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www.ufaw.org.uk

Animal Welfare 2021, 30: 71-80
ISSN 0962-7286
doi: 10.7120/09627286.30.1.071

Keel-bone fractures are associated with bone quality differences in laying hens

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Abstract

*This study aimed to investigate the relationship between bone quality in terms of metabolism, homeostasis of elements, bone mineral density (BMD), and microstructure and keel-bone fractures in laying hens (*Gallus gallus domesticus*). One hundred and twenty 17 week old Lohmann White laying hens with normal keel bones were individually housed in furnished cages for 25 weeks. Birds were then euthanased and dissected to assess keel-bone status at 42 weeks. Serum and keel-bone samples from normal keel (NK) and fractured keel (FK) hens were collected to determine the previously mentioned bone quality parameters. The results showed FK hens to have higher levels of the components of osteocalcin, greater alkaline phosphatase activity in serum and keel bones, and greater tartrate-resistant acid phosphatase (TRAP) activity in keel bones, compared to NK hens. Additionally, FK hens also had higher concentrations of Li, B, K, Cu, As, Se, Sn, Hg, and Pb, but lower concentrations of Na, P, and Ca. Moreover, FK hens showed decreased bone microstructural parameters including bone volume/tissue volume, trabecular number, degree of anisotropy, connectivity density, and BMD, but increased trabecular separation. Meanwhile, no differences were detected in serum TRAP activity, trabecular thickness, bone surface, or bone surface/bone volume. Results showed laying hens with keel-bone fractures to have differences in bone metabolism, elements of homeostasis, bone microstructure parameters, and BMD. These results suggest that keel-bone fractures may be associated with bone quality.*

Keywords: *animal welfare, bone metabolism, bone microstructure, bone mineral density, keel-bone fracture, laying hen*

Introduction

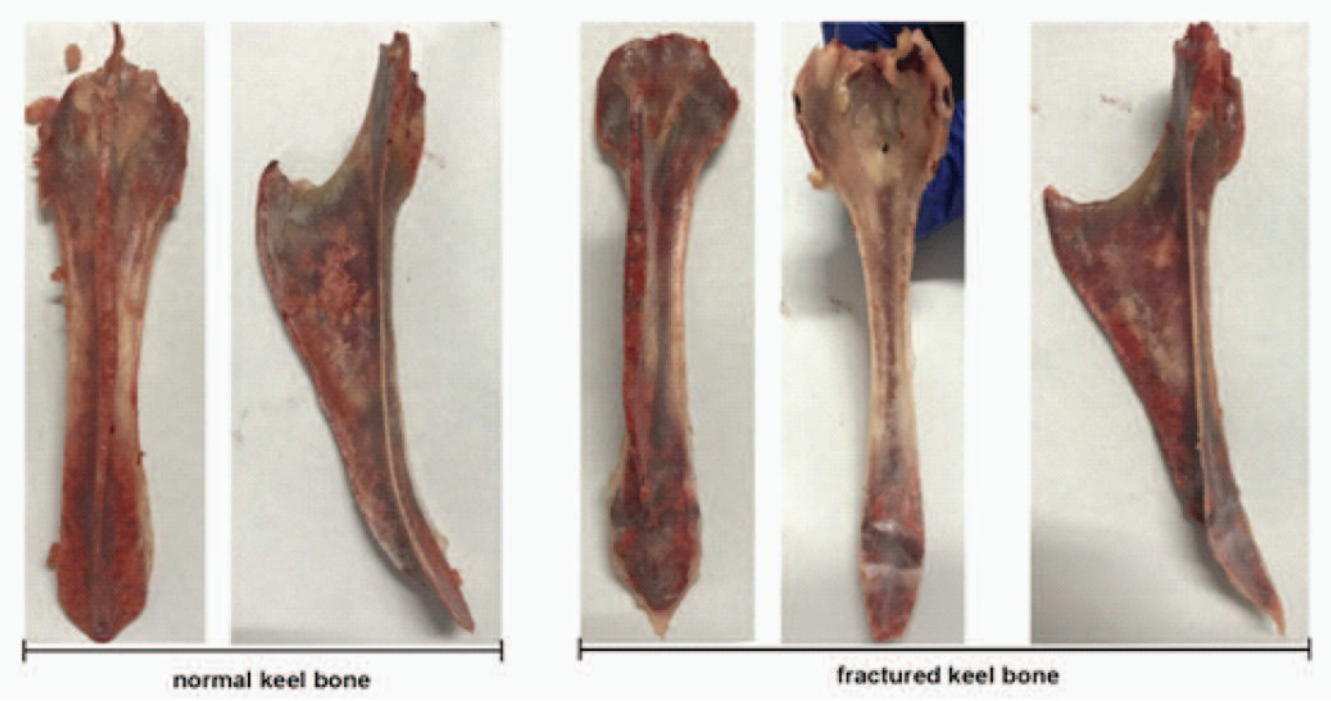
In modern egg production systems where, as an alternative to conventional cages, laying hens (*Gallus gallus domesticus*) are housed in furnished cages or in non-cage systems, keel-bone fractures are one of the biggest threats to health and welfare (Riber *et al* 2018). The keel is a structural bone that extends axially from the sternum over the midline in avian species, playing a vital role in flying and respiration (Casey-Trott *et al* 2015). In addition to impinging upon bird behaviours, such as sitting, standing, sleeping, and perching (Nasr *et al* 2012; Casey-Trott & Widowski 2016), numerous studies have reported that keel-bone fractures also led to a decrease in production performance and eggshell quality (Nasr *et al* 2012; Rufener *et al* 2019; Wei *et al* 2020), as well as causing stress and inflammatory responses in laying hens (Wei *et al* 2019).

Keel-bone fractures may also require a lengthy healing due to additional fractures occurring during the recovery period (Richards *et al* 2011). Serum osteocalcin (OC) level and alkaline phosphatase (ALP) activity are crucial biological indexes, commonly used in the evaluation of osteoblast activity during bone remodeling in rats, sheep, and humans (Seibel 2006). Increase and decrease in OC was generally associated with an increase and decrease in osteogenesis, respectively. After complete recovery of damaged bone, the OC levels return to normal (Harris *et al* 2001). Similarly, ALP activity in serum and bone increases as a result of bone damage and fracture healing (Hatayama *et al* 2012; Kubo *et al* 2012). Tartrate-resistant acid phosphatase (TRAP) is often used as an important indicator of osteoclast and macrophage activity (Schleicher *et al* 2013). Decreased TRAP activity inhibits the ossification of cartilage tissue, while excessive activity can accelerate bone metabolism, which has been shown to cause osteoporosis in rats (Angel *et al* 2000). Additionally, the activity of ALP and TRAP also reflects bone turnover levels during remodeling in laying hens fed omega-3 polyunsaturated fatty acid diets (Tarlton *et al* 2013). Therefore, OC, ALP and TRAP are not only essential regulators of bone metabolism but may also be used as important predictors of bone health and quality in animals.

Appropriate amounts of macro and trace minerals are known to be essential for maintaining the integrity of cell

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Figure 1



Normal (NK) and fractured (FK) keel-bone samples used in the present study.

ultrastructure and normal enzyme activity, as well as regulating the normal physiological processes in both humans and animals (Soetan *et al* 2010). However, various diseases and/or harmful external stimuli can affect the body, inducing imbalanced ion metabolism and leading to alterations in certain tissue and organ mineral concentrations or compositions (Soetan *et al* 2010). Furthermore, bone development and the overall health of laying hens depend, essentially, on appropriate amounts of dietary minerals. Previous studies found that in laying hens, levels of dietary calcium (Ca) and phosphorus (P) influence bone mineral content (BMC), bone mineral density (BMD), bone strength and turnover as well as bone microarchitectural damage (Onyango *et al* 2003; Olgun & Aygun 2016). The high incidence of keel-bone fractures in commercially cage-housed laying hens may occur as a result of Ca deficiency or other nutritional disorders (Olgun & Aygun 2016). Additionally, zinc (Zn), manganese (Mn), lead (Pb), selenium (Se), and other elements have been found to be closely associated with bone health and adequate mineral composition (Swiatkiewicz & Koreleski 2008; Stefanello *et al* 2013; Brito *et al* 2014).

Various studies have focused on the effects of keel-bone fractures on behaviour, welfare, production performance, and egg quality (for reviews, see Riber *et al* 2018; Wei *et al* 2020), as well as potential solutions to reducing incidence of keel-bone fractures in laying hens (Hardin *et al* 2019) but, as yet, there have been no studies investigating the relationship between keel-bone fractures and bone metabolism and microstructure parameters. The aim of this study therefore was to investigate differences in metabolism, mineral elements, and the microstructure of keel bones for laying hens individually housed in furnished cages, both with and without keel-bone fractures, in order to gain a better understanding of the underlying relationship between keel-bone fractures and differences in bone metabolism and quality-related indexes. Our hypothesis was that differences in bone metabolism indicators, concentrations of specific elements, microstructural parameters and BMD would show an association with keel-bone fractures in laying hens.

Materials and methods

Ethical statement

Animal care and treatment procedures in this experiment complied with the Institutional Animal Care and Use Committee of the Northeast Agricultural University (ethical number IACUCNEAU20150616).

Study animals and management

A total of one hundred and twenty 17 week old Lohmann White laying hens that were individually housed in furnished cages and had normal keel bones were used in the present study. Each cage measured 0.7 × 0.5 × 0.7 m (length × width × height) and was furnished with two perches, a nesting box, a nipple drinker, and a feeding trough. These were installed within a semi-enclosed hen house and showed a temperature and relative humidity of 21 to 26°C and 45 to 70%, respectively. Lighting consisted of a combination of natural and artificial light with the artificial photoperiod lasting 16 h (0500 to

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2100h) and utilising a light intensity of 18 to 22 lux. The remaining 8 h were dark. Hens were fed commercial egg-laying diet with a metabolisable energy content of 2,800 kcal per kg and crude protein content of 16.08% with *ad libitum* access to feed and water throughout the entire experimental period (17 to 42 weeks of age).

Assessment of keel-bone damage and sample collection

For the assessment of keel-bone damage, palpation of live hens took place as did visual observation post-dissection. Keel bones were divided into three broad groups: normal; fractures; and deviations. As per the objective of our study, normal (NK) and fractured keels (FK) were investigated (see Figure 1). NK included keels with an absence of fractures or that had a deviation degree less than 0.5 cm measured from a theoretical two-dimensional straight plane (either the transverse or sagittal planes) (Casey-Trott *et al* 2015) while FK consisted of keels showing sharp bends, clear shearing or displacement and bumps (Casey-Trott *et al* 2015). Each laying hen's keel bone was firstly assessed via palpation at 42 weeks of age. Blood samples were then taken from the wing vein of every study subject. Serum was obtained after centrifugation at 3,000 rpm for 15 min at 4°C and stored in the freezer at –20°C. These serum samples (n = 10 from both NK and FK hens) were selected for bone metabolism indicator analysis. Finally, all hens were euthanased by cervical dislocation before the entire keel bone was excised from the body, and connective muscle and soft tissue removed. Keel bones then underwent visual assessment. There were 65 hens with FK, 28 hens with DK (deviated keels), and 27 hens with NK. Thereafter, forty-four NK and FK keel bones (n = 22 each) were randomly selected for the keel-bone samples. These were stored at –80°C for determination of bone metabolism indicators (n = 10 each), analysis of elements' concentration (n = 6 each), and measurement of micro-computed tomography (Micro-CT) (n = 6 each).

Bone metabolism indicators analysis

From each NK and FK bone (n = 10 each) a 1 g segment was removed (approximately 2 cm in length), 2 cm from the caudal border of keel bone and weighed. These bone samples were then pulverised, freeze-dried and extracted in accordance with Tarlton *et al* (2013), and extract aliquots stored at –80°C for further use. The concentration of OC in serums and keel-bone extracts from NK and FK hens (n = 10 each) were assessed using commercially available enzyme-linked immunosorbent (ELISA) kits (Shanghai Jinma Laboratory Equipment Corporation Ltd, Shanghai, China) as per the manufacturer's instructions. ALP and TRAP levels in both serum and keel-bone extracts from NK and FK hens (n = 10 each) were measured using commercially available ELISA kits (Nanjing Jiancheng Technology Ltd, Nanjing, China).

Keel-bone mineral elements' analysis

From each NK and FK bone sample (n = 6 each) a 1 g piece was removed (approximately 2 cm in length), 2 cm from the caudal border of keel bone, and the sample was weighed to determine the mineral content of 29 elements, including lithium (Li), boron (B), sodium (Na), magnesium (Mg), aluminum (Al), silicon (Si), potassium (K), calcium (Ca), titanium (Ti), vanadium (V), chromium (Cr), manganese (Mn), iron (Fe), cobalt (Co), nickel (Ni), copper (Cu), zinc (Zn), arsenic (As), selenium (Se), strontium (Sr), molybdenum (Mo), cadmium (Cd), stannum (Sn), antimony (Sb), barium (Ba), mercury (Hg), thallium (Tl), and lead (Pb), using inductively coupled plasma mass spectrometry (ICP-MS, Thermo iCAP Q, MA, USA). Precisely, each piece of 1 g keel bone was dissolved in solution containing 5 ml of 65% (w/w) HNO_3 and 2 ml of 30% H_2O_2 (w/v) and then diluted in deionised water into 10 ml of the final volume. The samples were then dissolved once more in a 1,800 W microwave digestion system as follows: 3 min at 100°C, 10 min at 150°C, and 45 min at 180°C. Finally, all dissolved samples were diluted into 50 ml of final volume with ultra-pure water and mixed thoroughly for ICP-MS analysis. The operating parameters of ICP-MS were as follows: frequency 27.12 MHz, reflect power 1,550 W, carrier gas flow rate 1.05 l per min, plasma gas flow rate 14.0 l per min, an auxiliary gas rate 0.8 l per min, S/C temperature 2.7°C, sampling depth 6.0 mm, nebuliser pump 40 revolutions per min, oxide ions (156/140) < 2.0%, and doubly charged (70/140) < 3.0%. The content of all mineral elements was calculated with blanks and standards.

Keel micro-computed tomography (Micro-CT) analysis

The NK and FK bone samples (n = 6 each) were cut into 1.3 cm long pieces from 2 cm from the caudal border of the keel bone for the Micro-CT analysis and each subsequent sample was soaked in a solution of 4% paraformaldehyde for three days. Imaging was performed using Micro-CT (μCT 50, Switzerland Scanco Medical AG, Zürich, Switzerland) at 16 μm voxel size with a source potential of 70 kV, a current of 200 μA, an integration time of 300 ms, and a 0.5 mm aluminum filter. The scanned images were reconstructed using Mimics 19.0 and ABA special bone analysis software (Healthcare Locus SP, Connecticut, USA) to determine mean BMD, bone volume/tissue volume (BV/TV), trabecular thickness (TbTh), trabecular separation (TbSp), trabecular number (TbN), bone surface (BS), bone surface/bone volume (BS/BV), degree of anisotropy (DA), and connectivity density (ConnDn).

Statistical analysis

Statistical analysis software SPSS 22 for Windows (SPSS Inc, Chicago, IL, USA) was used for data analysis. Total obtained data were tested for normality using the Kolmogorov-Smirnov test. Statistical differences between laying hens with normal and fractured keels were analysed using an independent *t*-test. The results were expressed as means (± SEM), and the different levels considered statistically significant at $P < 0.05$. Correlations between bone metabolism indicators, microstructure parameters, and mineral elements were analysed using Spearman's correlation test. Values > 0.50 were considered to indicate a strong correlation.

https://doi.org/10.7120/09627286.30.1.071 Published online by Cambridge University Press

Table 1 Mean (± SEM) osteocalcin concentration and alkaline phosphatase and tartrate resistant acid phosphatase activity in the serum and keel bone of laying hens (n = 10).

Indicators (units)	NK[1]	FK[2]	Effects
Serum			
OC[3] (ng l^{-1})	393.51 (± 8.72)	449.61 (± 16.45)	**, $t_{1,18}$ = –3.01
ALP[4] (U l^{-1})	1,373.88 (± 60.95)	1,676.14 (± 30.85)	**, $t_{1,18}$ = –4.43
TRAP[5] (U l^{-1})	136.67 (± 4.04)	146.39 (± 5.63)	ns, $t_{1,18}$ = –1.40
Bone			
OC[3] (ng l^{-1})	460.90 (± 11.98)	554.52 (± 7.67)	**, $t_{1,18}$ = –4.02
ALP[4] (U l^{-1})	1,979.68 (± 32.68)	2,091.14 (± 34.09)	*, $t_{1,18}$ = –2.36
TRAP[5] (U l^{-1})	151.48 (± 6.28)	176.58 (± 4.70)	**, $t_{1,18}$ = –3.20

[1] NK: normal keel laying hens;
[2] FK: fractured keel laying hens;
[3] OC: osteocalcin;
[4] ALP: alkaline phosphatase;
[5] TRAP: tartrate resistant acid phosphatase;
Means in the same row differ significantly (*t*-test; two-tailed);
* $P < 0.05$; ** $P < 0.01$.

Results

Bone metabolism indicators in serum and keel bones

OC concentration in serum and keel bones and the ALP and TRAP activity of NK and FK hens are shown in Table 1. FK hens showed a higher serum OC content (P = 0.008; $t_{1,18}$ = –3.01) and serum ALP activity (P =0.001; $t_{1,18}$ = –4.43) compared to NK hens; however, there was no difference in serum TRAP activity (P = 0.178; $t_{1,18}$ = –1.40) between NK and FK hens. Similarly, the concentrations of bone OC (P = 0.001; $t_{1,18}$ = –4.02) and activities of bone ALP (P = 0.028; $t_{1,18}$ = –2.36) and bone TRAP (P = 0.004; $t_{1,18}$ = –3.20) in FK hens were higher than those in NK hens.

Concentrations of mineral elements in keel bone

The concentrations of 29 mineral elements in the NK and FK laying hen groups are shown in Table 2. The data indicated that for a total of 12 mineral elements concentrations changed significantly. Compared to the NK group, FK hens had higher concentrations of Li (P = 0.037; $t_{1,10}$ = –2.41), B (P = 0.046; $t_{1,10}$ = –2.28), K (P = 0.022; $t_{1,10}$ = –2.70), Cu (P = 0.025; $t_{1,10}$ = –2.64), As (P = 0.014; $t_{1,10}$ = –3.49), Se (P = 0.016; $t_{1,10}$ = –2.90), Sn (P = 0.042; $t_{1,10}$ = –2.33), Hg (P = 0.011; $t_{1,10}$ = –3.11), and Pb (P = 0.004; $t_{1,10}$ = –4.13), with lower concentrations seen for Na (P = 0.017; $t_{1,10}$ = 2.85), P (P = 0.046; $t_{1,10}$ = 2.28), and Ca (P = 0.038; $t_{1,10}$ = 2.54). Figure 2 shows the relative concentration of these statistically significant elements expressed as ratios of FK to NK.

Microstructural parameters of keel bone

BMD and other microarchitectural parameters of keel bones in NK and FK laying hens acquired from Micro-CT images are shown in Table 3. In comparison to NK hens, the FK group had lower BV/TV (P = 0.011; $t_{1,10}$ = 3.30), TbN (P = 0.002; $t_{1,10}$ = 4.69), DA (P = 0.037; $t_{1,10}$ = 2.51), ConnDn (P = 0.005; $t_{1,10}$ = 3.85) and BMD (P = 0.049; $t_{1,10}$ = –2.32), with a higher TbSp (P = 0.033; $t_{1,10}$ = –3.14). However, no significant differences were found between NK and FK hens for TbTh (P = 0.510; $t_{1,10}$ = 0.69), BS (P = 0.880; $t_{1,10}$ = 0.16), and BS/BV (P = 0.418; $t_{1,10}$ = –0.85).

Correlation analysis

The relationships between bone metabolism and bone structure-related indexes and minerals were determined by Spearman's correlation analysis (Table 4; see supplementary material to papers published in *Animal Welfare*: https://www.ufaw.org.uk/the-ufaw-journal/supplementary-material). The results showed a positive correlation between OC content and ALP activity and a negative correlation between OC concentration and Ti and Zn levels. Furthermore, the ALP activity was positively correlated with OC concentration and TRAP activity, while the TRAP activity had a positive correlation with ALP activity, Ti, Mn, Zn, and Ba levels. However, a negative correlation was observed between TRAP activity and ConnDn. The BMD was positively correlated with TbN, B, Ca, Ba and Zn concentrations and negatively correlated with BV/TV, TbSp, and ConnDn. Also, positive correlations were detected between the BV/TV and TbN and ConnDn, and negative correlations with BMD, TbSp, B, K, As, Sb, and Pb. The TbTh had a negative correlation with Al content, while the TbSp had a positive correlation with B, K, Se, and Sb concentrations. In addition, a negative correlation was observed between TbSp and BV/TV, TbN, ConnDn, and Co. TbN was positively correlated with BV/TV, ConnDn, Na, and Co levels, and negatively correlated with TbSp, B, K, Se, Sn, Sb, and Pb. ConnDn positively correlated with BV/TV, TbN, and Co, and negatively correlated with BMD, TRAP, TbSp, K, Sb, and Pb.

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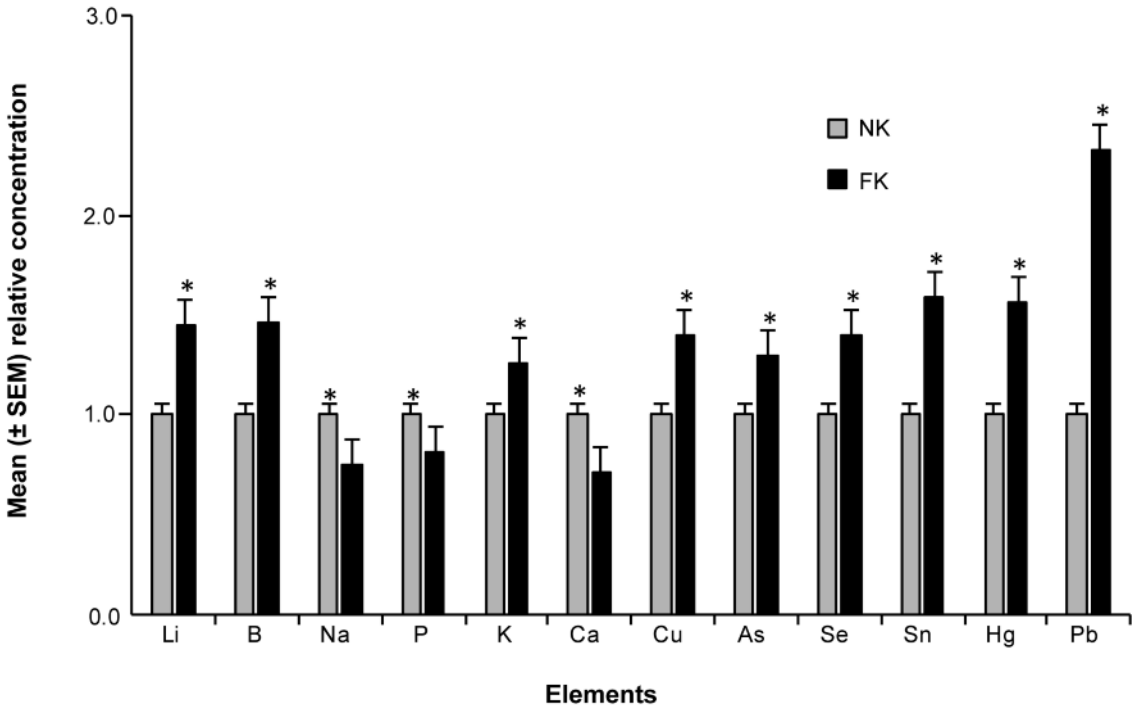
Table 2 Mean (± SEM) concentrations of 29 mineral elements in the keel bones of laying hens (n = 6).

Elements	Unit	NK	FK	Effects
Li	μg kg^{-1}	53.27 (± 5.70)	77.38 (± 8.22)	*, $t_{1,10}$ = –2.41
B	mg kg^{-1}	2.46 (± 0.23)	3.59 (± 0.44)	*, $t_{1,10}$ = –2.28
Na	mg kg^{-1}	1,430.35 (± 73.53)	1,068.37 (± 103.55)	*, $t_{1,10}$ = 2.85
Mg	mg kg^{-1}	2,080.96 (± 68.33)	1,985.67 (± 83.13)	ns, $t_{1,10}$ = 0.89
Al	mg kg^{-1}	26.12 (± 2.86)	30.50 (± 2.42)	ns, $t_{1,10}$ = –1.17
Si	mg kg^{-1}	53.72 (± 2.29)	48.87 (± 3.87)	ns, $t_{1,10}$ = 1.08
P	mg kg^{-1}	76,904.19 (± 4,065.86)	62,448.86 (± 4,857.27)	*, $t_{1,10}$ = 2.28
K	mg kg^{-1}	2,842.29 (± 245.61)	3,592.33 (± 129.97)	*, $t_{1,10}$ = –2.70
Ca	mg kg^{-1}	154,840.10 (± 7,545.80)	110,095.10 (± 5,898.41)	*, $t_{1,10}$ = 2.54
Ti	mg kg^{-1}	390.65 (± 15.64)	310.97 (± 39.17)	ns, $t_{1,10}$ = 1.89
V	mg kg^{-1}	0.22 (± 0.04)	0.21 (± 0.01)	ns, $t_{1,10}$ = 0.17
Cr	mg kg^{-1}	1.12 (± 0.25)	1.00 (± 0.15)	ns, $t_{1,10}$ = 0.41
Mn	mg kg^{-1}	10.72 (± 0.58)	12.63 (± 1.54)	ns, $t_{1,10}$ = –1.16
Fe	mg kg^{-1}	136.59 (± 8.72)	169.96 (± 16.39)	ns, $t_{1,10}$ = –1.80
Co	μg kg^{-1}	34.63 (± 6.20)	23.27 (± 1.31)	ns, $t_{1,10}$ = 1.79
Ni	μg kg^{-1}	100.14 (± 8.32)	101.59 (± 11.34)	ns, $t_{1,10}$ = –0.10
Cu	mg kg^{-1}	0.86 (± 0.07)	1.20 (± 0.11)	*, $t_{1,10}$ = –2.64
Zn	mg kg^{-1}	145.33 (± 14.18)	160.17 (± 12.14)	ns, $t_{1,10}$ = –0.80
As	mg kg^{-1}	0.13 (± 0.01)	0.17 (± 0.01)	**, $t_{1,10}$ = –3.49
Se	mg kg^{-1}	0.28 (± 0.02)	0.38 (± 0.03)	*, $t_{1,10}$ = –2.90
Sr	mg kg^{-1}	71.88 (± 8.99)	67.85 (± 8.47)	ns, $t_{1,10}$ = 0.33
Mo	mg kg^{-1}	0.20 (± 0.01)	0.26 (± 0.04)	ns, $t_{1,10}$ = –1.35
Cd	μg kg^{-1}	3.84 (± 0.35)	5.02 (± 0.59)	ns, $t_{1,10}$ = –1.74
Sn	μg kg^{-1}	19.11 (± 2.61)	30.33 (± 4.05)	*, $t_{1,10}$ = –2.33
Sb	μg kg^{-1}	15.27 (± 0.91)	23.07 (± 4.06)	ns, $t_{1,10}$ = –1.88
Ba	mg kg^{-1}	38.26 (± 6.69)	37.89 (± 3.00)	ns, $t_{1,10}$ = 0.05
Hg	μg kg^{-1}	0.71 (± 0.02)	1.10 (± 0.13)	*, $t_{1,10}$ = –3.11
Tl	μg kg^{-1}	1.42 (± 0.08)	1.55 (± 0.12)	ns, $t_{1,10}$ = –0.91
Pb	mg kg^{-1}	0.97 (± 0.13)	2.26 (± 0.28)	**, $t_{1,10}$ = –4.13

NK: normal keel laying hens;
FK: fractured keel laying hens;
Means in the same row differ significantly (*t*-test; two-tailed);
* $P < 0.05$; ** $P < 0.01$.

https://doi.org/10.7120/09627286.30.1.071 Published online by Cambridge University Press

Figure 2



Mean (± SEM) changes in the concentration of 12 elements in laying hens with fractured keel (FK) relative to laying hens with normal keel (NK), (n = 6). * $P < 0.05$.

Table 3 Mean (± SEM) microarchitectural parameters of laying hens' keel bones from Micro-CT images (n = 6).

Parameters (units)	NK[1]	FK[2]	Effects
BMD (mg HA cm^{-3})	651.39 (± 7.40)	624.75 (± 8.76)	*, $t_{1,10} = -2.32$
BV/TV (%)	58.42 (± 2.67)	43.37 (± 3.70)	*, $t_{1,1} = 3.30$
TbTh (mm)	0.24 (± 0.02)	0.22 (± 0.01)	ns, $t_{1,10} = 0.69$
TbSp (mm)	0.25 (± 0.02)	0.63 (± 0.30)	*, $t_{1,10} = -3.14$
TbN (mm^{-1})	4.08 (± 0.26)	2.05 (± 0.34)	**, $t_{1,10} = 4.69$
BS (mm^2)	980.11 (± 83.14)	960.24 (± 96.99)	ns, $t_{1,10} = 0.16$
BS/BV (mm^{-1})	8.51 (± 0.48)	9.08 (± 0.45)	ns, $t_{1,10} = -0.85$
DA	2.04 (± 0.10)	1.71 (± 0.09)	*, $t_{1,10} = 2.51$
ConnDn (mm^{-3})	23.79 (± 2.50)	11.87 (± 1.82)	**, $t_{1,10} = 3.85$

[1] NK: normal keel laying hens;
[2] FK: fractured keel laying hens;
BMD: bone mineral density;
BV/TV: bone volume/tissue volume;
TbTh: trabecular thickness;
TbSp: trabecular separation;
TbN: trabecular number;
BS: bone surface;
BS/BV: bone surface/bone volume;
DA: degree of anisotropy;
ConnDn: connectivity density;
Means in the same row differ significantly (*t*-test; two-tailed);
* $P < 0.05$; ** $P < 0.01$.

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Discussion

Keel-bone fractures are an important health and welfare problem in all laying hen production systems. The objective of this study was to investigate the relationship between keel-bone fractures and bone quality-related factors, including bone metabolism indicators, homeostasis of elements, BMD, and microstructural parameters in caged laying hens. Our results suggest a correlation between keel-bone fractures and differences in bone quality.

During fracture healing, a balance between bone resorption (by osteoclasts) and bone formation (by osteoblasts) is essential. Serum OC levels and the activity of ALP represent important biochemical indicators for evaluating osteoblast activity during bone remodelling (Seibel 2006). A relatively smaller protein OC is produced by osteoblasts during the period of matrix mineralisation which is then released into circulation before being incorporated into the bone matrix (Harris *et al* 2001). Elevated and reduced OC levels have been associated with increased and decreased bone formation, respectively. However, once the bone has fully repaired OC levels return to normal (Harris *et al* 2001). Osteoblasts also produce bone ALP and their activity is reflected in the amount of synthesised ALP, consequently this molecule is considered a reliable indicator of bone metabolism (Kress 1998). Recent studies have shown ALP's bone and serum activity to be elevated during bone injury, fracture healing, and bone diseases (Hatayama *et al* 2012; Kubo *et al* 2012). These findings are consistent with our results, where OC levels and ALP activity were noticeably higher in the serum and bone of laying hens with FK. This also indicates that elevated osteoblast activity in FK hens may occur as a result of being triggered during fracture healing.

TRAP is commonly used to monitor osteoclast and macrophage activity, being abundantly expressed in bone, liver and spleen tissues as well as produced in osteoclasts and in activated dendritic cells and macrophages (Schleicher *et al* 2013). Seebeck *et al* (2005) found that changes in TRAP activity mainly depend on the stage of fracture healing in sheep. During bony callus formation, TRAP activity was seen to decrease, indicating the lower level of bone resorption, and thus the promotion of bone formation (Seebeck *et al* 2005). Subsequently, TRAP activity increases slightly as the newly formed bone starts to remodel (Seebeck *et al* 2005). In the present study, TRAP activity was detected more in the bones of FK hens than NK; however, serum TRAP activity showed no difference between either group. This may indicate that the remodelling of newly formed bone started with the increased bone resorption regulated by osteoclasts and decreased bone formation regulated by osteoblasts, ie in order for the healing of keel-bone fractures to be completed in laying hens. Otherwise, elevated TRAP activity may increase the susceptibility for keel-bone fractures, since elevated TRAP increases bone resorption which, in turn, causes osteoporosis, characterised by reduced bone strength and rigidity (Angel *et al* 2000). Thus, increased TRAP levels can increase the risk of fractures.

It has been previously documented that deficient or excessive amounts of a variety of macro and trace mineral elements can cause physiological and immunological imbalances, thereby reducing the performance of animal production (Perry *et al* 1991; Adedokun & Adeola 2013). Moreover, mineral elements are also important for bone development and health in poultry. Recent studies have shown the importance of Ca and P in maintaining hens' bone quality and strength (Rath *et al* 2000; Adedokun & Adeola 2013). Low dietary Ca levels stimulate the secretion of thyroid hormones and vitamin synthesis, which subsequently activate the release of bone minerals, therefore, adequate dietary Ca is essential to reduce bone turnover (Rath *et al* 2000). Furthermore, a lack of Ca in poultry diets has been reported as causing poor nutrient absorption, thereby impairing Ca absorption in the intestines (Perry *et al* 1991) and reducing bone strength (Onyango *et al* 2003). Reduced bone strength decreases bone quality and is associated with an increased prevalence of keel-bone fractures in laying hens (Stratmann *et al* 2016). In the present study, low levels of Ca and P were clearly apparent in the keel bones of FK hens, indicating that reduced Ca and P might be related to reduced keel-bone strength and quality. Or, that fractures lead to reduced Ca and P content.

Both humans and animals require sufficient mineral content due to the essential role they play in regulating a variety of physiological processes (Soetan *et al* 2010). A lack of mineral content, including K, Ca, P, and Pb seriously affect human health and was also found to reduce production performance and bone quality in farm animals (Onyango *et al* 2003; Brito *et al* 2014). It has previously been confirmed that K can prevent bone damage and osteoporosis by helping preserve Ca content in bones and maintaining a normal pH value (New *et al* 2000). Additionally, dietary supplements of K can improve Ca balance, reduce bone absorption, and increase bone formation (New *et al* 2000). In the present study, increased K content in the keel bones of FK hens might be related to stimulation of fracture healing by promoting bone formation. Sodium, usually in the form of sodium salts, plays an important regulatory role in healthy bone development. Nevertheless, a high Na level is considered a risk factor for osteoporosis, being positively associated with the activity of osteoclasts (Teucher *et al* 2008). Noticeably reduced levels of Na in FK hens were detected, suggesting a possible association with the fracture healing stages. Osteoblast activity increased as indicated by elevated ALP levels, while the activity of osteoclasts decreased as indicated by reduced TRAP activity during the bone remodelling. Also, the low observed Na content may be associated with the promotion of the fracture healing process by improving osteoblast activity. Similarly, Pb is also related to decreased bone quality and density, which may result in osteoporosis and a greater likelihood of fracture occurrence (Brito *et al* 2014). The Pb content in FK hens was elevated in the current study, which may be one of the determinant factors for keel-bone fractures. Additionally, heavy metal elements, Sn and Hg, induce a degree of biological toxicity in living tissues and organs,

https://doi.org/10.7120/09627286.30.1.071 Published online by Cambridge University Press

both in humans and animals, which causes damage to the body (Su 2014). Our results showed Sn and Hg content in the keel bones of FK hens to be significantly higher than in NK hens, which might contribute to the severity of keel-bone fractures. Therefore, the homeostasis of macro and trace mineral elements plays a crucial role in maintaining bone quality and health.

Use of Micro-CT images represents a valuable method of assessing bone microstructure and mass. Certain key bone structural parameters, including BV/TV, TbN, TbTh, TbSp, DA, and ConnDn have generally been utilised to analyse the microstructure of rodent trabecular bones (Bouxsein *et al* 2010). Trabecular bone volume fraction (BV/TV [in %]) represents the percentage of cancellous space occupied by trabecular bone in the volume in question (Aguado *et al* 2017), and is commonly used as a predictor of fracture risk as well as being strongly associated with bone strength and quality (Mittra *et al* 2005; Karim & Vashishth 2011). Recent studies in chickens exposed to hypo-activity have found that a reduced BV/TV ratio due to increased TbSp and decreased TbN in the tibia and femur, altered the trabecular microstructure and caused bone loss, finally resulting in decreased bone mass and quality (Aguado *et al* 2015, 2017). Additionally, TbSp, TbTh, DA, and ConnDn also play important roles in fracture resistance, and increased TbTh and BV/TV are related to higher bone strength (Mittra *et al* 2005; Karim & Vashishth 2011). On the other hand, bone loss is primarily influenced by TbN and ConnDn (Mittra *et al* 2005). In this study, FK hens had a patently high level of TbSp and low levels of BV/TV, TbN, and ConnDn, which indicates that changes in bone microstructure parameters could be an underlying mechanism of keel-bone fractures via a reduction of bone quality. The BMD reflects bone mineral content since it is closely associated with bone strength and rigidity, and increased BMD may lead to stronger bones and a greater ability to resist fractures and deformities in birds (Flis *et al* 2019). In the current study, there was a decreased BMD in the keel bones of FK hens. Thus, this result may indicate that a decreased BMD could be associated with keel-bone fractures accompanied by reduced bone strength and rigidity in laying hens.

In this study, a number of associations were found between the bone metabolism indexes, microstructure parameters, BMD, and mineral elements. The important indexes of osteoblast activity, OC and ALP, were positively correlated, which could promote bone formation. A common indicator of osteoclast activity, TRAP, was positively correlated with ALP, Mn, and Zn, which indicates an association with the different stages of bone healing. Specifically, Zn stimulates osteoblasts and inhibits osteoclasts during the healing process to improve bone quality (Scrimgeour *et al* 2007); therefore, the increase in Zn concentration may promote bone formation by increasing ALP activity and decreasing TRAP activity. Furthermore, BMD was positively correlated with TbN, B, Ca, and Zn concentrations, which positively affected bone strength and quality. This is consistent with previous findings that the addition of Mn and Zn in the diet of laying hens improves bone quality (Swiatkiewicz & Koreleski 2008), and elevated BMD causes stronger bone strength with a better fracture and deformity resistance in birds (Flis *et al* 2019). Similarly, the reduced BV/TV was positively correlated with TbN, while being negatively correlated with TbSp in FK laying hens. This is consistent with the results of Aguado *et al* (2015, 2017), who found that hypo-activity decreased BV/TV and TbN, and increased TbSp in hens' tibia and femur, leading to bone loss and reduced bone mass and quality. Therefore, bone loss and weakness cause bone damage that are correlated with bone metabolism, homeostasis of chemical elements, microstructure and BMD in laying hens.

Animal welfare implications

Keel-bone fractures are prevalent in laying hens in all housing systems, and impact negatively on welfare, production performance and egg quality. Hence, the present study sought to investigate whether the occurrence of keel-bone fractures is associated with differences in bone quality. The results will help better understand the underlying relationship between keel-bone fractures and bone quality in laying hens.

Conclusion

In conclusion, the present study provides evidence that keel-bone fractures are associated with differences in the concentrations and activities of bone metabolism-related indexes, the concentrations of certain mineral elements, and bone microstructural parameters, as well as BMD in laying hens. These results help elucidate the relationship between differences both in bone quality and keel-bone health in laying hens.

Acknowledgements

This study was supported by National Natural Science Foundation of China (grant number 31672466 and 31972608). No potential conflicts of interest are reported by the authors.

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