Brazilian Journal of Poultry Science
Revista Brasileira de Ciência Avícola

e-ISSN: 1806-9061 2024 / v.26 / n.2 / 001-008

http://dx.doi.org/10.1590/1806-9061-2024-1931

Original Article

Effect of Different Cage Densities and Age on Keel Bone Damage and Some Hormones in Laying Hens

■Author(s)

Uysal A[I] https://orcid.org/0000-0003-2803-2410
Laçin E[I] https://orcid.org/0000-0002-8417-6710

[I] Department of Animal Science, Faculty of Veterinary Medicine, Atatürk University, 25240 Erzurum, Turkey.

■Mail Address

Corresponding author e-mail address
Ayşe Uysal
Department of Animal Science, Faculty of Veterinary Medicine, University of Atatürk, Erzurum-25240, Turkey.
Phone: +90 442 231 7181
Email: mrsvetayse@gmail.com

■Keywords

Cage density; Conventional cage; Keel bone damage; Laying hens; Welfare.

Submitted: 15/March/2024
Approved: 26/June/2024

ABSTRACT

This study aimed to determine the effect of three distinct cage densities (750 cm²/hen, 535 cm²/hen, and 375 cm²/hen) on the keel bone damage of brown (Hyline Brown, HB) and white (Isa Tinted, IT) laying hens by x-ray method. Moreover, osteocalcin (OC), calcitonin (CT), and parathormone (PTH) hormones were examined by taking blood from a total of 162 laying hens (54 laying hens from each period) at the 35th, 51st and 60th weeks. The research took place from laying hen ages of 20 to 60 weeks. A total of 396 laying hens (198 HB, 198 IT) were used in the research. Scoring was done at the end of the experiment, using the x-ray images of the chest area of the laying hens at the 35th, 51st, and 60th weeks (162 laying hens). A '0' score was attributed to images with deviation and fracture; while those without them were attributed a score of '1'. The hormones examined were not affected by cage density. Only PTH hormone differed according to age ($p<0.05$) and genotype (IT > HB) ($p<0.001$). It was determined that keel bone damage (deviation, $p=0.009$) was greater at low cage density, and age had no effect on damage occurrence. The study showed that the cage density applied in conventional cage systems may affect keel bone damage.

INTRODUCTION

The keel bone is a flat and large bone that is responsible for body support and is located in the lower part of the bird. The keel bone consists of three parts: cranial, middle and caudal (Can *et al*., 2010; Saraiva *et al*., 2019; Baur *et al*., 2020). This bone has a structure that narrows from the cranial end to the caudal end. The caudal end end of this bone remains cartilaginous even after its ossification process is completed (Casey-Trott *et al*., 2015; Saraiva *et al*., 2019; Baur *et al*., 2020). It is reported that the ossification process of the keel bone is incomplete at the beginning of the laying period, and continues until about 40 weeks of age. (Saraiva *et al*., 2019). By means of the pectoral muscles that attach to it, the keel bone helps birds to fly and flap their wings. Furthermore, as it is connected with the abdominal muscles, the keel bone also has an important role in lung ventilation (Hardin *et al*., 2019; Saraiva *et al*., 2019; Wei *et al*., 2021). Because the breastbone protrudes from the chicken's body, it is the first area affected by impact or trauma (Casey-Trott *et al*., 2015).

Keel bone damage, which has increased in many countries in the last two decades and is thought to cause pain (Casey-Trott *et al*., 2016; Katinka *et al*., 2019; Habig *et al*., 2021) or suffering in hens, is considered one of the most important welfare problems of the laying hen industry (Habig *et al*., 2021; Zhang *et al*., 2022). In addition, keel bone damage has been reported to cause reluctance or decrease in the

natural behaviors of chickens, such as wing flapping, perching, walking, standing, and lying (Dedousi *et al*., 2020; Wei *et al*., 2021). The etiology of keel bone damage remains unclear. Studies have argued that keel bone damage is a multifactorial problem related to trauma, high egg production, breed selection, chicken age, osteoporosis, early laying, nutrition, and late maturation of the keel bone (Kittelsen *et al*., 2020; Laçin & Ayse, 2020; Rufener *et al*., 2020; Kittelsen *et al*., 2023).

In recent years, the X-ray method has been widely preferred for the diagnosis of keel bone damage (Richards *et al*., 2011). It has been stated that fractures of the keel bone are detected with the X-ray method with an accuracy of 82.4% (Jung *et al*., 2022).

It is reported that the main cause of keel bone damage is the rearing system. In general, the use of perches in enriched cages has been reported to increase the risk of keel bone damage compared to conventional cages (Sosnowka *et al*., 2021). Cage density in conventional cages is known to be an important stress factor in laying hens (Ozeturk *et al*., 2022). It has been reported that as cage density increases, egg production and weight in laying hens decrease, the age of sexual maturity is delayed, and the feed utilization rate is negatively affected (Kum *et al*., 2006; Yörük *et al*., 2008; Yardım *et al*., 2021). In addition, it has been stated that at high cage densities, there is a deterioration in the feather quality of chickens, a decrease in immune system function (Zhang *et al*., 2022), and keel bone fractures (Pulcini *et al*., 2023). It is also stated that under high cage densities, the air quality inside the cage is worse, and the immune system of the laying hens is negatively affected (Yildiz *et al*., 2007; Ozenturk & Yıldız, 2021).

Some hormones play a role in the calcium metabolism. Parathormone (PTH) plays a role in the calcium metabolism in bone by stimulating the PTH receptor (PTHR). Furthermore, PTH is the main hormone that plays a role in regulating phosphorus levels throughout the body and acts as a regulator in the healing of fractures and bone formation (Dale *et al*., 2015). Calcitonin (CT) increases as blood calcium levels increase (hypercalcemia), and accelerates the transfer of calcium to the bone (PTH antagonist). The CT hormone prevents bone resorption by ensuring the transition of calcium from the blood to the bones (with the help of vitamin D), and reduces urinary calcium excretion (Güzel, 2008; Atik *et al*., 2009; Topaloğlu *et al*., 2017). Osteocalcin (OC) hormone is one of the hormones responsible for the structure of bone, and is synthesized by osteoblasts. Like PTH and CT, OC is also a hormone that regulates blood calcium metabolism (Güzel 2008).

The majority of scientific studies on keel bone damage in laying hens have been carried out in alternative breeding systems (cage-free system). It remains unclear whether this damage is observed with the use of conventional cage systems, which dominate the egg sector in Türkiye. Therefore, this study was conducted to determine whether cage density in conventional cage systems has an impact on keel bone damage.

MATERIALS AND METHODS

Ethical approval

Permission for this study was obtained from the Local Ethics Committee for Animal Experiments of Atatürk University (decision n. 2021-3/82, dated 14.04.2021). Furthermore, a work permit was obtained from the Food and Livestock Application and Research Center for the conduct of the study (permit n. 36643897-000-E.2100056848, dated 25.02.2021). Permission for the use of the x-ray method in the diagnosis of keel bone damage was obtained from the Animal Hospital of Atatürk University Veterinary Faculty (permit n. 36643897-000-E.2100066648, dated 03.03.2021). Moreover, permission to work in the laboratory for the determination of the hormones involved in chicken bone development was obtained from the Department of Biochemistry (permit n. 36643897-000-E.2100056912, dated 25.02.2021).

This study was carried out at the Poultry Unit of Atatürk University Food and Livestock Research and Application Center. Laying hens were raised in a coop consisting of 3 blocks, 2 rows, and 4 floors of battery cages. There were 240 cages, 120 in the front row and 120 in the back row, with a total of 720 cages, located in a hen house with 3 blocks. These cages had a base slope of 7° that allowed the eggs to fall onto the egg belt. All cages were made of galvanized sheets and wire. There were a total of 2 nipples in two opposing cages. All cages were of the same size. Ventilation in the hen house was provided by ventilation chimneys, side-wall windows, and negative-pressure fans (140 cm x 140 cm). An automatic lighting schedule of 16L:8D (light/dark) was applied. During winter, heating was provided by placing wintering hives inside the henhouse. The temperature and humidity of the house were measured with fixed thermometers (hygro guard 30 novasina) to determine whether the ambient temperature was within or outside the comfort zone

of the chickens. Feed was distributed once a day in the henhouse at 09:00 in the morning, by means of automation.

Animals and study design

In this study, a total of 396 laying hens were used, comprising 198 Isa Tınted hybrids and 198 Hy-Line Brown hybrids. The hybrids, which were transferred to the poultry unit at the pullet stage, were fed *ad libitum* with starter and grower feeds. The trial was started when the hybrids reached 20 weeks of age, and continued until 60 weeks of age. In this study, low (LCD, 5 hens/cage; area per hen 750 cm^2), medium (MCD, 7 hens/cage; area per hen 535.71 cm^2), high (HCD, 10 hens/cage; area per hen 375 cm^2) cage densities were used. Before the chickens were placed in the cages, the animals were weighed individually to ensure uniformity in terms of live weight. Brown and white hybrids of similar live weights were randomly placed in the cages. Live weight uniformity levels of 94% and 93% were achieved for the white and brown hybrids, respectively. Among all of the hybrids (brown + white), 87% uniformity was achieved in terms of live weight. After groups of 5, 7, and 10 hens per cage were established, subgroups were formed with 9 repetitions. In total 54 cages were used, 27 of which faced the windows, while the other 27 faced the central aisle. A total of 396 hybrids, comprising 198 brown and 198 white hybrids, were placed in these cages.

To examine CT, OC and PTH hormones, blood was taken from the wing vein (Vena cutanea ulnaris) of laying hens at 35, 51, and 60 weeks. Blood was taken from a total of 162 laying hens, 54 laying hens in each period. One laying hen from each experimental group was randomly selected. The blood samples were maintained for 10 minutes at room temperature from the time of venipuncture, and were centrifuged at 3500 rpm for 10 minutes. At the 60^{th} week, the serum samples stored at -80°C in a refrigerator were thawed at +4°C for 24 hours, and transferred to the laboratory of the Biochemistry Department of Atatürk University Veterinary Faculty for ELISA analyses.

Measurement procedure of chicken OC, PTH, and CT levels with ELISA kits

Basic principle

Double-antibody sandwich ELISA (enzyme-linked immunosorbent assay) kits were used in this study. The standard or sample was added to the plate wells coated with the specific monoclonal antibody, and then biotin-labelled secondary antibodies were added. Finally, streptavidin-HRP solution was added and the plate was incubated for complex formation. At the end of the incubation period, the plate was washed to remove the enzymes that did not participate in complex formation. A blue colour developed upon reactions during incubation with chromogen A and B. With the addition of the reaction termination solution, the blue colour turned yellow and the intensity of this yellow colour was read with an ELISA reader at a wavelength of 450 nm.

X-rays

A total of 54 brown and white laying hens, one from each cage, at the three time points, and a total of 162 chickens (54x3) at weeks 35, 51 and 60, were transferred to the Radiology Unit of the Animal Hospital of Atatürk University Veterinary Faculty for radiographic examinations. The animals were transported by placing 3 or 5 chickens in a cardboard box with ventilation holes on each corner and the top, depending on the cage densities of the groups (5, 7 and 10 hens per cage). In this study, the chickens were not anesthetized for x-ray imaging. During imaging, the animals were held still using sandbags filled with mosaic stones (Fig 1). The chickens were maintained in lateral recumbency on the cassette for X-ray imaging, and the sandbags were placed on their wings and feet. Laterolateral radiographs were then taken with an X-ray device (46 kV and 2.4 m) set at a distance of 80 cm from the keel bone of the chickens. Keel bone damage was scored based on the x-ray images obtained at the end of the trial. Deviations and fractures in the chest region were examined and scored as follows: for deviation, 0: no deviation; 1: there is a deviation; for fracture, 0: no fracture, 1; there is a fracture (Fig 2). At the end of the study period (60th week), 54 randomly selected chickens were slaughtered for the inspection of the breast area (Fig 3). The purpose of this procedure was to confirm the reliability of the x-ray method and increase the visibility of the breast damage.

Statistical Analysis

The analytical and descriptive analyses of the study data were performed with the SPSS v. 18 software. For the statistical evaluation of the periodically measured parameters, the Repeated Measures of the General Linear Model (GLM) procedure was applied to assess the hormones (CT, OC and PTH) involved in bone development. Fracture and deviation data associated

with keel bone damage were tested using multiple logistic regression analysis.

RESULTS

It was determined that age, breed, and cage density did not have a significant effect on the hormones CT and OC (p>0.05). Table 1 shows that the effect of the breed on PTH was very significant (p<0.001), and the effect of age was significant (p<0.05). The mean PTH levels of the HB and IT layer hens were 52.79 pg/ml and 73.30 pg/ml, respectively. The mean PTH levels at weeks 35, 51 and 60 were 56.11, 56.87 and 76.15 pg/ml, respectively. Furthermore, it was determined that cage density did not have a significant effect on PTH levels (p>0.05) (Table 1).

Table 1 – Variance analysis results of PTH (pg/ml) hormone at different densities, hybrids, and times.

Cage densities	Hybrid (H)	Age (A)	Average	SEM
LCD	Hy-Line Brown	35	51.59	14.01
		51	44.97	14.01
		60	52.30	14.01
	Isa Tinted	35	61.24	14.01
		51	70.76	14.01
		60	61.83	14.01
MCD	Hy-Line Brown	35	62.72	12.54
		51	63.12	12.54
		60	53.33	12.54
	Isa Tinted	35	55.61	12.54
		51	49.73	12.54
		60	105.35	12.54
HCD	Hy-Line Brown	35	49.67	12.54
		51	42.68	12.54
		60	54.707	12.54
	Isa Tinted	35	55.796	12.54
		51	69.98	12.54
		60	129.37	12.54
p<				
CD	NS			
H	***			
A	*			
CD x H	NS			
CD x A	NS			
H x A	*			
CD x H x A	NS			

LCD (5 hens/cage): low cage densities; MCD (7 hen/cage): medium cage densities; HCD (10 hen/cage): high cage densities; CD: cage densities; H: hybrid; A: Age; NS: not significant; SEM: standard error; Hy-Line Brown: brown hybrid; Isa Tinted: White hybrid; ***: p<0.001; *: p<0.05.

As shown in Table 2, the effect of cage density on keel bone fracture was found to be statistically insignificant (p>0.05). Based on the coefficients, it was ascertained that the rate of keel bone fractures decreased at MCD (medium cage density) compared to LCD (low cage density) (p>0.05). Moreover, it was determined that age did not have a significant effect on keel bone fracture (p>0.05) (Table 2).

Table 2 – Regression coefficients and odds ratios of factors affecting keel bone fracture in multiple logistic regression analysis.

Factors	p	Coefficients	SE	Probability ratios
Cage densities	0.739			
LCD		0.248		1
MCD	0.619	-0.141	0.500	1.282
HCD	0.791		0.531	0.869
Age	0.504			
35th week				
52th week	1.000	<0.001	0.543	1.000
60th week	0.324	0.498	0.505	1.646
Constant	<0.001	-1.794	0.488	0.166

LCD (5 hens/cage): low cage densities; MCD (7 hen/cage): medium cage densities; HCD (10 hen/cage): high cage densities; SE; standard error.

The effect of cage density on keel bone deviation was found to be very significant (p=0.009); It was determined that the deviation rate decreased with increased cage density. The effect of age on keel bone deviation was found to be insignificant, but when the probability ratios were examined, it was observed that the deviation rate increased with advancing ages (at week 35: 1; at week 52: 1.227; at week 60: 2.286) (Table 3).

Table 3 – Regression coefficients and probability ratios of factors affecting keel bone deviation (C or S shape) in multiple logistic regression analysis.

Factors	p	Coefficients	SE	Probability ratios
Cage densities	0.009			
LCD				1
MCD	0.161	-0.571	0.407	0.565
HCD	0.002	-1.423	0.463	0.241
Age	0.132			
35th week				1
52th week	0.652	0.205	0.453	1.227
60th week	0.058	0.827	0.437	2.286
Constant	0.137	-0.572	0.384	0.564

LCD (5 hens/cage): low cage densities; MCD (7 hen/cage): medium cage densities; HCD (10 hen/cage): high cage densities; SE; standard error.

Figure 1 - X-ray unit and restraint of laying hen.

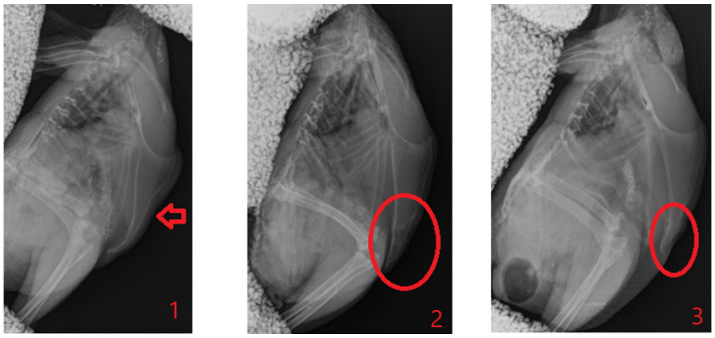

Figure 2 – X-ray image of hybrids with keel bone damage (1: deviation; 2 and 3: fracture).

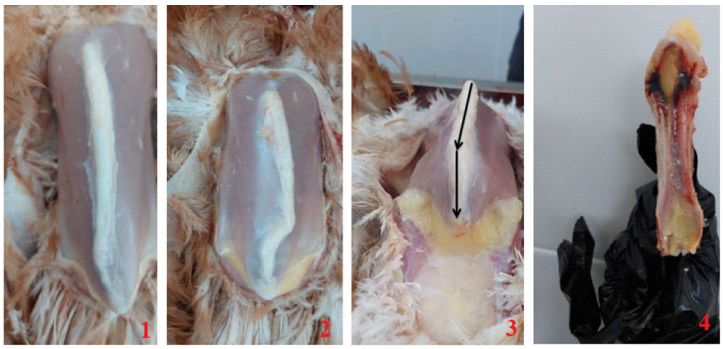

Figure 3 – Hybrids with keel bone damage (photos 1 and 2 are of different hybrids, photos 3 and 4 are of the same hybrid).

DISCUSSION

In this study, it was determined that keel bone deviation was more common in low cage density (p=0.009). This result can be interpreted as a result of more collisions between birds and with poultry equipment, since chickens are freer at low cage density, and trauma generally lies at the root of keel bone damage. In a study on the subject, it was stated that keel bone deviation increased due to increased movement (Fleming *et al*., 2004). However, Vits *et al*. (2005) stated that keel bone damage increased as cage density increased. In another study, it was reported that sternum fractures were more common in high cage densities due to movement restriction (Montalcini *et al*., 2023). There are also studies arguing that the effect of cage density on keel bone damage is insignificant (Abrahamsson, 1993; Habig *et al*., 2013). Fawcett *et al*. (2020) stated that cage density does not affect keel bone damage. When the effect of age on keel bone deviation was assessed, it was observed that the deviation rate increased with advancing age (probability rate at week 35: 1; odds ratio at week 52: 1.227; and probability rate at week 60: 2.286) (p>0.05). Fawcett *et al*., (2020) found that, as in the current study, sternum fracture was not affected by age, but the deviation rate was slightly increased with increasing ages. Another study conducted on the subject reached results consistent with the current study, and it was determined that keel bone damage increases with increasing age (Stratman *et al*., 2015). Several studies have reported that keel bone damage (fracture and deviation) increases with age (Kappeli *et al*., 2011; Petrik *et al*., 2014; Toscano *et al*., 2015; Stratmann *et al*., 2016; Casey-Trott *et al*., 2017; Wei *et al*., 2019; Baur *et al*., 2020; Saraiva *et al*., 2020; Habig *et al*., 2021).

It was determined that the effects of age, breed, and cage density on CT and OC levels were insignificant (p>0.05). As in the current study, another study determined that the effect of age on the OC and CT hormone in laying hens was insignificant (Wei *et al*., 2021). Contrary to the results of the current study and the previous study, it has also been argued that the effect of age on OC and CT hormones is significant (Güzel, 2008). In another study, serum OC levels were reported to decrease with age (Regmi *et al*., 2017). In a study by Wang *et al*. (2020), while the serum CT levels of chickens housed at high and low cage densities did not differ and were similar to those determined in the present study, the serum OC levels were higher at higher cage densities (p<0.05), which contradicts the present study. While the effects of breed (p<0.001) and age (p<0.05) on PTH levels were significant, the effect of cage density was found to be insignificant (p>0.05). These levels were considered to be associated with the egg production of chickens, given that PTH is secreted at low blood calcium levels. During the study period, the HBs laid more eggs than the Its, and are predicted to have had higher blood calcium levels. In a study investigating the serum PTH levels of White Plymouth Rock and White Cornish hens between the ages of 22 and 40 weeks, it was determined that serum PTH levels were higher in the White Plymouth Rocks during the peak egg production period (Preda *et al*., 2013). In the present study, the mean PTH levels at weeks 35, 51 and 60 were 56.11, 56.87 and 76.15 pg/ml, respectively. Another study reported that blood PTH levels do not change with age (Wei *et al*., 2021). In a study on the serum PTH levels of laying hens housed at low and high stocking densities, serum PTH levels were found to be lower at high stocking density, which contradicts the result of the present study (p<0.01) (Wang *et al*., 2020). Serum PTH levels were also found to be higher in Arbor Acres male broilers housed at normal and low cage densities (Ma *et al*., 2020).

CONCLUSIONS

In the present study, keel bone fracture was not affected by cage density and age; and its deviation was not affected by age, but by cage density. It has been determined that keel bone deviation is more common in the low cage density. Furthermore, among the studied hormones with a function on bone development (OC, CT and PTH), breed and age only had an effect only on PTH. Cage density did not affect these hormones.

ACKNOWLEDGMENTS

This manuscript is part of a PhD thesis. I would like to thank TÜBITAK for their support with the 2211 Domestic Postgraduate Scholarship Program during my doctoral education.

Author contributions

AU, EL: conceptualization, investigation, data curation, writing – original draft preparation, formal analysis, supervision, writing – review and editing, methodology, investigation, visualization; AU: supervision, visualization. All authors reviewed the results and approved the final version of the paper.

Funding

This study was supported by Atatürk University Scientific Research Projects Coordination Unit (BAP Unit) as project code TSA-2021-9527.

Data availability statement

Will be available upon request.

Conflicts of interest

The authors declare no conflict of interest.

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