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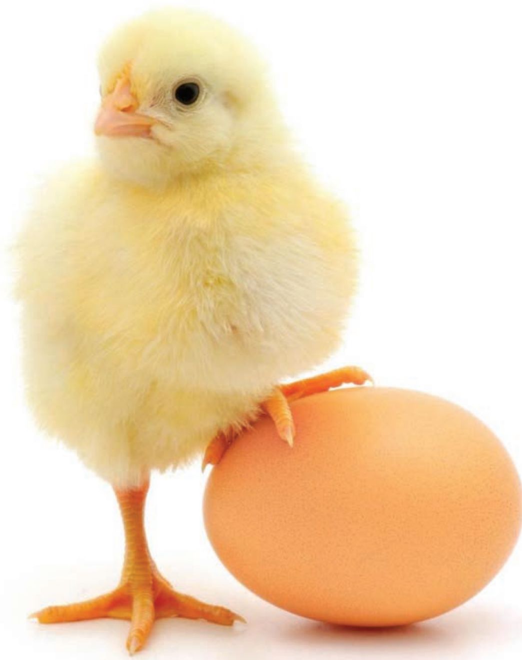

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Research Article
Effect of Stocking Density and Dietary Antimicrobial Inclusion of Male Broilers Grown to 35 Days of Age[1] Part 2: Blood Physiological Variables

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Abstract
Objective: This study investigated the effects of recommended stocking densities and dietary antimicrobial inclusion of male broilers grown to 35 days of age on selected blood physiological variables. **Materials and Methods:** In each study, a total of 1024 1-day-old Ross×Ross 708 male chicks were randomly distributed into 32 pens based on 4 assigned stocking density treatments. The treatments consisted of 4 densities (27, 29, 33, 39 kg m^{-2}) and 2 diets (AGP+, ABF) arranged in a 4×2 factorial with eight replicates. Conventional (antimicrobial-growth-promoters, AGP+) and antibiotic free (ABF) diets were equally assigned to each pen with feed and water provided *ad libitum*. Blood samples were collected from the brachial wing vein of 3 birds per pen on day 15, 28 and 35, which were then analyzed immediately for whole blood physiological variables. Blood plasma samples were analyzed for T_3, T_4 and corticosterone. **Results:** Results show there was no effect of stocking density on any of the selected physiological variables. However, in comparison to broilers fed with ABF diet, broilers with AGP+ had significant higher levels of pO_2, sO_2, SaO_2, Ca^{2+} and K^+ which were within physiological ranges. In addition, blood glucose and plasma corticosterone concentrations were not affected by treatments, suggesting an absence of physiological stress. **Conclusion:** In conclusion, Stocking densities up to 39 kg m^{-2} with appropriate environmental management regardless of antimicrobial addition in the diets may be suitable for both poultry integrators and contract growers to enhance broilers production efficiency without compromising the welfare of broilers grown to 35 days of age.

Key words: Stocking-density, antimicrobial, blood-gases, broilers, welfare

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

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INTRODUCTION

The poultry industry is making rapid progress in improving the efficiency of broiler growth. Advances include improved genetics and nutrition along with changes in environmental management, resulting in more rapid broiler growth. Consumer demand for breast meat has driven a shift in market composition towards increased market weights. Over the past five decades, consumption of poultry meat has increased dramatically, which is expected to continue in the future due to the relative price-competitiveness compared to other meat products[1-3]. In addition, poultry meat production globally will further grow by 2.4% per year over the next 20 years and the total production in 2030 will be around 160 million tons that will have a share of 39% in total meat production[4]. However, as the demand for animal protein products increases, increasing animal production and production efficiencies will be critical to the continued viability of the U.S. poultry and livestock industries.

Significant expansion in conventional (antimicrobial-growth-promoters, AGP+) and antibiotic-free (ABF) broiler production is expected to continue as demand for poultry products continues to increase and feed prices remain stable. However, there is worldwide concern over the present state of antibiotic resistance (AMR) among zoonotic bacteria that potentially circulate among food-producing animals including poultry[5-8]. This has resulted in the general public's perception that antibiotic use by human beings and in food animals selects for the development of AMR among food-borne bacteria that could complicate public health therapies[9-13]. Consumer perceptions of increased production efficiency generally assume negative effects on health and welfare of poultry. With increasing movement away from antimicrobial growth promoters (AGPs) coupled with increasing market weights, modification of traditional methods of housing management and environmental control is necessary to maintain or enhance production efficiency. Stocking density is commonly cited as a primary concern regarding health and welfare of broilers.

As the demand for poultry and associated products increases, increasing production and production efficiencies will be critical to the continued viability of domestic poultry industries in the United State. A larger part of this concern centers on the question of whether stocking density elicits adaptive responses that are characteristic of physiological stress. It is important to recognize that many poultry production management strategies including stocking density and dietary antimicrobial inclusion during the course of poultry production may potentially have an adverse impact on blood homeostasis and welfare of birds, which can result in production inefficiencies, compromised animal well-being and increased animal morbidity and mortality[14-16].

Hematological analyses in combination with other biochemical evaluations have been used to assess the health status of animals and they are the indicators of internal organ health and systemic homeostasis[16,17]. Changes in acid-base balance are the early manifestation or clinical signs of many diseases in both domestic animals including chicken and human beings[18-20]. Stress responses are also integrally involved with acid-base balance in several species[21]. In addition, changes in major selected blood variables are routinely used to determine various influences of environmental, nutritional and pathological factors[17]. Hence, it is necessary to examine these main effects of stocking density, dietary antimicrobial inclusion and their interaction, if any on selected blood physiological variables of broilers grown to 35 days of age to ensure the health and welfare of broilers. This study examined the main effects of stocking density, dietary antimicrobial inclusion and their interaction if any on selected blood physiological variables of welfare/well-being in optimum housing environments of male broilers grown to 35 days of age. This will also provide information needed to prevent costly restrictions on the poultry industry, while helping them to provide optimum and humane production environments since establishing proper welfare practices are central to international trade negotiations of meat products.

MATERIALS AND METHODS

Bird husbandry: All procedures relating to the use of live birds in this study were approved by the USDA-ARS Animal Care and Use Committee at the Mississippi State location. In addition, unnecessary discomfort to the birds was also avoided by using proper housing and handling techniques[22]. The present study used the same group of birds examined in our recent study[23]. This experiment was repeated two times and in each 1024 1-day-old male chicks were obtained from a commercial hatchery. Chicks were equally and randomly allocated to 32 groups inside 32 pens based on stocking density treatment assigned. Conventional (antimicrobial-growth-promoters, AGP+) and antibiotic free (ABF) diets were equally assigned to each pen to give 4 by 2 treatments with 4 replicates. Each pen was equipped with one tube feeder and nipple drinkers. Lighting was provided with 5000K LED bulbs. Light intensity settings were verified from the center and four corners of each room at bird level (30 cm) using a photometric sensor (LI-210, Li-Cor, Lincoln, NE) for each intensity adjustment. The light bulbs were cleaned weekly to minimize dust build-up, which would otherwise reduce their intensity. Chicks were vaccinated for Marek's, Newcastle and infectious bronchitis diseases at the hatchery. The chicks remained in their respective rooms from 1-day-old throughout the experimental period (1-35 day of age). All birds were fed the same diet throughout the study. Birds were provided a 3-phase feeding program (starter: 1-14 day; grower: 15-28 day; and finisher: 29-35 day of age). Diets were formulated to meet or exceed NRC[24] nutrient recommendations for each feeding phase. Starter feed was provided as crumbles and subsequent feeds were provided as whole pellets. Feed and water were offered *ad libitum*. Temperature on day 1 was maintained at $32\pm1.1^\circ C$ and was decreased as the birds progressed in age until $15.6^\circ C$ was reached at 35 day of age. Bedding material was sourced from a commercial cooperator to mimic commercial conditions and litter microflora. To prevent cross-contamination between AGP+ and ABF pens, shoe covers were used any time any personnel entered pens and shoe covers were changed when going between AGP+ and ABF-treatment pens.

Experimental treatments: The experimental design was a 4×2 factorial consisting of 4 Densities (kg m^{-2}) [27, 29, 33, 39] and 2 Diets [antibiotic free (ABF), conventional (antimicrobial-growth-promoters, AGP+)]. The AGP diet contained 2 additives of *salinomycine* and *bacitracin* based on manufacture's recommended levels. The ABF diet did not contain any antimicrobial and used play sand as inert filler. In addition, used litter was obtained from AGP and ABF commercial farms to simulate commercial conditions for both conventional and ABF groups and then placed in respective diet treatment pen. The AGP and ABF treatments pens were partitioned to avoid cross contamination of litters.

Measurements

Blood collections and chemical analyses: On day 15, 28 and 35 within 45 sec after birds were caught, blood samples were collected between 0800 and 0900 h from a brachial vein of 3 randomly selected birds from each pen. The birds were then returned to the appropriate pens without unnecessary discomfort using proper housing and handling techniques[22]. Blood samples (3 mL) were collected directly into heparinized (50 IU mL^{-1}) monovette syringes. Blood samples were drawn directly from the syringes into a blood gas electrolyte analyzer (ABL-80 CO-OX Flex, Radiometer America, Westlake, OH) for immediate analysis of pCO_2, pO_2, HCO_{3-}, pH, Hct, Hb, sO_2 and electrolytes (Na^+, K^+, Ca^{2+} and Cl^-). This ABL-80 CO-OX Flex blood gas electrolyte analyzer was set to reflect a broiler body temperature of $41.5^\circ C$ as per the manufacturer's instructions. The mean corpuscular hemoglobin concentration (McHc) in grams per deciliter and arterial oxygen saturation (SaO_2), which is the amount of oxyhemoglobin (O_2Hb) in blood expressed as a percent of the total amount of hemoglobin able to bind oxygen (O_2Hb+deoxyhemoglobin) were calculated as previously described[17]. The needle mounted on each monovette syringe was then removed, a cap was placed over the needle port and the syringes containing the blood samples were plunged into ice. After all birds were bled, the iced samples were transferred to the laboratory and centrifuged at $4,000\times g$ for 20 min at $4^\circ C$. Two milliliters of each of the plasma samples from the syringes were stored in 2.5 mL graduated tubes at $-20^\circ C$ for later chemical analyses. On the day of plasma analyses, plasma samples were removed from the freezer, thawed and analyzed for corticosterone using a universal microplate spectrophotometer (Bio-Tek Instruments Inc., Winooski, VT) with ELISA reagent assay test kits (EIA-CS Kit, Enzo Life Sciences, Farmingdale, NY), according to the manufacturer's instructions. Levels of plasma triiodothyronine (T_3) and thyroxine (T_4) concentrations were measured using a universal microplate spectrophotometer (Bio-Tek Instruments Inc.) with ELISA reagent assay test kits from ALPCO Diagnostics (Salem, NH) according to the manufacturer's instructions.

Statistical analysis: The experimental design was a randomized complete block design. Treatment structure was a 4×2 factorial arrangement with the main factors consisting of 4 stocking densities (kg m^{-2}) [27, 29, 33, 39] and 2 Diets [antibiotic free (ABF), conventional (AGP+)]. Individual sample data within each of the replicate units were averaged before analysis and data from the two trials were pooled and analyzed together. A mixed model ANOVA employing PROC MIXED procedure of SAS software[25] was used to analyze the data. Trial was a random effect, whereas the stocking density and diets were the fixed effect. The main effects of stocking density and diets and the interaction of these 2 factors on blood physiological variables were tested and pen was considered the experimental unit. Means comparisons on d 15, 28 and 35 were assessed by least significant differences and statements of significance were based on $p\leq0.05$ unless otherwise stated. Analyses of variance combined across days were performed to obtain treatment comparisons averaged across days and to test for treatment interactions with equal variances between days. In addition, ANOVAs for each of the blood sampling days interval was performed.

RESULTS

The data in this study represented combined effects of treatments (Stocking densities, diets) over day, since there was no effect of treatments for each of the sampling days (15, 28, 35). The effects of stocking density and dietary antimicrobial inclusion on blood gases and acid-base balance are presented in Table 1. As shown in Table 1, there was no effect of stocking density noted on blood gases and acid-base balance. However, in comparison with broilers fed antibiotic free diet (ABF), broilers fed antimicrobial growth promoter (AGP) up to 35 days of age had higher levels of pO_2 ($p<0.001$), sO_2 ($p<0.001$) and SaO_2 ($p<0.001$). The effects of stocking density and dietary antimicrobial inclusion on blood electrolytes and angap levels are presented in Table 2. There was no effect of stocking density noted on any of the blood electrolytes and angap levels. However, in comparison with broilers fed ABF diet, broilers fed AGP diet had higher levels of Ca^{2+} ($p<0.001$) and K^+ ($p<0.001$). Moreover, the influence of stocking density and dietary antimicrobial inclusion on selected blood metabolites are presented in Table 3. There was no effect of stocking density noted on selected blood metabolites but in comparison with broilers fed ABF, broilers fed AGP had higher level of T_3 ($p<0.002$).

DISCUSSION

Previous study evaluated the main effects of stocking density, dietary antimicrobial inclusion and their interaction if any on selected blood plasma biochemical, enzymatical

Table 1: Combined main effects of stocking density and dietary antimicrobial inclusion of male broilers grown to 35 days of age on BW, pH, blood gases, sO_2, SaO_2, hematocrit, hemoglobin and McHc[1]

	Stocking density (kg m^{-2})						Diets[2]			
Treatments/variables	27	29	33	39	SEM[3]	p-value	AGP+	ABF	SEM[3]	p-value
BW (kg)	2.015	1.984	1.995	1.976	0.015	0.547	2.038	1.947	0.010	0.483
pH	7.300	7.300	7.300	7.310	0.007	0.749	7.310	7.300	0.005	0.264
pCO_2 (mm Hg)	57.330	56.560	56.640	56.250	2.507	0.992	56.160	57.220	1.773	0.676
pO_2 (mm Hg)	48.590	49.790	49.940	48.970	0.828	0.609	52.120^a	46.520^b	0.566	0.001
HCO_{3-} (mm Hg)	25.360	25.110	25.250	25.440	0.974	0.974	25.300	25.300	0.689	0.993
sO_2 (%)	22.990	23.390	23.750	23.150	0.682	0.873	25.700^a	20.940^b	0.482	0.001
SaO_2 (%)	70.570	71.500	72.070	71.100	0.932	0.718	73.230^a	69.650^b	0.659	0.001
Hb (g dL^{-1})	7.930	8.010	7.940	7.860	0.152	0.923	8.020	7.850	0.108	0.251
Hct (%)	24.700	24.920	24.730	24.480	0.460	0.926	24.980	24.440	0.326	0.249
McHc (%)	32.100	32.120	32.110	32.100	0.020	0.778	32.120	32.100	0.014	0.321

[a-b]Means within a row that lack common superscripts differ significantly ($p\leq 0.05$). [1]Table values are least squares means of eight replicates for each treatment. [2]Diets: 1 = conventional (AGP+), 2 = antibiotic free (ABF). [3]Pooled SEM for main effects (n = 8)

Table 2: Combined main effects of stocking density and dietary antimicrobial inclusion of male broilers grown to 35 days of age on blood electrolytes and Angap[1]

	Stocking density (kg m^{-2})						Diets[2]			
Treatments/variables	27	29	33	39	SEM[3]	p-value	AGP+	ABF	SEM[3]	p-value
Ca^{2+} (mEq L^{-1})	3.07	3.04	3.04	3.04	0.012	0.564	3.08^a	3.04^b	0.002	0.001
Na^+ (mEq L^{-1})	148.63	148.67	148.34	148.27	0.482	0.962	148.33	148.53	0.343	0.631
K^+ (mEq L^{-1})	4.39	4.37	4.37	4.36	0.063	0.714	4.59^a	4.35^b	0.033	0.001
Cl^- (mEq L^{-1})	105.42	105.66	105.41	105.51	0.244	0.958	105.62	105.42	0.243	0.672
Anion Gap (mEq L^{-1})	22.82	22.71	22.67	22.53	1.077	0.986	22.62	22.63	0.756	0.967

[a-b]Means within a row that lack common superscripts differ significantly ($p\leq 0.05$). [1]Table values are least squares means of eight replicates for each treatment. [2]Diets: 1 = conventional (AGP+), 2 = antibiotic free (ABF). [3]Pooled SEM for main effects (n = 8)

Table 3: Combined main effects of stocking density and dietary antimicrobial inclusion of male broilers grown to 35 days of age on blood GLU, Osmo, CS, T_3 and T_4[1]

	Stocking density (kg m^{-2})						Diets[2]			
Treatments/variables	27	29	33	39	SEM[3]	p-value	AGP+	ABF	SEM[3]	p-value
GLU (mg dL^{-1})	250.380	249.2200	244.290	244.700	2.340	0.176	240.770	243.520	1.6670	0.074
Osmo (mOs kg^{-1})	311.080	0.10.74	310.330	309.720	0.942	0.762	310.110	310.820	0.6660	0.457
CS (pg mL^{-1})	2507.600	2302.1000	2542.900	3299.900	641.270	0.427	3320.700	3246.200	453.44	0.314
T_3 (ng mL^{-1})	3.747	3.7500	3.604	3.617	0.136	0.560	3.763^a	3.616^b	0.0960	0.002
T_4 (mg dL^{-1})	1.775	1.8250	1.779	1.792	0.071	0.888	1.756	1.729	0.2450	0.082

[a-b]Means within a row that lack common superscripts differ significantly ($p\leq 0.05$). [1]Table values are least squares means of eight replicates for each treatment. [2]Diets: 1 = conventional (AGP+), 2 = antibiotic free (ABF). [3]Pooled SEM for main effects (n = 8)

variables and electrolytes levels of male broilers grown to 35 days of age[23]. In continuation of the study with the same group of birds, we examined selected blood physiological welfare indicators variables. The results under ANOVA combined over days (day 1-35 of age) indicate that there was no significant effect of stocking density on all selected blood physiological variables especially those which are directly relate to acid-base balance that can lead to acidosis or alkalosis. The acid-base status of poultry is challenged daily by environmental factors such as light program, temperature, humidity and air quality among others and they influence respiratory and metabolic activities[26-30]. The primary organ systems used in acid-base homeostasis in birds are the lungs and kidneys, supported by the gastrointestinal tract and cardiovascular system that participate in thermoregulatory processes through modulation of heat dissipation and oxygen transport[31]. This is based on the fact that 3 systems (buffer, respiratory and renal systems) function interdependently to regulate and maintain acid-base balance. However as indicated in Table 1, in comparison with broilers fed antibiotic free diet (ABF), broilers fed conventional (antimicrobial growth promoters [AGPs]) diet, had significant ($p<0.05$) higher levels of pO_2, sO_2 and SaO_2. The increased pO_2 sO_2 and saO_2 observed in broilers fed AGP+ diet may be due to adequate blood oxygenation, which may prevent the risk of hypoxia by increases in systemic venous pO_2 and sO_2[32]. When oxygen intake is low (low pO_2, sO_2) relative to BW, the heart essentially pushes the blood through the lungs with more pressure to increase the amount of oxygen available for the bird's metabolism. However, because both lung and cardiovascular volume within lung tissue is fixed in birds, unlike in mammals, a point is eventually reached whereby the lungs may no longer accommodate more blood being supplied by the heart and this may have a negative effect on the body (poor oxygenation).

As shown in Table 2, broilers fed AGP diet had significantly higher levels of Ca^{2+} ($p<0.001$) and K^+ ($p<0.001$) in comparison with those fed ABF diet may be associated with broilers higher BW (2.038 vs 1.947 lbs.). Calcium is an essential electrolyte for many physiological and biochemical processes, the strength and integrity of the skeletal tissues and its deficiency can lead to skeletal deformations rickets and tibial dyschondroplasia, fractures and neural weakness among others[33]. In addition, Ca^{2+} is the most abundant electrolyte in the body of poultry that consisting of 1.5% of its body weight, serve as a transmitter of an intracellular signal and improved growth performance and tibia characteristics in broilers[34-36]. Physiologically, calcium provides the structural strength of the poultry skeleton by the formation of calcium salts. Moreover, Ca^{2+} plays vital roles in many of the biochemical reactions within the body via its concentration in the extracellular fluid. In addition, calcium is also critical for muscle contraction, nerve signaling, blood clotting and maintaining normal heart function. Also, K^+ has been shown to be more involved in many metabolic processes, amino acid absorption and transport, protein synthesis and acid-base balance[15,37,38]. Most of the body K^+ content resides in the intracellular space of skeletal muscle[38-40]. Furthermore, it has been shown that K^+ is required for the formation of a complex of amino acyl-s-RNA, ribosome(s), messenger RNA and amino acid transport[41]. It has been documented that K^+ is the monovalent cation which regulates protein synthesis in the cell and that relatively small decreases in intracellular potassium result in a reduction in protein synthesis[41].

As shown in Table 3, the plasma T_3 levels of birds fed AGP diet were significantly higher in comparison with those fed ABF diet but no effect on T_4 concentration. Thyroid hormones especially tri-iodothyronine (T_3) and thyroxine (T_4) are the most important humoral factors that influence all major metabolic pathways of protein, carbohydrates and lipid metabolism and are involved in the regulation of the basal metabolism of the majority of tissues including liver, heart, kidney and brain among others[42]. It has been documented that T_3 is the main physiological thyroid hormone regulating oxygen consumption and daily activities, particularly on growth and it is metabolically more active than T_4. It has been reported that the T_3 hormone is closely associated with feeding and is also a key factor influencing conversion of T_4 to T_3 and that a higher T_3 level is associated with increased protein deposition[43]. It is generally believed that T_4 is the predominant thyroid hormone in circulation but it has little biological activity in comparison with physiological T_3 that has more metabolically active activities[44]. Therefore the high level of T_3 in birds fed AGP diet may relate to feed intake due to antimicrobial that led to relate higher BW that support the classical role of the thyroid hormones in metabolic and physiological activities on growth and development. Concentrations of glucose and corticosterone among others have been suggested to be sensitive indicators of physiological responses in stressed broiler chickens[44,45]. In this study, there were no effects of treatments on blood glucose and plasma corticosterone concentrations, suggesting that these treatments and their interaction did not present stressors to broilers in this study. All the changes that we observed in birds fed AGP diet were due to the antimicrobial in the diet. It has been documented that most growth promoters use in animal nutrition such as antimicrobial act by balancing the intestinal flora and influencing intestinal villi, thereby improve nutrient digestibility, health, growth and productive performances among others[46,47].

CONCLUSION

From physiological perspectives, nonsignificant effect of stocking density on all selected blood physiological welfare indicators variables are in broad agreement with those reported in the literature and within the range of body physiology homeostasis in broilers, which indicates that the welfare of these broilers was not compromised. Also, some selected blood physiological welfare indicators were markedly affected by dietary antimicrobial that were relate to roles of inclusion growth promoters such as antimicrobial in animal nutrition without compromising the welfare of broilers. Plasma corticosterone and glucose levels were not affected by treatments or their interaction. Furthermore, the results of this study supplement current knowledge of the blood gases, electrolytes and enhancing our knowledge of homeostasis variation and the range of various blood metabolites in developing male broilers.

SIGNIFICANCE STATEMENTS

Our study adds to the accumulating evidence that stocking density and antimicrobial we used in this study do not cause organs and muscle damage and stress indicators in broiler chickens. Therefore, stocking densities up to 39 kg m^{-2} with appropriate environmental management regardless of antimicrobial addition in the diets may be suitable for both poultry integrators and contract growers to enhance broilers production efficiency without compromising the welfare of broilers grown to 35 days of age. This will also provide information needed to prevent costly restrictions on the poultry industry, while helping them to provide optimum and humane production conditions.

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