

ANIMAL WELL-BEING AND BEHAVIOR

Effects of alpha-lipoic acid on the behavior, serum indicators, and bone quality of broilers under stocking density stress

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ABSTRACT The objective of present study was to investigate the effects of alpha-lipoic acid (ALA) dietary supplementation on the behavior, physiological and oxidant stress indicators, and bone quality in broilers under high stocking density (HSD) stress. A total of one thousand eight hundred 22-day-old *Arbor Acres* male broiler chicks were randomly allocated to 18 pens (2.97×2.03 m) in 3 groups: 14 birds/m² (NSD, normal stocking density) or 18 birds/m² (HSD) or 18 birds/m² plus 300 mg/kg ALA dietary supplement (HSD + ALA, high stocking density + alpha-lipoic acid). Each treatment had 6 replicates, and the experiment lasted 3 wk. The HSD group was significantly lower than the NSD group ($P < 0.05$) in the frequency of eating, walking, and preening behavior. The alkaline phosphatase activity and serum calcium content were significantly higher, and the parathyroid hormone (PTH) level was significantly lower in the HSD group than in the NSD group ($P < 0.05$). When compared with the NSD group, the HSD group showed an increase ($P < 0.05$) in serum

heterophil/lymphocyte ratio (H/L ratio), corticosterone (CORT), malondialdehyde (MDA) content, and catalase (CAT) activity, whereas a decrease ($P < 0.05$) in total antioxidant capacity (T-AOC), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px) concentrations. The HSD group was also significantly lower ($P < 0.05$) than the NSD group in the tibia and femur breaking strength, bone mineral density, and BMC. Importantly, the addition of ALA into the diets of the HSD group enabled the HSD + ALA group to recover to the levels of NSD group ($P > 0.05$) in the standing and preening behavior, alkaline phosphatase activity, PTH concentration, H/L ratio, CAT, T-AOC, MDA, SOD, and GSH-Px. These results indicate that the increase of stocking density lowered the bone quality, increased the physiological and oxidative stress indicators, and modified the behavior of broilers, whereas ALA dietary supplementation could counteract the reduction in the performance and physiological responses of broilers under high-density environmental stress.

Key words: broilers, stocking density, alpha-lipoic acid, behavior, bone quality

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INTRODUCTION

In the last decades, poultry farmers have been exploring different alternatives to pursue higher production efficiency, reduce costs, and simultaneously meet various requirements of customers in different markets (Estevez, 2007; Shao et al., 2015). Broiler production showed the characteristics of intensive and high stocking density (HSD) regime and exceeded the recommended

density of broilers. For example, the recommended density is 0.070 m²/chicken (36 kg/m²) by the United States national poultry commission and 0.073 m²/chicken (30 kg/m²) by the royal society for the prevention of cruelty to animals and the European Union, with body-weight being calculated by weight per unit area (Ravindran et al., 2006; Robins et al., 2011). When these recommended densities are exceeded, broilers may suffer prolonged stress that is associated with serious health issues (Pettit-Riley and Estevez, 2001; Onbacsilar and Aksoy, 2005; Estevez, 2007; Mosca et al., 2015). Nowadays, quality and safety are the primary concerns in the meat market, and the negative impacts of HSD on broiler production have gained increasing attention from researchers (Dozier et al., 2006; Estevez, 2007).

Several studies have reported that HSD could induce adverse effects on broilers, such as reduced growth

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performance (Estevez, 2007; Zhang et al., 2009) and breast fillet yield (Bilgili and Hess, 1995; Dozier III et al., 2005). Dozier III et al. (2005) mentioned that an increase of stocking density beyond 30 kg/m² leads to the decrease of growth and the increase of footpad lesions and skin scratches. Other studies have shown that male broilers (2.7 kg) exhibited lower body weight, worse feed efficiency, and reduced breast fillet yield with the stocking density increased from 10.5 to 13.2 birds/m² (Bilgili and Hess, 1995). In addition, HSD has been reported to change hormone release, induce poor leg condition, increase disease susceptibility and physiological stress, and cause changes in behavior (Ravindran et al. 2006; Estevez, 2007; Simsek et al. 2009). Ravindran et al. (2006) had also reported that HSD could increase crowding and induce stress in chicks, leading to changes in their metabolism. Simsek et al. (2009) found that crowding conditions (up to 22 birds/m²) could lead to oxidative stress and decrease bursa weight in broilers. Moreover, HSD has been shown to affect the bone quality of broilers, as indicated by increased tibia curvature and decreased tibia breaking strength, because of insufficient rearing space, which may lead to lameness and cause animal welfare issues (Buijs et al., 2012). In general, HSD has different effects on the physiological stress level and metabolism of broilers (Ravindran et al. 2006; Estevez, 2007).

Alpha-lipoic acid (ALA), a sulfur-containing compound, is an essential coenzyme in mitochondria (Lu et al., 2017). In in vivo systems, ALA directly and indirectly regenerates other antioxidants, such as glutathione, ascorbate, and vitamin E (Biewenga et al., 1997). Alpha-lipoic acid has been demonstrated to improve the growth performance and antioxidant capability of broilers (Chen et al., 2011). In our previous work, ALA dietary addition was shown to alleviate the effect of chronic ammonia stress on broilers (Lu et al., 2017). In this study, we hypothesized that ALA dietary addition can also reduce the HSD stress in broilers. To test this hypothesis, we evaluated the impact of ALA supplementation on the behavior, serum indicators, and bone quality of the broilers under HSD stress.

MATERIALS AND METHODS

Experimental Design, Diets, and Management

This experiment was carried out at the Institute of Animal Husbandry and Veterinary Science, Henan Academy of Agricultural Sciences (Zhengzhou). The present study was approved by Henan Academy of Agricultural Sciences and conducted according to the Guidelines for Experimental Animal Research of the Ministry of Science and Technology, PR China (GB 14925-2010; General Administration of Quality Supervision, Inspection and Quarantine of the People's Republic of China, 2010).

The birds (*Arbor Acres*) used in this study were purchased from a commercial hatchery (Wellhope Agri-

Tech Joint Stock Co. Ltd., Kaifeng, Henan, China) and fed with a corn and soybean meal basal diet formulated in the mashed form. At 22 D of age, 1,800 male broilers with a similar bodyweight (BW) were selected and randomly assigned to 18 pens, furnished with tube feeders and nipple drinkers. The experiment consisted of 3 groups (6 pens for each group): normal stocking density (NSD) (35 kg/m²) (positive control), HSD (45 kg/m²) (negative control), and high stocking density + alpha-lipoic acid (HSD + ALA) (experimental group), corresponding to 14 (84 birds/pen), 18 (108 birds/pen), and 18 (108 birds/pen) birds/m², respectively. Each treatment had 6 replicates, and the experiment lasted 3 wk.

The pens were 80 cm above the ground (2.97 m long × 2.03 m wide × 0.7 m high). The mesh of the floor lasted 1.5 × 1.0 cm (inner diameter) is commercially used in China. The amount of feeding and drinking space available per bird was kept constant in the 2 density treatments by blocking a proportion of the feeding area and drinking nipples within the cage.

Corn-soybean meal basal diets were formulated to contain adequate nutrient levels as defined by the NRC standards (NRC, 1994). The experimental HSD + ALA diet was formulated by mixing the basal diet with 300 mg/kg ALA (Shanxi Xintai Pharmaceutical Co., Ltd., China). The compositions of the experimental diets are presented in Table 1.

Throughout the experiment, the feed and water were provided *ad libitum*. The light regime was 23 h of light

Table 1. Composition and calculation analysis of the basal diets (% dry basis).

Items	Days of age	
	1–21 D	22–42 D
Ingredients (%)		
Corn	57.30	61.60
Soybean meal	37.80	33.50
Soybean oil	1.90	2.50
Salt	0.30	0.30
Limestone	1.65	1.58
Choline chloride	0.10	0.10
CaHPO ₄	0.44	0.15
Vitamin premix ¹	0.02	0.02
Trace-mineral premix ²	0.20	0.20
L-Lysine	0.08	0.02
DL-methionine	0.19	0.10
Phytase		0.02
Total	100	100
Proximate compositions (%)		
Metabolic energy (MJ/kg)	12.57	12.99
Crude protein	21.50	20.05
Calcium	1.02	0.91
Available phosphorus	0.46	0.41
Lysine	1.15	1.00
Methionine	0.52	0.42
Methionine + cystine	0.85	0.77
Threonine	0.88	0.81
Tryptophan	0.29	0.27

¹Vitamin premix (per kilogram of diet): vitamin A, 12,000 IU; vitamin D₃, 2,000 IU; vitamin E, 20.75 mg; vitamin K₃, 2.65 mg; vitamin B₁, 2 mg; vitamin B₂, 5 mg; vitamin B₆, 2 mg; vitamin B₁₂, 0.025 mg; biotin, 0.0325 mg; folic acid, 1.25 mg; pantothenic acid, 12 mg; niacin, 50 mg.

²Mineral premix (per kilogram of diet): Mn, 100 mg I, 0.35 mg; Se, 0.15 mg; Zn, 75 mg; Cu, 8 mg; Fe, 80 mg; Co, 0.2 mg.

and 1 h of dark (Downs et al., 2006; Lewis et al., 2009; Tang 2012; Li et al., 2015; Qin et al., 2018). All the management procedures followed commercial settings, including the normal immunization and disinfection, normal commercial feeding management, standard ventilation of the chicken house, daily checking of birds, and timely recording of the growth performance.

Sample Collection and Analysis

Behavioral Observation In behavioral observation, cameras were used to record the behavior of the observed animals in each pen to avoid possible interferences that can occur during direct observation. Briefly, 5 cameras (Noldus observer XT, Version 7.0, Noldus Information Technology BV, Wageningen, the Netherlands) were installed around and in the middle of each of the 18 pens to obtain a uniform overview of the functional areas (Bergmann et al., 2017). Video recordings were performed twice a day to shoot the target birds for 2 h continuously during the last 4 D (4–7 D) in week 6 as previously reported (Neves et al., 2015). The target sampling method was used for observation, and 4 target chickens were labeled with biological dye at each replicate group (Eklund and Jensen, 2010). Table 2 shows the definition of the observed broiler chicken behaviors as reported by Buijs et al. (2010). Behavior frequency is defined as the number of times for the behavior per unit time. The behavior occurrence proportion is defined as the percentage of each behavior in the sum of observed behaviors.

The behavior of each bird was recorded by instantaneous sampling once every 60 s, and the interindividual distance between each animal was measured at the same (instantaneous) time when the video was paused.

Serum Parameters After fasting for 12 h on day 42 of the experiment, 4 birds from each pen were bled by venipuncture, followed by placing the blood collected in each tube in an ice bath immediately and transporting all the samples to the laboratory after collection. Next, one drop of blood was expelled from each heparinized tube to make a thin smear on a clean microscope slide, followed by air-drying, staining the dried blood smear with the Wright's

stain (Janssen Chimica, Beerse, Belgium), counting 100 leukocytes on each smear under a microscope, and calculating the heterophil/lymphocyte (H/L) ratios. The average of 4 samples from 1 pen was recorded for data analysis. Serum samples were obtained by gentle centrifugation at $3,000 \times g$ and 4°C for 10 min, followed by storage at -20°C until further analysis. Serum calcium (Ca), phosphorus (P), parathyroid hormone (PTH), catalase (CAT), corticosterone (CORT), superoxide dismutase (SOD), alkaline phosphatase (ALP), malondialdehyde (MDA), total antioxidant capacity (T-AOC), and glutathione peroxidase (GSH-Px) were analyzed with clinical chemistry assay kits according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Bone Properties At the end of the experiment, tibias, femurs, and humerus were obtained from 2 bird/pen, sealed in plastic bags, and stored at -20°C until further analysis. After carefully removing the cartilage, fat, muscle tissue, and other tissues on the bone, the bone fresh weight, length, and diameter were measured. After defatting (sequential 36-h Soxhlet extraction first with ethyl alcohol and then with diethyl ether) and drying (100°C , 24 h), the left tibia, femur, and humerus were weighed and then ashed in a muffle furnace (600°C , 18 h). The contents of Ca and P were determined respectively by the EDTA titration and ammonium metavanadate colorimetric methods. The right bone was used to measure the fracture strength via the 3-point bending test using a testing machine (TMS-Pro, Food Texture Analyzer, FTC). Force was applied to the center of the bone with a load cell capacity of 50 kg and a crosshead speed of 10 mm/min, which was held by 2 supports at 4.0 cm apart. Bone mineral density (BMD) and bone mineral content (BMC) were measured by a Stratec XCT scanner (Model 922010, Norland Medical Systems Inc., Fort Atkinson, WI) with XMENU software version 5.40 C.

Statistical Analysis

The experiment was conducted as a randomized complete block design with 6 replicates. Each pen was considered an experimental unit. The data followed the normal distribution and were analyzed by one-way analysis of variance using SPSS statistical software (Ver. 22.0 for Windows, SPSS, Inc., Chicago, IL). Tukey's multiple range test was used to determine the means and differences among treatments. Data are presented as mean \pm SE of 6 replicates. Significant differences among treatments were determined at $P < 0.05$.

RESULTS

Behavior

The effects of stocking density and ALA on the frequency of behaviors of broilers are shown in Figure 1. The HSD group was significantly lower ($P < 0.05$) than the NSD group in the frequency of eating, walking,

Table 2. Definition of the recorded behaviors in the broilers.

Behavior	Definition
Eating	Birds were scored as eating when they pecked at the feed;
Drinking	Drinking when they were pecking at the drinkers and subsequently tilted their head back;
Standing	Birds were scored as standing when they were not moving and their body did not touch the floor;
Walking	Walking or running in a normal or nonaggressive manner, without exhibiting any other defined behavior;
Preening	Preening consisted of moving the beak over the feathers;
Stretching	One wing on the same side of the body is extended
Sitting	(half extended) and one leg is extended without moving; Elbows or chest were on the ground, with the head hanging on the neck motionless or swinging briefly to the ground.

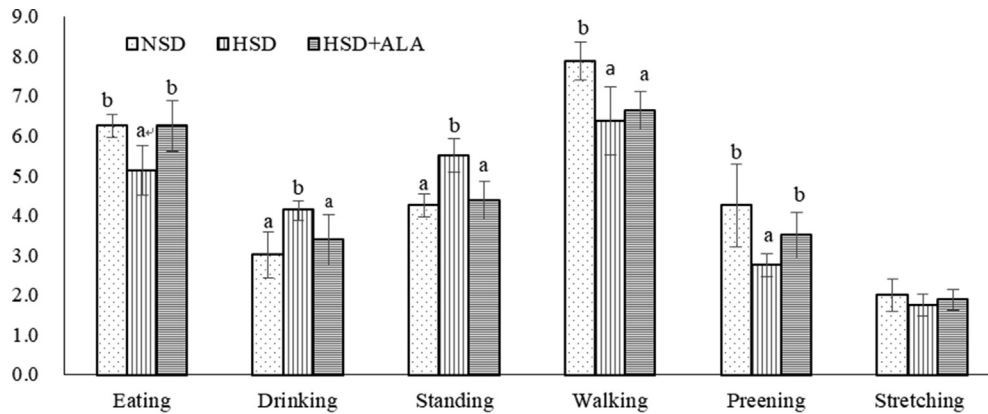


Figure 1. Effect of stocking density and alpha-lipoic acid on the frequency of behaviors of the broilers. NSD, normal stocking density; HSD, high stocking density; HSD + ALA, high stocking density + alpha-lipoic acid. ^{a,b}Means within the same row without similar superscripts are significantly different ($P < 0.05$). Data are presented as mean \pm SE of 6 replicates.

and preening but higher ($P < 0.05$) in the frequency of standing and drinking. Meanwhile, the HSD + ALA group recovered to the level of the NSD group ($P > 0.05$) in the frequency of standing, preening, eating, and drinking. The 3 groups showed no significant differences in the frequency of stretching behavior ($P > 0.05$).

The effects of the stocking density and ALA on the percentage of behaviors of the broilers are presented in Table 3. The HSD group was significantly higher than the NSD group ($P < 0.05$) in the percentage of drinking and standing, whereas lower ($P < 0.05$) in the percentage of eating, walking, preening, and stretching. Meanwhile, the HSD + ALA group recovered to the level of NSD group ($P > 0.05$) in the proportion of stretching and standing behavior of broilers.

Serum Parameters

Table 4 shows the effect of stocking density and ALA on the serum Ca and P concentrations and ALP activity of the broilers. The HSD group was seen to be higher ($P < 0.05$) than the NSD group in serum ALP and Ca concentration, whereas lower ($P < 0.05$) in serum PTH concentration. Adding ALA to the HSD group was shown to reduce the content of ALP (390.10) to the level of the NSD group (275.78 vs. 284.51) ($P < 0.05$). Furthermore, the HSD + ALA group was

Table 3. Effect of stocking density and alpha-lipoic acid on the percentage of behaviors of the broilers.

Items	NSD ¹	HSD ¹	HSD + ALA ¹	P value
Eating	20.37 \pm 1.14 ^b	19.72 \pm 0.72 ^a	19.23 \pm 0.78 ^a	0.023
Drinking	4.78 \pm 0.12 ^a	6.32 \pm 0.46 ^b	6.34 \pm 0.59 ^b	0.031
Standing	24.10 \pm 0.36 ^a	26.11 \pm 0.77 ^b	24.32 \pm 1.12 ^a	0.013
Walking	6.11 \pm 0.43 ^b	4.40 \pm 0.44 ^a	3.97 \pm 0.21 ^a	0.043
Preening	0.86 \pm 0.11 ^b	0.54 \pm 0.07 ^a	0.62 \pm 0.09 ^a	0.022
Stretching	0.35 \pm 0.08 ^b	0.23 \pm 0.03 ^a	0.30 \pm 0.04 ^b	0.019
Sitting	41.44 \pm 0.68 ^a	44.70 \pm 1.61 ^b	46.34 \pm 0.08 ^b	0.011

^{a,b}Means within the same row without similar superscripts are significantly different ($P < 0.05$). Data are presented as mean \pm SE of 6 replicates.

¹NSD: normal stocking density; HSD: high stocking density; HSD + ALA: high stocking density + alpha-lipoic acid.

significantly lower than the HSD group in serum Ca but higher in PTH concentration ($P < 0.05$). However, the 3 groups showed no significant differences in P concentration ($P > 0.05$).

Physiological and Oxidant Stress Index

The effects of stocking density and ALA on the physiological and oxidant stress indicators of broilers are shown in Table 5. The HSD group was seen to be significantly higher ($P < 0.05$) than the NSD group in blood H/L ratio, CORT level, MDA concentration, and CAT activity but significantly lower ($P < 0.05$) in serum T-AOC, SOD, and GSH-Px activity. Meanwhile, the HSD + ALA group was significantly higher ($P < 0.05$) than the HSD group in serum T-AOC, SOD, and GSH-Px activity. Moreover, when compared with the HSD group, the HSD + ALA group significantly decreased ($P < 0.05$) the H/L ratio, CAT activity, and MDA level to the similar values of the NSD group.

Bone Development

The effects of stocking density and ALA on tibia, femur, and humerus are shown in Tables 6–8, respectively. In Table 6, stocking density and ALA were shown to have a strong impact on the tibia development of broilers. The HSD group was significantly lower ($P < 0.05$) than the NSD group in the diameter, weight, BMD, BMC, and strength of tibial bone but higher ($P < 0.05$) in the tibia phosphorus content. No significant differences were observed among the 3 groups ($P > 0.05$) in the length, Ca, and ash of the tibia bone. The weight of tibia was significantly increased ($P < 0.05$) in the ALA + HSD group vs. the HSD group and similar to that of the NSD group.

In Table 7, the HSD group was shown to be significantly lower ($P < 0.05$) than the NSD group in the femoral diameter, BMD, BMC, strength, phosphorus, and ash content of the broilers. No significant differences were found among the 3 groups ($P > 0.05$) in femoral length, weight, and calcium level. However, the HSD

Table 4. Effect of stocking density and alpha-lipoic acid on the serum Ca and P concentrations and ALP activity of the broilers.

Items	NSD ¹	HSD ¹	HSD + ALA ¹	P-value
Serum Ca ² (mmol/L)	2.91 ± 0.14 ^b	3.31 ± 0.07 ^a	2.79 ± 0.13 ^b	0.014
Serum P ² (mmol/L)	2.06 ± 0.33	1.95 ± 0.18	1.98 ± 0.14	0.713
Serum ALP ² (U/L)	275.78 ± 29.99 ^a	390.10 ± 21.58 ^b	284.51 ± 27.15 ^a	0.023
Serum PTH ² (ng/mL)	80.29 ± 5.84 ^b	65.65 ± 6.16 ^a	88.99 ± 6.73 ^b	0.031

^{a,b}Means within the same row without similar superscripts are significantly different ($P < 0.05$). Data are presented as mean ± SE of 6 replicates.

¹NSD: normal stocking density; HSD: high stocking density; HSD + ALA: high stocking density + alpha-lipoic acid.

²Serum Ca: Serum calcium, Serum L: Serum phosphorus, Serum ALP: Serum alkaline phosphatase activity, Serum PTH: Serum parathyroid hormone.

+ ALA group was significantly higher ($P < 0.05$) than the HSD group and similar to the NSD group in femoral BMD and phosphorus.

In Table 8, the HSD group was seen to be lower ($P < 0.05$) than the NSD group in humeral strength and phosphorus. However, there were no significant differences among the 3 groups in the other humeral indices.

DISCUSSION

Behavior

Behavior can usually reflect the comfort of animals with the environment, and several behavioral parameters can serve as reliable indicators for the health and welfare status of broilers (Erasmus, 2017). Beerda et al. (2000) stated that behavior is an important index for the adaptation of animals to their physical condition and social environment. In the present study, the frequency and percentage of behaviors of eating, walking, and preening were shown to be significantly lower in the HSD group than in the NSD group, which were similar to the results reported by Buijs et al. (2010), who found that sitting and preening bouts had a shorter duration in the higher density group. This finding can be partially attributed to the fact that HSD can cause a poor leg condition, such as lameness, leading to less movement, and thus reduced preening. Some studies have also shown that lameness could limit the physical access of broilers to feeders (Feddes et al., 2002; Dozier

III et al., 2005), indicating that the feeding behavior of broilers is decreased as the stocking density is increased. In addition, we found that the frequency and percentage of drinking and standing were both higher in the HSD group than in the NSD group. Dawkins et al. (2004) also mentioned that housing conditions are very crucial in intensive breeding, and the increase of stocking density could decrease the dissipation of body heat and limit air circulation, which may be the cause for the increased drinking behavior.

In the current study, the ALA + HSD group showed an increase in the frequency of eating and preening behavior of broilers. Previous studies have reported that HSD could increase crowding and cause oxidative stress in broilers (Ravindran et al., 2006; Simsek et al., 2009). Furthermore, ALA has been shown to scavenge free radicals, replenish endogenous antioxidants in chicken birds (Packer et al., 2001), and improve their antioxidant capability (Chen et al., 2011). A possible explanation for the positive effects of ALA in the current study is that ALA can enhance the antioxidant capacity and reduce the environmental stress of broilers.

Serum Parameters

Alkaline phosphatase is considered as an early marker of bone formation, and the function of osteoblasts can be evaluated based on their activity (Gallea et al., 2001). Moreover, the PTH can regulate the level of calcium and phosphorus in blood and enhance bone absorption. In the present study, we

Table 5. Effect of stocking density and alpha-lipoic acid on the antioxidant stress index of the broilers.

Items	NSD ¹	HSD ¹	HSD + ALA ¹	P-value
CORT ² (ng/mL)	47.65 ± 1.10 ^a	60.21 ± 0.90 ^b	58.69 ± 1.82 ^b	0.042
H/L ² ratio	0.43 ± 0.01 ^a	0.50 ± 0.03 ^b	0.40 ± 0.03 ^a	0.026
CAT ² (U/mL)	1.44 ± 0.04 ^a	1.61 ± 0.06 ^b	1.39 ± 0.04 ^a	0.017
T-AOC ² (U/mL)	7.42 ± 0.90 ^b	5.09 ± 0.85 ^a	7.58 ± 0.89 ^b	0.033
MDA ² (mmol/ml)	1.56 ± 0.09 ^a	1.76 ± 0.05 ^b	1.52 ± 0.07 ^a	0.012
SOD ² (U/mL)	255.55 ± 10.63 ^b	223.86 ± 9.93 ^a	263.26 ± 12.99 ^b	0.027
GSH-Px (U/mL)	443.11 ± 18.07 ^b	422.31 ± 13.41 ^a	458.41 ± 15.59 ^b	0.035

^{a,b}Means within the same row without similar superscripts are significantly different ($P < 0.05$). Data are presented as mean ± SE of 6 replicates.

¹NSD: normal stocking density; HSD: high stocking density; HSD + ALA: high stocking density + alpha-lipoic acid.

²CORT: corticosterone; CAT: catalase; SOD: superoxide; MDA: malondialdehyde; H/L ratio: heterophil/lymphocyte ratio; T-AOC: total antioxidant capacity; GSH-Px: glutathione peroxidase.

Table 6. Effect of stocking density and alpha-lipoic acid on the tibia characteristics of the broilers.

Items	NSD ¹	HSD ¹	HSD + ALA ¹	P value
Tibia length (mm)	97.60 ± 3.12	92.43 ± 2.42	93.89 ± 2.17	0.600
Tibia diameter (mm)	9.24 ± 0.33 ^b	8.49 ± 0.27 ^a	8.78 ± 0.49 ^a	0.025
Tibia weight (g)	13.26 ± 1.68 ^b	11.55 ± 1.41 ^a	12.95 ± 1.22 ^b	0.136
Tibia BMD ² (g/cm ²)	0.21 ± 0.02 ^b	0.14 ± 0.01 ^a	0.16 ± 0.01 ^a	0.022
Tibia BMC ² (g)	1.93 ± 0.26 ^b	1.42 ± 0.19 ^a	1.65 ± 0.23 ^a	0.025
Tibia Strength (N)	431.45 ± 50.72 ^b	399.88 ± 59.84 ^a	405.08 ± 27.78 ^a	0.041
Tibia Ca ² (%)	17.11 ± 0.94	16.83 ± 0.32	17.28 ± 0.41	0.474
Tibia P ² (%)	7.12 ± 0.25 ^a	8.03 ± 0.44 ^b	7.90 ± 0.11 ^b	0.019
Tibia ash (%)	46.20 ± 1.00	45.38 ± 0.70	46.17 ± 1.64	0.416

^{a,b}Means within the same row without similar superscripts are significantly different ($P < 0.05$). Data are presented as mean ± SE of 6 replicates.

¹NSD: normal stocking density; HSD: high stocking density; HSD + ALA: high stocking density + alpha-lipoic acid.

²Tibia BMD: Tibia bone mineral density; Tibia BMC: Tibia bone mineral content; Tibia Ca: Tibia calcium; Tibia P: Tibia phosphorus.

observed higher serum ALP and Ca concentrations but lower serum PTH concentration in the HSD group vs. the NSD group, with no significant difference between the 2 groups in the serum phosphorus level. Our observations were similar to the results of [Özbey and Esen \(2007\)](#) in that stocking density could significantly reduce the calcium level of rock partridges (*Alectoris graeca*). However, our results were different from the study of [Tong et al. \(2012\)](#) in that the serum ALP, Ca, and P concentrations were not significantly affected by the stocking density. This discrepancy could be attributed to differences in hybrid, stocking density level, diet, etc.

In the present study, the HSD + ALA group was significantly lower than the HSD group in the serum content of ALP and Ca but notably higher in the PTH content. Alpha-lipoic acid has been shown to eliminate free radicals, increase the production of glucose and ascorbate, increase the activity of nitric oxide synthase, and create an acidic environment in the body to promote the absorption and digestion of calcium and phosphorus ([Shay et al., 2009](#)). This well supports the current finding that high density reduces the frequency of feeding behavior, and ALA dietary supplementation can alleviate the density stress by restoring the normal behavior frequency and promoting Ca/P absorption.

Physiological and Oxidant Stress Index

In our study, the HSD group was significantly higher than the NSD group in serum MDA and CAT levels but lower in T-AOC, SOD, and GSH-Px activity. Similar results were also found in several previous studies ([Simsek et al., 2009](#); [Simitzis et al., 2012](#)). For instance, [Macias-Barragan et al. \(2014\)](#) showed that oxidative damage could decrease the content of glutathione in mice. High stocking density can reduce the body's antioxidant capacity and produce lipid peroxidation and oxidative stress products, resulting in further deterioration of tissues and cells in the body.

The H/L ratio is a reliable indicator of stress in poultry, and a high H/L value is associated with increased stress in chickens. Thus, H/L ratio and CORT are considered as the main adaptive stress response indices of poultry ([Puvadolpirod and Thaxton, 2000](#); [Lentfer et al., 2015](#)). In the present study, the serum CORT concentration and the H/L ratio of broilers were significantly higher in the HSD group than in the NSD group, and the addition of lipoic acid in the diet of broilers reared under the high-density environment (ALA + HSD group) tended to reduce the CORT content and decrease the H:L ratio. This agreed with the observations of several previous studies.

Table 7. Effect of stocking density and alpha-lipoic acid on the femur characteristics of the broilers.

Items	NSD ¹	HSD ¹	HSD + ALA ¹	P-value
Femur length (mm)	72.78 ± 1.44	71.66 ± 3.00	72.48 ± 1.65	0.653
Femur diameter (mm)	10.69 ± 0.67 ^b	10.17 ± 0.20 ^a	10.28 ± 0.62 ^a	0.033
Femur weight (g)	10.17 ± 1.31	9.22 ± 0.86	9.40 ± 1.30	0.356
Femur BMD ² (g/cm ²)	0.14 ± 0.02 ^b	0.11 ± 0.02 ^a	0.16 ± 0.02 ^b	0.031
Femur BMC ² (g)	1.13 ± 0.16 ^b	0.72 ± 0.10 ^a	0.88 ± 0.15 ^a	0.032
Femur strength (N)	352.23 ± 26.32 ^b	300.57 ± 38.57 ^a	304.92 ± 29.13 ^a	0.023
Femur Ca ² (%)	16.70 ± 0.86	16.54 ± 0.40	16.25 ± 0.52	0.477
Femur P ² (%)	7.11 ± 0.39 ^b	6.66 ± 0.13 ^a	7.05 ± 0.23 ^b	0.022
Femur ash (%)	46.05 ± 1.27 ^b	44.31 ± 0.89 ^a	45.68 ± 1.65 ^{a,b}	0.083

^{a,b}Means within the same row without similar superscripts are significantly different ($P < 0.05$). Data are presented as mean ± SE of 6 replicates.

¹NSD: normal stocking density; HSD: high stocking density; HSD + ALA: high stocking density + alpha-lipoic acid.

²Femur BMD: Femur bone mineral density; Femur BMC: Femur bone mineral content; Femur Ca: Femur calcium; Femur P: Femur phosphorus.

Table 8. Effect of stocking density and alpha-lipoic acid on the humerus characteristics of the broilers.

Items	NSD ¹	HSD ¹	HSD + ALA ¹	P-value
Humerus length (cm)	67.93 ± 1.44	66.80 ± 1.45	67.13 ± 1.96	0.485
Humerus diameter (cm)	8.03 ± 0.28	7.98 ± 0.21	8.33 ± 0.23	0.051
Humerus weight (g)	6.27 ± 0.73	6.08 ± 0.83	6.72 ± 0.45	0.289
Humerus BMD ² (g/cm ²)	0.15 ± 0.02	0.13 ± 0.02	0.14 ± 0.02	0.166
Humerus BMC ² (g)	0.97 ± 0.16	0.88 ± 0.10	0.78 ± 0.13	0.092
Humerus strength(N)	479.98 ± 43.57 ^b	372.32 ± 49.92 ^a	398.40 ± 64.06 ^a	0.038
Humerus Ca ² (%)	19.45 ± 0.71	18.42 ± 1.21	19.06 ± 0.46	0.144
Humerus P ² (%)	8.80 ± 0.48 ^b	8.07 ± 0.49 ^a	8.12 ± 0.45 ^a	0.031
Humerus ash/%	52.90 ± 2.11	51.63 ± 1.62	52.27 ± 1.53	0.480

^{a,b}Means within the same row without similar superscripts are significantly different ($P < 0.05$).

Data are presented as mean ± SE of 6 replicates.

¹NSD: normal stocking density; HSD: high stocking density; HSD + ALA: high stocking density + alpha-lipoic acid.

²Humerus BMD: Humerus bone mineral density; Humerus BMC: Humerus bone mineral content; Humerus Ca: Humerus calcium; Humerus P: Humerus phosphorus.

Skomorucha et al. (2009) had reported that increasing stocking density lowered physiological indices, including CORT and H/L ratio in chickens. Das and Lacin (2014), Nazar et al. (2015) and Onbasilar et al. (2008) have found that the H/L ratio increases with the increase of stocking density in broilers. A possible explanation for this is the higher level of stress in the HSD group, which results from the deterioration of the housing environment, causing the poultry to show abnormal behavior, leading to a higher metabolic rate under the circumstances of an increasing stocking density.

In the present study, ALA dietary supplementation was shown to significantly decrease the H/L ratio, CAT, and MDA content but increase the T-AOC, SOD, and GSH-Px activity in the serum of the HSD group. Our results agree with the study of Bai et al. (2011), who found that the lipoic acid can improve the antioxidant status of sows and the activity of antioxidant enzyme in the serum of piglets. Lu et al. (2016) have also shown that ALA supplementation increased the SOD and GSH-Px activity and decreased the MDA content in the serum of broilers to different extents under ammonia stress conditions. These results indicated that lipoic acid can increase the activity of antioxidant enzyme and alleviate the oxidative damage caused by oxidative stress, probably because of the fact that, after entering cells, ALA can be partially reduced to dihydro-lipoic acid and exist in the body in the forms of ALA and dihydro-lipoic acid, which have metal-chelating capacity and can scavenge reactive oxygen species (Biewenga et al., 1997).

Bone Development

Bone (femur, tibia, and humerus) quality, length, calcium content, and phosphorus content are important indicators for the bone metabolism and strength. The ash content, also one of the indices for the bone mineral level, is positively correlated with bone strength. In this study, stocking density had a profound impact on tibia and femur quality. However, the HSD group showed no significant difference from the NSD group in the humerus quality of broilers but exhibited a significant reduction

in the BMD and BMC indices and the strength of tibia and femur. These are consistent with the study of Talaty et al. (2009), who showed that tibia breaking strength was lower in the HSD-housed chickens than in the NSD-housed chickens. Buijs et al. (2012) and Hall (2001) have also indicated that as density increases, bone quality decreases, with a decrease in tibial shear strength and an increase in tibial curvature, probably because of a decrease in the activity of birds caused by the high density, thus impairing the health of their legs. Foutz et al. (2007) stated that changes in bone quality are an indirect index for changes in behavior. Bradshaw et al. (2002) also mentioned that some leg deformities are painful and can decrease the walking ability of broilers. Increased density may result in shorter sitting bouts, forcing birds to adjust their sitting posture more often. These results also provide evidence that increased density causes changes in the behavior of broilers.

In the present study, lipoic acid supplementation is shown to relieve the negative effects of high-density stress on bone development, possibly because of the fact that ALA can effectively improve the antioxidant status of broilers (Bai et al., 2011), change their behaviors (Foutz et al., 2007), alleviate the negative effects of high-density stress (Simsek et al., 2009; Simitzis et al., 2012) and finally favor bone formation. Currently, rare studies have been performed on the effect of lipoic acid on bone growth, and its positive effects in antioxidative capacity and Ca/P absorption (Shay et al., 2009) may contribute to the improved bone quality.

CONCLUSION

The results of the present study demonstrate evidence that high-density environmental stress in broilers changed the performance of their main behaviors, decreased their tibia and femur development, and induced physiological and oxidative stress. However, ALA supplementation in the diet can alleviate the negative effects on broilers under high-density environmental stress. Further studies will focus on the effects of ALA on the productivity, intestinal health, and

proteomics of broilers under stocking density chronic stress.

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