Untargeted metabolomics study on the effects of rearing ducks in cages on bone quality

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ABSTRACT The cage rearing model of the modern poultry industry makes the bones of birds more vulnerable to deterioration. In this study, at 8 wk of age, a total of 60 birds were randomly allocated to 2 groups, including the floor rearing group (FRD) and cage rearing group (CRD), and their body weight was measured every 2 wk. At the age of 20 wk, the tibia, femur, and humerus were collected from each group (n = 12) to determine the bone quality parameters such as weight, size, bone mineral density (BMD), breaking strength, cortical thickness, and area, ash content, calcium (Ca) content, and phosphorus (P) content. Meanwhile, the serum metabolome composition of both groups was detected by untargeted metabolome technology. The results showed that there were no significant differences in body weight, bone weight, and size between the 2 groups (P > 0.05), but the humerus mineral density and the breaking strength, cortical bone thickness, cortical bone area percentage of tibia, femur, and humerus of CRD was significantly lower than those of FRD (P < 0.05), indicating that the cage rearing system caused the deterioration of bone quality. Based on nontarget metabolomics, 49 metabolites were correlated with bone quality parameters, and 10 key metabolites were strongly correlated, including erucic acid, citric acid, and ketoleucine. In addition, the KEGG analysis showed that the caged system mainly perturbed amino acid metabolism, lipid metabolism, and energy metabolism, which led to changes in related metabolite levels, produced ROS, and altering energy supply, thus leading to a deterioration of bone quality of cage rearing ducks. Therefore, our findings were helpful to further understand the potential mechanism of the deterioration of duck bone quality in cage rearing system, provided a theoretical basis for reducing the occurrence of poultry osteoporosis, and ensuring the healthy development of poultry breeding.

Key words: rearing system, metabolomics, bone quality, duck

INTRODUCTION

As modern poultry farming has become more standardized, the traditional floor rearing system has been gradually replaced by a cage rearing system. The cage rearing system is easier and more convenient to administer than a floor rearing system, and the farmer can get higher returns by increasing the poultry density (Duncan, 2001). Cage-reared birds have limited movement and are more susceptible to bone loss and bone density reduction. Furthermore, poor bone quality is more likely to generate bone fractures and increased mortality (Webster, 2004; Meng et al., 2017). It was estimated that bone problems are responsible for 13% of all bird deaths in cages (Onbasilar et al., 2016). Broken bones can puncture muscles and blood vessels, contaminating and degrading meat. Infection, low productivity, and poor meat quality due to fractures negatively impact the economics and welfare of poultry farming (Hughes et al., 1993).

Bones are subjected to different forces or combinations of forces (axial compression or stretching, shearing, twisting, or bending) during normal function. The fracture occurs when the forces exceed the ultimate strength of the bone. The maximum bone strength is related to the mechanical properties of bone and varies significantly among species and individuals (Sabater Gonzalez, 2019). The macroscopic mechanical properties of bone are jointly affected by its density, macroscopic size, microstructure, and material properties (Ammann and...
Bone quality parameters are related to age, sex, hormonal, nutritional, and environmental factors. Research shows that hens raised on the floor system had stronger bones than their contemporaries maintained in cages (Newman and Leeson, 1998). Additionally, some bone quality parameters, such as cortical bone density, cortical bone thickness, and bone ash content, were higher in cage-reared birds than floor-reared birds (Regmi et al., 2015). Cage-reared birds are restricted in movement, while floor rearing allows more space for load-bearing exercises, such as wing-flapping, walking, or running, which in theory could change the properties of bones and make them stronger (Newman et al., 1998). Some studies have demonstrated that mechanical loading positively affects bone formation and bone quality (Robling et al., 2008; Ming et al., 2013). On the other hand, lack of exercise has been reported to lead to osteoporosis in birds (Fleming et al., 2006).

Generally, the deterioration of bone quality is affected by many factors, and the main reason is the bone loss caused by the imbalance of bone metabolism. The self-regulation process of bone tissue to adapt to changes in the external environment is called bone metabolism, composed of 2 parts: bone formation led by osteoblasts and bone resorption led by osteoclasts. When bone metabolism is in a negative balance, the rate of bone formation is lower than that of bone absorption, resulting in irreversible bone loss and increased risk of bone fragility and fracture (Blair et al., 2002; Armas and Recker, 2012). The bone formation and resorption indices can indirectly reflect bone metabolic rate. Bone formation indexes can be byproducts produced in bone formation and the enzymes secreted by osteoblasts. Bone resorption indexes can be collagen metabolites and enzymes secreted by osteoclasts.

Untargeted metabolomics can perform qualitative and quantitative analysis of small molecule metabolites in serum simultaneously. Many metabolites are involved in bone turnover, which may have complex interactions. The metabolome can dynamically and comprehensively display the status of bone metabolism, which may provide more information for bone quality assessment. Therefore, metabolomics has been widely used in human osteoporosis studies to determine biochemical markers of bone metabolism in serum or urine to evaluate fracture risk and bone quality (Lv et al., 2016). For example, You et al. used metabolomics to investigate the relationship between plasma metabolome and BMD in Taiwanese women and identified 7 metabolites associated with low BMD (You et al., 2014). Other researchers have also found some metabolic markers associated with osteoporosis in humans through metabolomics (Zhu et al., 2016; Miyamoto et al., 2018). In addition, researchers also used metabolomics to reveal the effects of drugs on osteoporosis (Liu et al., 2012; Pan et al., 2016).

In this study, the bone quality of cage rearing ducks and floor rearing ducks was evaluated by measuring bone weight, size, BMD, breaking strength, cortical thickness and area, ash content, calcium (Ca) content, and phosphorus (P) content to explore the bone deterioration condition of ducks after caged rearing. At the same time, LC-MS metabolomic analysis was applied to find out marker metabolites associated with bone mass, to reveal the critical factor affecting the quality of bone and explore the mechanism of this effect to provide a theoretical basis for reducing the occurrence of poultry osteoporosis, and also provide a reference for the study of human osteoporosis.

MATERIALS AND METHODS

Materials

The animal use protocol listed below has been reviewed and approved by the Sichuan Agricultural University Animal Ethical and Welfare Committee. A total of 60 female ducks of Nonghua variety used in the experiment were provided by the Waterfowl Breeding Farm of Sichuan Agricultural University. Before 8 wk of age, all ducks were reared in a floor rearing system and were fed ad libitum with the same basal diets (Dietary Nutrients are shown in Supplementary Table 1). In-floor rearing system, 90 ducks were housed per floor pen (78 m²) with 5 cm thick sawdust bedding covering the concrete floor. The floor was equipped with a feeder, nipple drinker, and laying nest. At 8 wk of age, the ducks were randomly divided into the floor rearing group (FRD) and cage rearing group (CRD). Each duck was given 260 g standard layer duck mash diets per day (Dietary Nutrients are shown in Supplementary Table 1), and the individual body weight was measured every 2 wk. FRD continues to be fed in the floor rearing system. Another group of ducks was reared in a cage rearing system, where the stainless mesh bed with 1.0 cm diameter holes was set at the height of 50 cm above the ground. Single cage size 300*450*500 mm and was equipped with long feeder and nipple drinker. The temperature of the ducks’ room was maintained between 20 and 30°C. In the 20th wk, 12 ducks of similar weight from each group were selected and euthanized at the am. After exsanguination, the left tibia, femur, and humerus were collected and stored in a freezer at -20°C until analysis. In addition, 6 ducks from each group were randomly selected from the above ducks. Before euthanasia, blood samples were individually collected from the wing vein and then were centrifuged at 4,500 r/min for 10 min at 4°C to obtain serum.

Bone Size and Weight

Bone length, midpoint diameter, and midpoint perim-eter of the tibia, femur, and humerus were measured and recorded. We measured the length and midpoint diameter with vernier calipers. Wrap a cotton thread around the bone midpoint 5 times, then we measure the length
of the cotton thread with a ruler to calculate the perimeter. The bone weight was determined with an electronic balance (1/10,000).

**Bone Mineral Density**

The bones were thawed at room temperature and then taken to the Wenjiang Branch, West China Hospital, for dual-energy X-ray absorptiometry (DXA). Tibia, femur, and humerus were carefully arranged on the bottom of a box filled with water such that the bones were lined up perpendicular to the gantry. Scans were performed and analyzed using the minor animal mode (version 8.10) of the DXA device (Lunar iDXA, GE Medical Systems Ultrasound & Primary Care Diagnostics LLC, Madison, WI).

**Bone Strength**

After fundamental characteristics analysis, these bones were subjected to strength tests. The bending test of bone mid diaphysis was performed on a testing machine (TA. XTplus, stable microsystems, Vienna court Lammas Road, Godalming, Surrey, UK) equipped with an interchangeable load cell (Range of forces from 0 to 60,000 g) for bone mechanical properties assay. The distances between the 2 round support bars were 3 cm for the tibia, 5 cm for the femur, and 5 cm for the humerus. The load was applied in the midpoint of the bone at a 2 mm/min displacement rate until fracture.

**Cortical Bone Area and Thickness**

The bones were cut with a precision saw and collected at the midpoint of the bone with a thickness of 4 mm. After being directly scanned on a microscope, the resultant images were analyzed using ImageJ’s public domain software program (National Institutes of Health, MD). The cortical bone area and percentage of cortical bone area in the cross-sectional area of bone were calculated. The cortical bone thickness was determined with a vernier caliper. Four points were selected in the bone section to measure the thickness with a vernier caliper, then the average value was calculated as the cortical bone thickness of the bone.

**Bone Ash, Calcium, and Phosphate Contents**

The percentage of ash was expressed relative to the fat-free dry bone weight. Rapid determination of calcium by EDTA complexometric titration was used to analyze the content of Ca, and the phosphorus vanadium molybdate yellow colorimetric method was used to analyze the content of P (Zhang, 2007). After the samples were mixed with ammonium vanadomolybdate reagent, the content of P was determined by spectrophotometer (VLBL00D1-3020, Thermo Fisher Scientific Oy Ratastie 2, FI-01620 Vantaa, Finland). The standard curve was drawn with standard phosphorus concentration as abscissa and absorbance as ordinate (as shown in Supplementary Figure 1).

**Preparation of the Serum Samples for Metabolomics Assay**

Six serum samples from each group (CRD and FRD) were selected and thawed at 4°C. The 100 μL sample was added to the EP tube with 400 μL methanol (-20 °C) and vortex for 60 s. After being centrifuged at 12000 rpm for 10 min at 4 °C, all the supernatant was transferred into another 2 mL centrifuge tube and was concentrated to dry in a vacuum. Then the sample was dissolved with 150 μL 2-chlorobenzaldehyde (4 ppm) 80% methanol solution, and the supernatant filtered through a 0.22 μm membrane to obtain the prepared samples for LC-MS. Quality control (QC) samples were pooled by 20 μL from each sample, and the rest of the samples were used for the LC-MS analysis.

**LC-MS Analysis**

LC-MS analyses were performed using a liquid chromatograph (UltiMate 3000, Thermo Fisher Scientific, MA) equipped with an ACQUITY UPLC HSS T3 (150 × 2.1 mm, 1.8 μm, Waters) column maintained at 40°C. The mobile phase consisted of 0.1% formic acid in water (B1), and 0.1% formic acid in acetonitrile (A1) or 5 mM ammonium formate in water (B3), and acetonitrile (A3) was carried with elution gradient as follows: 0–1 min, 2% A1/A3; 1–9 min, 2–50% A1/A3; 9–12 min, 50–98% A1/A3; 12–13.5 min, 98% A1/A3; 13.5–14 min, 98–2% A1/A3; 14–20 min, 2% A1-positive model (14–17 min, 2% A3-negative model). The ESI-MSn experiments were executed on the mass spectrometer (Q Exactive Plus, Thermo Fisher Scientific, MA). The following parameters were employed: positive ion spray voltage 3.50 kV, normalized collision energy was 30 eV. Sheath gas 30 arbitrary units, auxiliary gas 10 arbitrary units, and capillary temperature 325°C. The analyzer scanned over a mass range of m/z 81-1,000 for a full scan at a mass resolution of 70,000. Data-dependent acquisition (DDA) MS/MS experiments were performed with an HCD scan. The normalized collision energy was 30 eV. Dynamic exclusion was implemented to remove some unnecessary MS/MS spectra information.

**Metabolomic Data Analysis**

After the original data is converted into MZXML format, a series of operations, including peaks identification, peaks filtration, and peaks alignment, were conducted using the XCMS package of R (v3.3.2). The obtained data matrix was exported to Microsoft Excel for analysis. Ion peaks with a variation coefficient of over 30% were deleted, and the batch normalization of the intensity of the data was carried out. Simac 14.1 (Umetrics, Sweden) was used to perform principal
component analysis (PCA) and orthogonal projections to latent structures-discriminant analysis (OPLS-DA) on the metabolic data of all serum samples to assess the metabolite diversity between and within-group samples. Metabolites were annotated using the HumanMetabolome Database (HMDB) (http://www.hmdb.ca), Metlin (http://metlin.scripps.edu), massbank (http://www.massbank.jp/), LipidMaps (http://www.lipidmaps.org), mzcloud (https://www.mzcloud.org) and the self-built standard product Database. The screening criteria for differential metabolites were \( P \)-value \( \leq 0.05 \) and Variable Importance in Projection (VIP) \( \geq 1 \). Agglomerate hierarchical clustering was performed for each data set using the PHEATMAP package in R (v3.3.2).

**Differential Metabolites and Metabolic Pathway Correlated With Bone Quality**

The associations between differential metabolites and bone quality were evaluated by Spearman rank correlation analysis. Differential metabolites with a correlation coefficient greater than 0.5 and a \( P \)-value less than 0.05 were imported into the METPA database (Kanehisa and Goto, 2000). KEGG enrichment was performed with differential metabolites to analyze metabolic pathways related to differential metabolites.

**Statistical Analysis**

All bone quality parameter data were analyzed using the SPSS software, version 21 (International Business Machines Corporation, Armonk, NY). A nonparametric test was used for the analysis of group differences. The data were presented as means \( \pm \) SEM, and differences were considered statistically significant at \( P < 0.05 \). The associations were evaluated by Spearman correlation analysis.

**RESULTS AND ANALYSIS**

**Effects of Different Rearing Systems on Duck BW and Bone Quality Parameters**

As shown in Figure 1A, the bodyweight of CRD increased from wk 8 to wk 10 and decreased to wk 12, followed by another increase to wk 20. The bodyweight of FRD decreased slightly from wk 8 to wk 10 and increased gradually to 20 wk of age. Compared with CRD, the bodyweight of FRD in 10 wk was lower (\( P < 0.05 \)). The bodyweight of FRD controlled on 12, 14, 16, 18, and 20 wk of age was higher than CRD but did not differ between the 2 flocks (\( P > 0.05 \)).

The morphology of the tibial, femur, and humerus of CRD and FRD at 20 wk of age was presented in Figure 1C. The bone morphometry was not significantly

![Figure 1](image-url)  
**Figure 1.** Effects of rearing systems on body weight and bone quality parameters in ducks. (A) The body weight development in cage rearing ducks and floor rearing ducks from 8 to 20 wk of age. All values are expressed as Means \( \pm \) SEM, \( n = 30 \). (B) Radiographic images of the tibia, femur, and humerus of CRD and FRD at 20 wk of age. C, Tibia, femur, and humerus of CRD and FRD at 20 wk of age. Abbreviations: CRD, cage rearing ducks; FRD, floor rearing ducks.
affected by different rearing systems. There were no significant differences between FRD and CRD in weight, length, diameter, and perimeter of the tibia, femur, and humerus (P > 0.05) (Supplementary Table 2). The X-ray films of bones of CRD and FRD are shown in Figure 1B, and the results showed that the CRD exhibited a lower BMD of humerus than FRD (P < 0.05), however, there was no significant difference in BMD of femur and tibia between the 2 groups (P > 0.05). In addition, as shown in Table 1, rearing systems significantly affected the bone-breaking strength, cortical thickness, percentage of the cortical area, and calcium content. The breaking strength, percentage of the cortical area, and cortical thickness of the tibia, femur, and humerus of FRD were significantly higher than CRD (P < 0.05). However, there was no significant (P > 0.05) difference in bone ash content, calcium content, and phosphorus content between FRD and CRD.

**Correlation Analysis of Bone Quality Parameters**

In the current study, a correlation analysis developed using all bone quality parameters sets (different rearing systems) for tibia femur and humerus showed a positive correlation between humerus breaking strength, percentage of humerus cortical area, tibia cortical thickness, tibia breaking strength, percentage of tibia cortical area, humerus cortical thickness, and percentage of femur cortical area. In addition, the tibia BMD was positively correlated with femur BMD and humerus BMD. The femur breaking strength was positively correlated with the femur cortical thickness and tibia breaking strength (Figure 2, Supplementary Table 3).

### Table 1. Effects of different rearing systems on bone quality parameter.

| Bone quality parameter | Tibia | CRD  | FRD  | Femur | CRD  | FRD  | Humerus | CRD  | FRD  |
|------------------------|-------|------|------|-------|------|------|---------|------|------|
| BMD                    | 0.33 ± 0.12 | 0.29 ± 0.02 | 0.34 ± 0.18 | 0.27 ± 0.02 | 0.26 ± 0.03   |
| Breaking strength (kg)  | 34.21 ± 6.76 | 47.75 ± 9.56 | 38.03 ± 7.02 | 57.7 ± 12.6 | 32.83 ± 7.68 |
| Percentage of cortical area (%) | 47.97 ± 3.61 | 53.04 ± 2.68 | 37.6 ± 3.7 | 44.18 ± 2.36 | 27.41 ± 2.33 |
| Cortical thickness (mm) | 0.81 ± 0.08 | 0.9 ± 0.08 | 0.74 ± 0.1 | 0.83 ± 0.08 | 0.61 ± 0.04 |
| Bone ash content (%)    | 62.62 ± 3.44 | 60.29 ± 2.44 | 56.5 ± 6.13 | 53.91 ± 1.65 | 63.28 ± 1.73 |
| Calcium content (%)     | 25.06 ± 1.32 | 25.13 ± 0.55 | 22.49 ± 2.35 | 21.57 ± 0.45 | 23.97 ± 0.75 |
| Phosphorus content (%)  | 11.05 ± 0.58 | 11.24 ± 0.33 | 9.7 ± 1.05 | 9.41 ± 0.3 | 10.57 ± 0.51 |

Note: All values are expressed as means ± SEM, n = 12. a,b Means within rows with no common superscript differ significantly (P < 0.05). Abbreviations: BMD, bone mineral density; CRD, cage rearing ducks; FRD, floor rearing ducks.

### Multivariate Statistical Analysis of Metabolomics Assay

The comprehensive metabolomic analysis was performed of sera from ducks in 2 groups, and the base peak chromatogram obtained is shown in Supplementary Figure 2. To distinguish cage rearing ducks from the floor rearing ducks, the metabolic profile obtained from LC-MS was plotted using PCA and OPLS-DA. In the PCA score (Figure 3A,B), all QC samples showed a high degree of aggregation, indicating the excellent stability of the analytical platforms, and the notable differences ascended from an intrinsic discrepancy between groups rather than
instrumental movement. As shown in Figure 3A, B, the clustering trend displayed that the CRD was separated evidently from FRD, demonstrating that the serum metabolic state of ducks changed obviously after cage rearing. To select differential metabolites, the obtained metabolic profile of the serum sample was resolved by a more sophisticated OPLS-DA, in which the clustering of CRD was separated from the FRD in Figure 3C, D. The corresponding loading plot to identify biomarkers is shown in Figure 3E, F. The ions farther away from the origin point contribute significantly to the clustering of the 2 groups.

Screening and Correlation Analysis of Differential Metabolites

The base peak chromatogram detected 3,935 and 4,582 metabolites in positive and negative ion modes, respectively. Then the variable importance in the projection VIP value >1 of the first principal components of the OPLS-DA model, combined with the P-value < 0.05 of the t-test, was used to find the differential metabolites. The total number of different metabolites between the FRD and CRD was 1401, as shown in Supplementary Table 4, in which 61 metabolites are annotated. A heat-map was constructed to visualize the changes of these metabolites in different groups, 33 molecules were down-regulated, and 28 were up-regulated in CRD (Figure 4A). The correlation analysis among annotated differential metabolites is shown in Figure 4B and Supplementary Table 5.

Metabolites Correlated With the Bone Quality Parameter

The correlation analysis was performed to investigate the relationship between the annotated differential metabolites and bone quality parameters. As shown in Figure 5 and Supplementary Table 6, we found 49
differential metabolites, and they were correlated with 21 bone quality parameters, respectively. Among them, 39 metabolites were weakly correlated with bone quality parameters ($0.80 > \text{correlation coefficients} > 0.50$ and $P < 0.05$), and 10 metabolites strongly correlated with bone quality parameters (correlation coefficients $> 0.80$ and $P < 0.05$) were found as follows: 1,8-cineole, 5-valerolactone, citric acid, 12-keto-leukotriene B4, erucic acid, ketoleucine, d-arabitol, 8-hydroxyquinoline, methylmalonic acid, and 2-hydroxybutyric acid.

Metabolic Pathway Perturbation Affecting Bone Quality

Differential metabolites were imported into the MetaboAnalyst 4.0, and potential metabolic pathways were enriched through metabolic pathway concentration and topological analysis. The results showed that 33 metabolic pathways were enriched according to the differential metabolites in the serum (Figures 6A). 27 pathways were enriched by the differential metabolites correlated with bone quality parameters (correlation coefficients $> 0.80$ and $P < 0.05$) were found as follows: 1,8-cineole, 5-valerolactone, citric acid, 12-keto-leukotriene B4, erucic acid, ketoleucine, d-arabitol, 8-hydroxyquinoline, methylmalonic acid, and 2-hydroxybutyric acid.

DISCUSSION

The study analyzed the impact of different systems used for keeping birds on their bone condition. Because floor rearing ducks have more space to exercise, their bones should be stronger than those reared in a cage (Newman et al., 1998). Regmi et al. (2015) found that the humeri cortical bone density, tibia cortical bone thickness, and humeri cortical bone thickness were improved in the conventional floor rearing system. Our results also suggest that the humeri mineral density of FRD had significantly more significant than the CRD, but the difference in tibia and femur density between the 2 groups was not significant. For caged ducks, the femur and tibia still support the body, and both perches and stand contribute to strong bones, but the wings cannot flap as freely as they do in the floor system, so the humeri of caged ducks are less exercised and prone to bone loss. Additionally, compared to CRD, the bones of FRD were significantly stronger in terms of breaking force, cortical thickness, and percentage of cortical area. The decreased breaking force of FRD can be attributed to cortical bone damage caused by bone loss. Correlation analysis results showed that the fracture strength of both femur and humerus was positively correlated with cortical bone thickness, which also supported this point.

The mechanical pressure and environmental stress brought by different rearing systems can cause changes in the metabolic process through various hormones and cytokines to regulate the growth and regeneration of bird bones (Rodriguez-Navarro et al., 2018). Various metabolites are involved in the regulatory process. In this study, 49 differential metabolites of CRD and FRD were correlated with bone quality parameters. Among them, 10 metabolites were strongly correlated. Several studies have reported the effects of these metabolites on bone. Erucic acid, one of the monounsaturated fatty acids, regulates the differentiation of mesenchymal stem cells into osteoblasts rather than adipocytes by accelerating osteoblast marker genes (Takahashi et al., 2020). N-Methyl pyrrolidinone enhances the kinase activity of the BMP receptor complex (Miguel et al., 2009). Creatine can increase BMD and provide a benefit for increasing
regional bone mineral content (Chilibeck et al., 2005; Chilibeck et al., 2015). Hydroxybutyric acid plays an important role in guiding bone regeneration (Kose et al., 2003). In our study, the contents of the above metabolites were lower in cage-reared ducks, which may be associated with the deterioration of the bone quality of these ducks. Moreover, the mutation of Cytochrome P450 3A4 (CYP3A4) was found to be significantly associated with low bone mass (Fukushima-Uesaka et al., 2004), and the CYP3A4 activity can be measured by the levels of 6beta-hydroxytestosterone (de Koning et al., 2014). We found that ground ducks had a high level of 6beta-hydroxytestosterone, indicating that different rearing systems may affect the activity of CYP3A4, resulting in the difference in bone quality. Picolinic acid, a catabolite of tryptophan, is neuroprotective and promotes osteogenesis in vitro (Ding et al., 2020). Duque et al. (2020) demonstrate that Picolinic acid positively affects bone in vivo by increasing bone formation, bone mass, and bone strength in mice. Here we show that Picolinic acid was positively correlated with bone quality parameters. Wang et al. reported that 8-hydroxyquinoline derivatives have antioxidant activities and could protect mesenchymal stem cells cultured in vitro against \( \text{H}_2\text{O}_2 \) induced

**Figure 5.** Heatmap of correlation coefficient matrix between annotated differential metabolites and bone quality parameter of tibia, femur, and humerus. Strong correlations are indicated by large circles, whereas small circles indicate weak correlations. The blue indicates a positive correlation, and the red shows a negative correlation. Metabolites strongly correlated with bone quality parameters are marked with a red frame.
oxidative stress (Wang et al., 2010). However, our experiment found that the level of 8-hydroxyquinoline was negatively correlated with bone quality parameters, so the effect of 8-hydroxyquinoline on bone in organisms still needs further study. In addition, Polyunsaturated fatty acids and their derivatives have critical roles in regulating various biological processes and affecting bone metabolism through involvement in the inflammatory process (Poulsen et al., 2007). Our study found that the content of Pimelic acid and 12-keto-leukotriene B4 in the serum of cage reared ducks was significantly higher than that of floor reared ducks, indicating that the oxidation process of fatty acids in cage reared ducks was intensified. To the best of our knowledge, ROS and other free radicals will be generated in the body because of fatty acid oxidation, which is often associated with the principle of oxidative stress and the pathophysiology of osteoporosis (Cervellati et al., 2014; Geng et al., 2019).

To further understand the mechanism of the influence of different rearing systems on bone quality, we conducted enrichment analysis of 45 metabolites significantly associated with bone quality parameters and found that the cage reared system caused a disturbance of 27 metabolic pathways. As shown in Figure 7, some of these metabolites are involved in multiple metabolic pathways and interact to form a complex network.

**Figure 6.** KEGG enrichment bubble diagram. (A) Pathway analysis of the 61 differential metabolites of serum. (B) Pathway analysis of the differential metabolites correlated with bone quality parameter. The ratio is the number of differential metabolites in the corresponding pathway to the total number of identified metabolites. The higher the value, the higher the degree of enrichment of differential metabolites in this pathway. The color of the point represents the P-value of the hypergeometric test. The smaller the value is, the greater the reliability of the test is, and the more statistically significant it is. The size of the point represents the number of differential metabolites in the corresponding pathway, and the larger the point, the more differential metabolites in the pathway.

**Figure 7.** Schematic diagram of metabolite interaction. Red indicates that metabolites are upregulated in cage reared ducks, and blue indicates down-regulated. The 2 metabolites connected by a solid line can be directly transformed, while the dotted line indicates indirect transformation.
Previous studies have shown that amino acids regulate bone growth by stimulating the secretion of hormones such as insulin and growth hormone (Yang et al., 2010). The disorder of amino acid synthesis and metabolism pathway will cause the change of amino acid content in ducks. Arginine stimulated IGF-I production and collagen synthesis in osteoblast-like cells and deaminated to produce nitric oxide (NO). NO in appropriate concentrations can protect against bone loss and promote osteoblast proliferation, while high concentrations inhibit osteoblast proliferation (Chevalley et al., 1998; Hao et al., 2005). This study showed an increased level of arginine in the serum of the cage reared ducks, inferred that the generation of NO produced from arginine was raised, which may be partly responsible for decreased bone quality. Glutamine, the most bountiful and flexible amino acid in the body, can interconvert with glutamate and stimulate osteoclasts through glutamate receptors leading to bone resorption (You et al., 2014; Zhou et al., 2019). This may explain the negative correlation between plasma Glutamine levels and bone quality parameters in the present study.

Energy metabolism and bone metabolism have a mutual feedback relationship (Confavreux, 2011). The published papers indicated that hormones produced by the bone could affect energy metabolism, insulin resistance, obesity, and the development of diabetes (Motyl et al., 2010). In contrast, a large amount of energy is needed to dissolve crystalline calcium phosphate or hydroxyapatite and degrade fibrous collagen during bone remodeling (Lv et al., 2016). Here we found that citric acid, a key metabolite of the TCA cycle, was down-regulated in cage rearing ducks, which may indicate a reduction in energy supply. Meanwhile, perturbations of energy metabolic pathways can also affect bone quality by changing metabolite levels. Citric acid is abundant in vertebrate bones and can accelerate calcium and phosphorus absorption in bones (Kim et al., 2017; Ikeda et al., 2021).

**CONCLUSIONS**

In summary, there were no significant differences in body weight, bone weight, and size between the 2 groups \((P > 0.05)\), but the breaking strength, cortical bone thickness, cortical bone area percentage ofibia, femur, and humerus, humerus mineral density and the calcium content of CRD \((P < 0.05)\), indicating that the cage rearing system caused the deterioration of bone quality. Based on non-target metabolomics, 49 metabolites were correlated with bone quality parameters, and 10 key metabolites were strongly correlated, including erucic acid, citric acid, and ketoleucine. In addition, the KEGG analysis showed that the caged system mainly perturbed amino acid metabolism, lipid metabolism, and energy metabolism, which led to changes in related metabolite levels, produced ROS, and altered energy supply, leading to the deterioration of bone quality of caged ducks. Therefore, our findings were helpful to further understand the potential mechanism of the deterioration of duck bone quality in cage rearing system, provided a theoretical basis for reducing the occurrence of poultry osteoporosis, and ensuring the healthy development of poultry breeding.

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**DISCLOSURES**

The authors declare no conflict of interest.

**SUPPLEMENTARY MATERIALS**

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.jsp.2021.101604.

**REFERENCES**

Ammann, P., and R. Rizzoli. 2003. Bone strength and its determinants. Osteoporos. Int. 14:13–18.

Armas, L. A. G., and R. R. Recker. 2012. Pathophysiology of osteoporosis new mechanistic insights. Endocrinol. Metab. Clin. North Am. 41 475+-.

Blair, H. C., M. Zaidi, and P. H. Schlesinger. 2002. Mechanisms balancing skeletal matrix synthesis and degradation. Biochem. J. 364:329–341.

Cervellati, C., G. Bonaccorsi, E. Cremonini, A. Romani, E. Fila, M. C. Castaldini, S. Ferrazzini, M. Giganti, and L. Masari. 2014. Oxidative stress and bone resorption interplay as a possible trigger for postmenopausal osteoporosis. Biomed Res. Int. 2014:8.

Chevalley, T., R. Rizzoli, D. Manen, J. Caverzasio, and J. P. Bonjour. 1998. Arginine increases insulin-like growth factor-I production and collagen synthesis in osteoblast-like cells. Bone 23:103–109.

Chilibeck, P., M. Chrusch, K. Chad, K. S. Davison, and D. Burke. 2005. Creatine monohydrate and resistance training increase bone mineral content and density in older men. J. Nutr. Health Aging 9:352.

Chilibeck, P. D., D. G. Candow, T. Landeryou, M. Kaviani, and L. Paus-Jenssen. 2015. Effects of creatine and resistance training on bone health in postmenopausal women. Med. Sci. Sports Exerc. 47:1587–1595.

Confavreux, C. B. 2011. Bone: from a reservoir of minerals to a regulator of energy metabolism. Kidney Int. 79:121:S14–S19.

de Koning, D. A., M. Mooij, T. N. Johnson, and S. N. de Wildt. 2014. Developmental changes in the processes governing oral drug absorption. Pages 25–42 in Pediatric Formulations. Springer, New York, NY.

Ding, K., M. E. McGee-Lawrence, H. Kaiser, A. K. Sharma, J. L. Pierce, D. L. Irisik, W. B. Bollag, J. Xu, Q. Zhong, W. Hill, X.-M. Shi, S. Fulzele, E. J. Kennedy, M. Elsalanty, M. W. Hamrick, and C. M. Isakles. 2020. Picolinic acid, a tryptophan oxidation product, does not impact bone mineral density but increases marrow adiposity. Exp. Gerontol. 133:110885.

Duncan, I. J. 2001. The pros and cons of cages. World’s Poult. Sci. J. 57:381–390.

Duque, G., C. Vidal, W. Li, A. Al Saedi, M. Khalil, C. K. Lim, D. E. Myers, and G. J. Guillemin. 2020. Picolinic acid, a catabolite.
