Chapter 7
Relationship Between Bone Morphology and Bone Quality in Female Femurs: Implication for Additive Risk of Alternative Forced Molting

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Abstract Calcium (Ca) storage in bone has a relationship with eggshell production. Forced molting by feeding restriction for older hens improves eggshell quality but leads to a decline in the bone quality. Dietary minerals improve the eggshell and bone quality; however, the effect on bone quality post-molting has not been clarified. This study evaluated the effects of dietary Ca and minerals on the eggshell and bone quality during both pre- and post-molting periods. The bone quality was evaluated by measurement of bone density and Fourier transform infrared spectroscopy (FT-IR) analysis. The eggshell quality was evaluated by morphological observation of the mammillary cores by scanning electron microscope and FT-IR analysis. The high Ca concentration feed group showed a low bone density post-molting and a high carbonate/phosphate ratio pre-molting. In high mineral concentration feed group, the eggshell strength, the thickness, and the proportion of large mammillary core areas were significantly higher ($p < 0.05$) than control group post-molting. These results suggest that the eggshell strength increases as the proportion of mammillary core areas increases. Furthermore, the high carbonate/phosphate ratio promoted a decrease in bone density. Therefore, the concentration of dietary mineral is strongly related with the maintenance of eggshells and bone quality post-molting.

Keyword Hens · Fourier transform infrared spectroscopy (FT-IR) · Scanning electron microscope (SEM) · Carbonate/phosphate ratio (C/P ratio) · Bone density · Mammillary cores · Calcium (Ca)

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7.1 Introduction

A high egg laying ratio of laying hens is maintained by the metabolic mechanism of calcium (Ca) which is the main ingredient of eggshell. Approximately 70% of Ca derived from feed is accumulated in the marrow bones, a characteristic feature of birds, and approximately 68% of Ca in the bones is used for eggshell formation (Itoh 1971). Therefore, Ca storage in bones has a direct relationship with eggshell production. Consequently, the decline in Ca metabolism of the aging hens which leads to an increase in the number of broken eggs is a key issue in the poultry industry. In Japan, forced molting by feeding restriction of hens is applied in the latter stages of the laying period to improve the hen-day egg production and the eggshell quality and to extend the laying period (Roland and Bushong 1978). However a decrease in bone quality and bone density is caused by forced molting (Mazzuco and Hester 2005). Therefore, it is necessary to address the decline of bone quality in post-molting. Several studies have reported that dietary minerals and the particle size of Ca can lead to an improvement in the bone and eggshell quality (Calvo et al. 1982; Guinotte et al. 1991; Mekada et al. 1976). However, the effect on bone quality in post-molting has not yet been clarified. This study evaluated the effects of the particle size of Ca and the mineral components contained in feed on the eggshell and bone quality in fasting hens during pre- and post-molting periods.

7.2 Material and Method

7.2.1 Breeding Test

Sixteen egg-laying hens (Julia) were equally divided into four feeding groups, a control group (Group C) and three experimental groups (Group D, E, and F): Ca concentration ($CaCO_3$) was 3.8% $CaCO_3$ for Group C and 4.6% $CaCO_3$ for each experimental group by weight. $CaCO_3$ particle size was 40% $CaCO_3$ of 1–1.4 mm diameter for Groups C, D, and F. Group E was fed with powdered $CaCO_3$ less than 0.3 mm diameter. All groups, except Group F, received the same concentration of mineral additives, a mineral premix containing manganese sulfate, iron (III) sulfate, zinc sulfate, copper (II) sulfate, calcium iodate, and cobalt (II) sulfate. Group F received a richer mineral concentration.[1] The hens were fed from the age of 160 days old. Feed was given every day and there was no restriction on water intake by the hens. Forced molting by feeding restriction was applied for 8 days from the age of 503 days old to 510 days old. After forced molting, hens were refeed according to their group's respective feeding specification. All experiments were conducted in

[1] The amount of minerals added to feed is proprietary information and subject to a confidentiality agreement.

accordance with the regulations of the Kagawa University Animal Care and Use Committee.

7.2.2 Sample Collection

When the sample hens were 458 days old, two hens from each groups (a total of eight hens) were slaughtered, and femur samples were collected. The samples were regarded as pre-molting samples (PRE). When the hens were 512 days old, two hens from each groups (a total of eight hens) were slaughtered after 24 h of refeeding, and their femur samples were collected. These samples were regarded as post-molting samples (POST). The femur heads were fixed in 4% paraformaldehyde phosphate buffer solution (pH 7.4) and demineralized with 0.5 M EDTA (pH 8.0) for 3 weeks. Femur heads were embedded in paraffin and sectioned to obtain 10 μm sections.

7.2.3 Bone Density

The sections were stained by the Azan-Mallory method. The trabecular and bone marrow areas in femoral cancellous bone were observed at 10× magnification under a fluorescence microscope (Keyence Corp., Japan), and their central parts, 5500 μm away from periosteum, were analyzed using the software WinROOF version 7.4 (Mitani Corp., Japan). The area ratio of the trabecular to total area was determined as bone density.

7.2.4 Fourier Transform Infrared Analysis of the Femoral Cortex

The qualitative characteristics of the femoral cortex were determined using Fourier transform infrared spectroscopy (FT-IR) (Jasco Corp., Japan) analysis. The femoral cortex was powdered and mixed with potassium bromide (KBr). Spectroscopic analysis was based on two parameters: the calcification and the carbonate/phosphate ratio (C/P ratio) of the samples, both in PRE and POST. The calcification was calculated as the ratio of the area of the phosphate bands (900–1200 cm^{-1}) to the area of the amide I bands (1585–1720 cm^{-1}). The C/P ratio was calculated as the ratio of the area of the carbonate bands (850–890 cm^{-1}) to that of the phosphate bands (900–1200 cm^{-1}) (Boskey and Camacho 2007).

7.2.5 Eggshell Quality Test

The eggshell quality tests were performed twice in both PRE and POST. Eggshell thickness was determined three times on the eggshell equator for eight eggs per group. Eggshell strength was determined by an eggshell strength meter (Fujihira Industry Co., Ltd., Japan). The eggshells of 501 and 680 days old hens were used as morphological samples. The eggshells were cut into about 5 mm squares, and the eggshell membrane was dissolved with a mixture of 6% sodium hypochlorite, 4.12% sodium chloride, and 0.15% sodium hydroxide (Radwan et al. 2010). The mammillary cores were observed at 200× magnification under a scanning electron microscope (SEM) (JCM-6000: JEOL Ltd., Japan). The areas of approximately 100 mammillary cores were analyzed per piece, and 4 pieces per group were examined. Thereafter, the histogram of the areas of the mammillary cores was produced.

In the FT-IR analysis, the eggshells, including their membranes, were powdered and mixed with KBr. Spectroscopic analysis was calculated using the ratio of the peak at 873 cm^{-1} to the peak at 713 cm^{-1}, a parameter to evaluate the carbonate purity of the eggshells (Rodriguez-Navarro et al. 2015).

7.2.6 Statistics

Statistical processing was done using the IBM SPSS version 19 software (IBM Corp., USA). The significant differences among the groups were analyzed by the Tukey's HSD and Games-Howell post hoc tests. The level of significance was set at 5%, and the trend level was set at 10%.

7.3 Results

7.3.1 Bone Density

The trabecular areas in POST from all groups were significantly lower than those in PRE ($p < 0.05$). The area ratio of the trabecular to total area in PRE did not differ between any groups. However, the area ratio of the trabecular to total area in POST was significantly lower in Groups D and E than those in Groups C and F ($p < 0.05$) (Fig. 7.1a, b).

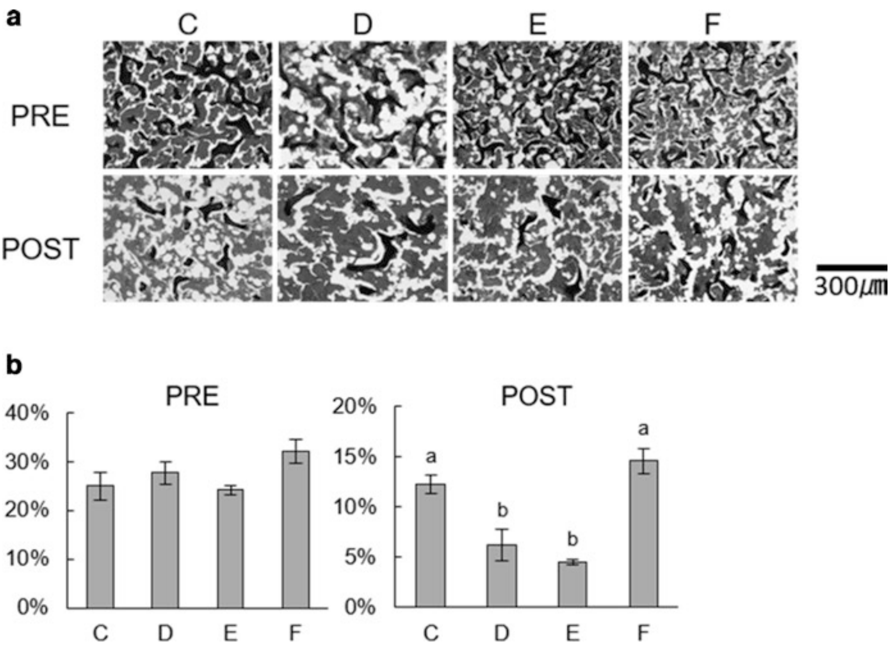


Fig. 7.1 (**a**) The trabecular and bone marrow areas in PRE and POST (black area, trabecular area; gray area, bone marrow area; white area, background). (**b**) The area ratio of the trabecular to total area in PRE and POST (a, b bars with different superscripts within a group are different at $p < 0.05$, $n = 4$)

7.3.2 Fourier Transform Infrared Analysis of Femoral Cortex

The calcification of the femoral cortex in Group F significantly decreased in POST ($p < 0.05$), whereas that in the other groups showed a decreasing trend ($p < 0.1$) (Fig. 7.2a). The C/P ratio of Groups D and E were significantly higher than Group F in PRE ($p < 0.05$) (Fig. 7.2b).

7.3.3 Egg Quality Test

Eggshell strength in Group F was significantly higher than those in Group C in POST ($p < 0.05$) (Fig. 7.3a). Eggshell thickness in Group F was significantly higher than those in Groups C and E in POST ($p < 0.05$) (Fig. 7.3b). The mammillary cores were observed as shown in Fig. 7.4a, and the proportions of the area of the eggshell mammillary cores did not differ in any group in PRE (Fig. 7.4b). There was a significant difference between Group C and Group F in POST with respect to their mammillary core areas larger than 6000 μm^2 ($p < 0.05$) (Fig. 7.4b). The ratio of the peak at 873 cm^{-1} to the peak at 713 cm^{-1} did not differ in any groups in PRE and POST (Fig. 7.5).

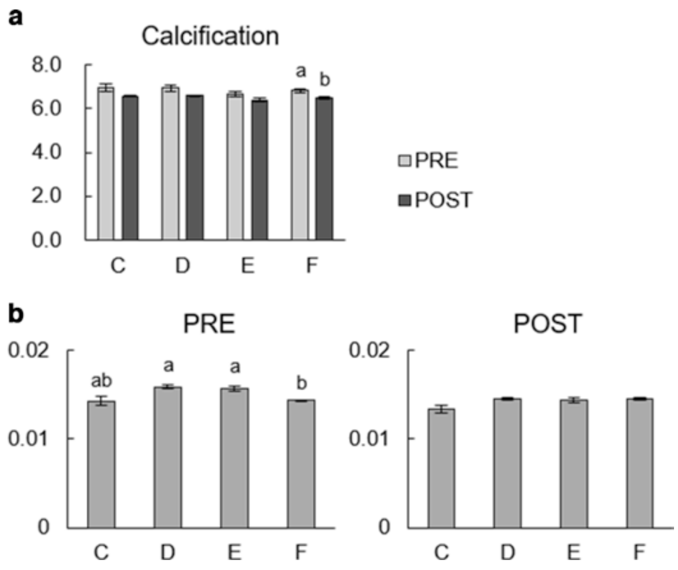


Fig. 7.2 (**a**) The calcification of the femoral cortex (a, b bars with different superscripts within a group are different at $p < 0.05$, $n = 6$). (**b**) The C/P ratio of the femoral cortex (a, b bars with different superscripts within a group are different at $p < 0.05$, $n = 6$)

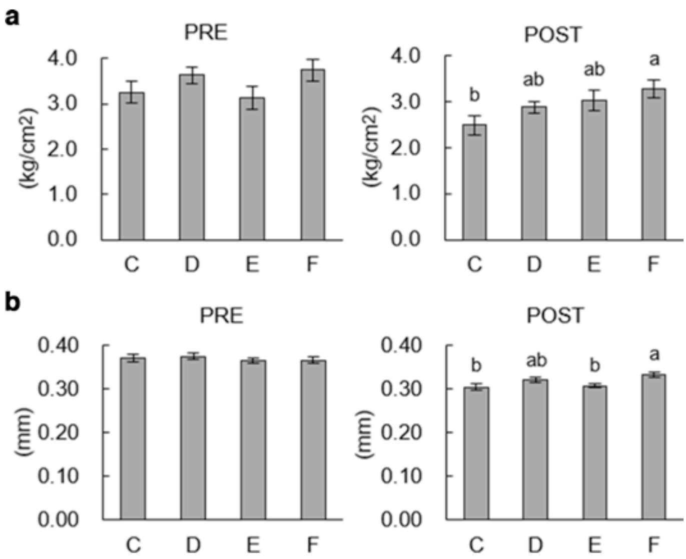


Fig. 7.3 (**a**) Eggshell strength in PRE and POST (a, b bars with different superscripts within a group are different at $p < 0.05$, $n = 16$). (**b**) Eggshell thickness in PRE and POST (a, b bars with different superscripts within a group are different at $p < 0.05$, $n = 16$)

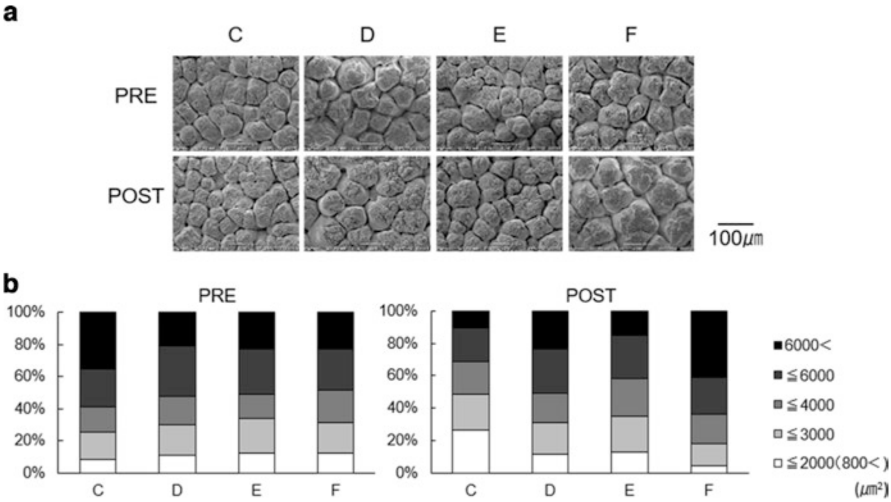
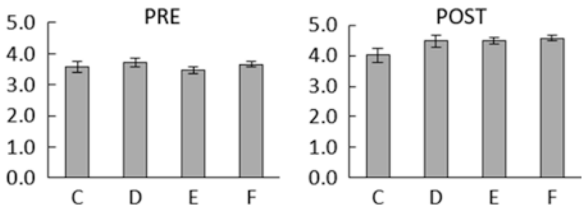


Fig. 7.4 (**a**) SEM microphotographs of eggshell mammillary core in PRE and POST. (**b**) Histogram of the areas of mammillary cores in PRE and POST ($n = 4$)

Fig. 7.5 The ratio of the peak at 873 cm^{-1} to the peak at 713 cm^{-1} (a parameter to evaluate the carbonate purity of the eggshell) in PRE and POST ($n = 6$)



7.4 Discussion

In this study, we focused on the effects of the mineral components and the particle size of $CaCO_3$ contained in feed and we investigated the morphology and quality of bone and eggshell in PRE and POST. The area ratio of the trabecular to total area is an index for evaluating bone density (Hagino, 2005). It was thought that a decrease in bone density of the femoral cancellous bone was due to the influence of forced molting because of the area ratio of the trabecular to total area diminished in all groups in POST (Fig. 7.1b). Thus, three experimental groups' feeds did not suppress the decline of the bone density.

Calcification is an index for evaluating bone strength, and high calcification indicates that the storage function of mineral in bone is high (Boskey and Camacho 2007). The decline of bone strength of the femoral cortex was also caused by the impact of forced molting because the calcification decreased in all groups in POST

(Fig. 7.2a). Furthermore, when the hardness of the part of the cortex of the femoral diaphysis was determined by Vickers hardness tester, the result of bone hardness and bone calcification were linked (data not shown), and further analysis is necessary about bone strength.

The C/P ratio is used as an index of the purity of bone minerals, and a low C/P ratio indicates a high degree of crystal purity (Malluche et al. 2012). The finding of this study suggested that crystal purity was low in PRE in Groups D and E (Fig. 7.2b). The main constituent of bone minerals are present in the bone as calcium phosphate and undergo a phase change to convert to stable apatite due to maturation. Apatite in bones replaces ions, such as carbonate ions(CO_3^{2-}) and phosphate ions, in the crystal lattice. Apatite containing a lot of CO_3^{2-} is reported to have high solubility and increase bone dissolution (Suetsugu 1996; Gourion-Arsiquaud et al. 2012). This suggested that the solubility of apatite was high in Groups D and E in PRE because a ratio of carbonate in Groups D and E were higher than Group F. Therefore, we think that the reduction of bone density was promoted further in Groups D and E in POST. These differences were thought to be due to the difference in the ratio of Ca and other mineral concentration in feed.

Eggshell strength and thickness in Group F were significantly higher than those in Group C in POST (Fig. 7.3a, b). Since zinc and manganese contained in mineral additives in feed have a relationship in eggshell formation, it was conclusive that the high mineral concentration feed improves eggshell quality and supports the previous research which mineral addition in feed improve eggshell quality (Mekada et al. 1976). Eggshell is formed by mammillary core formation on the eggshell membrane and deposition of Ca as a starting point from the mammillary cores. It was reported that as the number of large mammillary cores where several mammillary cores fused increases, the eggshell strength increases (Solomon 2010; Stefanello et al. 2014). As a result of the large number of mammillary core areas larger than 6000 μm^2 in POST, it was thought that the eggshell strength was linked the size of mammillary cores (Fig. 7.4b). Therefore, in addition to evaluating conventional eggshell strength, the morphological observation of the mammillary cores which is the microstructure of eggshell suggested that it is a new means to evaluate eggshell quality. The mechanism of action which mineral components in feed change the form of eggshell structure requires further investigation.

In the FT-IR analysis of eggshells, it was reported that the purity of carbonate changes during eggshell formation (Rodriguez-Navarro et al. 2015). We examined if the eggshell crystal quality could be evaluated after laying, however, the influence of feed on eggshell crystal quality was not detected.

The present result suggested that high mineral concentration feed improved eggshell quality, and high Ca concentration feed promoted the reduction of bone density in POST. In conclusion, the present study has demonstrated that the ratio of the minerals except Ca and the Ca concentration in the feed is important for maintenance of eggshell quality and bone quality in pre- and post-molting. It is necessary to analyze the bone metabolism in order to investigate the effect of feed in the future.

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