Journal of Emergy, Life Cycle and System Analysis in Agriculture

Journal homepage: jelsa.uoz.ac.ir

The impact of embryonic thermal manipulation on the intestinal microbiota, morphology, and long bone characteristics of male broiler chickens

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ARTICLE INFO

Article history:
Received: 11 July 2022
Accepted: 5 November 2022
Available online: 12 November 2022

Keywords:
Chronic heat stress
Global warming
Ross strain
Thermal manipulation
Thermotolerance

ABSTRACT

Climate change is caused by global warming, which is our most pressing environmental concern. However, some of these modifications will have negative effects on animal welfare and the quality and quantity of poultry products. We examined the effects of different periods of thermal manipulation (TM) during embryogenesis on the European production efficiency index (EPEI), intestinal microbiota and morphology, and long bone characteristics of Ross (308) broilers strain exposed to Chronic Heat Stress (CHS). Consequently, 608 fertile eggs were utilized in a completely randomized design comprising four treatments and four replicates. 7 to 16 days were spent incubating experimental groups with different TM (for control (0 h), 6, 12, and 18 hours). Humidity and temperature were maintained at 65% and 39.5°C. After hatching, male chicks were chosen, housed under standard conditions, and then subjected to chronic heat stress (CHS) between 28 and 42 days later. Mortality in the TM-treated groups was significantly ($P \leq 0.05$) lower than in the control group during CHS, and mortality was lowest after 12 hours of treatment. The EPEI was greater in treated chickens at 12 and 18 hours compared to untreated chickens ($P \leq 0.015$). The treatments have no effect on the intestinal microbiota ($P \geq 0.05$). The tibial length ($P \leq 0.05$) and width ($P \leq 0.048$) of birds given 12- and 18-hour treatments increased significantly. ≤ TM caused significant changes in the villus's height and area of the villus ($P \leq 0.05$). TM-treated birds had higher villus height than control. It can be concluded that TM may increase the height of villus and long bone characteristics and decrease the mortality rate in broilers exposed to CHS due to adaptation and thermotolerance.

Highlights

- The study investigates the effects of thermal manipulation (TM) during embryogenesis on broiler chickens under chronic heat stress (CHS).
- TM reduced mortality and increased production efficiency index (EPEI) of chickens exposed to CHS compared to control.
- TM did not affect the intestinal microbiota but enhanced the intestinal morphology and long bone characteristics of chickens.
- The paper demonstrates that TM is a potential strategy to improve the welfare and performance of broiler chickens under global warming.

1. Introduction

The global warming phenomenon is a serious challenge facing poultry production in tropical and subtropical regions. Heat stress begins when the ambient temperature rises above the thermoneutral zone, which ranges between 16 and 25 ˚C for poultry species. Heat stress is one of the most challenging environmental conditions influencing commercial poultry. Feed consumption, growth rates, feed efficiencies, and survival abilities all decrease as environmental temperature rises (Mashaly et al., 2004;

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https://doi.org/10.22034/aes.2022.350864.1041

Zaboli et al., 2019). The Broilers are very sensitive to heat stress due to feathering and the absence of sweat glands (Yahav et al., 2009). On the other hand, genetic selection has improved the relative weight of breast muscle, body weight gain, and feed efficiency. However, this is not accompanied by an adequate increase in cardiovascular and respiratory system function, which consequently causes the birds to be incapable of regulating their body temperature properly (Havenstein et al., 2003). Heat stress is common in tropical and subtropical areas like Iran, where spring and summer temperatures range from 35 °C to 45 °C. According to Akbarian et al. (2013), global warming makes the effects of heat stress worse.

Overall, the combined complications of intensive genetic selection, global warming, and expanding poultry production in the tropical climate caused economic losses. Therefore, various techniques have been tested to overcome this problem, such as nutritional and genetic methods, but most are costly and have low efficiency. Recently, thermal manipulation (TM) (postnatal and prenatal) has been developed and proposed as a new technique. It can improve performance and decrease mortality by reducing metabolic rate, body temperature, and thyroid activity (Loyau et al., 2015; Zaboli et al., 2016; 2022). Some studies (Yahav and Mcmurtry, 2001; De Basilio et al., 2001; Zaboli et al., 2022; 2016) explain that postnatal exposure of 3- or 5-day-old chicks (for 24 h at 37.5-38 °C) enables chickens to regulate body temperatures efficiently during heat challenges later and can diminish the mortality rate by about 50%. Furthermore, TM during embryogenesis has been tested in many studies (Moraes et al., 2004; Collin et al., 2007). The treatment combining 39.5 °C with 65% RH for 12 h/d between days E7 and E16 of embryogenesis appears to enhance the thermotolerance of broiler chickens without negative effects (Loyau et al., 2015; Zaboli et al.,2022; 2016).

Heat stress adversely impacts the intestinal epithelial structures, such as alteration in the digestibility and metabolism of various nutrients; disruption in the structure and function of the intestinal epithelium (Burkholder et al, 2008); and alteration of the normal and protective microbiota (Bailey et al, 2004). Burkholder et al (2008) noted that heat stress significantly decreased the intestinal bacterial populations of birds (Burkholder et al., 2008). In addition, the TM improved intestinal morphology (Temim et al., 2000; Uni et al., 2001). Bone weakness and skeletal disorders associated with rapid growth in the new genotype lead to economic losses and animal welfare issues (Kim et al., 2011). Also, heat stress intensifies the disorders and decreases long bone length and width (Bruno et al., 2007; Zaboli et al., 2017). So, we hypothesized that TM might improve long -bone-related traits and intestinal microbiota.

The profitable role of TM in broilers under acute heat stress has already been described. However, little is known about how TM affects performance under chronic heat stress (CHS) and how it affects the long bone characteristics, the shape of the intestine, and the microbiota in Ross strain broilers.

Therefore, the present investigation aimed to evaluate the effect of TM on performance, long bone traits, intestinal morphology, and microbiota of male broiler chickens exposed to CHS from 28 to 42 days of age.

2. Materials and Methods

2.1. Experimental procedures

The experimental protocol was approved by the Tarbiat Modares University Animal Care Protocol. Hence, 608 fertile eggs were used in a completely randomized design of four treatments with four replicates. Experimental groups with different TM (for control (0 h), 6, 12, and 18 hours) were incubated at 65% humidity and 39.5°C from 7 to 16 days of incubation for 0, 6, 12, and 18 hours. After hatching, the male broiler was selected, housed in standard conditions, and then exposed to CHS from day 28 to day 42 (Zaboli et al., 2016). To induce CHS, all groups in experimental pens were exposed from 10:00 AM to 4:00 PM to 32-36 °C and 55% RH, with overnight temperatures of 28 2°C and 46 5% RH. The average time it takes for the ambient temperature to rise from 27 to 32 °C is about 30 minutes. To avoid potential incubator influences, all eggs were incubated in the same incubator at 37.8 °C, 56% RH, and were turned once per hour from E0 to E7 (Zaboli et al., 2022). At 7 days of incubation, infertile and embryo mortalities were removed after candling. On day 19 of incubation, all eggs were relocated to a hatcher with 37.8 °C and 56% RH.

Newly hatched chicks were recorded every hour. Chicks with dry feathers, at almost 180 minutes after hatching, were taken out of the incubator for evaluating and sexing. Male broiler chickens of the same weight were carefully chosen, and 12 male broiler chickens were randomly distributed into the experimental cages. This experiment was performed only on male birds because of their better sensitivity to heat stress than females (Piestun et al., 2008).

All Ross Management Guide recommendations were used to feed all the chicks. Water and feed were provided *ad libitum* under 23-hour lighting. The temperature was 32 °C in the first week and then decreased to 24 °C. At the end of the experiment, the birds were euthanized by CO2 asphyxiation, and cervical dislocation was accomplished for dissection and then sampled.

2.2. The European production efficiency index

The European Production Efficiency Index (EPEI) was calculated using the equation as follows (Hajati et al., 2015):

$$EPEI = \frac{BW\ (kg) \times survived\ percentage}{PP \times FCR} \times 100$$

where PP is the production period length (days).

2.3. Intestinal Microbial Population

The contents of the ileum and cecum were used to look at the total number of Lactobacillus, Coliforms, and aerobic bacteria that were still alive. Therefore, 1 g of Ileal and Cecal contents of two birds in each replicate were separately collected into the sterile tubes for serial dilution. The culture medium for microbial enumeration was de

Man, Rogosa, and Sharpe agar for Lactobacillus and Mac Conkey agar for Coliforms. Plate count agar was used for the total count of bacteria under aerobic conditions. The bacterial numbers were expressed as log10 CFU per gram of DM.

2.4. Intestinal Morphometric Parameters

The region from Meckel′s diverticulum to a point 40 mm proximal to the Ileocecal junction was defined as an intestinal ileum segment. Two birds in each replicate were randomly selected for measuring after slaughter as described by Burkhulder et al. (2008) with a little modification (Burkhulder et al., 2008). In brief, two-centimeter tissue samples were taken from the midpoint of the aforementioned section, then rinsed with saline and immersed in a phosphate-buffered formalin solution. Two portions per sample were cut perpendicular to the longitudinal axis of the intestine by microtome (Sakura SRM 200, Tokyo, Japan) and embedded in paraffin wax. Transverse sections were cut (3 µm), stained with hematoxylin-eosin, and analyzed under a light microscope to determine morphometric indices, according to Burkholder et al. (2008). The morphometric parameters (all expressed in micrometer) measured included villus height and area, crypt depth, and villus width at the top and base. The ten longest and straightest villus and associated crypts were measured from each segment. Measurements for the villus height were taken from the tip of the villus to the villus-crypt junction. The crypt depth was defined as the depth of the invagination between adjacent villus, and the villus width was measured at the top and bottom of the villi. The mean of 10 measurements per sample was used as the average value for further analysis.

2.5. Long Bone Measurement

Tibia, femur, and humerus were evaluated at 42 d of age. Also, 12 birds per group were individually weighed, and after slaughtering, the bones were removed and frozen. For analysis, the bones were boiled in water, and the adhered muscle was scraped off with scissors and blades. After cleaning, the bones were defatted in ether for 24 h and then dried in an oven with forced ventilation at 105 °C for 75 h. After 12 h at room temperature, the bones were weighed, and length and width (in the medial portion) were measured with a caliper.

2.6. Statistical Analyses

Data were subjected to statistical analysis using SAS (SAS Institute, 2002), and means were separated by Duncan's multiple range tests ($P < 0.05$). Mortality data was subjected to chi-square ($\chi 2$) analysis. Statistical significance is considered as ($P < 0.05$).

3. Resalts

3.1. European Production Efficiency Index (EPEI)

The performance data showed no difference between the TM-treated groups and those with control (no published). EPEI was affected by treatments significantly ($P \leq 0.05$), and EPEI was higher in chickens treated for 6, 12, and 18 hours than in control. The lowest EPEI was found in the control group. The treatments statistically decreased mortality in the treated birds compared to the control ($P \leq 0.05$). The lowest mortality was observed in 6 and 12 hour treated groups (Table 1).

Table 1. The effect of TM on mortality percent and EPEI of male broiler under CHS

Treatments	Mortality (%)			EPEI
	1-28 days	28-42 days	1-42 days	
18	7	7	13	355[a]
12	4	5	9	350[a]
6	4	5	9	350[a]
control	3	19	20	320[b]
SEM	--	--	--	72.1
p-value	0.562	0.067	0.056	0.015

[a,b] Means within columns with different superscript letters are significantly different ($P < 0.05$). Treatments: In the control group, eggs were incubated at 37.8°C and 56% RH; other treatments (6, 12, and 18 hours) incubated at 65% humidity and 39.5°C from 7 to 16 days of incubation for 6, 12, and 18 hours a day. After hatching, housed in standard conditions, all treatments were subjected to CHS from day 28 to 42.

3.2. Intestinal Microbiota

The results concerning the effect of the TM on ileal and cecal microbiota in the main parts of the digestive tract on day 42 of the experimentation are given in Table 2. statistical analysis showed that Total Anaerobes, *Lactobacilli*, and *coliforms* were not significantly influenced by treatment ($P > 0.005$).

3.3. Intestinal Morphology

The results of intestinal morphology are listed in Table 3. There was a significant impact on villus height ($P<0.05$). CHS significantly decreased villus height in the control group, while the TM improved it. The villus area was significantly affected among the groups due to the TM. The lowest villus area was observed in the PRE and PO3 groups, respectively. Villus width and crypt depth of the ileum did not differ among groups ($P>0.05$).

3.4. Long -Bone Parameters

The results of tibia, femur, and humerus length, width, and weight are shown in Table 4. Tibia length was affected and improved significantly by treatments ($P\leq0.05$). Birds experienced TM exhibiting higher value than the control group. The 12 hours group also showed the highest value. There was a significant improvement in tibia width in the 18 groups ($P\leq0.05$).

Furthermore, TM affected Femur length statistically. The highest and lowest value was observed in 18 hours of treatment and control, respectively. No changes were found in the tibia, femur weight, and humerus parameters.

Table 2. The effect of TM on Ileal and Cecal microbial population (log10 cfu/g of DM) of male broiler under CHS

	Ileum			Cecum		
Treatments	***Lactobacillus***	***Coliforms***	**Aerobic bacteria**	***Lactobacillus***	**Coliforms**	**Aerobic bacteria**
18	90.7	7.022	60.9	21.10	10.13	70.10
12	88.7	6.99	71.9	31.10	10.14	74.10
6	90.7	7.05	81.9	35.10	10.10	76.10
Control	87.7	7.02	67.9	20.10	10.13	73.10
SEM	0.006	0.015	0.022	0.012	0.012	0.066
P-value	0.15	0.099	0.101	0.19	0.22	0.25

[a,b] Means within columns with different superscript letters are significantly different ($P < 0.05$). Treatments: In the control group, eggs were incubated at 37.8°C and 56% RH; other treatments (6, 12, and 18 hours) incubated at 65% humidity and 39.5°C from 7 to 16 days of incubation for 6, 12, and 18 hours a day receptivity. After hatching, housed in standard conditions, all treatments were subjected to CHS from day 28 to 42.

Table 3. The effect of TM on ileal morphology of male broiler under CHS

Treatments	**Villus height (µM)**	**Villus area (μM^2)**	**Villus width (µM)**	**Crypt depth (µM)**
18	950.11±9[a]	0.086±0.002[c]	95.60±8	120.25±2.3
12	946.41±69[b]	0.092±0.002[b]	95.93±8	122±3.9
6	956.92±7[a]	0.096±0.004[a]	96.8±7	122.60±2.1
Control	916.11±11[c]	0.095±0.003[a]	97.65±6	122.44±5.5
SEM	9.29	0.003	1.8	1.9

[a,b] Means within columns with different superscript letters are significantly different ($P < 0.05$). Treatments: In the control group, eggs were incubated at 37.8°C and 56% RH; other treatments (6, 12, and 18 hours) incubated at 65% humidity and 39.5°C from 7 to 16 days of incubation for 6, 12, and 18 hours a day receptivity. After hatching, housed in standard conditions, all treatments were subjected to CHS from day 28 to 42.

Table 4. The effect of TM on the long bone characteristics of male broiler under CHS treatments

Variables	**6**	**12**	**18**	**Control**	**SEM**	***p-value***
			Tibia			
Length	100.2[b]	102.91[a]	101.68[ab]	99.21[c]	1.51	0.051
Width	10.98[b]	11.25[b]	11.78[a]	10.95[b]	0.15	0.052
Weight	8.85	8.85	8.75	8.8	0.13	0.87
			Femur			
Length	72.01[ab]	71.9[ab]	73.8[a]	70.59[b]	0.93	0.028
Width	7.61	7.61	7.43	7.51	0.096	0.059
Weight	7.3	7.25	7.15	7.25	0.054	0.054
			Humerus			
Length	67	66.5	68.05	67.22	0.69	0.21
Width	7.08	6.00	7.85	7.52	0.098	0.22
Weight	4.45	4.75	4.65	4.58	0.046	0.33

[a,b] Means within columns with different superscript letters are significantly different ($P < 0.05$). Treatments: In the control group, eggs were incubated at 37.8°C and 56% RH; other treatments (6, 12, and 18 hours) incubated at 65% humidity and 39.5°C from 7 to 16 days of incubation for 6, 12, and 18 hours a day receptivity. After hatching, housed in standard conditions, all treatments were subjected to CHS from day 28 to 42.

4. Discussion

4.1. European Production Efficiency Index (EPEI)

Results showed that treated groups exhibited higher EPEI. Despite no significant difference in mortality from 0 to 28, 28 to 42, and 0 to 42 days of age. However, mortality was decreased statistically in treated chicks compared to control. Some studies also reported that treated birds performed better (Piestun et al., 2011,2009, 2008). Moreover, decreased mortality in TM-treated chicks agreed with Yahav and Hurwitz (1996) and Zaboli et al. (2016) findings. Thermotolerance is a physiological response in TM-treated birds. Tzschentke and Basta (2002) reported that exposure to a warmer prenatal incubation temperature resulted in an elevated neuronal hypothalamic warm sensitivity through an increased proportion of cold-sensitive neurons and a reduced proportion of warm-sensitive neurons in comparison with the control group (Tzschentke et al., 2002). So, a significant increase in EPEI and a lower mortality rate could indicate the greater resistance of TM-treated chicks to CHS due to the epigenetic temperature adaptation. Furthermore, this trial might reduce metabolic rate, thyroid hormone, and body temperature (Zaboli et al., 2016). Probably these changes positively affect EPEI.

4.2. Intestinal microbiota

In the gastrointestinal tract (GIT), the microbiota plays a major role in elevating nutrient absorption and amplifying the immune system, so the microbiota affects both the growth and health of the chicken (Choi et al., 2015). We hypothesized that TM could improve performance and intestinal morphology (Uni et al., 2001) and thereby positively change microflora in chickens exposed to CHS. Uni et al. and Temmim et al. (2000) reported that the birds treated by the TM pattern exhibited better brush border characteristics than the control (Uni et al., 2001; Temim et al., 2000). Bailey et al. noted that heat stress could alter the normal microbiota in the GIT (Bailey et al., 2004). Thus, it reinforced our hypotheses that TM could improve microflora. On the contrary, our results showed no significant changes in the Ileal and Cecal microbial populations. The mortality of sensitive birds can explain in the control group, and CHS for two weeks may create adaptation in the control group.

4.3. Intestinal morphology

Intestinal morphology condition could be considered as a proper response for TM experiments. The small intestine is the main site of the digestive system that is affected by environmental conditions. Moreover, it gives information about the health and performance of birds. The most important part of the brush border is the enterocytes located on top of the villus and is responsible for observation and disability (Yahav et al., 2007). Burkholder et al. (2008) showed that heat stress for 24 hours did not affect the villus height. They suggested that the short duration of the stressor, and the resistance of the ileum to structural change, could be possible responses to remain unchanged. We can conclude that improved villus height in treated birds caused increased performance during CHS. TM could induce thermotolerance and prevent the adverse effects of CHS on intestinal morphology. Likewise, Uni et al. (2001) reported an improvement in the enterocyte proliferation and the activity of brush border enzymes 48 hours after thermal conditioning. Meanwhile, TM reduces body temperature, and as a result, the treated bird could help the improvement of intestinal morphology (Zhu et al., 2002; Piestun et al., 2009).

4.4. Long Bone Parameters

performance improvements in poultry production are associated with skeletal disorders and are the major negative factor affecting poultry production. Furthermore, heat stress intensifies skeletal problems (Yalçin et al., 1996). Burno et al. (2007) reported that heat stress caused a decrease in both the tibia and humerus length and width. As a result, alterations in development caused by pre- and post-hatch treatment affected bone development. Small differences in incubation temperature applied throughout incubation have been shown to influence the growth of the long bone in the birds (Brookes et al., 1972). Raising the temperature of the eggs by 1°C, from 37.5 to 38.5°C, during ED 4 to 7 could increase the length of tibia and tarsus bones in Leghorns (Hammond et al., 2007). Moreover, our result showed that TM could lead to the development and improvement of a long bone, but the mechanisms are unclear (Maltby et al., 2006; Brookes et al., 1972; Zaboli et al., 2017). Otherwise, the results from this trial showed that TM decreased mortality and improved performance and also decreased the metabolic rate and body temperature (Zaboli et al., 2016). These changes may help improve long bone development (Collin et al., 2012). So that the negative impact of CHS is reduced.

5. Conclusion

In summary, the application of TM during the development and maturation of the thermal regulation system of the male broilers may induce positive effects on the intestinal morphology, long bone development, and EPEI, thus preventing the negative effects of CHS on broiler chickens in the first week of CHS to reduce mortality and improve EPEI. Overall, TM can affect intestinal microbiota and improve long bone characteristics. Notwithstanding, the underlying mechanisms of TM's long-lasting effect on broiler chickens' physiological responses remain to be elucidated in the future.

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