Changes of blood parameters associated with bone remodeling following experimentally induced fatty liver disorder in laying hens


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ABSTRACT Studies have demonstrated that obesity and osteoporosis are linked disorders in humans. This study examined the hypothesis that excessive lipid consumption affects bone metabolism in laying hens. A total of one hundred 63-wk-old laying hens were randomly divided into 2 treatments and fed either a regular layer diet (control) or a high energy and low protein diet (HE-LP; experimental treatment) for 80 d. Egg production, feed intake, and BW were recorded at various days during the treatment. At d 80, ten randomly chosen birds per treatment group were killed. Abdominal fat weight, liver weight, and liver fat content were determined. Serum levels of total calcium, inorganic phosphate, and alkaline phosphatase were measured using a biochemical analyzer. Serum concentrations of osteocalcin, leptin-like protein, and estrogen were measured by enzyme-linked immunosorbent assay. Tibia length and width were measured using a vernier caliper; density of the right tibias was determined using an x-ray scanner; and mechanical properties of the left tibias were analyzed using a material testing machine. The expression of osteocalcin and osteoprotegerin mRNA in the keel bone was analyzed by real-time PCR. The concentration of osteocalcin protein in the keels was measured using western blot. Compared with control hens, hens fed the HE-LP diet had lower egg production, lower feed intake, greater liver fat content, and greater abdominal fat pad mass (P < 0.05). Feeding the HE-LP diet increased serum alkaline phosphatase activity, osteocalcin, leptin-like protein, and estrogen concentrations (P < 0.05), and decreased the keel osteocalcin concentrations (P < 0.05). There were significant positive correlations between the serum concentrations of leptin-like protein, estrogen, and osteocalcin regardless of treatment (P < 0.05). The results indicated that HE-LP diet induced a fatty liver disorder in laying hens with an upregulation in bone turnover and exacerbated skeletal damage. The data supported a role for lipid metabolism in skeletal health of laying hens.

Key words: osteocalcin, fatty liver disorder, nutrition, osteoporosis, hen

INTRODUCTION

Bone remodeling, the formation and resorption, is essential to maintain skeletal physiological and structural integrity (bone homeostasis) during the lifespan of humans and other animals. The process is mediated mainly by osteoblast and osteoclast cells, functioning in bone formation and resorption, respectively (Matsuo and Irie, 2008). An imbalance between the 2 processes may result in osteoporosis. Osteoporosis is a bone disease characterized by progressive loss of mineralized structural bones, leading to excessive skeletal fragility and susceptibility to low-trauma fracture among the elderly (Zhao et al., 2008). Similarly, osteoporosis is common in caged laying hens during the laying phase. Osteoporosis affects hen well-being by increasing skeletal fragility and susceptibility to fracture (Webster, 2004; Whitehead, 2004).

Previous studies have indicated that osteoporosis is associated with obesity (Rosen and Bouxsein, 2006; Zhao et al., 2007). Recently, it has further reported that visceral fat has a deleterious effect on bone integrity (Janicka et al., 2007; Gilsanz et al., 2009; Zagarins et al., 2009). Finite element analysis shows that the amount of visceral and total abdominal fat inversely associates with bone strength and bone mineral density (Science Daily, 2012). These findings are contrary to previous reports that fat mass positively favors bone density and prevents osteoporosis (Reid, 2002, 2008). As in humans, chicken fatty liver syndrome or hepatic steatosis occurs when lipoproteins accumulate in the liver because hepatic lipogenesis exceeds the rate of fat mobilization, as a result of imbalance nutrition by...
excessive consumption of high-energy diets (Hermier et al., 1994; Lee et al., 2010). The pathophysiological characters of the fatty liver syndrome and its associated damages in chickens are similar to those caused by nonalcoholic fatty liver syndrome in humans (Ayala et al., 2009; Makovicky et al., 2011). Clinical studies have reported that patients with nonalcoholic fatty liver syndrome are at a high risk of developing osteoporosis or osteopenia (Pardee et al., 2012; Yilmaz, 2012). Similarly, change of fat metabolism may affect skeletal health in laying hens.

Osteocalcin (OC), a major noncollagenous protein in bones, is synthesized by osteoblasts and odontoblasts (Ugar and Karaca, 1999). Osteocalcin has been used as a biomarker of osteoblast activity for evaluating bone remodeling in humans and rodents (Christenson, 1997; Ugar and Karaca, 1999; Seibel, 2005). Recently, OC has also been considered as a regulator of energy metabolism in mammals (Lee et al., 2007; Ferron et al., 2008; Ferron et al., 2010). Two forms of OC are found in the blood: carboxylated OC and uncarboxylated OC (unOC; Poser et al., 1980; Gundberg et al., 1984). Carboxylated OC, containing γ-carboxyglutamate, binds strongly to hydroxyapatite. Most of carboxylated OC is incorporated into the bone matrix and involved in bone mineralization (Price and Nishimoto, 1980; Ducy et al., 1996), and the rest is released into the blood (Price and Nishimoto, 1980). Uncarboxylated OC, containing γ-glutamic, enters into the blood without binding to hydroxyapatite (Price et al., 1976; Ferron et al., 2010) and is the activated form regulating energy metabolism (Lee et al., 2007; Ferron et al., 2010). During bone remodeling, transformation from carboxylated OC to unOC is enhanced by an acidic environment of the resorption lacuna (Ferron et al., 2010).

Leptin and estrogen (ES) are 2 important adipokines in lipoprotein metabolism and bone remodeling. In mammals, leptin is synthesized by adipocytes and acts as an inhibitor of appetite and a promoter of energy expenditure. Previous studies have shown that leptin has dual roles in regulating bone remodeling, stimulating perosteal bone formation through its anabolic effects on osteoblasts and inhibiting bone formation through its central effects (Hamrick and Ferrari, 2008). In birds, leptin cDNA as well as its receptor genes have been identified (Taouis et al., 1998; Dunn et al., 2000), but the leptin gene failed to be detected (Carre et al., 2006). Despite controversy over the avian leptin gene (Ohkubo and Adachi, 2008; Scanes, 2008; Sharp et al., 2008; Pitel et al., 2010), leptin-like protein has been detected in chicken (Neglia et al., 2008). Studies have demonstrated that avian leptin-like protein inhibits appetite (Denbow et al., 2000), favors energy expenditure (Cassy et al., 2001), and benefits reproduction (Cassy et al., 2004), but its function in bone integrity is still unclear (Mauro et al., 2010).

Estrogen affects food consumption and energy expenditure via inhibiting adipose metabolism (Cooke and Naaz, 2004). In mammals, ES also inhibits bone resorption (Oursler et al., 1991) and stimulates bone formation (Oursler et al., 1993). Pharmacologically, ES has been used for preventing and treating postmenopausal osteoporosis in women (Gallagher, 2001). However, ES in laying hens may result in bone loss, which differs from its functions in humans. Estrogen stimulates osteoblasts to form medullary bones rather than structural bones (Wilson and Thorp, 1998). Laying hens progressively lose their mineralized structural bones, starting at onset of the sexual maturity and continuing throughout the layer phase (Webster, 2004).

A change of fat metabolism, such as obesity and fatty liver disorder, may affect bone integrity, leading to osteoporosis in laying hens. The objective of this study was to examine the effects of fatty liver disorder induced by a high energy and low protein diet on bone metabolism and the changes in blood biochemical markers associated with bone health.

**MATERIALS AND METHODS**

**Birds and Diets**

The study was conducted under the guideline approved by the Animal Care and Use Committee of the Nanjing Agricultural University. A total of one hundred 63-wk-old Hy-line Brown laying hens (1.8 to 2.1 kg of BW) were housed in 2-bird cages (40 cm × 35 cm × 35 cm, length × width × height), each providing 700 cm² of floor space per hen. There were 5 cages per replication and 5 replications per treatment (50 birds were held in 25 cages/treatment). The cages were randomly allocated to control and experimental treatments. The control hens were fed with a regular layer diet, and the experimental hens were fed a high energy and low protein diet (HE-LP) for 80 d (Table 1). Feed and water were provided ad libitum. During the experimental period, hens were illuminated for 16 h/d.

**Sample Collection**

Egg production and dead birds were recorded daily. Feed intake was recorded and calculated from 1 to 6, 35 to 40, and 65 to 70 d during the treatment. Briefly, trough feeders were emptied before the test. During the test, paper was placed individually under feed troughs to collect wasted feed. A weighed portion of feed was added daily to the troughs. At the end of the test, feed intake was calculated as described by Pohle and Cheng (2009).

Body weight was measured at d 0 (immediately before the treatment) and d 80. A 3-mL blood sample was collected via the brachial vein from 20 birds at d 0, 60, and 80 (n = 10/treatment/time). The sampled birds were randomly taken among the cages balanced by treatments and bleeding twice was avoided by placing a yellow plastic leg ring on the shank for identifi-
cation. Blood samples were kept at room temperature for 30 min and then centrifuged at $1,500 \times g$ for 15 min at room temperature. The supernatant serum was collected and stored at $-70^\circ C$ for subsequent measurements. At d 80, following cervical dislocation, the liver, the abdominal fat pad, the tibias, and a tissue sample (1.5 × 1 cm$^2$) of the front portion of the keel bone from each sampled bird were collected ($n = 10$/treatment). The keel bone samples were immediately frozen in liquid nitrogen and stored at $-70^\circ C$ for real-time PCR and Western blot analysis.

**Analysis of Fat Content of the Liver and Abdominal Fat Pad**

Liver DM was determined immediately after collection using the oven-drying method, at 105$^\circ C$ for 24 h (Bax et al., 2012). Relative weight of the liver and abdominal fat pad mass was calculated using the following formula:

$$\text{Relative liver weight or relative the abdominal fat pad weight} = \frac{\text{liver DM or abdominal fat pad (g)}}{\text{BW (kg)}}.$$  

Liver fat content was determined by using the Soxtec Auto Extraction Unit (Foss Soxtec 2050, Hillerod, Denmark).

**Bone Radiography and Biomechanical Properties**

The soft tissue of the tibias was removed. The length from tibia spine to inferior articular surface and the diameter at the narrowest point of the tibia shaft were measured using a vernier caliper. The density of the right tibias was measured using a HF50-R32 x-ray apparatus (44 kV and 4 mA, Wandong Medical Equipment Co., Ltd., Beijing, China). Briefly, the tibias were placed on a 35 × 43 cm x-ray plate and a 16-step standard scale with 0.25-mm increment aluminum step-wedge was also exposed on the plate at the same time. The image was collected using Regius model 110 Computed Radiography (Konica Minolta Medical and Graphic Inc., Tokyo, Japan) and was analyzed with Image J software (Zhou et al., 2009). The mechanical properties of the left tibias were examined immediately after collected using a material testing instrument (Lloyd Instruments Ltd., Wokingham, UK) according to the 3-point bending test (Norddin et al., 1995; Barnett et al., 2009). Briefly, the bone was placed on a bending jig at the 2 points separated by 6 cm, keeping the midpoint of the bone at the center (Barnett et al., 2009). The weight load was applied to the midpoint of the shaft in an anterior-posterior direction at a deformation rate of 5 mm/min until failure. Mechanical data was collected, and the force deflection curve was analyzed with Nexygen plus material test and data analysis software. The value for force at failure point (N), force at yield point (N), and stiffness (N/m) were recorded.

**Blood Biochemical Analysis**

The values of serum total calcium, inorganic phosphate, and total alkaline phosphatase (ALP) activity were measured using an automated clinical biochemistry analyzer (Hitachi 7600–020, Hitachi Ltd., Tokyo, Japan) following the manufacturer’s instructions. Serum concentrations of ES, leptin-like protein, and total OC were determined using ELISA based on the manufacturer’s instructions (Bio-Swamp Immunoassay R&D Center, Shanghai, China). Briefly, samples along with each relative standard were loaded to the wells (2 wells per sample) of microplates precoated with a capture antibody, incubated at 37$^\circ C$ for 30 min, and then added detecting antibody labeled with horseradish peroxidase at 37$^\circ C$ for 30 min. Following washing, the color reaction was induced by adding 50 $\mu$L of 3,3',5,5'-tetramethylbenzidin substrate solutions, and the reaction was terminated by adding 50 $\mu$L of sulfuric acid solutions. The color reaction was measured spectrophotometrically at 450 nm and a reference wavelength of 630 nm. The concentrations of samples were determined by comparing with the optical density of the standard curve. The detection ranges of ES, leptin-like protein, and OC were 0 to 640 pg/mL, 0 to 80 ng/mL, and 0 to 48 ng/mL, respectively.

**Real-Time PCR**

The samples of keel bone were ground in liquid nitrogen for the real-time PCR analysis. Briefly, total
RNA was extracted using Trizol reagent per instructions (Invitrogen, Carlsbad, CA). Extracted RNA was then quantified using a spectrophotometer (Eppendorf BioPhotometer Plus, Hamburg, Germany). The cDNA of OC, osteoprotegerin (OPG), and β-actin was synthesized from 1 µg of total RNA at 37°C for 1 h using Quantscript RT Kit (Tiangen Biotech Co. Ltd., Beijing, China): 1 × RT mix, 0.25 mM dNTP, 1 µM oligo-dT15, and 0.5 µL of Quant Reverse Transcriptase to make a total of 10 µL.

The levels of mRNA of OC and OPG were determined using fluorescent quantitative real-time PCR with the double standard curve method (Xu et al., 2011). In the study, β-actin gene was used as a housekeeping gene. The sequence of primers was designed using primer 5.0 and synthesized by TaKaRa Biotechnology Co. (Dalian, China; Table 2). For generation of the double standard curves, OC, OPG, and β-actin of the plasmids were used as standard curves, each containing copy numbers were calculated. Serial 10-fold dilutions of plasmids were used as standard curves, each standard sample containing a known amount of copy numbers in the range from 10^3 to 10^7 copies.

Real-time PCR reaction was performed using ABI 7300 instrument (Applied Biosystems, Foster City, CA) with SYBR Premix Ex Taq (TaKaRa Biotechnology Co.). In a total volume of 20 µL, each reaction contained 1 × SYBR Premix Ex TaqII, 0.2 µM each primer, 1 × ROX Reference Dye, and 2 µL of sample cDNA. The samples were amplified with a precycling hold at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5 s, annealing and extending at 60°C for 31 s, and then PCR products were run for dissociation curve to confirm the specificity of the primers and to ensure that samples were free of DNA contamination. To ensure accuracy and consistency, all samples were measured in duplicates. The results were presented as the ratio of the gene copy numbers between OC mRNA, OPG mRNA, and β-actin mRNA, respectively.

### Western Blot Analysis of OC Protein

Protein was extracted from grounded keel bone using RIPA Lysate per instructions (Beyotime, Shanghai, China). Briefly, 200 µg of protein per sample was separated with a 15% SDS-PAGE. After electrophoreses, the proteins were electrophoretically transferred to nitrocellulose membranes for 1 h at 4°C at a constant voltage of 100 V (Bio-Rad, Hercules, CA). The membranes were incubated with 5% defatted milk in TBST solution (Tris buffered saline with Tween-20, 10 mM Tris, 0.15 mM NaCl, 0.05% Tween-20, pH 7.4) for 1.5 h to block nonspecific binding, and then incubated overnight at 4°C with a polyclone antibody against chicken OC at a 1:5,000 dilution, followed by a horseradish peroxidase conjugated secondary antibody at a 1:5,000 dilution for 1.5 h. Visualization of the site of antigen-antibody complex was carried out with chemiluminescence solution (Immobilon Western Chemiluminescent HRP Substrate, Millipore, Billerica, MA). Immunoreactive bands were detected using the Bio-Rad GelDoc 2000 imaging system (Bio-Rad). For internal control, mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used. The bands were analyzed by Quantity One software. The OC value was represented as the ratio of the optical density of OC band to the density of the related GAPDH band.

### Statistical Analysis

Statistical analysis was performed using SPSS 16.0 with one-way ANOVA. Data were expressed as the mean ± SE. The least significant difference method was used, and the nonparametric tests were performed if data lacked homogenous variances. The P < 0.05 was accepted as a significant difference. The coefficient between the concentrations of serum OC, blood biochemistry, and bone strength parameters were analyzed using Pearson correlations.

### RESULTS

#### Egg Production, Lipid Storage, and Bone Profiles

One bird of the experimental group died during the study, and pathological examination showed the liver...
and liver blood vessels were crisp (distortion of tissue architecture resulting from chronic inflammation) and broken. However, there was no difference between treatments on hen mortality \( (P = 0.31; \text{data not shown}) \).

Average egg production was reduced in HE-LP hens compared with that of control hens, especially at wk 5, 7, and 9 to 11 \( (P < 0.01, \text{Figure 1}) \).

Compared with control hens, HE-LP hens consumed less feed at all of the tested periods during the treatment \( (\text{controls vs. HE-LP hens}: 99.5 \pm 1.2 \text{ g vs. } 96.5 \pm 1.1 \text{ g at 1 to } 6 \text{ d}, P = 0.09; 97.7 \pm 0.9 \text{ g vs. } 94.3 \pm 1.1 \text{ g at 35 to } 40 \text{ d}, P = 0.03; 99.3 \pm 1.3 \text{ g vs. } 94.7 \pm 1.2 \text{ g at 65 to } 70 \text{ d}, P = 0.01, \text{Table 3}) \). There was no difference in BW before \( (\text{d } 0, \text{controls vs. HE-LP hens}: 1.85 \pm 0.05 \text{ kg vs. } 1.85 \pm 0.04 \text{ kg}, P = 0.99) \) and after the treatment \( (\text{d } 80, 1.88 \pm 0.06 \text{ kg vs. } 1.92 \pm 0.06 \text{ kg}, P = 0.66, \text{Table 3}) \). In addition, there was no difference in the relative liver weight between control hens and HE-LP hens \( (P = 0.31) \). However, compared with control hens, HE-LP hens had a greater abdominal fat pad weight \( (P = 0.007) \) and liver fat content \( (P = 0.03) \).

There were no treatment effects on hens’ tibia length, width, strength, and density \( (\text{Table 4}) \).

**OC and OPG mRNA Expression and OC Protein Concentrations in the Keel Bone**

The expression of both OC mRNA and OPG mRNA were not different among hens from the 2 treatments \( (P = 0.39 \text{ and } P = 0.34, \text{respectively; Figure 2}) \). The concentrations of OC protein of the keel bone were decreased in HE-LP hens compared with those of control hens \( (P = 0.05, \text{Figure 3}) \).

**Bone Remodeling-Associated Factors**

There were no differences in blood concentrations of calcium and phosphorus among hens from the 2 treatments \( (P > 0.05; \text{data not shown}) \). However, HE-LP hens had higher serum ALP activity \( (P = 0.03) \), OC \( (P = 0.05) \), leptin-like protein \( (P = 0.02) \), and ES concentrations \( (P = 0.001) \) at d 80 than those of control hens; and the serum ALP activity \( (P = 0.08) \) and OC concentrations \( (P = 0.10) \) also tended to increase in HE-LP hens at d 60 \( (\text{Figure 4}) \). There was an age effect on the concentrations of serum OC, leptin-like protein, and ES in control hens but not HE-LP hens. The concentrations of serum OC, leptin-like protein, and ES gradually declined from d 0 to 80 during the treatment \( (\text{from } 63 \text{ to } 75 \text{ wk of age}) \), but ES concentrations were not different \( (\text{Figure 4}) \).

The concentrations of serum OC were positively correlated with the concentrations of serum leptin-like protein regardless of treatment \( (\text{control hens: } r = 0.87, P = 0.01; \text{HE-LP hens: } r = 0.82, P = 0.04; \text{Table 5}) \). The concentrations of serum OC also had a positive relationship with the concentrations of serum ES in HE-LP hens \( (r = 0.67, P = 0.02) \). Positive correlations between OC and bone density \( (r = 0.70, P = 0.01) \), bone strength parameters including force at failure point of the tibia \( (r = 0.59, P = 0.04) \), and force at yield point bone strength \( (r = 0.59, P = 0.04) \) were found for control hens.

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**Table 3.** Effects of high energy and low protein diet on feed intake and lipid storage

<table>
<thead>
<tr>
<th>Group</th>
<th>BW (kg/bird)</th>
<th>Feed intake (g/bird)</th>
<th>Relative abdominal fat pad mass (g/kg)</th>
<th>Relative dry liver mass (g/kg)</th>
<th>Liver fat content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.88 ± 0.06</td>
<td>99.3 ± 1.3</td>
<td>34.8 ± 2.5</td>
<td>5.8 ± 0.3</td>
<td>24.9 ± 2.0</td>
</tr>
<tr>
<td>HE-LP</td>
<td>1.92 ± 0.06</td>
<td>94.7 ± 1.2*</td>
<td>50.0 ± 4.3**</td>
<td>6.3 ± 0.5</td>
<td>33.5 ± 3.2*</td>
</tr>
<tr>
<td>n³</td>
<td>10</td>
<td>50</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>P-value</td>
<td>0.66</td>
<td>0.01</td>
<td>0.007</td>
<td>0.31</td>
<td>0.03</td>
</tr>
</tbody>
</table>

1Values indicated mean ± SE at d 80 during the process of the treatment.
2HE-LP: hens fed high energy and low protein.
3Sample numbers per group.
*P ≤ 0.05, **P ≤ 0.01, compared with the controls.

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**Figure 1.** The effect of a high energy and low protein (HE-LP) diet on egg production. Compared with control hens, HE-LP hens had lower egg production at wk 5, 7, and 9 to 11 during the treatment. **P < 0.01 compared with the levels of control hens. Data were expressed as the mean ± SE.
In addition, there were several correlations among the measured indexes regardless of treatment. Serum ALP activity had a negative correlation with the tibia stiffness \( (r = -0.58, P = 0.01; \text{Table 6}) \); concentrations of leptin-like protein had a positive correlation with both liver fat content \( (r = 0.48, P = 0.04) \) and abdominal fat pad mass \( (r = 0.46, P = 0.05) \); and the concentrations of both serum OC and ES had positive correlations with leptin-like protein and abdominal fat pad mass but not liver fat content, respectively \( (P < 0.01) \).

**DISCUSSION**

Fatty liver is a chronic condition associated with a positive energy balance, resulting in excessive fat accumulating in the liver cells, which lose their functions gradually and are destroyed. Fatty liver syndrome is frequently seen in chickens, especially in layers and broiler breeders (Harms et al., 1982; Hansen and Walzem, 1993; Hermier, 1997; Scheele, 1997). The liver is a premium source for synthesizing proteins and peptides, which may function as hormones or enzymes that are directly or indirectly involved in energy and bone metabolism (Watkins et al., 1997; Liu et al., 2004). The present results indicate that the HE-LP diet successfully induced fatty liver disorder in laying hens with an upregulated bone turnover, exacerbating skeletal damage. The data support our hypothesis that changes in fat metabolism decrease skeletal health and aggravate the development of osteoporosis in laying hens.

There was no difference in hen mortality between treatments. However, compared with control hens, HE-LP hens had a significantly higher liver fat content, higher abdominal fat pad weight, lower egg production, and higher concentrations of leptin-like protein and ES. These pathophysiological changes indicate that fatty liver disorder had been successfully induced in HE-LP hens, similar to the findings reported in a previous study (Julian, 2005). However, relative liver mass was not significantly affected by treatment. Although the pathological examination was not conducted in HE-LP hens, the present and previous studies may suggest that hepatic steatosis is the main symptom rather than an increase in liver mass in the hens with fatty liver disorder (Ayala et al., 2009). Based on the pathological changes, the hyperlipidemic chicken has been claimed as a useful model of nonalcoholic steatohepatitis in human research (Ayala et al., 2009).

Osteocalcin is a special marker of bone turnover in humans and rodents (Christenson, 1997; Ugar and Karaca, 1999; Seibel, 2005). Similar to the findings in human and rodent studies, the current results support that OC is involved in bone remodeling in laying hens.

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**Table 4.** Effects of high energy and low protein diet on tibia properties

<table>
<thead>
<tr>
<th>Group</th>
<th>Length (mm) ± SE</th>
<th>Diameter (mm) ± SE</th>
<th>Force at failure point (N) ± SE</th>
<th>Force at yield point (N) ± SE</th>
<th>Stiffness (N/m) ± SE</th>
<th>Radiographic density (mm Al) ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11.5 ± 0.16</td>
<td>0.71 ± 0.01</td>
<td>171.0 ± 12.6</td>
<td>122.5 ± 11.6</td>
<td>367,419 ± 17,911</td>
<td>3.3 ± 0.4</td>
</tr>
<tr>
<td>HE-LP</td>
<td>11.4 ± 0.13</td>
<td>0.74 ± 0.01</td>
<td>154.4 ± 8.3</td>
<td>112.5 ± 7.5</td>
<td>324,675 ± 45,514</td>
<td>3.1 ± 0.5</td>
</tr>
<tr>
<td>n</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>P-value</td>
<td>0.70</td>
<td>0.10</td>
<td>0.29</td>
<td>0.48</td>
<td>0.92</td>
<td>0.61</td>
</tr>
</tbody>
</table>

1Values represent mean ± SE at d 80 during the process of the treatment.  
2HE-LP: hens fed high energy and low protein.

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**Figure 2.** The effect of a high energy and low protein (HE-LP) diet on osteocalcin (OC) mRNA and osteoprotegerin (OPG) mRNA expression in laying hens. A. The ratio of OC mRNA to β-actin mRNA. B. The ratio of OPG mRNA to β-actin mRNA. Data were expressed as the mean ± SE.
The concentrations of serum OC significantly decreased with age in control hens but not in HE-LP hens, coinciding with the reports that osteoporosis is progressively aggravated with age (Webster, 2004; Whitehead, 2004). In the present study, bone density had a positive correlation with serum OC concentrations in control hens at d 80 posttreatment. The relationship was absent in the HE-LP hens, which had greater concentrations of serum OC than control hens. It suggests the pathophysiological changes associated with fatty liver disorder interfere with the homeostasis of OC in regulating bone turnover, by which bone health is reduced.

In supporting the hypothesis, Liu et al. (2003, 2004) reported that long-term dietary lipids intake had a significant adverse effect on bone metabolism by changing the production of regulatory factors involved in bone remodeling in birds. Watkins et al. (1997) also reported that dietary fat affected bone formation rate in the tibia of chickens by controlling the production of regular factors in bones.

There were not significantly differences in the levels of both OC and OPG mRNA between hens from the 2 treatments. The present results indicate that the biological process associated with the HE-LP diet induced changes of bone remodeling in hens is most likely at the level of translation, synthesizing proteins, rather than transcription. In HE-LP hens, compared with control hens, serum OC concentrations were increased but bone OC concentrations were decreased, which suggests there was a greater bone turnover and OC was released from the bone matrix into the blood. The increased serum OC concentrations could be in the form of unOC, although we did not measure the OC subtypes. Previous studies have evidence that carboxylated OC is synthesized and incorporated into bone matrix during bone formation, whereas unOC is released into the blood during bone resorption (Ferron et al., 2010). The current results may indicate that the HE-LP diet caused an increase in bone resorption. Similar to the current results, Liu et al. (2004) reported that dietary lipids caused a decrease in mineral content in the tibia by increasing bone resorption.

Alkaline phosphate is another bone formation marker in animals including chickens (Christenson, 1997; Seibel, 2005). However, unlike mammals, there is little detectable ALP in the liver and none in the lung, skeletal muscle, and the heart in chickens (Christenson, 1997; Seibel, 2005). In chickens, elevated ALP activity has been predominantly related to increased osteoblastic activity and used as a marker for evaluating skeletal health and bone disease, such as skeletal growth, nutritional secondary hyperparathyroidism, rickets, fracture repair, and osteomyelitis (Lumeij and Westerhof, 1987). In the current study, there was a negative correlation

### Table 5. The correlations of serum osteocalcin (OC) concentrations with various bone remodeling associated measures

<table>
<thead>
<tr>
<th>Item</th>
<th>Control hens</th>
<th>HE-LP&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Serum OC concentration</td>
<td>Serum OC concentration</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Item</td>
<td>r&lt;sup&gt;2&lt;/sup&gt;</td>
<td>P-value</td>
</tr>
<tr>
<td>Total calcium</td>
<td>−0.24</td>
<td>0.25</td>
</tr>
<tr>
<td>Inorganic phosphate</td>
<td>0.13</td>
<td>0.36</td>
</tr>
<tr>
<td>Alkaline phosphate</td>
<td>0.39</td>
<td>0.13</td>
</tr>
<tr>
<td>Leptin-like protein</td>
<td>0.87</td>
<td>0.001**</td>
</tr>
<tr>
<td>Estrogen</td>
<td>0.46</td>
<td>0.09</td>
</tr>
<tr>
<td>Force at failure point</td>
<td>0.59</td>
<td>0.04*</td>
</tr>
<tr>
<td>Stiffness</td>
<td>0.59</td>
<td>0.04*</td>
</tr>
<tr>
<td>Force at yield point</td>
<td>0.30</td>
<td>0.20</td>
</tr>
<tr>
<td>Density</td>
<td>0.70</td>
<td>0.01**</td>
</tr>
</tbody>
</table>

<sup>1</sup>HE-LP = hens fed high energy and low protein.
<sup>2</sup>The correlations were analyzed by Pearson correlation.

*P < 0.05; **P ≤ 0.01.
between ALP activity and bone stiffness, indicating that increased ALP activity in HE-LP hens was more likely to indicate higher bone remodeling with weaker bone strength. A similar correlation between ALP activity and bone strength was proposed by Hassanabadi et al. (2007). The study demonstrated that a lower calcium diet induced a significantly increased ALP activity in laying hens.

**Table 6.** The correlations of fat deposition, blood measures, and bone strength

<table>
<thead>
<tr>
<th>Item</th>
<th>Liver fat content</th>
<th>Fat pad mass</th>
<th>ALP</th>
<th>OC</th>
<th>Leptin-like protein</th>
<th>ES</th>
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<td>Liver fat content</td>
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</tbody>
</table>

1ALP: alkaline phosphate activity; ES: estrogen; OC: osteocalcin.
2The correlations were analyzed by Pearson correlation.
3*P ≤ 0.05; **P ≤ 0.01.

Figure 4. The effects of a high energy and low protein (HE-LP) diet on serum concentrations of alkaline phosphates (ALP), osteocalcin (OC), estrogen (ES), and leptin-like protein in laying hens. A. Serum ALP concentration; B. serum osteocalcin (OC) concentrations; C. serum ES concentrations, and D. serum leptin-like protein concentrations. #P < 0.05 and ##P < 0.01 compared with the levels of control hens. *P < 0.05 and **P < 0.01 compared with the level at d 0 within the same treatment. Data were expressed as the mean ± SE.
Adipocytes are one of the sources of ES synthesis (Siiteri, 1987). Serum ES concentrations undergo age-related changes, being positively correlated with age-related declines in egg production and molting in laying hens (Beck and Hansen, 2004). In the present study, control hens displayed an age-related decline in the concentrations of ES. Compared with control hens, HE-LP hens had a higher ES concentration at d 80 posttreatment. In addition, there was a positive correlation between ES and serum OC concentrations in HE-LP hens. It may suggest the fat intake-associated change in blood ES concentrations related to osteoblast activities, resulting in increasing blood OC concentrations. The results further support the hypothesis that both ES and OC functionally integrate with osteoblast activity (Chen et al., 2002). However, the cellular mechanisms that regulate the effects of HE-LP diet on ES metabolism in laying hens are unclear. It may be related to fatty liver disorder and the resultant liver damage. A previous study reported that the high ES concentration was a unique feature of fatty liver hemorrhagic syndrome in laying hens (Hansen and Walzem, 1993). Hepatic steatosis has deleterious effects on ES metabolism in humans and laying hens (Adlercreutz, 1970; Haghighi-Rad and Polin, 1981). In both humans and birds, ES enhances osteoblast activity, but its effects are distinctly different for each species. Unlike its effects on bone remodeling in humans (Reid, 2002), ES increased bone formation but failed to improve bones strength in chickens (Wilson and Thorp, 1998).

In control hens but not in HE-LP hens, the concentrations of serum leptin-like protein decreased with age. Similar results were reported in a study conducted in humans and found that circulating leptin concentrations were inversely related to age, with around a 53% decrease in subjects over 60 yr of age (Ostlund et al., 1996). The effects of age on the concentrations of leptin-like protein were not observed in HE-LP hens. In addition, compared with control hens, the leptin-like protein concentrations were significant higher in HE-LP hens at d 80 posttreatment. The reasons for the increase in leptin-like protein concentrations in HE-LP hens could be related to increased liver fat content and abdominal fat pad mass as a result of imbalance in fat metabolism. There was a significant correlation between the concentrations of leptin-like protein and serum OC in both control hens and HE-LP hens. This further suggests that there is a direct linkage between adipokines and bone metabolism in laying hens.

In conclusion, the present study provides evidence that fatty liver disorder can be induced in laying hens by feeding the HE-LP diet. Although HE-LP hens had a high bone turnover, their bone health was not improved, even aggravating the incidence of skeletal damage. The positive correlations between the concentrations of ES, leptin-like protein, and serum OC further suggest that adipokines and bone metabolism are directly linked in regulating bone remodeling in laying hens. These data provide evidence that is valuable for modifying diets to control bone damage and osteoporosis in laying hens.

ACKNOWLEDGMENTS

This work was supported by the Program for Postgraduates Research Innovation of University of Jiangsu Province (CXXZ11-0681), National Natural Science Foundation of China (No. 30972234 and 31272618), and Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

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REFERENCES


