reported that bone quality was negatively correlated with the quality and production of eggshells (Cransberg et al., 2001; Eusemann et al., 2018b; Hocking et al., 2003; Kim et al., 2005; Rufener et al., 2019). However, some research suggested that such relationship was typically weak or even absent (Alfonso-Carrillo et al., 2021; Gebhardt-Henrich et al., 2015; Jendral et al., 2008; Rennie et al., 1997). In addition to being influenced by each other, eggshell and bone qualities are affected by a range of factors such as environment, physiological status, and diet (Cufadar et al., 2011; Feng et al., 2023; Fu et al., 2021b; Khanal et al., 2020a). Thus, this thesis explored the relationship between eggshell and bone quality under three conditions.

Firstly, the effects of bone changes on eggshell quality were explored by altering a conventional caging system (CCS) to an aviary system (AVS). Both pullets (16 weeks of age) and laying hens (30-73 week of age) exhibited better bone qualities in AVS than in CCS (Casey-Trott et al., 2017b; Casey-Trott et al., 2017a; Khanal et al., 2021; Leyendecker et al., 2005). In this thesis, chapter 3 supported this notion and indicated that such benefits could even last throughout the end of the laying period (95 weeks of age). The results of the statistical analysis of a mixed model are also similar. These results suggested that changing CCS to AVS is effective in improving bone quality. Chapter 3 also showed that there was a positive relationship between eggshell and bone quality, and consistent with previous studies we showed that AVS simultaneously improved bone and eggshell quality (Ahammed et al., 2014; Leyendecker et al., 2005). Enhanced bone quality may indicate a more active bone remodeling, which may be a possible cause of eggshell alteration as bone resorption is a major calcium source for eggshell calcification (Clunies et al., 1993). Moreover, increasing exercise, along with improving well-being, may also positively impact

some other pathways, independent of bone and eggshell improvements, awaiting further research.

Secondly, the relationship between bone and eggshell quality was explored in hens with a similar breeding background, environment, age and diet, but showing a difference in eggshell quality. Such experiments are highly warranted since there is a high level of variability in eggshell quality in the late laying period (Bain et al., 2016). Chapter 4 showed that negative correlations between bone and eggshell quality were observed in the individuals with different eggshell quality. This was consistent with a previous study that reported that low-quality bones were associated with high-quality eggshells (Kim et al., 2005).

Finally, chapter 5 explored the effects of dietary addition with calcium metabolism regulators (calcitriol and quercetin) on eggshell and bone quality as well as the relationship between eggshell and bone quality. This chapter found that there were positive correlations between eggshell and bone quality except for the indicators related to the mammillary layer, suggesting that eggshell and bone quality can be enhanced simultaneously through improving calcium metabolism. This was consistent with a previous study that demonstrated the calcitriol addition improved eggshell and bone quality at the same time (Frost et al., 1990).

This thesis demonstrated that the relationships between eggshell and bone quality were discrepant under different conditions. Overall, bones look like a calcium reservoir, while eggshells seem to be a calcium consumer. When the quality of bone improves, the eggshell formation is promoted. However, when the calcium requirement of eggshell calcification increases, bones are deprived of calcium and subsequently weaken. Although the correlations between eggshell and bone quality are not consistent in different conditions, the better eggshell quality is always linked to an increased uterine calcium transport and an intensive bone resorption. The differences in bone formation may be the main reason for the different correlations between eggshell and bone quality. For example, in chapter 4, the hens were subjected to the same enviroment and diet. The bone formation may be not enough to restore the reduced bone mass caused by enhanced bone resorption in hens with strong shells, which eventually results in a reduced bone quality. However, in chapter 5, the hens fed with calcitriol or quercetin had a higher level of bone formation, which may receive more compensation of bone mass and lead to a relatively better bone quality.

1.2. Differences between humerus and femur

As mentioned in "*1.1. Comparison of eggshell and bone relationship among three conditions*", the description of bones mentioned two important bones: the humerus and the femur. The differences between these two bones are discussed in more detail in this section to understand the relationship between bone and eggshell quality more comprehensively. The humerus and femur are two special bones remained in avian evolution. Both of them suffer high risks of fracture in aged hens (Clunies et al., 1992; Gregory et al., 1989; Hanlon et al., 2022), being paid to significant attention. The humerus is a type of pneumatic bone and mainly consists of cortical bone, while the

femur is comprised of cortical and medullary bone. Thus, they may respond differently to different environments, diets and eggshell calcification intensities.

In chapter 3, AVS increased the quality of the humerus but failed to affect that of the femur. The primary skeletal development of layers is finalized before or during the early stage of egg laying (Hanlon et al., 2022; Rath et al., 2000). The positive effect of AVS on bone quality has been reported in pullets (Campbell et al., 2016b; Casey-Trott et al., 2017c). Thus, hens housed in AVS may have better bone quality compared to CCS at the beginning of laying period. Following the laying period, it is possible that the calcium in femur is gradually consumed due to the high calcium demand for eggshell calcification. This conjecture is supported by the upregulation of femoral TRAP that is related to the capacity of bone resorption (Hayman et al., 1996). However, the high calcium demand for eggshell calcification in AVS did not affect the humerus since no evidence of more bone resorption in the humerus was noted.

In chapter 4, there were negative correlations between eggshell quality and the components of both bones. An enlarged diameter and more structural damages of endocortical bones in the femur of hens with high eggshell breaking strength (HBS) suggested an increased bone resorption (Shen et al., 2016; Sheu et al., 2015). This indicated that the low femoral component in the HBS group may be associated with an increased bone resorption. However, enlarged endocortical diameter, reduced skeletal minerals and upregulated bone formation genes' expression (RUNX 2 and OCN) were observed in the humerus of the HBS, indicating that the low humeri component in the HBS may be associated with a decreased bone formation.

In chapter 5, dietary addition with calcitriol or quercetin increased femur quality but did not influence humerus quality. During the growth stage of eggshell calcification, increased expression of TRAP and decreased expression of ALP in the femur of calcitriol and quercetin supplementation groups suggested a higher bone resorption (Hayman et al., 1996) and a lower bone formation (Nizet et al., 2020), which may benefit the calcium acquisition of eggshell calcification. Additionally, the expressions of ALP, OPN and TRAP were upregulated in calcitriol and quercetin groups during the initiation stage of eggshell calcification, which could promote bone turnover to increase bone mass (Hayman, 2008; McKee et al., 1992; Nizet et al., 2020). Although dietary calcitriol and quercetin addition enhanced the expression of bone resorption-related genes such as TRAP and CTSK (Dacke et al., 2015) in the humerus, there was no association with bone loss. This may be due to the relatively short treatment period, and longer periods are needed to evaluate the risk of bone loss.

Therefore, in conclusion, compared with the humerus, the femur is more susceptible to the changes in environment, eggshell calcification intensity and diet. This is consistent with a previous study that reported more rapid bone loss in the femur than in the humerus of hens subjected to a calcium-deficient diet (Clunies et al., 1992). The rapid response toward external changes may be attributed to medullary bones that are more easily resorbed and formed due to their low quantity and randomly distributed collagen content (Dacke et al., 1993). This suggests that the femur is able to provide calcium more easily for maintaining mineral homeostasis. In contrast, the upregulation of bone absorption genes expression was rarely observed in the humerus.

Thus, the effect of eggshell calcification on the humerus may be alleviated, even be exempt due to the maintenance of mineral homeostasis by the femur.

Considering the rapid response of the femur to high calcium demands, is it reasonable to discuss the correlation between bone and eggshell quality using only the femur? The quality of femur exhibited positive correlations with eggshell quality under relatively short-term treatments (12 weeks, in chapter 5) due to its rapid response to calcium changes. However, the relationship between its quality and eggshell quality was weak under long-term experiments (from pullet to over 80 weeks of age, in chapter 3 and chapter 4). Thus, the femur seems suitable for being the major bone taking into account when evaluating the relationship between bone and eggshell quality under short-term treatments.

Considering the insensitive response of the humerus to high calcium demands, does the correlation between humerus and eggshell quality still make sense? Chapter 5 demonstrated that the humerus quality did not show any significant correlation with eggshell quality under the relatively short-term treatments (12 weeks). However, under the relatively long-term impacts (from pullet to over 80 weeks of age, in chapter 3 and chapter 4), correlations between humerus and eggshell quality were observed, while these correlations were not consistent. This suggested that the effect of eggshell calcification on the humerus could take a longer time. Additionally, the quality of the humerus may provide complementary information to understand the relationship between bones and eggshells in long-term experiments.

1.3. Underlying mechanism of eggshell quality reduction in aged laying hens

Eggshell quality reduction in aged laying hens is caused by multiple factors. This PhD thesis demonstrated that it is linked to at least three factors, including low bone resorption, uterine tissue damages and a low metabolic capacity of calcium.

Poor quality bones in CCS were presented in many studies (Casey-Trott et al., 2017a; Khanal et al., 2020a; Khanal et al., 2021), as also in chapter 3 of this PhD thesis. However, the effect of rearing systems on bone remodeling have rarely been reported in the past studies. Bone remodeling involves the resorption of old bone by osteoclasts and the formation of new bone by osteoblasts, both are responsible for skeletal development and mineral maintenance. Poor quality bones in CCS may indicate a lower bone remodeling, including less bone resorption. As shown in chapter 3, the expression levels of bone resorption-related genes (TRAP and CTSK) were lower in CCS, suggesting bone resorption was less (Dacke et al., 2015). Active bone resorption surface and the percentage of active osteoclasts were increased with eggshell calcification (van de Velde et al., 1984), contributing to free up calcium for eggshell formation (Clunies et al., 1993; Comar et al., 1949). The lower bone resorption may be unfavorable for the calcium transport from skeletons to the uterus, ultimately limiting eggshell calcification.

Under the same environment and given the same diet, more femoral bone resorption damages, such as irregular erosions in the endocortical surface and demineralized regions in the intracortical region (Shen et al., 2016; Sheu et al., 2015), were presented

in the HBS group compared to the low eggshell breaking strength (LBS) group. This also illustrated that the formation of high-quality eggshells requires more bone resorption. Additionally, altering rearing system (from CCS to AVS) and supplementing calcitriol or quercetin in diets could enhance bone resorption, which may be a reason for the increased eggshell quality. Thus, the reduced eggshell quality in aged hens may be related to the decreased bone resorption.

The variation coefficients of eggshell breaking strength increased with age, resulting in an increase in the proportion of individuals with low eggshell breaking strength, which may be one main reason for increased breakage eggs in aged laying hens (Alfonso-Carrillo et al., 2021; Bain et al., 2016; Sirri et al., 2018). Chapter 4 compared the uterine transcription profiles and physiological characteristics of hens in the HBS and LBS groups. The results showed that there was an increased amount of apoptotic events and a more severe tissue damage in the uterus of the LBS group. Additionally, the uterine transcription profiles showed that the increased apoptosis in the LBS was possibly due to cytosolic calcium overload. Calcium is a common signaling molecule that regulate many cellular processes, including cell survival and cell death (Berridge, 2012). The substantial transport of calcium in uterine cells may present a significant challenge to cell survival. Cytotoxic calcium overload can trigger intrinsic cell death at both the endoplasmic reticulum and mitochondria (Bruce, 2018). Excessive damage may result in tissue damages. An intact and healthy uterus is essential for maintaining uterine calcium transport. Previous studies reported that uterine deterioration was observed in aged laying hens after high-intensity laying cycles, impacting the synthesis of the organic matrix and ion transport in the uterus (Feng et al., 2023; Park et al., 2017; Park et al., 2018). In our study, the blockage of uterine calcium transport due to tissue damages limited eggshell calcification, causing slowly growing mammillary knobs and effective layer, eventually leading to a weak ultrastructure and reduced mechanical properties. Additionally, chapter 5 showed that the uterus was healthier in the dietary calcitriol and quercetin supplementation groups, which may be responsible for eggshell quality improvements. Thus, the deterioration in uterine tissues is one major reason for eggshell quality reduction in aged laying hens. The current study was focused on the brown-egg layer, since the brown-egg flock generally had a poorer eggshell quality compared to the white-egg one at similar laying rates (Akbari Moghaddam Kakhki et al., 2020b; Hocking et al., 2003; Jansen et al., 2020a). Compared to the brown-egg line, the white-egg line had lower eggshell weight (Singh et al., 2009), and it was also more sensitive to calcium changes (Jansen et al., 2020b). It indicates that the eggshell quality may reduce more when the calcium transport was hindered. However, the white-egg line tolerated higher level of oxidative stress than the brown-egg line (Akbari Moghaddam Kakhki et al., 2020a), indicating it exhibited enhanced stress resistance. Additionally, the bone- and egg laying-associated blood parameters of white- and brown-egg lines differed over the course of the pre- and egg-laying period and the daily egg laying cycle (Habig et al., 2021). These two lines also varied in their adaptability to different environments (Küçükyılmaz et al., 2012). Thus, whether the occurrence of uterine injury is a factor in the eggshell quality reduction of white-egg layers needs further investigation.

Eggshell quality reduction was characterized as a lower eggshell weight and a thinner eggshell thickness (Gautron et al., 2021; Rodriguez-Navarro et al., 2002). Thus, the decreased deposition of calcium bicarbonate on eggshells may be a possible mechanism of eggshell quality reduction. In chapter 5, increasing dietary calcium level did not improve eggshell quality and instead increased breakage eggs, which coincided with a previous study (Wang et al., 2021a). This indicated that the reduced eggshell quality in aged laying hens may be more related to the calcium metabolic disorder than to insufficient dietary calcium. Several studies have consistently reported disruptions in calcium metabolism and its regulatory mechanisms in aged laying hens, leading to a decline in eggshell quality (Al-Batshan et al., 1994; Bar et al., 1988; Franco-Jimenez et al., 2005). A compromised calcium metabolism was found to be associated with a disorder in vitamin D_3 metabolism (Bar, 2008), coupled with decreased estrogen levels and receptors (Hansen et al., 2003; Liu et al., 2018) during the late laying period. In chapter 5, dietary addition with calcitriol or quercetin raised calcium retention of hens and uterine calcium transport, resulting in an increased calcium concentration of uterine fluid to promote eggshell deposition, ultimately enhancing eggshell quality. Thus, a disorder in calcium metabolism may contribute to the deterioration in eggshell quality of aged laying hens. Additionally, the current study only observed the single effect of quercetin or calcitriol on laying hens. Further comparative studies exploring the effects of quercetin supplementation alone, calcitriol supplementation alone, and a combination of quercetin and calcitriol supplementation could provide valuable insights into any additive effects on eggshell and bone quality.

1.4. Possible mechanism of bone quality reduction in aged laying hens

In this thesis, the deterioration in bone quality was shown to be associated with rearing systems, intensive eggshell calcification and a reduced calcium metabolic capacity.

Different rearing systems offer different freedom of movement to hens. CCS limits the movement of hens, while other systems (e.g., AVS) provides more space and facilities to increase the opportunities of locomotion (Campbell et al., 2016a, 2016b). Limitation of exercise is detrimental to skeletal development during childhood and adolescence while accelerating bone loss in adulthood (Gregson et al., 2010; Zymbal et al., 2019). Additionally, the pullets also exhibited a lower mineralization in bones when they subjected to exercise restriction (Khanal et al., 2020b; Khanal et al., 2021). Similarly, chapter 3 found that the individual housed in CCS had bones with low minerals and mechanical properties at the end of laying period. This suggested that the CCS or its constraints on movement may be a potential factor contributing to decreased bone quality in aged laying hens. Altering CCS to AVS alleviated the deterioration of bone quality of aged laying hens, which was in accordance with previous studies (Casey-Trott et al., 2017b; Leyendecker et al., 2005). Additionally, the egg production of AVS (89.48%) for the whole laying period were significantly higher than that of CCS (85.76%). However, the previous studies showed that the hens

housed in AVS had higher feed intake and FCR (feed:egg) compared to those housed in CCS (Ahammed et al., 2014; Matthews et al., 2015). It indicated that the hens consumed more dietary calcium in the AVS, which may be one of the reasons for the increase of bone mass and the promotion of eggshell calcification.

In addition to physiological calcium loss due to aging, the metabolic bone damage related to eggshell formation may also exacerbate bone mineral loss, inducing osteoporosis in birds (Cransberg et al., 2001; Wilson et al., 1998). In chapter 4, there were negative correlations between eggshell and bone quality in aged hens under the same environmental and dietary conditions. Previous reports also indicated that the more and better eggshells were produced, the more susceptible the bone was to fracture (Eusemann et al., 2018b; Kim et al., 2005; Rufener et al., 2019). This indicated that overabundant eggshell deposition led to weak bones. Bone quality is determined by bone remodeling. As we mentioned earlier, the formation of high-quality eggshells is coupled with enhanced bone resorption. Increased bone resorption accelerates the loss of bone mass and the weakening of bone structure. During eggshell formation, there is a pronounced osteoblastic activity in the bone after bone resorption, facilitating the remodeling of new bone for the subsequent cycle of eggshell calcification (van de Velde et al., 1984). Chapter 4 found that the adverse effects of eggshell calcification on bone quality may not solely be attributed to the increased bone resorption, it may also entail a reduction in bone formation to meet the elevated calcium demands for eggshell calcification. Therefore, the reduction in bone quality of aged laying hens may be related to more eggshell deposition.

In this thesis, weakened bones generally presented a lower bone mass, which was in agreement with previous reports (Bain et al., 2016; Fleming et al., 2006). This indicated that a disorder in calcium metabolism of aged hens may be one reason for reduced bone quality. As age advanced, the calcium metabolism capability of hens decreased, and the femur ash also declined, while both were simultaneously increased after molting (Al-Batshan et al., 1994). Additionally, in chapter 5, dietary supplementation of calcitriol or quercetin to improve calcium metabolism of aged hens could promote bone formation and enhance bone quality. These supplementations emphasized the importance of calcium metabolism capability of hens to bone quality.

2. General conclusion

This thesis found that better bones (by altering rearing system from CCS to AVS) may lead to high-quality eggshells, better eggshells may result in low-quality bones, and an improved calcium metabolism (by supplementing calcitriol or quercetin in diets) could increase eggshell and bone quality simultaneously (Figure 6-1).

The relationships between eggshell and bone quality were discrepant under different conditions (Figure 6-1). A better-quality shell is often accompanied by an increased uterine calcium transport and an intensive bone resorption. The varied impacts of different conditions on bone formation may be the main reason for diverse correlations between eggshell and bone quality.

The femur responds more quickly to external changes than the humerus. The femur is more suitable for evaluating the relationship between bone and eggshell quality in short-term treatments, while the humerus provides complementary information to understand the relationship between bones and eggshells in long-term experiments.

The reduced eggshell quality in aged laying hens may be related to the less bone resorption, the uterine tissue damages and the lower calcium metabolic capacity of hens.

The deterioration in bone quality may be associated with rearing systems (the opportunities of movement), the high intensity eggshell calcification and the reduced calcium metabolic capacity of hens.

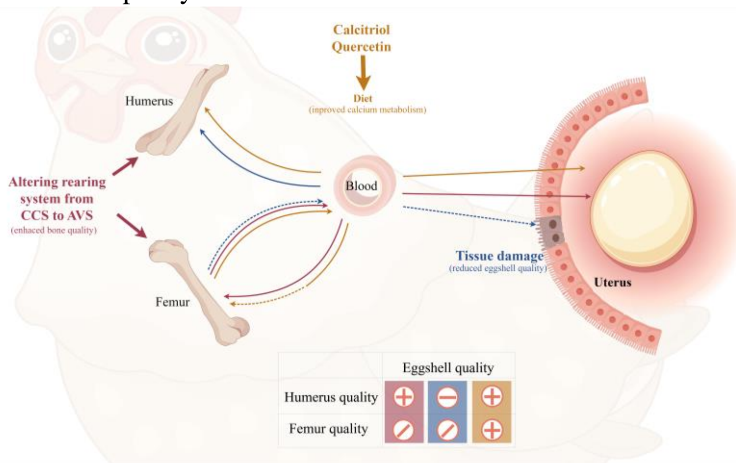

Figure 6-1. A schematic model displaying the calcium transport in uterus and bones under three conditions: different rearing systems (red), different eggshell quality (blue), dietary supplementation of calcium regulators (brown). Solid lines indicate an increase in calcium transport while dashed lines represent a decrease in calcium transport. CCS, conventional caging system; AVS, aviary system. +, positive correlation; -, negative correlation; /, without correlation.

3. Perspectives

This thesis indicated that the relationships between bone and eggshell quality are discrepant in different cases. The femur is suitable for short-term evaluations, while the humerus is suitable for long-term evaluations. This thesis provides references for the selection of specific bone types as the subject in future laying hen research. This difference is mainly due to the presence of medullary bone in the femur. The relationship between medullary bone and eggshell calcification, as well as the pathway of their interaction need further exploration in the future, which would contribute to a better understanding of the relationship between bone and eggshell quality.

The relationship between bone and eggshell quality primarily manifests in composition changes. The changes in hormones may serve as regulatory factors in the calcium transport between the skeleton and the uterus. In the future, the hormones

(such as calcitriol and estrogen in chapter 5) involved in signaling should be screened first by *in vitro* and *in vivo* assays. To determine the candidate hormones that are involved in the crosstalk between the uterus and bones, the changes in hormones were first examined in hens with different conditions (e.g. different bone quality or eggshell quality), and then the hormone changes were tested in the uterine cells or organoids under different physiological states (e.g. with or without apoptosis and damages). The effects of the candidate hormones on the differentiation of osteoclasts and remodeling of bone organoid will be further determined. Next, the effectiveness of the screened hormones should be validated by activating and inhibitory their receptors and potential downstream pathways. Finally, the regulation of the changes in uterus and bones on the secretion of hormones should be explored. More knowledge on these signaling pathways will help to provide potential targets for eggshell and bone quality modulation and new insights for the interaction between organs (skeletons and uterus).

This thesis suggested possible mechanisms of eggshell and bone quality reductions. Some strategies that enhance the movement (such as increasing space allowance, supplementing enrichments), maintain the health of uterine tissues (such as providing balanced diets, supplementing antioxidants in diets) and improve the metabolic capacity of calcium (such as applying an appropriate split feed system, increasing limestone particles) of hens may benefit the qualities of eggshell and bone. It is worthwhile to further explore and evaluate these strategies in the future, thereby providing opportunities to the elongation of the egg-laying cycle.

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Appendices

Scientific publications

Scientific publications

Adapted from the thesis:

1. **Fu Y**, Wang J, Schroyen M, Chen G, Zhang HJ, Wu SG, Li BM and Qi GH (2022). Effects of rearing systems on the eggshell quality, bone parameters and expression of genes related to bone remodeling in aged laying hens. ***Frontiers in Physiology***, 2022;13:962330. (**Chapter 3**)
2. **Fu Y**, Zhou JM, Schroyen M, Zhang HJ, Wu SG, Qi GH and Wang J. Decreased eggshell strength caused by impairment of uterine calcium transport coincide with higher bone minerals and quality in aged laying hens. ***Journal of Animal Science and Biotechnology***, 2024; 15:37. (**Chapter 4**)
3. **Fu Y**, Zhou JM, Schroyen M, Lin J, Zhang HJ, Wu SG, Qi GH and Wang J. Dietary supplementation with calcitriol or quercetin improved eggshell and bone quality by modulating calcium metabolism. ***Animal Nutrition***, 2024 (In Press). (**Chapter 5**)

Related topic:

1. **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***, 2021,15(3):100163.
2. **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. ***Poultry Science***, 2021, 100(7):101102.
3. Zhou JM[#], **Fu Y**[#], Uchechukwu EO, Wang J, Zhang H, Li XB, Qi GH and Wu SG. Supplementation of serine in low-gossypol cottonseed meal-based diet improved egg white gelling and rheological properties by regulating ovomucin synthesis and magnum physiological function in laying hens. ***Journal of Integrative Agriculture***, Available online 22 September 2023. (Co-first authors)
4. **Fu Y**[#], Zhao DR[#], Wang J, Zhang HJ, Wu SG and Wang J. Effect of dietary chloride levels on ion transport in blood and uterus during the growth phase of calcification. (Submitted to the journal, co-first authors)