EFFECTS OF STOCKING DENSITY ON THE PERFORMANCE, TIBIA MINERALIZATION, AND THE EXPRESSION OF HYPOTHALAMIC APPETITE GENES IN BROILER CHICKENS

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Abstract

The current study investigated the effects of stocking density (SD) on the performance, tibia mineralization, and the hypothalamic appetite genes expression in broilers. A total of 2,800 1-d-old male broilers (Cobb 500) were distributed in a completely randomized design to 1 of 5 SD treatments with 8 replicate cages for each treatment. The SD treatments were 12.5, 15.0, 17.5, 20.0, and 22.5 birds/m², corresponding to 50, 60, 70, 80, and 90 birds per cage (4 m²/cage), respectively. The concentration of tibia phosphorus was determined by the ammonium metavanadate colorimetric method and the mRNA abundance in different tissues was measured by the real-time quantitative PCR method. The data were analyzed by the one-way and/or two-way analysis of variance and polynomial contrasts were used to determine the effect of increasing SD. Feed intake linearly decreased ($P<0.05$) with increasing SD during d 1–42 production period. On d 42, body weight and tibia breaking strength were significantly lower in the groups of 17.5, 20.0 and 22.5 birds/m² than in the groups of 12.5 and 15 birds/m² ($P<0.01$). Concentrations of ash and phosphorus in the tibia of broilers linearly decreased ($P<0.03$) with increasing SD on d 42. The SD of 22.5 birds/m² decreased the mRNA abundance of neuropeptide Y (*NPY*), NPY-receptor (*NPYR*) 1, and *NPYR2* ($P<0.05$), while it increased melanocortin receptor 4 mRNA abundance ($P=0.012$) in the hypothalamus of broilers as compared with the SD of 12.5 birds/m² on d 21 and 42. The mRNA abundance of hypothalamic cocaine and amphetamine-regulated transcript (*CART*), corticotrophin-releasing factor (*CRF*), and CRF-receptor 1 (*CRFR1*) were higher ($P<0.05$) in the group of 22.5 birds/m² than in the group of 12.5 birds/m² on d 21. We concluded that increasing stocking density beyond 15 birds/m² (corresponding to the 45 kg/m² at 42 days of age) suppressed final BW and bone mineralization of broilers raised in multitier cage system. Hypothalamic NPY and CRF signaling might be involved in the anorexigenic effect of HSD.

Key words: stocking density, tibia mineralization, feed intake, broiler

Stocking density (SD) has critical implications for the broilers meat industry, because higher returns can be obtained as the number of birds per unit space increases (Mench, 2018). However, high stocking density (HSD) increases ammonia production, footpad dermatitis, litter moisture, stress, and preening in the litter-flooring system for broilers, resulting in the decrease of performance (Cengiz et al., 2018; Sanchez-Casanova et al., 2019; McKeith et al., 2020). Stocking densities under which broiler chickens are kept vary in different countries and husbandry systems (Verspecht, 2011). In Europe, the allowed stocking density is 33 kg/m² for broilers in a litter-flooring system, but a maximum HSD of 42 kg/m² is prescribed by the European Union (EU) Council Directive under the best practice managerial conditions, including NH₃ and CO₂ concentrations, temperature, and humidity in the housing system (European Commission, 2007). In China, more and more farms adopt a multilayer cage rearing system to save land and labor costs. This does not permit the broilers touching the feces, and the manure belt is regularly cleaned, which improve the air quality in broilers house and reduce the incidence of poultry disease (Li et al., 2017). However, no literature was found about the effects of SD on the broilers performance in this multilayer battery cage-housing system.

High stocking density negatively influences the skeletal development in broilers, as shown by an increase in leg deformations (Buijs et al., 2012; Sun et al., 2018). The leg deformation increases the risk of fracture during catching and transportation, which creates problems during processing (Averós and Estevez, 2018; Rashidi et al., 2019; Tahamtani et al., 2020). Several studies showed that HSD decreased shear strength of the tibia of broilers, but there is some discrepancy in the effect of HSD on the concentrations of calcium (Ca) and phosphorus (P) in tibia of broilers reared in the litter-floor system (Baitshotlhi et al., 2014; Sun et al., 2018). Broiler chickens reared in cages have a higher prevalence of leg deformations and gait problems than those housed in the litter-bedded floor system (Li et al., 2017; Averós and Estevez, 2018). However, little literature was found about the effects of SD on tibia Ca and P deposition in broilers housed in the multilayer cage rearing system.

Previous studies found that HSD decreases the feed intake of broilers (Wang et al., 2014; Madilindi et al., 2018; Cengiz et al., 2018; Sanchez-Casanova et al., 2019; Magnuson et al., 2020; McKeith et al., 2020); however, scanty literature was reported about the mechanism of this action. Hypothalamus plays a critical role in the appetite regulation (Harrold et al., 2012; Everaert et al., 2019). Two distinct neuronal populations in the arcuate nucleus of the hypothalamus (ARC) are involved in the regulation of appetite and feed intake (Everaert et al., 2019). Neuropeptide Y (NPY) and agouti-related peptide (AgRP), which are colocalized in the ARC, both exert orexigenic effects, while pro-opiomelanocortin (POMC), and cocaine and amphetamine-regulated transcript (CART), which are also colocalized in distinct but adjacent subsets of neurons within the ARC, have anorexigenic effects (Harrold et al., 2012; Yi, 2016; Everaert et al., 2019). Additionally, in the paraventricular nucleus of the hypothalamus, corticotropin-releasing factor (CRF) family neuropeptides, including CRF and urocortin (UCN) 3, also have an

anorexigenic effect (Maniam and Morris, 2012). This biological function is mediated by two G-protein-coupled receptors, corticotropin-releasing factor receptor (CRFR) 1 and CRFR2 (Stengel and Taché, 2014). Thus, we hypothesized that hypothalamic neuropeptides are involved in the feed intake regulation of broilers in response to HSD.

The objective of this study was to investigate the effects of SD on the performance, bone mineralization of broilers reared in the multilayer cage-housing system, and the expression of potential hypothalamic appetite genes involved in the feed intake regulation of broilers.

Material and methods

All procedures in our study were approved by the Animal Care and Use Committee of Sichuan Agricultural University (SAU-20170034). The study was undertaken following the Guidelines for Experimental Animals established by the Ministry of Science and Technology (Beijing, China).

Birds, experimental design, and management

This research was conducted in a closed broiler house owned by Yuguan Ecological Food Company, Sichuan, China, during the autumn of 2017. In the closed house, a 4-tier H-type cages system was used and there was an automatic cleaning manure belt under each tier of cages. After acclimatizing for 24 h, a total of 2,800 1-d-old male broilers (Cobb 500) were distributed in a completely randomized design to 1 of 5 SD treatments with 8 replicate cages for each treatment. The SD treatments were 12.5, 15.0, 17.5, 20.0, and 22.5 birds/m², corresponding to 50, 60, 70, 80, and 90 birds per cage (4 m²/cage), respectively. Experimental birds were reared in the second tier of this multilayer cages system during the whole experiment (d 1–42). All broilers were fed the same diets (Table 1) in the starter (d 1–21) and grower (d 22–42) periods. The diets were formulated according to the nutrient requirements for the broilers recommended by the Feeding Standard of Chicken (Ministry of Agriculture of the People's Republic of China, 2004). Each replicate cage (200 × 200 × 50 cm) had a fiberglass feeder outside, nine drinkers, and one plastic bottom floor mesh. The broilers were allowed *ad libitum* access to the diets and water. The manure belts were automatically cleaned twice per week during d 1–21 and three times per week during d 22–42. The ventilation rate was controlled to keep the air movement at 4.75 cubic meters per hour, per kilogram body weight in the house. The NH₃ and CO₂ concentrations, temperature, and humidity in the house were regulated according to the Environmental Management in the Broiler House (www.aviagen.com). The other management procedures, including lighting schedule and vaccination, were conducted according to the Cobb Broiler Management Guide (www.cobb-vantress.com).

Table 2. Primers for real-time PCR¹

Gene	Accession No.	Orientation	Primer sequence (5'-3')
β -actin	NM_205518.1	Forward	GAGAAATGTGCGTGACATCA
		Reverse	CCTGAACCTCTCATTGCCA
NPY	M87294.1	Forward	CATGCAGGGCACCATGAG
		Reverse	CAGCGACAAGGCGAAAGTC
AgRP	XM_004950992.1	Forward	GGTTCTTCAACGCCTTCTGCTA
		Reverse	TTCTTGCCACATGGGAAGGT
POMC	NM_001031098.1	Forward	GCCAGACCCCGCTGATG
		Reverse	CTTGTAGGCGCTTTTGACGAT
CART	XM_003643097.2	Forward	GCTGGAGAAGCTGAAGAGCAA
		Reverse	GGCACCTGCCCCGAACCT
NPYR1	NM_001031535.1	Forward	TAGCCATGTCCACCATGCA
		Reverse	GGGCTTGCTGCTTTAGAGA
NPYR2	NM_001031128.1	Forward	TGCCTACACCCGCATATCG
		Reverse	GTTCCCTGCCCCAGGACTA
NPYR5	NM_001031130.1	Forward	GGCTGGCTTTGTGGGAAA
		Reverse	TTGTCTTCTGCTTGCGTTTGT
CRF	NM_001123031.1	Forward	TCAGACCAAGAGCCATACA
		Reverse	GCTCTATAAAAATAAAGAGGTGACATCAGA
UCN3	XM_001231710.2	Forward	GGGCCTTCCGTCTCTACAATG
		Reverse	GGTGAGGGCCGTGTTGAG
CRFR1	NM_204321.1	Forward	CTGCTGTCTTGTGCTGGGAAT
		Reverse	ATCCTCCCCGGATTGAC
CRFR2	NM_204454.1	Forward	GGATCAAATACAACACCACAAAAAAT
		Reverse	GGCCCATGTCCCATTGC
MC3R	XM_004947236.1	Forward	GCCTCCCTTTACGTTACATGT
		Reverse	GCTGCGATGCGCTTCAC
MC4R	NM_001031514.1	Forward	CCTCGGGAGGCTGCTATGA
		Reverse	GATGCCCAGAGTCACAAACACTT

NPY, neuropeptide Y; AgRP, agouti-related peptide; POMC, pro-opiomelanocortin; CART, cocaine and amphetamine-regulated transcript; NPYR, NPY receptor; CRF, corticotrophin-releasing factor; UCN3, urocortin 3; CRFR, CRF receptor; MC3R, melanocortin receptor 3; MC4R, melanocortin receptor 4.

¹Primers for UCN3, MC3R, and MC4R genes were described in Wang *et al.* (2017).

Growth performance and carcass traits

Body weights (BW) and feed intake for each replicate cage were measured on d 1, 21, and 42. Body weight gain (BWG) and average daily feed intake (ADFI) were calculated during starter (d 1–21), grower (d 22–42), and overall (d 1–42) phases. Mortality was recorded daily. Any bird that died was weighed and the weight was used to adjust the feed conversion ratio (FCR). The FCR was calculated by dividing total feed intake by live weight plus dead birds in each phase. On d 42, one bird per replicate was randomly selected, fasted for 8 h, and weighed. All selected birds were hung on shackles by both legs, and were electrically stunned using a waterbath stunner with a space current of 100 milliamperes. The birds were then sacrificed by

cervical dislocation. After exsanguination, the head, feathers, feet, digestive organs, abdominal fat (comprising fat surround the abdominal organs), and internal organs (including heart, liver, spleen, bursa, and lung) were removed from the birds. The carcass was then spray-washed, immersion chilled at 2°C for 30 min, and allowed to drain for 5 min effectively. The eviscerated carcass was weighed and the eviscerated carcass yield was calculated as a percentage of live body weight. The breast muscle, leg muscle, and abdominal fat were weighed, and the breast muscle, leg muscle, and abdominal fat yields were calculated as percentages of eviscerated carcass weight.

Samples collection

On d 21 and 42, one bird per replicate cage was selected according to the average BW within the cage after an 8 h of fasting. All selected birds were electrically stunned and then were sacrificed by cervical dislocation as described in growth performance and carcass traits section. The left tibia was then dissected and stored at -20°C for tibia breaking strength and tibia calcium and phosphorus contents analyses. For the selected birds in the low stocking density (LSD) group of 12.5 birds/m² and the HSD group of 22.5 birds/m², the hypothalamus was collected as described by Shipp et al. (2015). The collected hypothalamus was snap-frozen in liquid nitrogen for the mRNA abundance analysis. On d 42, approximately 500 g of fresh excreta samples (mixtures of feces and urine) were collected from the bottom tray of each cage into plastic bags and stored immediately at -20°C until analysis.

Excreta ammonia emissions analysis

Ammonia emission of each excreta sample was determined, as described by Moore et al. (1996). In brief, the total sampled manure from each cage was thawed and then homogenized, after which 100 g subsamples in triplicate were placed in 2-L plastic containers equipped with air inflows and outflows. The samples were incubated at 22°C and ammonia-free air (the air passed through two consecutive 1 M hydrochloric acid (HCl) traps and one trap containing deionized water) was continuously passed through each chamber. The ammonia volatilized from the excreta was trapped in two consecutive boric acid traps containing 100 mL of 0.32 N boric acid solution each. After 24 h post-incubation, the traps were titrated with 0.10 N HCl to determine the ammonia content. The ammonia emission was expressed as milligrams per 100 g of excreta.

Tibia breaking strength and minerals analyses

Tibia breaking strength (TBS) was determined, as described by Lei et al. (2011). Briefly, tibia samples were boiled in deionized water for 10 min, and all soft tissue was removed. Tibia breaking strength was measured using a texture analyzer (Model TA.XT Plus, Stable Micro Systems Ltd., UK). Samples were placed on vertical brackets set 40 mm apart, and a 10 mm compression rod was positioned near the center of the bone. The instrument was equipped with a 20-kg-load cell, and a crosshead speed of 50 mm/min was used during the breaking strength determination. Tibia samples were then dried for 12 h at 105°C and ashed in a muffle furnace (Model Sx2-SRJX, Shenyang Electric Furnaces Co., Shenyang, China) at 550°C for 8 h. The tibia ash samples were subjected to digestion in nitric acid (reagent grade,

70.0% purity) in a microwave digestion system (Mars 6, CEM Corporation, Matthews, NC, USA). The concentration of calcium (Ca) in tibia was determined using an atomic absorption spectrophotometer (ContrAA 700, Analytik Jena AG, Jena, Germany). The concentration of tibia phosphorus (P) was determined by the ammonium metavanadate colorimetric method (method 991.25, AOAC, 2000), using an ultraviolet and visible spectrophotometer (Model DU730, Beckman Coulter Inc., Brea, CA, USA) at 400 nm.

Total RNA extraction and real-time PCR assays

Total RNA was extracted from each hypothalamic sample using the Trizol reagent (Invitrogen, San Diego, CA, USA) following the manufacturer's instructions. Subsequently, the extracted RNA sample was transferred to the spin column and further purified using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) with the optional RNase-free DNase I (Qiagen) treatment. The RNA quality was evaluated by agarose-formaldehyde gel electrophoresis, and RNA concentration was quantified using a spectrophotometer (NanoDrop 2000; Thermo Fisher Scientific Inc., Madison, WI, USA) at 260/280/230 nm. The first-strand cDNA was reverse-transcribed from 200 ng of total RNA using the iScript™ Select cDNA Synthesis Kit (Bio-Rad Laboratories Inc., Hercules, CA, USA). Quantitative real-time PCR was performed in triplicate on an iCycler iQ™ Real-Time PCR Detection System (Bio-Rad Laboratories Inc.). Real-time PCR was performed in 10-μL volume reactions that contained 3 μL of 10-fold diluted cDNA, 1 μL each of 5 μM forward and reverse primers for the different gene (Table 2), and 5 μL 1 × iQ™ SYBR® Green Supermix (Bio-Rad Laboratories Inc.). The cycling program was 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s, and a final melting curve analysis. The β-actin was used as the reference gene, and the target and reference genes had similar and nearly 100% amplification efficiencies by performing a 10-fold dilution series experiment as described by Bai et al. (2014). Relative quantities of mRNA were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), and the samples from the LSD group on d 21 were used as the calibrator.

Statistical analysis

Normality tests were performed using the univariate procedure of SAS 9.2 (SAS Institute Inc., 2009) with normal and plot options, and Levene's tests were used to evaluate heterogeneity of variances. The effect of stocking density on the growth performance, ammonia emission, and tibia mineralization parameters was analyzed by one-way and/or two-way analysis of variance (ANOVA) using general linear model (GLM) procedure of SAS 9.2 (SAS Institute Inc., 2009). However, the data of gene expression was analyzed by two-way analysis of variance (ANOVA) using the GLM procedure of SAS 9.2 (SAS Institute Inc.). The statistical model included the effects of stocking density, sampling time, and their interaction. For all data, when the ANOVA was significant, *post hoc* comparisons of treatment means were carried out using Tukey's tests, and polynomial contrasts were used to test linear and quadratic responses for increasing stocking density. The replicate cage served as the experimental unit ($n = 8$). All statistical significances were set at $P < 0.05$.

Results

Growth performance and carcass traits

Table 3 shows the results of the growth performance of broilers. There was no significant difference ($P>0.5$) on initial body weight (D1) in all studied SD. Stocking density had significant effect ($P<0.01$) on BW at D21 and D42. The BW linearly decreased with the increase in SD at D21 and D42. The birds were heavier in the LSD than in the HSD at D1 and D42. The BWG and feed intake linearly decreased ($P<0.01$) with increasing SD from 12.5 to 22.5 birds/m² in the starter period (d 1–21). The BWG was significantly higher in the SD of 12.5 and 15.0 birds/m² than in the SD of 22.5 birds/m², whereas there was not significant difference between birds in SD of 12.5 and 15.0 birds/m² during d 1–21. The feed intake in the SD of 12.5 birds/m² was greater ($P<0.01$) than that in the SD of 15.0, 17.5 birds/m² and 22.5 birds/m². The BWG also linearly decreased ($P<0.01$) with increasing SD from 12.5 to 22.5 birds/m² in the grower period (d 22–42). The feed intake in the SD of 20 and 22.5 birds/m² was significantly lower than in the SD of 15.0 birds/m² during d 22–42. The BWG and feed intake linearly decreased ($P<0.05$) with increasing SD from 12.5 to 22.5 birds/m² during the entire experimental period (d 1–42). Birds in the lower SD consumed less feed and gained less weight than birds in higher SD which consumed more feed and gained more body weight during entire production period (d 1–42). The SD had no significant influence on the FCR during d 1–21, d 22–42, and d 1–42 ($P>0.10$) and also on mortality during the entire d 1–42 production period.

Table 3. Effects of stocking density on the performance of broilers¹

Item	Stocking density (birds/m ²)					SEM	P-value	
	12.5	15.0	17.5	20.0	22.5		linear	quadratic
BW (g)								
D 1	46.9	46.9	46.7	47.0	46.8	0.14	0.992	0.902
D 21	883 a	856 ab	846 bc	840 bc	819 c	7.11	0.002	0.709
D 42	3130 a	3129 a	3074 b	3028 c	3043 bc	12.4	0.001	0.272
BWG (g)								
D 1–21	836 a	809 ab	799 bc	793 bc	772 c	7.13	<0.001	0.540
D 22–42	2247 ab	2272 a	2227 b	2168 c	2224 b	13.7	<0.001	0.278
D 1–42	3083 a	3082 a	3027 b	2981 b	2996 b	12.4	0.001	0.272
Feed intake (g)								
D 1–21	1091 a	1062 b	1046 b	1040 bc	1019 c	6.48	<0.001	0.315
D 22–42	4107 abc	4172 a	4139 ab	4017c	4052 bc	42.5	0.356	0.017
D 1–42	5197	5234	5185	5074	5071	44.5	0.043	0.118
FCR (g:g)								
D 1–21	1.235	1.241	1.236	1.239	1.244	0.006	0.205	0.828
D 22–42	1.828	1.835	1.857	1.852	1.821	0.020	0.304	0.004
D 1–42	1.685	1.698	1.712	1.702	1.693	0.013	0.233	0.003
Mortality (%)								
D 1–42	1.18	2.50	1.54	3.50	2.50	1.89	0.341	0.463

BW = body weight; BWG = body weight gain; FCR = ratio of feed consumption: body weight gain.

¹Means ($n = 8$) within a row lacking a common lowercase differ ($P<0.05$).

Table 4. Effects of stocking density on the carcass traits of broilers¹

Item	Stocking density (birds/m ²)					SEM	P-value	
	12.5	15.0	17.5	20.0	22.5		linear	quadratic
Eviscerated carcass yield (%) ²	69.14	70.69	68.36	69.91	70.24	1.25	0.200	0.267
Breast muscle yield (%) ³	28.89	27.93	29.73	28.47	29.37	0.52	0.390	0.743
Leg muscle yield (%) ³	20.42	21.37	20.67	21.19	20.74	0.37	0.678	0.268
Abdominal fat yield (%) ³	1.79 b	1.68 b	1.83 b	2.34 a	2.19 a	0.11	<0.001	0.554

¹Means (n = 8) within a row lacking a common lowercase differ (P<0.05).

²Calculated as a percentage of live body weight.

³Calculated as a percentage of eviscerated carcass weight.

Results of the carcass traits of broilers are shown in Table 4. The SD had no significant effect (P>0.10) on the eviscerated carcass, breast muscle, and leg muscle yields of broilers on d 42. The abdominal fat yield linearly increased (P<0.01) with increasing SD in broilers on d 42. The broilers had higher abdominal fat yield in the SD of 20.0 and 22.5 birds/m² (P<0.05) than that in the SD of 12.5, 15 and 17.5 birds/m².

Excreta ammonia emission

Results of the excreta ammonia emission are shown in Figure 1. Ammonia emission from the excreta of broilers was higher (P<0.05) in the group of 22.5 birds/m² than in the SD of 20 birds/m² which was greater than that in the SD of 12.5, 15 and 17.5 birds/m². There was no significant difference in the ammonia emission from the excreta of broilers among the SD of 12.5, 15.0, and 17.5 birds/m² (P>0.22).

Figure 1. The ammonia emission from the excreta of broilers reared at different stocking density. Data are means $\pm$ S.E.M (n = 8). Bars lacking different letters are significantly different (P<0.05)

Table 5. Effects of stocking density on bone mineralization parameters in the tibia of broilers (dried-tibia basis)¹

Item	Stocking density (birds/m ²)					SEM	P-value	
	12.5	15.0	17.5	20.0	22.5		linear	quadratic
D 21								
TBS (kg)	18.67	14.38	17.66	19.16	17.79	1.48	0.556	0.484
Ash (%)	45.59	46.68	47.03	45.31	45.50	0.70	0.503	0.142
Ca (%)	15.55	15.82	16.16	15.28	15.16	0.29	0.176	0.081
P (%)	7.75	7.91	7.85	7.87	7.50	0.14	0.224	0.083
D 42								
TBS (kg)	44.74 a	42.34 a	28.62 b	30.78 b	29.89 b	2.71	<0.001	0.079
Ash (%)	50.10	49.04	48.43	48.62	47.80	0.69	0.026	0.599
Ca (%)	16.20	16.36	16.90	16.71	16.60	0.33	0.291	0.318
P (%)	8.17 ab	8.39 ab	8.54 a	8.11 b	7.42 c	0.15	0.001	<0.001

TBS, tibia breaking strength.

¹Means ($n = 8$) within a row lacking a common lowercase differ ($P < 0.05$).

Bone mineralization parameters

Table 5 shows the tibia mineralization results of broilers on d 21 and 42 of production period. The SD had no significant effect on TBS, and the contents of ash, calcium, and phosphorus in the tibia of broilers on d 21 ($P > 0.10$). However, on d 42, TBS, tibia ash and phosphorus contents linearly decreased with increasing SD from 12.5 to 22.5 birds/m² ($P < 0.05$). The TBS of broilers was lower in the SD 17.5, 20.0, and 22.5 birds/m² than that in the groups of 12.5 and 15.0 birds/m² ($P < 0.04$) on d 42. Only tibia phosphorus content was lower at the SD of 22.5 birds/m² treatment than that at the SD of 12.5 birds/m² treatment on d 42 ($P < 0.05$). No significant difference was observed in the tibia calcium content across all the studied SD treatments on d 42 ($P > 0.10$).

Hypothalamic gene expression

Table 6 shows the results of hypothalamic gene expression in broilers during d 21 and 42 of production period. The SD significantly influenced the mRNA expression of *NPY*, *NPYR1*, *NPYR2*, *UCN3*, and *MC4R* in the hypothalamus of broilers. The HSD of 22.5 birds/m² decreased the mRNA abundance of *NPY*, *NPYR1*, *NPYR2*, and *UCN3* ($P < 0.05$), while it increased *MC4R* mRNA abundance ($P = 0.012$) in the hypothalamus as compared to the LSD of 12.5 birds/m². The mRNA abundance of hypothalamic *NPYR1*, *MC3R*, and *MC4R* increased ($P < 0.05$) in broilers from d 21 to d 42. The interaction of the SD and age of broiler significantly influenced the mRNA expression of *CART*, *CRF*, and *CRFR1* in the hypothalamus of broilers. The HSD of 22.5 birds/m² increased the mRNA abundance of hypothalamic *CART*, *CRF*, and *CRFR1* in broilers on d 21 ($P < 0.05$), while it did not significantly affect these indices in broilers on d 42 as compared to the LSD of 12.5 birds/m² ($P > 0.10$). The SD, age of broiler, and their interaction had no significant influence ($P > 0.10$) on the mRNA expression of *AgRP*, *POMC*, and *CRFR2* in the hypothalamus of broilers.

Table 6. Effects of stocking density on orexigenic and anorexigenic neuropeptide and receptor mRNA expression in hypothalamus of broilers¹

Effects	SD		NPY	AgRP	POMC	CART	NPYR1	NPYR2	NPYR5	CRF	UCN3	CRFR1	CRFR2	MC3R	MC4R
		time													
LSD	D 21		1.07	1.00	1.00	1.01 b	1.00	1.00	1.00	1.02 b	1.03	1.00 b	1.06	1.02	1.07
	D 42		0.68	0.56	0.99	1.65 a	0.67	0.37	0.89	2.41 a	0.68	2.28 a	0.96	1.22	1.82
HSD	D 21		1.47	1.17	1.16	1.02 b	1.30	0.91	1.07	0.86 b	1.17	0.94 b	1.02	1.32	1.71
	D 42		0.87	1.01	1.19	0.94 b	0.96	0.46	1.28	1.00 b	1.00	1.19 b	1.13	1.54	2.07
SEM			0.17	0.17	0.20	0.11	0.11	0.10	0.16	0.18	0.11	0.11	0.14	0.11	0.19
SD															
LSD			1.27	1.09	1.08	1.01	1.15	0.95	1.04	0.94	1.10	0.97	1.04	1.17	1.39
HSD			0.78	0.79	1.09	1.30	0.81	0.42	1.08	1.71	0.84	1.74	1.05	1.38	1.95
SEM			0.11	0.12	0.16	0.08	0.07	0.08	0.12	0.12	0.08	0.08	0.10	0.08	0.14
Time															
D 21			0.87	0.78	1.00	1.33	0.83	0.68	0.94	1.71	0.85	1.64	1.01	1.11	1.45
D 42			1.17	1.09	1.18	0.98	1.13	0.68	1.18	0.93	1.08	1.06	1.08	1.43	1.89
SEM			0.12	0.13	0.15	0.07	0.08	0.08	0.13	0.12	0.08	0.07	0.10	0.07	0.13
P-value															
SD			0.009	0.113	0.969	0.058	0.007	<0.001	0.775	<0.001	0.032	<0.001	0.993	0.076	0.012
Time			0.100	0.101	0.446	0.004	0.015	0.993	0.185	<0.001	0.059	<0.001	0.620	0.010	0.037
SD × Time			0.543	0.457	0.954	0.004	0.983	0.438	0.355	0.003	0.461	<0.001	0.478	0.936	0.348

SD, stocking density; LSD, the SD of 12.5 birds/m²; HSD, the SD of 22.5 birds/m²; NPY, neuropeptide Y; AgRP, agouti-related peptide; POMC, pro-opiomelanocortin; CART, cocaine and amphetamine-regulated transcript; NPYR, NPY receptor; CRF, corticotrophin-releasing factor; UCN3, urocortin 3; CRFR, CRF receptor; MC3R, melanocortin receptor 3; MC4R, melanocortin receptor 4.

¹Means (*n* = 8) within a column lacking a common lowercase differ (*P* < 0.05).

Discussion

Growth performance

The results of the present study demonstrated that HSD significantly decreased feed intake, and BWG, but did not improve FCR during the entire experimental period. These results were consistent with the findings of the previous studies, conducted in the litter-flooring system, that reported reduced body weight gain and feed intake of chickens at HSD (Beloor et al., 2010; Shakeri et al., 2014; Madilindi et al., 2018; Li et al., 2019). Similarly, Abudabos et al. (2013) and Rambau et al. (2016) found no effect on FCR of increasing the stocking density of broilers from 30 to 40 kg/m². The poor growth performance of broilers under HSD presented in this study might be due to the reduction in feed intake (Feddes et al., 2002; Dozier et al., 2005), but not due to the poor feed efficiency (Benyi et al., 2012, 2015). There was higher amount of ammonia release from broiler excreta at SD of 20 and 22.5 birds/m² in the present study. Thus, it could be another explanation of the poor growth performance due to the poor air quality (microclimate conditions) at HSD (Dawkins et al., 2004; Meluzzi et al., 2008). The SD of 15 birds/m², corresponding to the 45 kg/m² at 42 days of age, had no detrimental effect on the performance of broilers. This stocking density was slightly higher than a maximum of 42 kg/m² prescribed by the EU Council Directive under the best practice managerial condition in a litter-flooring system (European Commission, 2007). The differences in the multilayer cage-housing system and litter-flooring system might cause this disparity in the optical stocking density (Swain et al., 2002; Li et al., 2015, 2017).

Carcass characteristics

In the present study, the HSD increased the percentage of abdominal adipose tissue weight in broilers. The increase in abdominal fat deposition might be due to the less locomotion of broilers at HSD (Buijs et al., 2009, 2012). The increment in abdominal fat depot at HSD was consistent with the previous findings that HSD increased abdominal fat percent in broilers (Beg et al., 2011; Siaga et al., 2017). However, few studies found no significant effect on abdominal fat deposition of high stocking density in broilers (Tong et al. 2012; Rashidi et al., 2019). These disparities might be due to the differences in the scale of SD, housing conditions, and dietary composition in these studies. Adipose tissue not only accumulates lipids, but also acts as an endocrine organ (Zhang et al., 2014). The increment in abdominal fat deposition is associated chances to enhance the secretion of the adipokines, including leptin and adiponectin (Resnyk et al., 2015; Mellouk et al., 2018). These adipokines can bind to their receptors in the hypothalamus, resulting in the downregulation of NPY signaling and the increase in feed intake (Yi and Tschöp, 2012; Zhang et al., 2014). Unfortunately, we did not determine the concentration of these adipokines secreted by adipose tissue in this study.

Bone mineralization

In this study, the higher SD (exceeding 17.5 birds/m²) reduced the TBS of broilers on d 42, and it is in line with the previous findings in the litter-floor rearing

system, which reported decrease in tibia strength which occurred with increasing stocking density (Buijs et al., 2012; Vargas-Galicia et al., 2017). We also found that tibia ash content linearly decreased with increasing SD and the SD of 22.5 birds/m² decreased tibia phosphorus concentration on d 42. These results differed from the previous studies that reported no effect of SD on tibia mineral content, Ca, and P concentration in broilers reared in the litter-floor system (Tablante et al., 2003; Simsek et al., 2011; Sun et al., 2018). One possible explanation may be that the broilers could access the Ca and P from the litter (Blake and Hess, 2014), but they did not get them in the multilayer cage rearing system.

Hypothalamic gene expression

In order to elucidate the underlying mechanisms of decreased feed intake by HSD, we determined the orexigenic and anorexigenic appetite-regulating genes expression pattern in broilers. Higher stocking density decreased the transcriptional expression of *NPY* and increased *CART* mRNA abundance. In parallel, the decrease in the mRNA abundance of hypothalamic NPY receptors *NPYR1* and *NPYR2* occurred in broilers reared in HSD treatment. These results were similar to the recent findings regarding the decrease in expression of hypothalamic NPY mRNA, an orexigenic neuropeptide, in chronic heat stressed chicks (He et al., 2019). Our findings suggested that hypothalamic NPY pathway also is involved in the feed intake regulation of broilers in response to HSD treatment. They further showed the simultaneous increases in the hypothalamic CRF and CRFR1 of broilers under HSD rearing on d 21. The CRF signaling exacerbates the anorexic phenotype in broilers in the presence of environmental stressors (Stengel and Taché, 2014; Wang et al., 2017). These results indicated that the CRF signaling is involved in the regulation of the decrease in feed intake of broilers reared in HSD (Dedic et al., 2018). On the other hand, we found that HSD increased hypothalamic MC4R mRNA expression. The central melanocortin system plays a pivotal role in the appetite regulation under resting conditions (Boswell and Dunn, 2015; Wang et al., 2017). The increase in hypothalamic MC4R mRNA abundance indicated the melanocortin system plays a potential role in the decrease in feed intake of broilers reared in HSD. However, no significant effect of HSD on the mRNA expression of MC3R and POMC (the agonist of MC4R) occurred in the hypothalamus of broilers. Thus, future research should be conducted to examine the role of melanocortin system in the upregulation of feed intake of broilers under HSD rearing system.

In conclusion, increasing stocking density beyond 15 birds/m² (corresponding to the 45 kg/m² at 42 days of age) suppressed final BW and bone mineralization for the broilers raised in multitier cage system. Hypothalamic NPY and CRF signaling might be involved in the anorexigenic effect of HSD.

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