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ANIMAL BIOSCIENCE

Effect of *in ovo* feeding of γ-aminobutyric acid combined with embryonic thermal manipulation on hatchability, growth, and hepatic gene expression in broilers

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Objective: This study investigated the effects of *in ovo* feeding of γ-aminobutyric acid (GABA) and embryonic thermal manipulation (ETM) on growth performance, organ indices, plasma biochemical parameters, hepatic antioxidant levels, and expression of lipid metabolism-related genes in broilers.
Methods: Two hundred and fifty eggs were assigned to one of four treatments: control eggs incubated under standard conditions (CON); eggs that received an *in ovo* injection of 10% GABA on day 17.5 of incubation (G10); thermally manipulated eggs between days 10 and 18 of incubation at 39.6°C for 6 h daily (TM); and eggs that received both treatments during incubation (G10+TM). After 28 days of rearing, five birds per treatment were selected for blood and organ sampling.
Results: No differences were found in hatchability or growth parameters among different treatment groups. Hepatic gene expression of catalase (*CAT*) and glutathione peroxidase 1 (*GPx1*) was upregulated (p = 0.046 and p = 0.006, respectively) in the G10+TM group, while that of nuclear factor erythroid 2–related factor 2 (*NRF2*) was upregulated (p = 0.039) in the G10 group. In addition, the relative gene expression of NADPH oxidase 1 (*NOX1*) was significantly lower (p = 0.007) in all treatment groups than that in the CON group. Hepatic fatty acid synthase (*FAS*) levels and average daily feed intake (ADFI) of last week showed a positive correlation (r = 0.50, p = 0.038). In contrast, the relative gene expression of the extracellular fatty acid-binding protein (*EXFAB*) and peroxisome proliferator-activated receptor-γ (*PPAR-γ*) were positively correlated (r = 0.48, p = 0.042 and r = 0.50, p = 0.031) with the overall ADFI of birds.
Conclusion: Taken together, the results of this study suggest that the combination of *in ovo* feeding of GABA and ETM can enhance hepatic antioxidant function in broilers.

Keywords: Antioxidant Status; Broiler; γ-Aminobutyric acid (GABA); *In ovo* Feeding; Thermal Manipulation

INTRODUCTION

The poultry production industry faces several challenges that negatively affect birds: stocking density, disease infection, and heat stress (HS). Recent literature indicates that these stressors are often associated with the overproduction of reactive oxygen species (ROS), leading to oxidative stress [1,2]. Birds have developed various antioxidant defense systems to adapt to and survive in the environment. Indeed, in avian species, cell signaling and stress adaptation are maintained by the redox status [3]. The redox balance can be regulated by various systems. The first step is to lower the action of enzymes such as NADPH oxidase (NOX), which is responsible for ROS production, thus limiting the production of free radicals by decreasing oxygen accessibility. The combined action of three antioxidant enzymes en-

sures another line of defense: catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) [4]. SOD catalyzes the dismutation of O_2^- into O_2 and H_2O_2. Furthermore, GPx and CAT catalyze the breakdown of H_2O_2 into H_2O and oxygen [5]. These defense systems are triggered by the activation of nuclear factor (erythroid-derived 2)-like 2 (NRF2), which is responsible for the expression of antioxidant and detoxification-related genes [6].

Several strategies have been proposed to alleviate oxidative stress in broiler chickens. One approach was dietary supplementation with γ-aminobutyric acid (GABA) [7]. GABA is a neurotransmitter that regulates vital functions such as blood pressure and respiration [8]. Supplementation with GABA is particularly effective against heat-induced oxidative stress [9]. To mitigate HS, previous studies have used GABA as a feed additive for broilers [10] or provided it to the drinking water of laying hens [11]. Furthermore, our previous study showed that *in ovo* GABA feeding can improve the antioxidant status in chicks exposed to acute HS [12].

Another approach against heat-induced oxidative stress is epigenetic temperature adaptation. This method can be applied during pre- or early- post-hatch ontogenesis to modulate gene expression and provide long-lasting adaptation to the environment [13]. The most recent of these techniques is embryonic thermal manipulation (ETM). Furthermore, studies have demonstrated that a particular pattern of incubation temperature can affect the metabolic rate of hatchlings. Indeed, the results showed that ETM improves defense mechanisms by enhancing the antioxidant and immune responses of broilers [14].

In the present study, we hypothesized that *in ovo* feeding of GABA combined with ETM could modulate post-hatch gene expression in broilers. Thus, we evaluated the growth performance, organ indices, plasma biochemical parameters, and hepatic expression of genes related to antioxidant defenses and lipid metabolism in broilers.

MATERIALS AND METHODS

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Gyeongsang National University (GNU-200916-C0058).

Incubation, *in ovo* feeding procedure and feeding trial
Two hundred and fifty hatching eggs were obtained from 37-week-old Indian River breeder hens housed at a local breeder farm (Hapcheon, Korea). Following standard incubation conditions, the eggs were incubated in an incubator (Rcom Co., Ltd., Gimhae, Korea). Briefly, from embryonic day (ED) 1 to 18, eggs were subjected to 37.8℃ and 56% relative humidity (RH), and from ED 18 until hatching, the incubation temperature was maintained at 36.8℃ with 70%

RH. On ED 10, after candling, non-fertilized eggs were removed from the incubator. The eggs were distributed in four groups of equal numbers (n = 48) with similar weights (61.0± 2 g) using the Solver module of Microsoft Excel (Microsoft Excel 2016; Microsoft Corp., Washington, USA). Each group consisted of eight replicates with six eggs. In our previous study, we found no significant differences (hatching and biological parameters) between the sham control (distilled water injected) and the non-injected control; therefore, we did not include a sham treatment in this trial. The groups were the followings: i) control eggs without *in ovo* injection and incubated at standard temperature (CON); ii) eggs injected at 17.5 days of incubation with 0.6 mL of 10% (0.1 g/mL) GABA (#A2129; Sigma-Aldrich Inc., St. Louis, CA, USA) dissolved in distilled water (G10); iii) thermally manipulated eggs exposed to 39.6℃ for 6 h (from 10:00 h to 16:00) daily from ED 10 to 18 (TM); and iv) eggs that received both previous treatments during incubation (G10+TM).

The solution and methodology used for the *in ovo* feeding procedure were the same as those previously described [15,16]. Briefly, a hole was drilled from the blunt end of the eggs and subsequently injected with a 1 mL syringe with a 23 G and 1-inch needle (KOVAX-SYRINGE; Korea Vaccine Co., Ltd. Seoul, Korea). Therefore, the injection targeted the amnion with a depth of approximately 2.5 cm.

Immediately after the injection, eggs were sealed using surgical tape (3M Micropore, Saint Paul, MN, USA) and incubated. The control eggs were removed from the incubator for the same period but returned without injection. After hatching, one-day-old broilers (n = 140) with similar weights were selected to equalize the starting weight between the cages and treatments. The birds were raised in battery brooders in a thermally controlled environment at 34℃±1℃ and 50% RH for one-day-old broilers, and then the temperature was gradually decreased to 22℃±1℃ on day 28. Commercial feed (Nonghuyp Feed Inc., Seoul, Korea) (Table 1) and water were provided *ad libitum* under continuous lighting. Each treatment consisted of five cages with seven chicks each.

Blood, tissue sampling, and plasma biochemical parameters analysis
On day 28, one bird from each cage was randomly selected for blood and tissue sampling. The birds were euthanized with carbon dioxide before sampling. Blood was collected by heart puncture and transferred into heparinized vacuum containers (#367874; BD Co., Ltd., Franklin Lakes, NJ, USA). The blood samples were then centrifuged at 2,000×g for 10 min at 4℃, and plasma was collected and stored at –20℃ for later analysis. Tissues were sampled and weighed (liver, spleen, bursa, and heart), and the liver samples were snap-frozen in liquid nitrogen and stored at –80℃ for further

Table 1. Feed composition of the experimental diets from 0 to 28 days of age[1]

Items	Starter (0 to 7 d)	Grower (8 to 21 d)	Finisher (22 to 28 d)
Ingredients (%)			
Corn grain	38.97	45.87	39.68
Wheat grain	15.00	15.00	25.00
Soybean meal (42.6% CP)	32.00	25.60	20.60
Corn gluten	3.00	2.64	3.00
Meat and bone meal	2.00	2.00	2.50
Animal fat	4.00	3.88	4.54
Salt	0.25	0.25	0.25
Tricalcium phosphate	1.30	1.04	0.86
Limestone	1.26	1.22	1.26
Sodium bicarbonate	0.00	0.02	0.00
L-threonine	0.12	0.16	0.16
Lysine	1.23	1.43	1.32
DL-methionine	0.33	0.34	0.29
Choline chloride (50.0%)	0.03	0.03	0.03
Premix[2]	0.20	0.20	0.20
Phytase	0.05	0.05	0.05
Anti-coccidia	0.01	0.01	0.01
Calculated nutrients (%)			
Crude protein	23.00	20.50	19.50
Crude fat	6.31	6.36	6.90
Crude fiber	3.01	2.80	2.68
Crude ash	5.99	5.34	5.02
Calcium	1.01	0.90	0.86
Available phosphorus	0.60	0.53	0.49
Digestible lysine	1.43	1.24	1.09
Digestible methionine+cystine	1.07	0.95	0.86
Copper (ppm)	82.21	81.04	80.78
Zinc (ppm)	100.27	96.63	97.33
Metabolizable energy (kcal/kg)	3,050	3,150	3,200

[1] Nonghuyp Feed Inc. (Seoul, Korea).
[2] Trace minerals and vitamins provided per kilogram of premix: vitamin A, 12,000,000 IU; vitamin D_3, 3,000,000 IU; vitamin E, 40,000 IU; vitamin K_3, 2,000 IU; vitamin B_1, 2,000 mg; vitamin B_2, 5,000 mg; vitamin B_6, 3,000 mg; vitamin B_{12}, 20 mg; niacin, 40,000 mg; pantothenic acid, 10,000 mg; folic acid, 1,000 mg, iron, 88,000 mg; copper, 72,600 mg; zinc, 60,000 mg; manganese, 66,000 mg; iodine, 990 mg; selenium, 220 mg; cobalt, 330 mg.

analysis. Plasma metabolite concentrations were measured using a VetTest Chemistry Analyzer (IDEXX Co., Ltd., West-brook, CT, USA) with dry-slide technology, following the manufacturer's instructions.

Real-time polymerase chain reaction for mRNA quantification

Following the manufacturer's protocol, total RNA was extracted from the liver using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA). The concentrations and purities of the samples were determined by measuring the optical density of each sample using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). Subsequently, reverse transcription was conducted using the PrimeScript first-strand

cDNA synthesis kit (Takara, Tokyo, Japan) following the manufacturer's instructions. The cDNA was then used for performing real-time polymerase chain reaction (PCR) using the StepOnePlus real-time PCR system (Life Technologies, Carlsbad, CA, USA) according to the following protocol: 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Each reaction well was composed of a 20 μL volume containing Power SYBR Green PCR master mix (Life Technologies, USA) and 10 pmol concentration of forward and reverse primers specific for each gene and cDNA. Table 2 lists the primer sequences used in this study. Gene quantification was normalized to the Ct values of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and *β-actin*. Relative expression was determined using the $2^{-\Delta\Delta ct}$ algorithm. The geometric means of both references were used to calculate the expression levels of the target genes.

Statistical analysis

Before the analysis, all assumptions related to the application of parametric tests were assessed. Data were analyzed via one-way analysis of variance (ANOVA) using the "general

Table 2. Oligonucleotide primer sequences for RT-qPCR

Gene	Sequence	Accession number
ACC	F: CACTTCGAGGCGAAAAAC	XM_015295697.2
	R: GGAGCAAATCCATGACCA	
CAT	F: ACCAAGTACTGCAAGGCGAA	NM_001031215.1
	R: TGAGGGTTCCTCTTCTGGCT	
EXFABP	F: GGAGGACCTTGCACATGA	NM_205422.1
	R: GTGTAGTTCCGCTCCCTA	
FAS	F: CAATGGACTTCATGCCTC	NM_205155.3
	R: GCTGGGTACTGGAAGACA	
GPx1	F: AACCAATTCGGGCACCAG	NM_001277853.2
	R: CCGTTCACCTCGCACTTCTC	
NOX1	F: GCGAAGACGTGTTCCTGTAT	NM_001101830.1
	R: GAACCTGTACCAGATGGACTTC	
NOX4	F: CCTCTGTGCTTGTACTGTGTAG	NM_001101829.1
	R: GACATTGGAGGGATGGCTTAT	
NRF2	F: CAGAAGCTTTCCCGTTCATAGA	NM_205117
	R: GACATTGGAGGGATGGCTTAT	
PPAR-γ	F: TCAGGTTTGGGCGAATGC	XM_040646063.1
	R: CGCTCGCAGATCAGCAGA	
SOD	F: AGGGGGTCATCCACTTCC	NM_205064.1
	R: CCCATTTGTGTTGTCTCCAA	
β-actin	F: ACCGGACTGTTACCAACA	NM_205518.1
	R: GACTGCTGCTGACACCTT	
GAPDH	F: TTGGCATTGTGGAGGGTCTTA	NM_204305.1
	R: GTGGACGCTGGGATGATGTT	

RT-qPCR, real-time quantitative polymerase chain reaction; *ACC*, acetyl-CoA carboxylase; *CAT*, catalase; *EXFABP*, extracellular fatty acid-binding protein; *FAS*, fatty acid synthase; *GPx1*, glutathione peroxidase 1; *NOX1*, nicotinamide adenine dinucleotide phosphate oxidase 1; *NOX4*, nicotinamide adenine dinucleotide phosphate oxidase 4; *NRF2*, nuclear factor erythroid 2-related factor 2; *PPAR-γ*, peroxisome proliferator-activated receptor gamma; *SOD*, superoxide dismutase; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

linear model procedure" in the SAS software (version 9.4; SAS Institute Inc., 2009). A Tukey's post hoc test was performed following a significant p-value to assess the differences among means. Concerning hepatic gene expression, orthogonal planned contrasts were made to evaluate specific comparisons using the "Contrast statement" of the SAS software [17]. Differences were considered statistically significant at p≤0.05, and p≤0.1 was considered a trend. Moreover, gene expression patterns were detected by hierarchical clustering [18] using the package "ComplexHeatmap" in R software version 4.0.3 (R Core Team, 2020). Finally, Pearson's correlation analysis was performed for the evaluated parameters to develop a correlation matrix using the "CORR procedure" in the SAS software.

RESULTS

Hatching parameters, growth performance, and organ indexes

No significant differences were observed in the hatching parameters (Table 3) among different treatment groups. *In ovo* feeding of GABA and ETM did not significantly affect hatchability or the average hatchling weight.

Body weight, average daily weight gain (ADWG), and average daily feed intake (ADFI) were similar in all treatment groups regardless of the period. However, the G10+TM group had an overall higher (p = 0.018) feed conversion ratio (FCR) than the CON group from days 8 to 21 of the post-hatch period (Table 4).

The organ index results are presented in Table 5. The absolute spleen, bursa, and heart weights did not differ across different treatment groups. In contrast, absolute liver weight was statistically different (p = 0.046) among the treatment groups, with the highest value recorded in the G10 group and the lowest in the G10+TM group. Significant differences were observed in the relative weight of the bursa among the treatment groups. Chicks in the G10+TM group had a higher (p = 0.008) relative bursa weight than those in the CON group did. Finally, the relative liver, spleen, and heart weights were

similar among all the treatment groups.

Plasma biochemical parameters

Table 6 presents the effects of *in ovo* feeding with GABA and thermal manipulation on the plasma biochemical parameters of broilers. Plasma glucose, triglyceride, and cholesterol levels were not affected by any treatment. However, plasma total protein levels were higher (p = 0.019) in chicks in the G10+TM group than those in the CON group.

Hepatic mRNA relative expression

Tables 7 and 8 show the results of the ANOVA procedure and contrast analysis performed on the hepatic mRNA relative expression of the genes studied. Significant differences among the treatment groups were revealed for the expression of antioxidant-related genes. The glutathione peroxidase 1 (*GPx1*) gene was significantly upregulated (p = 0.039) in chicks in the G10+TM group compared to those in the CON group. In contrast, significantly lower (p = 0.039) expression of the NADPH oxidase 1 (*NOX1*) gene was observed in the G10 and G10+TM treatment groups than in the CON treatment group. Furthermore, the contrast analysis results showed that the chicks from the G10+TM group exhibited higher (p = 0.046) *CAT* gene expression than the CON group chicks. Although not statistically significant, *NOX4* gene expression showed a decreasing tendency (p = 0.077) in the G10 group. In addition, orthogonal contrast analysis showed that the *NRF2* gene was significantly upregulated (p = 0.039) in birds fed GABA *in ovo* (G10). Although a numerically lower relative expression of the lipid metabolism gene in the G10+TM group was continuously observed, neither the ANOVA procedure nor the contrast analysis revealed significant differences among all groups.

Gene expression data were clustered into four groups based on Euclidean distances (Figure 1). Two large clusters were identified: the first was composed of *NOX1*, *NOX4*, extracellular fatty acid-binding protein (*EXFABP*), and peroxisome proliferator-activated receptor-γ (*PPAR-γ*), with *NOX1*, *NOX4*, and *EXFABP* being downregulated (bright gray bars), whereas

Table 3. Effects of *in ovo* feeding of γ-aminobutyric acid and embryonic thermal manipulation on hatchability parameters

| Parameters | Treatments[1] | | | | SEM | p-value |
	CON	G10	TM	G10+TM		
Number of fertile eggs	48	48	48	48	NA	NA
Number of hatchlings	46	43	45	41	NA	NA
Hatchability (%)	95.8	89.6	93.7	85.4	1.9	0.255
Hatchling weight (g)	40.9	40.4	39.7	40.0	0.23	0.296

Hatchability was calculated based on n = 8 repetitions per treatment.
SEM, standard error of the mean; NA, not applicable.
[1] The treatments are described as follows: CON, control eggs without *in ovo* injection and incubated at standard temperature; G10, eggs injected at 17.5 days of incubation with 0.6 mL of 10% γ-aminobutyric acid dissolved in distilled water; TM, thermally manipulated eggs exposed to 39.6°C for 6 h daily from embryonic day 10 to 18; G10+TM, eggs that received both previous treatments during incubation.

Table 4. Effects of *in ovo* feeding of γ-aminobutyric acid and embryonic thermal manipulation on growth, feed intake, and feed conversion ratio in broiler chickens

Parameters	Treatment[1]				SEM	p-value
	CON	G10	TM	G10+TM		
BW (g)						
Day old	40.3	40.2	39.9	40.0	0.07	0.149
7 d	155.1	154.3	152.2	167	0.10	0.161
21 d	909.1	901.7	879.8	881.7	8.10	0.528
28 d	1,450.2	1,493.8	1,431.3	1,419	18.3	0.526
ADWG (g)						
1 to 7 d	16.4	16.2	16.1	18.2	0.37	0.151
8 to 21 d	53.8	53.4	52	51.1	0.52	0.211
22 to 28 d	77.3	84.6	78.8	76.8	2.31	0.649
1 to 28 d	49.1	51.4	48.9	48.6	1.40	0.628
ADFI (g)						
1 to 7 d	15.4	14.8	14.6	16.9	0.35	0.084
8 to 21 d	67.6	69.1	66.1	67.3	0.58	0.337
22 to 28 d	116	125.8	119.9	112.1	1.96	0.067
1 to 28 d	66.3	69.9	66.8	65.4	1.20	0.095
FCR (g/g)						
1 to 7 d	0.94	0.91	0.91	0.92	0.01	0.585
8 to 21 d	1.25[b]	1.28[ab]	1.26[ab]	1.30[a]	0.02	0.018
22 to 28 d	1.51	1.49	1.52	1.51	0.03	0.984
1 to 28 d	1.23	1.23	1.23	1.25	0.02	0.873

Values were obtained from n = 5 repetitions per treatment.
SEM, standard error of the mean; BW, body weight; ADWG, average daily weight gain; ADFI, average daily feed intake; FCR, feed conversion ratio.
[1] The treatments are described as follows: CON, control eggs without *in ovo* injection and incubated at standard temperature; G10, eggs injected at 17.5 days of incubation with 0.6 mL of 10% γ-aminobutyric acid dissolved in distilled water; TM, thermally manipulated eggs exposed to 39.6°C for 6 h daily from embryonic day 10 to 18; G10+TM, eggs that received both previous treatments during incubation. Chicks obtained were grown for 28 days.
[a,b] Means in a row that possess different superscripts differ significantly (p < 0.05).

PPAR-γ expression was decreased or unchanged compared to the control. The second-largest cluster included *SOD*, *GPx1*, *NRF2*, and fatty acid synthase (*FAS*) genes, which were moderately upregulated (blue bars). Finally, *CAT* and acetyl-CoA carboxylase (*ACC*) were the only genes in separate clusters. *CAT* was robustly upregulated (dark blue bars) in the treatment groups relative to the control group.

Figure 2 presents the results of Pearson's correlation anal-

Table 5. Effects of *in ovo* feeding of γ-aminobutyric acid and embryonic thermal manipulation on absolute and relative organs weight in broiler chickens

Parameters	Treatments[1]				SEM	p-value
	CON	G10	TM	G10+TM		
Absolute weight (g)						
Liver	36.9[ab]	41.2[a]	35.6[ab]	33.3[b]	1.1	0.046
Spleen	1.2	1.1	1.4	1.01	0.2	0.460
Bursa	1.7	2.1	1.8	2.3	0.1	0.093
Heart	7.3	8.2	6.4	6.8	0.3	0.080
Relative weight (%)						
Liver	2.9	3.3	3.1	3.1	0.08	0.264
Spleen	0.09	0.09	0.12	0.09	0.02	0.442
Bursa	0.13[B]	0.17[AB]	0.15[B]	0.22[A]	0.01	0.008
Heart	0.57	0.66	0.54	0.65	0.02	0.138

Values were obtained from n = 5 repetitions per treatment.
[1] The treatments are described as follows: CON, control eggs without *in ovo* injection and incubated at standard temperature; G10, eggs injected at 17.5 days of incubation with 0.6 mL of 10% γ-aminobutyric acid dissolved in distilled water; TM, thermally manipulated eggs exposed to 39.6°C for 6 h daily from embryonic day 10 to 18; G10+TM, eggs that received both previous treatments during incubation. Chicks obtained were grown for 28 days.
[a,b] Means in a row that possess different superscripts differ significantly (p < 0.05).
[A,B] Means in a row that possess different superscripts differ significantly (p < 0.01).

Table 6. Effects of *in ovo* feeding of γ-aminobutyric acid and embryonic thermal manipulation on plasma biochemical parameters in broiler chickens

Parameters	Treatments[1]				SEM	p-value
	CON	G10	TM	G10+TM		
Glucose (mg/dL)	251.8	274.4	274.8	266.6	6.7	0.624
Triglycerides (mg/dL)	41.4	27.6	26.6	37.6	3.2	0.267
Total protein (g/dL)	2.48[b]	2.62[ab]	2.58[ab]	3.1[a]	0.1	0.019
Cholesterol (mg/dL)	114.8	96.2	88.2	111	6.8	0.506

Values were obtained from n = 5 repetitions per treatment.
SEM, standard error of the mean.
[1] The treatments are described as follows: CON, control eggs without *in ovo* injection and incubated at standard temperature; G10, eggs injected at 17.5 days of incubation with 0.6mL of 10% γ-aminobutyric acid dissolved in distilled water; TM, thermally manipulated eggs exposed to 39.6°C for 6 h daily from embryonic day 10 to 18; G10+TM, eggs that received both previous treatments during incubation. Chicks obtained were grown for 28 days.
[a,b] Means in a row that possess different superscripts differ significantly (p < 0.05).

Table 7. Effects of *in ovo* feeding of γ-aminobutyric acid and embryonic thermal manipulation on relative hepatic gene expression in broiler chickens

Genes	Treatments[1]				SEM	p-value
	CON	G10	TM	G10+TM		
SOD	1.00	1.58	1.28	2.36	0.33	0.522
CAT	1.00	1.59	3.22	4.41	0.61	0.171
GPx1	1.00[a]	1.31[ab]	1.59[ab]	2.04[b]	0.19	0.039
NOX1	1.00[B]	0.28[A]	0.52[AB]	0.43[A]	0.09	0.007
NOX4	1.00	0.41	0.55	0.57	0.11	0.274
ACC	1.00	2.26	2.17	0.41	0.52	0.573
FAS	1.00	1.89	1.02	0.84	0.17	0.124
NRF2	1.00	2.26	1.01	1.23	0.22	0.123
EXFABP	1.00	0.91	0.51	0.21	0.11	0.283
PPAR-γ	1.00	0.99	0.99	0.43	0.14	0.497

Values were obtained from n = 5 repetitions per treatment.
SEM, standard error of the mean; *SOD*, superoxide dismutase; *CAT*, catalase; *GPx1*, glutathione peroxidase 1; *NOX1*, nicotinamide adenine dinucleotide phosphate oxidase 1; *NOX4*, nicotinamide adenine dinucleotide phosphate oxidase 4; *ACC*, acetyl-CoA carboxylase; *FAS*, fatty acid synthase; *NRF2*, nuclear factor erythroid 2-related factor 2; *EXFABP*, extracellular fatty acid-binding protein; *PPAR-γ*, peroxisome proliferator-activated receptor-gamma.
[1] The treatments are described as follows: CON, control eggs without *in ovo* injection and incubated at standard temperature; G10, eggs injected at 17.5 days of incubation with 0.6 mL of 10% γ-aminobutyric acid dissolved in distilled water; TM, thermally manipulated eggs exposed to 39.6°C for 6 h daily from embryonic day 10 to 18; G10+TM, eggs that received both previous treatments during incubation. Chicks obtained were grown for 28 days.
[a,b] Means in a row that possess different superscripts differ significantly (p < 0.05).
[A,B] Means in a row that possess different superscripts differ significantly (p < 0.01).

ysis between relative gene expression, growth parameters, plasma biochemical parameters, and relative liver weight. The overall ADFI was positively correlated (red color) with both relative *EXFABP* (r = 0.48, p = 0.042) and *PPAR-γ* (r = 0.55, p = 0.031) gene expression. Interestingly, the same positive correlation was observed between ADFI during the final week of the trial (ADFI4wk) and *FAS* (r = 0.50, p = 0.038) gene expression.

DISCUSSION

Modern broilers have undergone decades of intensive genetic selection for rapid growth and muscle yield, rendering them particularly susceptible to oxidative stress [19]. Oxidative stress occurs when there is an overproduction of free radicals, such that the antioxidant system can no longer act as a neutralizer [20]. There are numerous options for combating oxidative stress in poultry. The first step is to limit ROS pro-

duction by reducing the activity of NOX enzymes. The NOX protein complex mainly transfers electrons from NADPH to oxygen molecules, resulting in O_2^- production [21]. The second is to increase the scavenging of free radicals through antioxidant defenses. In chickens, the first line of antioxidant defense consists of SOD, CAT, and GPx [22]. Activation of the *NRF2* gene is a critical step in the antioxidant modulation pathway [6]. NRF2 is involved in the synthesis of the three major antioxidant enzymes [1]. This study evaluated the hepatic gene expression of *CAT*, *SOD*, *GPx1*, and *NRF2* after ETM and *in ovo* feeding with GABA. Only birds in the G10 group had significantly higher hepatic gene expression of NRF2 than the CON group. Both *GPx1* and *CAT* were significantly upregulated only in the G10+TM group (Table 8).

Interestingly, *SOD*, *CAT*, and *GPx1* were upregulated in all treatment groups, with the G10+TM group always showing the highest expression (Table 7). Furthermore, all three

Table 8. Results of planned contrasts of hepatic gene expression analysis (p-value)

Genes	Planned contrast[1]		
	CON vs G10	CON vs TM	CON vs G10+TM
SOD	NS	NS	NS
CAT	NS	NS	0.046
GPx1	NS	0.092	0.006
NOX1	0.001	0.019	0.007
NOX4	0.077	NS	NS
ACC	NS	NS	NS
FAS	0.065	NS	NS
NRF2	0.039	NS	NS
EXFABP	NS	NS	0.087
PPAR-γ	NS	NS	NS

[1] The treatments are described as follows: CON, control eggs without *in ovo* injection and incubated at standard temperature; G10, eggs injected at 17.5 days of incubation with 0.6 mL of 10% γ-aminobutyric acid dissolved in distilled water; TM, thermally manipulated eggs exposed to 39.6°C for 6 h daily from embryonic day 10 to 18; G10+TM, eggs that received both previous treatments during incubation. Chicks obtained were grown for 28 days.
SOD, superoxide dismutase; NS, not significant; *CAT*, catalase; *GPx1*, glutathione peroxidase 1; *NOX1*, nicotinamide adenine dinucleotide phosphate oxidase 1; *NOX4*, nicotinamide adenine dinucleotide phosphate oxidase 4; *ACC*, acetyl-CoA carboxylase; *FAS*, fatty acid synthase; *NRF2*, nuclear factor erythroid 2-related factor 2; *EXFABP*, extracellular fatty acid-binding protein; *PPAR-γ*, peroxisome proliferator-activated receptor-gamma.

treatments downregulated the expression of *NOX1*. Our previous study highlighted that *in ovo* feeding of GABA increased the plasma total antioxidant activity in heat-stressed chicks [12]. Others have also reported enhanced GPx activity but reduced oxidation levels in birds fed GABA during stress [23]. The ability of GABA to mitigate oxidative stress is linked to its ability to promote glutamate levels [24], thus promoting the synthesis of antioxidant enzymes, such as GPx [25]. However, the mechanism by which GABA could modulate NRF2 remains unclear and requires further research. Additionally, ETM ameliorated heat-induced oxidative stress by downregulating the hepatic *NOX* gene family [26]. Another study evaluating the effects of cold-induced oxidative stress in broilers also revealed that ETM could lower the expression of *NOX4* in the liver, spleen, and heart [27]. Based on the concept of an antioxidant system integrated into cells, we hypothesized that a combination of antioxidants would be more effective than a single antioxidant [22]. Therefore, these findings indicate the combined effect of *in ovo* feeding and thermal manipulation on the enhancement of hepatic antioxidant capacity in broilers.

Although no significant results were found across different treatment groups regarding lipid metabolism-related genes, we found correlations among hepatic gene expression of *FAS*, *EXFABP*, *PPAR-γ*, and ADFI (Figure 2). *FAS* and *ACC* are two important genes that encode key enzymes in

de novo fatty acid synthesis in birds [28]. FAS utilizes malonyl-CoA, catalyzed by ACC, to produce long-chain fatty acids. Similar to our results, *FAS* expression increased with increased feed intake in chickens [29]. This might explain why the correlation was stronger between ADFI in the last phase of the trial because chicks increased their feed intake with age. PPAR-γ is a transcription factor involved in the regulation of adipogenesis [30]. EXFABP belongs to a family of fatty acid-binding proteins that mediate the metabolism and transportation of lipids. The positive correlation between these genes and the overall ADFI in this study may indicate higher lipid deposition in birds with the highest feed consumption.

Hierarchical clustering revealed that the gene panel could be separated into four clusters, with two larger clusters. The first cluster included a set of downregulated genes (*NOX1*, *NOX4*, *EXFABP*, and *PPAR-γ*). Since NOXs family genes are responsible for ROS production, we can hypothesize that limited lipid deposition is associated with reduced ROS production. The second largest cluster included the *SOD*, *GPx1*, *NRF2*, and *FAS* genes, which were predominantly upregulated in our treatment groups. The first line of antioxidant enzymes showed increased gene expression in G10, TM, and G10+TM groups. The synthesis of this array of protective molecules results from the activation of the NRF2 transcription factor to maximize antioxidant protection and maintain internal redox balance [20]. This might explain why these genes were in the same cluster. In addition, the higher feed intake recorded in both G10 and TM during the final phase of our trial accounted for the upregulation of *FAS*.

This study aimed to not only assess the effects of *in ovo* feeding of GABA and ETM in broilers, but also to elucidate their potential potentiating effects when treated together. Because all treatments were applied during incubation, the primary outcomes to be measured were the hatching parameters. No significant differences were observed in hatchability or hatchling weight across all the treatment groups. Previous results have shown that *in ovo* feeding of amino acids [31], especially GABA, does not reduce hatchability in broilers. This result indicates that a high intake of exogenous GABA during incubation is not detrimental to chicken embryos, and thus, *in ovo* feeding of GABA is safe and can be useful for the poultry industry.

Our results also highlight that the ETM used in this study did not reduce hatchability. On the other hand, the effect of ETM on hatchability is inconsistent in the literature, reporting a significant reduction [32], increase, or no effect [16]. The discrepancies in the results may be attributed to the duration and intensity of thermal manipulation. Indeed, some studies [33] have reported decreased growth and hatchability in embryos exposed to continuous ETM. Therefore, intermittent ETM may be more appropriate to avoid a drastic
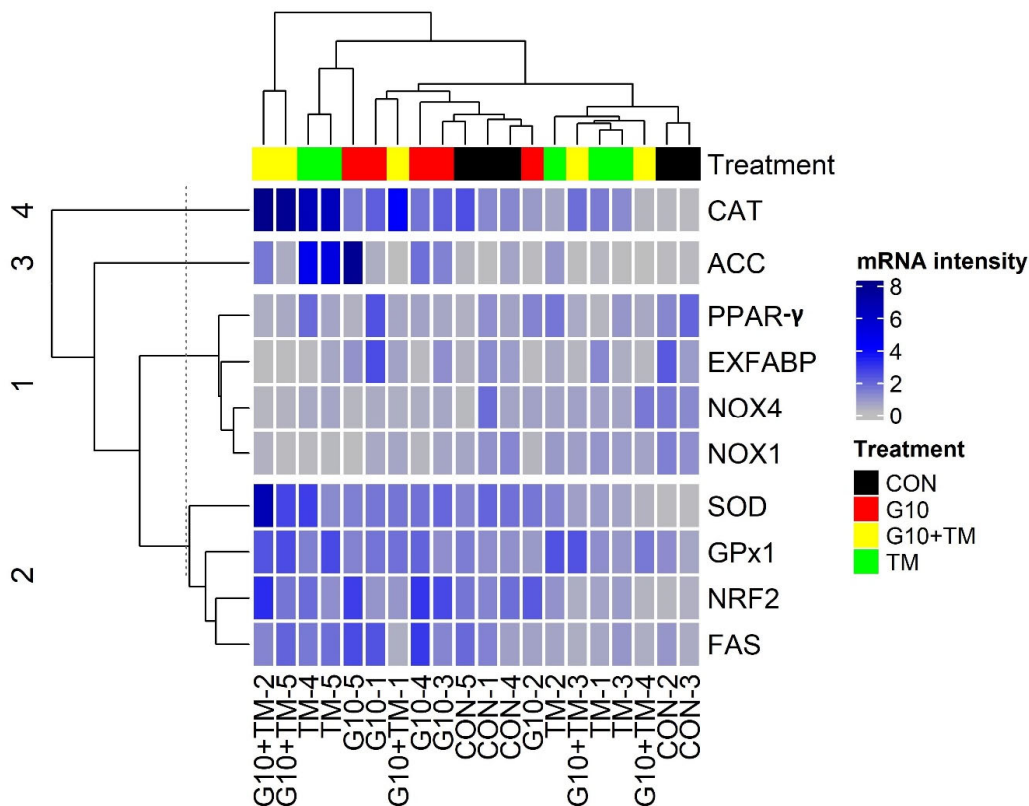
Figure 1. Hierarchical cluster tree of hepatic gene expression. The genes panel included antioxidant genes (*CAT*, *SOD*, *GPx1*, *NOX1*, *NOX4*, and *NRF2*) and lipid metabolism genes (*ACC*, *FAS*, *EXFABP*, and *PPAR-γ*). Each row represents a gene and each column represents an experimental unit belonging to a specific treatment. The treatments are described as follows: CON, control eggs without *in ovo* injection and incubated at standard temperature; G10, eggs injected at 17.5 days of incubation with 0.6 mL of 10% γ-aminobutyric acid dissolved in distilled water; TM, thermally manipulated eggs exposed to 39.6°C for 6 h daily from embryonic day 10 to 18; G10+TM, eggs that received both previous treatments during incubation. Gene expression analysis was performed using real-time quantitative polymerase chain reaction (RT-qPCR) with *GAPDH* and *β-actin* as reference genes. The tree was constructed using the package "ComplexHeatmap" of the R software version 4.0.3 (R Core Team, 2020). *CAT*, catalase; *ACC*, acetyl-CoA carboxylase; *PPAR-γ*, peroxisome proliferator-activated receptor-gamma; *EXFABP*, extracellular fatty acid-binding protein; *NOX4*, nicotinamide adenine dinucleotide phosphate oxidase 4; *NOX1*, nicotinamide adenine dinucleotide phosphate oxidase 1; *SOD*, superoxide dismutase; *GPx1*, glutathione peroxidase 1; *NRF2*, nuclear factor erythroid 2-related factor 2; *FAS*, fatty acid synthase.

reduction in hatchability. Finally, the combination of *in ovo* feeding of GABA and ETM, which does not significantly affect hatchability, indicates that both techniques can be implemented in this configuration without harming the embryo.

Post-hatch growth performance mostly did not show any differences between groups; only FCR during the 8 to 21 days period was increased in the G10+TM group. Studies evaluating *in ovo* feeding have consistently reported its significant effects on growth within the first two weeks of the rearing period. Growth performance has been reported to improve after *in ovo* feeding with various amino acids [34]. Although ADWG and FI were higher in the G10 group, no significant differences were found in our trial. Differences in nutrients, concentrations, and dosages may explain these results. Although the literature reports the merits of ETM, its beneficial effects on growth parameters have not been recognized. Likewise, ETM does not affect or reduce the growth performance [35]. Thus, the higher FCR recorded in the G10+TM

group may be associated with thermal manipulation rather than *in ovo* feeding, because the G10 group consistently performed better.

The organ index is an important indicator that reflects the developmental status of organs. The immune organ index is especially significant because it often correlates with the magnitude of immune function [36]. In this study, birds in the G10+TM group had the highest relative bursa weights. Similarly, Tang and Chen [37] found an increased bursa index and improved immune function in broilers supplemented with GABA at 14 and 21 days of rearing. Evidence suggests that GABA plays a role in immunity by modulating cytokine secretion and modifying immune cell secretion [38]. However, none of the trials evaluating the potential effects of GABA on the bursa have conducted an in-depth study to elucidate the mechanism underlying its action. Thus, although GABA effectively enhances the bursa index, the explanations on this topic remain speculative, and further research is needed.
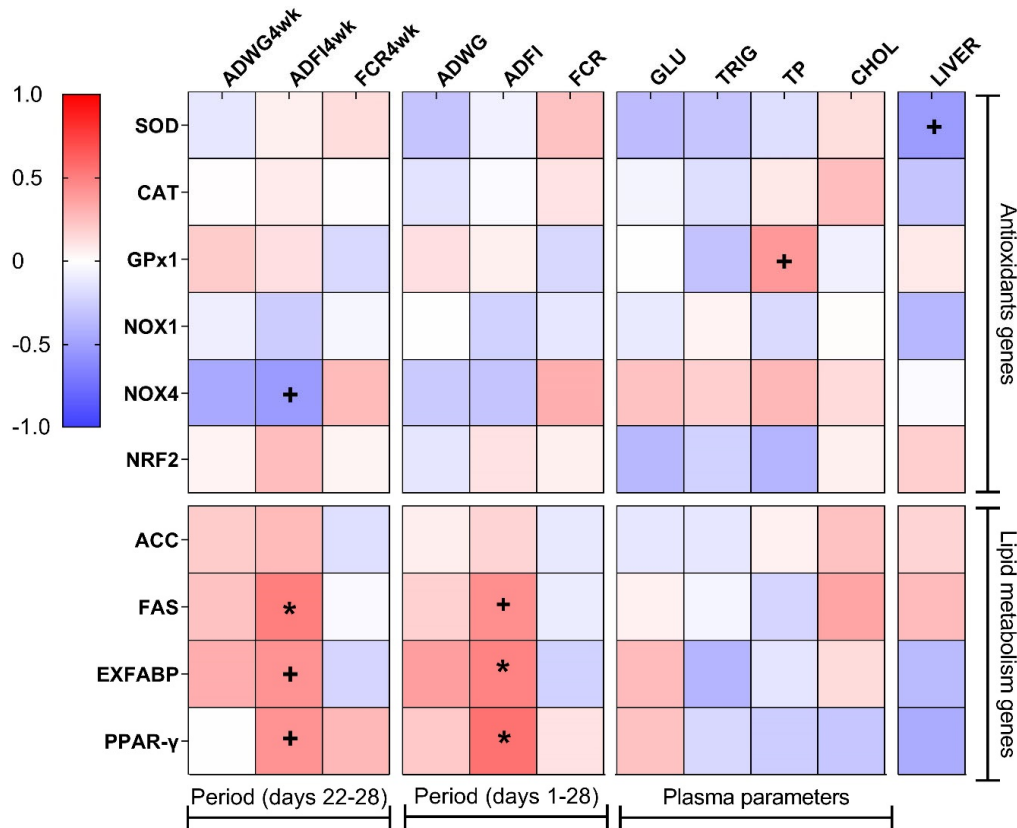
Figure 2. Pearson correlation heat map between the relative mRNA levels of the genes studied, the growth parameters, the plasma biochemical parameters, and the relative liver weight in broiler chickens. The red color indicates a positive correlation, the blue color indicates a negative correlation and the white color indicates no correlation. Pearson r values were calculated using the "CORR procedure" of the SAS software version 9.4 (SAS Institute Inc., 2009). ADWG4wk, average daily weight gain of the last week; ADFI4wk, average daily feed intake of the last week; FCR4wk, feed conversion ratio of the last week; ADWG, average daily weight gain; ADFI, average daily feed intake; FCR, feed conversion ratio; GLU, glucose; TRIG, triglycerides; TP, total protein; CHOL, cholesterol; LIVER, relative liver weight; *SOD*, superoxide dismutase; *CAT*, catalase; *GPx1*, glutathione peroxidase 1; *NOX1*, nicotinamide adenine dinucleotide phosphate oxidase 1; *NOX4*, nicotinamide adenine dinucleotide phosphate oxidase 4; *NRF2*, nuclear factor erythroid 2-related factor 2; *ACC*, acetyl-CoA carboxylase; *FAS*, fatty acid synthase; *EXFABP*, extracellular fatty acid-binding protein; *PPAR-γ*, peroxisome proliferator-activated receptor-gamma. + Indicates a trend at the 0.1 level. * Correlation is significant at $p < 0.05$ level.

In addition, the immunity-promoting abilities of ETM in poultry have already been demonstrated [26]. Thus, these results suggest the concomitant action of *in ovo* feeding of both GABA and ETM on the immune status of broilers.

In conclusion, the results of this study showed that *in ovo* GABA feeding and ETM increased the bursa index and improved hepatic antioxidant gene expression in broilers when treated together during incubation. Therefore, further investigation of the potential synergistic effect of the two methods for alleviating heat-induced oxidative stress in broilers will be of great interest.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

REFERENCES

1. Surai PF. Antioxidant systems in poultry biology: superoxide dismutase. J Anim Res Nutr 2016;1:8.
2. Goel A, Ncho CM, Choi YH. Regulation of gene expression in chickens by heat stress. J Anim Sci Biotechnol 2021;12:11. http://doi.org/10.1186/s40104-020-00523-5
3. Surai PF, Kochish II, Kidd MT. Redox homeostasis in poultry: regulatory roles of NF-κB. Antioxidants 2021;10:186. https://doi.org/10.3390/antiox10020186
4. Chueh CC, Lin LJ, Lin WC, et al. Antioxidant capacity of

banana peel and its modulation of Nrf2-ARE associated gene expression in broiler chickens. Ital J Anim Sci 2019;18:1394-403.

5. Chelikani P, Fita I, Loewen PC. Diversity of structures and properties among catalases. Cell Mol Life Sci 2004;61:192-208. https://doi.org/10.1007/s00018-003-3206-5

6. Ma Q. Role of nrf2 in oxidative stress and toxicity. Annu Rev Pharmacol Toxicol 2013;53:401-26. https://doi.org/10.1146/annurev-pharmtox-011112-140320

7. Ncho CM, Jeong C, Gupta V, Goel A. The effect of gamma-aminobutyric acid supplementation on growth performances, immune responses, and blood parameters of chickens reared under stressful environment: a meta-analysis. Environ Sci Pollut Res 2021;28:45019-28. https://doi.org/10.1007/s11356-021-13855-0

8. Cobb SR, Buhl EH, Halasy K, Paulsen O, Somogyi P. Synchronization of neuronal activity in hippocampus by individual GABAergic interneurons. Nature 1995;378:75-8. https://doi.org/10.1038/378075a0

9. El-Naggar K, El-Kassas S, Abdo SE, Kirrella AAK, Al Wakeel RA. Role of gamma-aminobutyric acid in regulating feed intake in commercial broilers reared under normal and heat stress conditions. J Therm Biol 2019;84:164-75. https://doi.org/10.1016/j.jtherbio.2019.07.004

10. Hu H, Bai X, Shah AA, et al. Interactive effects of glutamine and gamma-aminobutyric acid on growth performance and skeletal muscle amino acid metabolism of 22-42-day-old broilers exposed to hot environment. Int J Biometeorol 2016;60:907-15. https://doi.org/10.1007/s00484-015-1084-9

11. Choi YH. Effects of γ-aminobutyric acid on mortality in laying hens during summer time. J Agric Life Environ Sci 2019;53:131-9.

12. Ncho CM, Goel A, Jeong CM, Youssouf M, Choi YH. In ovo injection of gaba can help body weight gain at hatch, increase chick weight to egg weight ratio, and improve broiler heat resistance. Animals 2021;11:1364. https://doi.org/10.3390/ani11051364

13. Ncho CM, Gupta V, Goel A. Effect of thermal conditioning on growth performance and thermotolerance in broilers: A systematic review and meta-analysis. J Therm Biol 2021;98:102916. https://doi.org/10.1016/j.jtherbio.2021.102916

14. Al-Zghoul MB, Mohammad Saleh KM. Effects of thermal manipulation of eggs on the response of jejunal mucosae to posthatch chronic heat stress in broiler chickens. Poult Sci 2020;99:2727-35. https://doi.org/10.1016/j.psj.2019.12.038

15. Ncho CM, Goel A, Jeong CM, Gupta V, Choi YH. Effects of in ovo feeding of γ-aminobutyric acid on growth performances, plasma metabolites, and antioxidant status in broilers exposed to cyclic heat stress. Sustainability 2021;13:11032. https://doi.org/10.3390/su131911032

16. Goel A, Ncho CM, Jeong CM, Choi YH. Embryonic thermal manipulation and in ovo gamma-aminobutyric acid supple-mentation regulating the chick weight and stress-related genes at hatch. Front Vet Sci 2022;8:807450. https://doi.org/10.3389/fvets.2021.807450

17. Zhang H, Elliott KEC, Durojaye OA, Fatemi SA, WSchilling M, Peebles ED. Effects of in ovo injection of L-ascorbic acid on growth performance, carcass composition, plasma antioxidant capacity, and meat quality in broiler chickens. Poult Sci 2019;98:3617-25. https://doi.org/10.3382/ps/pez173

18. Slawinska A, Dunislawska A, Plowiec A, Gonçalves J, Siwek M. TLR-mediated cytokine gene expression in chicken peripheral blood mononuclear cells as a measure to characterize immunobiotics. Genes 2021;12:195. https://doi.org/10.3390/genes12020195

19. Soleimani AF, Zulkifli I, Omar AR, Raha AR. Physiological responses of 3 chicken breeds to acute heat stress. Poult Sci 2011;90:1435-40. https://doi.org/10.3382/ps.2011-01381

20. Surai PF, Kochish, II, Fisinin VI, Kidd MT. Antioxidant defence systems and oxidative stress in poultry biology: an update. Antioxidants 2019;8:235. https://doi.org/10.3390/antiox8070235

21. Kirkman HN, Gaetani GF. Catalase: a tetrameric enzyme with four tightly bound molecules of NADPH. Proc Natl Acad Sci 1984;81:4343-7. https://doi.org/10.1073/pnas.81.14.4343

22. Surai P, Kochish I, Fisinin V. Antioxidant systems in poultry biology: nutritional modulation of vitagenes. Eur Poult Sci 2017;81:214. https://doi.org/10.1399/eps.2017.214

23. Al Wakeel RA, Shukry M, Abdel Azeez A, Mahmoud S, Saad MF. Alleviation by gamma amino butyric acid supplementation of chronic heat stress-induced degenerative changes in jejunum in commercial broiler chickens. Stress 2017;20:562-72. https://doi.org/10.1080/10253890.2017.1377177

24. Chen Z, Tang J, Sun YQ, Xie J. Protective effect of γ-aminobutyric acid on antioxidation function in intestinal mucosa of Wenchang chicken induced by heat stress. J Anim Plant Sci 2013;23:1634-41.

25. Walls AB, Waagepetersen HS, Bak LK, Schousboe A, Sonnewald U. The glutamine-glutamate/GABA cycle: function, regional differences in glutamate and GABA production and effects of interference with GABA metabolism. Neurochem Res 2015;40:402-9. https://doi.org/10.1007/s11064-014-1473-1

26. Al-Zghoul MB, Sukker H, Ababneh MM. Effect of thermal manipulation of broilers embryos on the response to heat-induced oxidative stress. Poult Sci 2019;98:991-1001. https://doi.org/10.3382/ps/pey379

27. Saleh KM, Tarkhan AH, Al-Zghoul MB. Embryonic thermal manipulation affects the antioxidant response to post-hatch thermal exposure in broiler chickens. Animals 2020;10:126. https://doi.org/10.3390/ani10010126

28. Zaefarian F, Abdollahi MR, Cowieson A, Ravindran V. Avian liver: the forgotten organ. Animals 2019;9:63. https://doi.org/10.3390/ani9020063

29. Hicks JA, Trakooljul N, Liu HC. Discovery of chicken micro-RNAs associated with lipogenesis and cell proliferation. Physiol Genomics 2010;41:185-93. https://doi.org/10.1152/physiolgenomics.00156.2009

30. Wang G, Kim WK, Cline MA, Gilbert ER. Factors affecting adipose tissue development in chickens: a review. Poult Sci 2017;96:3687-99. https://doi.org/10.3382/ps/pex184

31. Ohta Y, Kidd MT. Optimum site for in ovo amino acid injection in broiler breeder eggs. Poult Sci 2001;80:1425-9. https://doi.org/10.1093/ps/80.10.1425

32. Narinç D, Erdoğan S, Tahtabiçen E, Aksoy T. Effects of thermal manipulations during embryogenesis of broiler chickens on developmental stability, hatchability and chick quality. Animal 2016;10:1328-35. https://doi.org/10.1017/S1751731116000276

33. Piestun Y, Halevy O, Yahav S. Thermal manipulations of broiler embryos-The effect on thermoregulation and development during embryogenesis. Poult Sci 2009;88:2677-88. https://doi.org/10.3382/ps.2009-00231

34. Kadam MM, Bhanja SK, Mandal AB, et al. Effect of in ovo threonine supplementation on early growth, immunological responses and digestive enzyme activities in broiler chickens. Br Poult Sci 2008;49:736-41. https://doi.org/10.1080/00071660802469333

35. Piestun Y, Halevy O, Shinder D, Ruzal M, Druyan S, Yahav S. Thermal manipulations during broiler embryogenesis improves post-hatch performance under hot conditions. J Therm Biol 2011;36:469-74. https://doi.org/10.1016/j.jtherbio.2011.08.003

36. Riras A, Fabricant J. Indication of immunodepression in chicken infected with various strain of Marck's dsease virus. Avian Dis 1988;32:1-8.

37. Tang J, Chen Z. The protective effect of gamma-aminobutyric acid on the development of immune function in chickens under heat stress. J Anim Physiol Anim Nutr (Berl) 2016;100:768-77. https://doi.org/10.1111/jpn.12385

38. Jin Z, Mendu SK, Birnir B. GABA is an effective immuno-modulatory molecule. Amino Acids 2013;45:87-94. https://doi.org/10.1007/s00726-011-1193-7