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Effects of In Ovo Feeding of γ-Aminobutyric Acid on Growth Performances, Plasma Metabolites, and Antioxidant Status in Broilers Exposed to Cyclic Heat Stress

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Abstract: γ-aminobutyric acid (GABA) is an amino acid used for mitigating the detrimental effects of heat stress in broilers. In addition, a growing body of literature suggests that the in ovo feeding of various nutrients can enhance the post-hatch thermotolerance of broilers. Therefore, we hypothesized that the supplementation of GABA during incubation might have positive effects in heat-stressed broilers. Chicks hatched from eggs were divided into three groups described as follows: chicks hatched from eggs incubated at normal temperature and then raised under thermoneutral temperature (CON); chicks hatched from eggs incubated at normal temperature but raised under cyclic heat stress (HS) (CON+HS); and chicks hatched from eggs injected with 60 mg of GABA dissolved in 0.6 mL of distilled water but raised under cyclic HS (G10+HS). The HS was applied between 28 and 31 days of age with ambient temperatures raised from 22 ± 1 °C to 33 ± 1 °C for 6 h daily. Compared to the CON group, average daily weight gain was significantly lower in the CON+HS but not in the G10+HS group. Feed intake was significantly decreased in both the CON+HS and G10+HS groups. Compared to the CON group, plasma corticosterone levels were significantly increased in the CON+HS group, but not the G10+HS group. Hepatic mRNA levels of the acetyl-CoA carboxylase gene (ACC) were significantly reduced in the G10+HS group compared to the CON group. In addition, positive Pearson correlation coefficients were found in mRNA levels between fatty acid synthase (FAS) and nicotinamide adenine dinucleotide phosphate oxidase 1 (NOX1) ($r = 0.55$, $p < 0.05$), NOX1 and NOX4 ($r = 0.65$, $p < 0.01$), and catalase (CAT) and superoxide dismutase (SOD) ($r = 0.62$, $p < 0.05$). Taken together, the results suggest that this study can serve as a basis for future work focusing on the in ovo feeding of GABA as a technique to combat heat stress in broilers.

Keywords: in ovo feeding; GABA; heat stress; broilers; antioxidant status

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1. Introduction

Due to global warming, high rearing temperature is becoming one of the most common issues in poultry production [1]. Heat stress (HS) occurs when the ambient temperature exceeds the optimal range recommended for growth. Due to the high selection for fast growth performance, broilers in the last phase of rearing are especially sensitive to HS [2]. Indeed, HS can impair the performance, metabolism, and health of broilers. The effects of HS can include reduced feed intake (FI), body weight gain (BW), and increased water consumption [3]. Excessively high ambient temperature is strongly correlated with high mortality rates [4]. In addition, previous reports have shown that HS induces oxidative damage by increasing the production of reactive oxygen species in cells [5,6].

Several strategies have been adopted to mitigate the deleterious effects of HS in poultry. The most common approaches include improvements in ventilation, the selection of

resistant strains, and nutritional management [7]. The latter consists of the dietary supplementation of various types of substances such as γ-aminobutyric acid (GABA). GABA is a four-carbon non-protein amino acid involved in inhibitory synaptic transmission [8]. In addition, GABA has been implicated in several regulatory functions such as memory, blood pressure, and respiration [9]. In broilers, GABA is used as a feed supplement to reduce the adverse effect of high environmental temperature [10]. Previous research demonstrated its effectiveness for increasing overall growth performance, improving nutrient absorption, and reducing oxidative stress in chickens under high temperatures [11,12].

Recently, in ovo feeding has been considered a cost-effective way to alleviate the drawbacks of post-hatch and incubational HS in broilers. Originally, in ovo feeding was developed as a tool for providing a continuous supply of critical nutrients during the first few days of the chick's life [13]. The methodology consists of injecting nutrients into the amnion of eggs so that by consuming the content of the amnion, the embryo can access nutrients before it starts to hatch [14]. The most recent advances involve testing the heat resistance of birds after the in ovo feeding of prebiotics [15] or amino acids [16]. Methionine–cysteine in ovo injection improved embryonic development, antioxidant status, and jejunum histomorphometry of broilers chicks exposed to incubational HS [17]. Furthermore, the damages (high corticosterone and HSP70 mRNA levels) occasioned by incubational HS were alleviated after the in ovo feeding of sulfur-containing amino acids [18]. Interestingly, evidence suggests that broilers' resistance toward post-hatch HS can also be improved by in ovo feeding. For example, the in ovo feeding of L-leucine was proven effective in affording thermotolerance in broilers under acute heat stress, primarily by changing amino acid metabolism up to market age [19]. In addition, in ovo injection of galactooligosaccharides reduced the harmful effects of hyperthermia on feed efficiency during the finisher feeding phase [20]. When injected in ovo, we found that GABA could improve the early heat resistance of broilers by enhancing antioxidant functions and regulating fatty-acid-metabolism-related genes in 10-day-old chicks [21].

There have been no studies addressing the potential long-term effects of in ovo feeding of GABA in broilers. Therefore, the objective of this study was to investigate the effects of in ovo feeding of GABA on the growth performances, organ indexes, blood biochemical parameters, antioxidant status, and gene expression of stress-related genes in broilers subjected to cyclic HS near market age.

2. Materials and Methods

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

2.1. Incubation and In Ovo Feeding Procedure

Three hundred eggs were obtained from a commercial breeder farm with 47-week-old Arbor Acres hens (Hapcheon, Korea). According to standard incubation conditions, the eggs were set for incubation in an incubator (Rcom Co., Ltd., Gimhae, Korea). Briefly, from embryonic day (ED) 1 to ED 18, eggs were subjected to 37.8 °C and 56% relative humidity (RH), and from ED 18 until hatching, the incubation temperature was maintained at 36.8 °C with 70% RH. On ED 10, unfertilized eggs were removed from the incubator after candling. On ED 17.5, the eggs were distributed into three groups of equal numbers (n = 60/group) with similar weight (66 ± 4 g) using the Solver module of Microsoft Excel (Microsoft Excel 2016; Microsoft Corp., Redmond, WA, USA). In our previous study, we found that there were no significant differences (hatching parameters and biological parameters) between a sham control (distilled water injected) and a non-injected control [21]; therefore, we did not include a sham treatment in this trial.

Moreover, the solution and methodology used for the in ovo feeding procedure were similar to those previously described [21]. The first two groups (total 120 eggs) were considered as control and were not injected. The third group was injected with 0.6 mL of 10% (0.1 g mL^{-1}) GABA (#A2129 Sigma-Aldrich Inc., St. Louis, MO, USA) dissolved

in distilled water. For in ovo injection, after a hole was made on the blunt end of each egg using a dental drill (Saeshin, Daegu, Korea), they were subsequently injected using a 1 mL syringe with a 23 G and 1-inch needle (Kovax-Syringe® Korea Vaccine Co. Ltd., Seoul, Korea), followed by being sealed using surgical tape (3M™ Micropore ™, Saint Paul, MN, USA) and returned to the incubator. The injection targeted the amnion. The control eggs were taken out of the incubator for the same amount of time without injection and then returned to the incubator. It should be mentioned that the incubation temperature was reduced to 20 °C due to an expected general blackout for 6 h between EDs 19 and 20 in the animal complex area.

2.2. Feeding Experiment and HS Challenge

After hatching, a total of sixty unsexed one-day-old broilers were raised in battery brooders under a thermally controlled environment at 34 ± 1 °C and 50% RH, and then the temperature was gradually decreased to 22 ± 1 °C on day 28. A commercially available feed, in crumbled form and water, were available ad libitum under continuous lighting. On day 28, the chicks were allocated into three different treatment groups: chicks hatched from non-injected eggs and reared at thermoneutral temperature (CON); chicks hatched from non-injected eggs and exposed to cyclic HS (CON+HS); and chicks hatched from GABA in ovo injected eggs and exposed to cyclic HS (G10+HS). Each treatment had 20 chicks kept in 5 cages with 4 chicks (unsexed) in each cage. They were then subjected to a daily cyclic HS challenge. An overview of the design is presented in Figure 1.

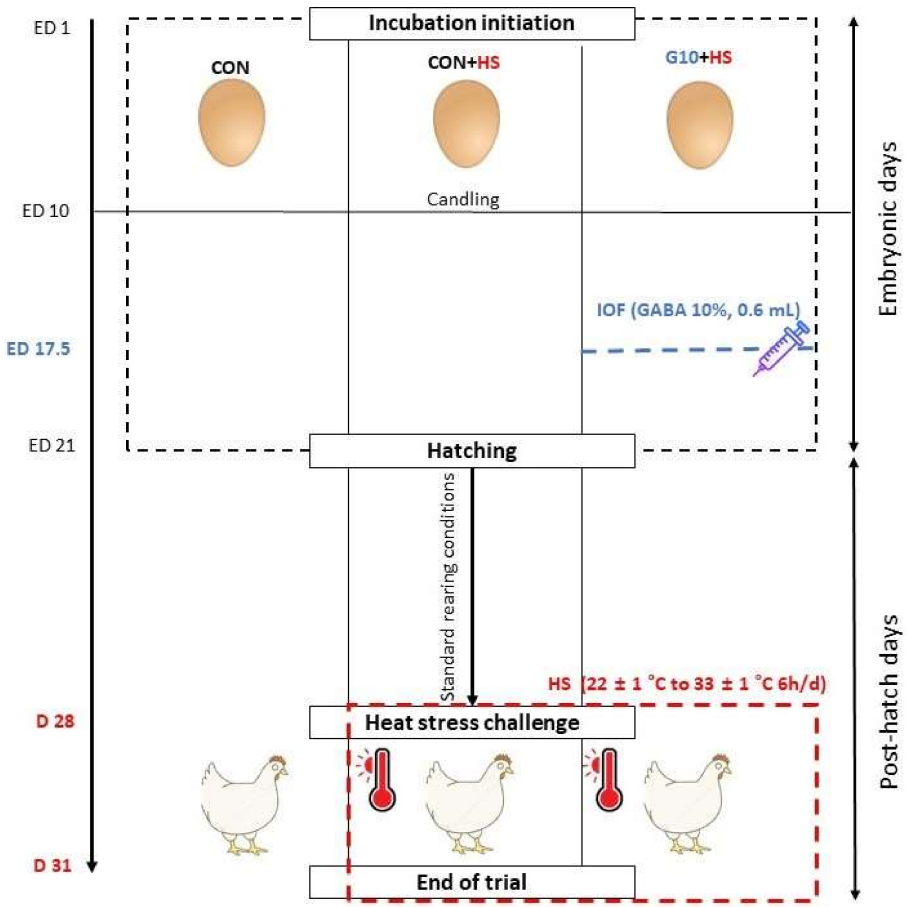

Figure 1. Study design. At 17.5 days of incubation, eggs were injected in ovo with or without 0.6 mL of GABA at 10%. Hatchlings obtained were raised until day 28 and subjected to cyclic heat stress (from 22 °C to 33 °C for 6 h daily) between 28 and 31 days of age or kept at a thermoneutral temperature (22 °C).

As previously executed [22], birds were challenged with a cyclic HS between 28 and 31 days of age with minor modifications. In one of two rooms, the ambient temperature was gradually increased from 22 ± 1 °C to 33 ± 1 °C over 6 h (from 10:00 a.m. to 4:00 p.m.) and then returned to 22 ± 1 °C over 1 h (Figure 2).

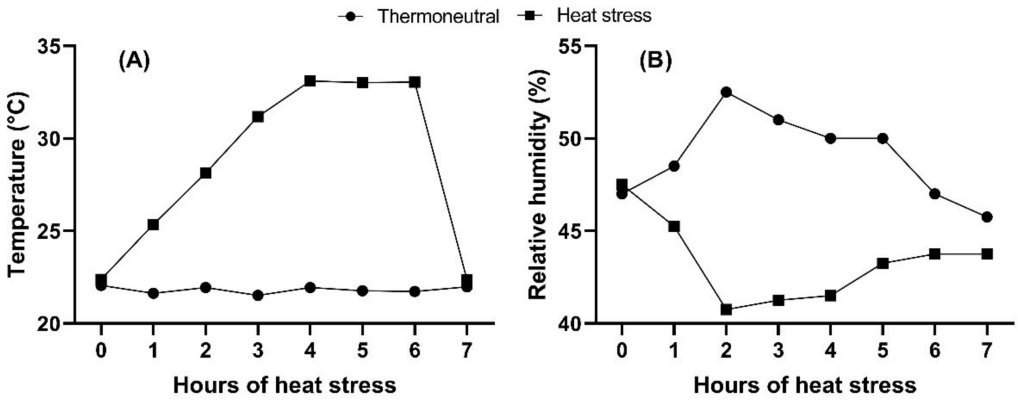

Figure 2. Average temperature (**A**) and relative humidity (**B**) variation in thermoneutral and heat stress environments. At 17.5 days of incubation, eggs were injected in ovo with or without 0.6 mL of GABA at 10%. Hatchlings obtained were raised until day 28 and subjected to cyclic heat stress (from 22 °C to 33 °C for 6 h daily) or kept at a thermoneutral temperature from days 28 to 31.

Chicks under thermoneutral temperature remained at 22 ± 1 °C until day 31. The body weight of individual birds and feed intake of each cage was measured weekly and at the end of the trial (day 31).

2.3. Rectal Temperature, Blood and Tissues Sampling

After the heat exposure on day 31, five birds close to the average body weight in each treatment were selected for blood and tissue sampling. The birds were euthanized with carbon dioxide before sampling. Blood was collected from heart puncture and transferred into heparinized vacuum containers (#367874, BD Co., Ltd., Franklin Lakes, NJ, USA). The blood samples were thereafter centrifuged at 2000× *g* for 10 min at 4 °C, and plasma was collected and then stored at −20 °C for later analysis. Organs were dissected free, and then weighed (the liver, spleen, bursa, heart, proventriculus, and gizzard), or the length was measured (the duodenum, jejunum, ileum, and caecum). Liver samples were snap-frozen in liquid nitrogen and stored at 2212−80 °C for further analysis. Rectal temperatures of birds selected for sampling were recorded before and after the cyclic HS exposure using a digital thermometer (HANNA instruments Inc., Padova, Italy) by inserting the probe 3 cm into the cloaca until the temperature readings stabilized.

2.4. Plasma Corticosterone, Metabolites, and Oxidative Stress Markers

Plasma corticosterone levels were detected by using an ELISA kit (ADI-901-097, ENZO Life Sciences, Inc., Farmingdale, NY, USA) according to the kit's instructions. Absorbance was measured at 405 nm by using a Multiskan FC microplate photometer (ThermoFisher Scientific, Inc., Waltham, MA, USA). Plasma metabolite concentrations were measured according to the manufacturer guide using a VetTest Chemistry Analyzer (IDEXX Laboratories, Inc., Westbrook, ME, USA) with dry-slide technology. Plasma samples were used for 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity assay (DPPH-RSA) and malondialdehyde analysis. DPPH-RSA was based on a method developed previously [23]. Briefly, plasma (20 μL) was diluted in 480 μL of 10 mmol/L sodium–potassium phosphate (pH 7.4) followed by the addition of 500 μL of 0.1 mmol/L (DPPH) free radical. Samples were incubated for 30 min in a dark environment. After incubation, samples were centrifuged briefly for 3 min at 6000× *g* followed by the absorbance reading at 520 nm. The percentage of inhibition was calculated based on the following formula: %

inhibition = [1 − (A1/A0)] × 100, where A0 is the absorbance of the control and A1 is the absorbance of test samples. Malondialdehyde concentrations were analyzed based on a previously developed method [24] with slight modifications. Briefly, 400 µL of plasma were added in tubes containing 400 µL of 40% trichloroacetic acid (TCA). Subsequently, 800 µL of 0.67% thiobarbituric acid (TBA) was added to the mixture, which was then kept in a boiling water bath at 95 °C for 45 min. After cooling on ice, samples were centrifuged at 6000× *g* for 30 s followed by an absorbance reading at 530 nm. The antioxidant balance was then calculated by dividing the DPPH-RSA value by the MDA.

2.5. Real-Time PCR for mRNA Quantification

Total RNA extraction from the liver was performed using Trizol™ reagent (ThermoFisher Scientific, Inc.), following the manufacturer's protocol. The concentrations and purities of the samples were confirmed by reading the optical density of each sample in a Nanodrop (ThermoScientific, Inc.). Subsequently, the reverse transcription reaction was conducted using a PrimeScriptTM first-strand cDNA synthesis kit (Takara, Tokyo, Japan), following the manufacturer's guide. The cDNA synthesized was then used to perform real-time PCR using a StepOnePlus™ system (Applied Biosystems, Inc., Waltham, MA, 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 20 µL volume containing Power SYBR™ green PCR master mix (ThermoScientific, Inc.), and a 10 pmol concentration of forward and reverse primer specific for each gene and cDNA. Information related to the primers is presented in Table 1. Target gene quantification was normalized to the Ct values of GAPDH. Relative expression was determined using the $2^{-\Delta\Delta ct}$ algorithm.

Table 1. Primer sequences used to evaluate the hepatic gene expression of broilers.

Gene	Sequence	Accession Number	Reference
ACC	F: CACTTCGAGGCGAAAAAC R: GGAGCAAATCCATGACCA	XM_015295697.2	This study
CAT	F: ACCAAGTACTGCAAGGCGAA R: TGAGGGTTCCTCTTCTGGCT	NM_001031215.1	[25]
FAS	F: CAATGGACTTCATGCCTC R: GCTGGGTACTGGAAGACA	NM_205155.3	This study
GAPDH	F: TTGGCATTGTGGAGGGTCTTA R: GTGGACGCTGGGATGATGTT	NM_204305.1	This study
GPx1	F: AACCAATTCGGGCACCAG R: CCGTTCACCTCGCACTTCTC	NM_001277853.2	[26]
HSP70	F: GCTGAACAAGAGCATCAATCCA R: CAGGAGCAGATCTTGCACATTT	AY143693.1	This study
HSP90	F: CCCGAGCAAGCTGGATTCT R: GGTCATCCCTATGCCGGTATC	NM_001109785	This study
NOX1	F: GCGAAGACGTGTTCCTGTAT R: GAACCTGTACCAGATGGACTTC	NM_001101830.1	[27]
NOX4	F: CCTCTGTGCTTGTACTGTGTAG R: GACATTGGAGGGATGGCTTAT	NM_001101829.1	[27]
SOD	F: AGGGGGTCATCCACTTCC R: CCCATTTGTGTTGTCTCCAA	NM_205064.1	[25]

Abbreviations: ACC, acetyl-CoA carboxylase; CAT, catalase; FAS, fatty acid synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPx1, glutathione peroxidase 1; HSP70, heat shock protein 70; HSP90, heat shock protein 90; NOX1, nicotinamide adenine dinucleotide phosphate oxidase 1; NOX4, nicotinamide adenine dinucleotide phosphate oxidase 4; SOD, superoxide dismutase.

2.6. Statistical Analysis

A replicate (cage) was considered an experimental unit for all the evaluated response parameters in this trial. A completely randomized design with 5 replicates per treatment was used. Each replicate was constituted of 4 unsexed birds. IBM SPSS Statistics for Windows software (IBM SPSS 25; IBM Corp., Armonk, NY, USA) was used to analyze all data via a one-way ANOVA. When the ANOVA was significant, a Tukey post hoc test was performed to assess the difference between means. As previously executed [28], before statistical analyses, the data in each group were subjected to the ROUT method of GraphPad prism 8 (GraphPad Software, Inc., La Jolla, CA, USA) to detect outliers. All percentage data below 20% and above 80% were arcsine-transformed. The data were tested for the normality of distribution using Shapiro–Wilk tests before applying ANOVA; therefore, the assumption for applying the ROUT method to potentially detect more than one outlier was met. The ROUT test revealed outliers only in the ACC mRNA levels. Results are presented as means ± SEM, and the significance level was set at $p < 0.05$. To detect potential associations between heat shock proteins, antioxidants and-fatty acid-metabolism-related gene expression, Pearson correlations between the relative mRNA levels of the genes studied were calculated to develop a correlation matrix.

3. Results

3.1. Growth Performances

Average daily body weight gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR) are shown in Table 2. In ovo GABA feeding did not affect the initial body weight of chicks. From days 1 to 28, chicks were raised under standard temperatures, and no significant differences in ADG, ADFI, and FCR were found among all treatments. After the cyclic HS, ADG was significantly reduced in the CON+HS compared to the CON group ($p < 0.05$), whereas ADFI was lower in both CON+HS and G10+HS groups ($p = 0.001$). However, there were no significant differences observed in FCR among all groups.

Table 2. Effects of the in ovo injection of γ-aminobutyric acid on growth parameters in broilers subjected to cyclic heat stress.

Parameters	Treatments			
	CON	CON+HS	G10+HS	*p*-Value
Initial body weight (g/bird)	45.1 ± 0.1	44.8 ± 0.2	45.0 ± 0.1	0.342
Bodyweight (g/bird)				
7 days	166 ± 4.3	177 ± 1.7	165 ± 5.2	0.111
21 days	981 ± 43.8	1011 ± 11.9	964 ± 48.4	0.684
28 days	1624 ± 65.0	1613 ± 39.1	1551 ± 82.0	0.697
31 days	1968 ± 77.5	1794 ± 96.7	1795 ± 119.7	0.295
ADG (g/birds)				
0 to 7 days	17.2 ± 0.6	18.8 ± 0.2	17.1 ± 0.7	0.102
8 to 21 days	58.2 ± 3	59.6 ± 0.9	57.0 ± 3.2	0.786
22 to 28 days	91.8 ± 4.5	86.0 ± 4.0	84 ± 2.7	0.496
28 to 31 days	86.0 ± 4.5 [a]	45.2 ± 2.4 [b]	61.0 ± 12.8 [ab]	0.011
ADFI (g/bird)				
0 to 7 days	18.9 ± 0.6	20.2 ± 0.4	18.8 ± 0.8	0.242
8 to 21 days	76.1 ± 3.0	77.4 ± 1.2	74.2 ± 3.6	0.73
22 to 28 days	142 ± 3.0	133 ± 5.6	132 ± 5.9	0.381
28 to 31 days	147 ± 7.8 [a]	94.1 ± 5.7 [b]	109 ± 9.1 [b]	0.001
FCR				
0 to 7 days	1.10 ± 0.02	1.07 ± 0.02	1.10 ± 0.01	0.537
8 to 21 days	1.29 ± 0.02	1.29 ± 0.01	1.31 ± 0.03	0.641
22 to 28 days	1.55 ± 0.05	1.55 ± 0.02	1.59 ± 0.04	0.788
28 to 31 days	1.72 ± 0.1	2.10 ± 0.1	1.97 ± 0.2	0.316

At 17.5 days of incubation, eggs were injected in ovo with or without 0.6 mL of GABA at 10%. Hatchlings obtained were raised until day 28 and subjected to cyclic heat stress (from 22 °C to 33 °C for 6 h daily) or kept at a thermoneutral temperature from days 28 to 31. Data were analyzed via one-way ANOVA (n = 5 cages/treatment). Means in the same row with different superscript letters are significantly different ($p < 0.05$). Abbreviations: ADG, average daily weight gain; ADFI, average daily feed intake; FCR, feed conversion ratio.

3.2. Organ Weight, Length, and Rectal Temperature

Table 3 shows the results of the organ weight in all experimental groups. There were no statistical differences between treatment groups for the proventriculus, gizzard, heart, bursa, liver, and spleen in relative and absolute weights.

Table 3. Effects of the in ovo injection of γ-aminobutyric acid on organ weights in broilers subjected to cyclic heat stress.

Absolute Organs Weight (g)	Treatments			
	CON	CON+HS	G10+HS	*p*-Value
Proventriculus	8.40 ± 1.4	8.70 ± 1.2	8.10 ± 1.1	0.959
Gizzard	15.5 ± 0.9	19.3 ± 2.3	18.1 ± 2.0	0.345
Heart	13.4 ± 0.4	12.1 ± 0.7	11.7 ± 0.6	0.132
Bursa	3.20 ± 0.2	3.30 ± 0.3	3.50 ± 0.5	0.827
Liver	56.7 ± 4.0	59.3 ± 3.2	52.2 ± 2.5	0.339
Spleen	2.10 ± 0.2	2.00 ± 0.1	1.90 ± 0.3	0.788
Relative Organs Weight (%)				
Proventriculus	0.4 ± 0.06	0.47 ± 0.06	0.44 ± 0.06	0.744
Gizzard	0.75 ± 0.02	0.88 ± 0.06	0.82 ± 0.06	0.151
Heart	0.65 ± 0.02	0.65 ± 0.04	0.64 ± 0.02	0.837
Bursa	0.15 ± 0.01	0.18 ± 0.02	0.18 ± 0.01	0.46
Liver	2.73 ± 0.2	3.2 ± 0.2	2.8 ± 0.1	0.099
Spleen	0.1 ± 0.01	0.1 ± 0.01	0.1 ± 0.01	0.896

At 17.5 days of incubation, eggs were injected in ovo with or without 0.6 mL of GABA at 10%. Hatchlings obtained were raised until day 28 and subjected to cyclic heat stress (from 22 °C to 33 °C for 6 h daily) or kept at a thermoneutral temperature from days 28 to 31. Data were analyzed via one-way ANOVA (n = 5 birds/treatment).

The organ lengths of the duodenum, jejunum, ileum, and caecum are given in Table 4. The CON+HS group exhibited a significantly shorter absolute duodenum length compared to the CON ($p < 0.05$). However, there were no significant differences in the absolute length of the jejunum, ileum, and caecum and the relative length of all organs.

Table 4. Effects of the in ovo injection of γ-aminobutyric acid on organ lengths in broilers subjected to cyclic heat stress.

Absolute Organ Length (cm)	Treatments			
	CON	CON+HS	G10+HS	*p*-Value
Duodenum	28.4 ± 0.75 [a]	24.3 ± 1.1 [b]	26.7 ± 0.86 [ab]	0.026
Jejunum	67.4 ± 2.6	59.9 ± 1.5	64.7 ± 4.7	0.286
Ileum	70.0 ± 1.1	59.9 ± 1.4	64.5 ± 4.8	0.095
Caecum	18.4 ± 0.77	15.7 ± 0.82	17 ± 0.53	0.122
Relative Organ Length (cm.kg^{-1})				
Duodenum	1.38 ± 0.05	1.31 ± 0.06	1.44 ± 0.03	0.232
Jejunum	3.26 ± 0.10	3.23 ± 0.10	2.92 ± 0.70	0.813
Ileum	3.40 ± 0.12	3.23 ± 0.10	3.49 ± 0.25	0.59
Caecum	0.89 ± 0.06	0.85 ± 0.04	0.92 ± 0.05	0.608

At 17.5 days of incubation, eggs were injected in ovo with or without 0.6 mL of GABA at 10%. Hatchlings obtained were raised until day 28 and subjected to cyclic heat stress (from 22 °C to 33 °C for 6 h daily) or kept at a thermoneutral temperature from days 28 to 31. Data were analyzed via one-way ANOVA (n = 5 birds/treatment). Means in the same row with different superscript letters are significantly different ($p < 0.05$).

Table 5 indicates the results related to rectal temperature. No significant differences in rectal temperature were observed between all treatments under standard housing temperature on day 28. Moreover, on day 31 after cyclic HS, chicks treated with CON+HS and G10+HS had significantly higher rectal temperature values compared to the CON group.

Table 5. Effects of the ovo injection of γ-aminobutyric acid on rectal temperature (°C) in broilers subjected to cyclic heat stress.

Broiler Age	Treatments			
	CON	CON+HS	G10+HS	*p*-Value
Day 28 (before HS)	41.5 ± 0.1	41.6 ± 0.1	41.6 ± 0.1	0.836
Day 31 (after HS)	41.6 ± 0.1 [a]	43.2 ± 0.1 [b]	43.4 ± 0.1 [b]	<0.001

At 17.5 days of incubation, eggs were injected in ovo with or without 0.6 mL of GABA at 10%. Hatchlings obtained were raised until day 28 and subjected to cyclic heat stress (from 22 °C to 33 °C for 6 h daily) or kept at a thermoneutral temperature from days 28 to 31. Data were analyzed via one-way ANOVA (n = 5 birds/treatment). Means in the same row with different superscript letters are significantly different ($p < 0.05$).

3.3. Plasma Corticosterone, Metabolites, and Oxidative Stress Markers

After cyclic HS exposure, the plasma DPPH-RSA of the CON+HS was significantly decreased ($p < 0.05$) compared to the CON group (Table 6). Malondialdehyde levels and the antioxidant balance in the plasma appeared to be similar among all treatment groups.

Table 6. Effects of the ovo injection of γ-aminobutyric acid on plasma oxidative stress markers in broilers subjected to cyclic heat stress.

Parameters	Treatments			
	CON	CON+HS	G10+HS	*p*-Value
DPPH-RSA (%)	49.6 ± 1.0 [a]	44.6 ± 1.9 [b]	45 ± 0.9 [ab]	0.036
MDA (nmol/mL)	0.67 ± 0.1	0.67 ± 0.1	0.61 ± 0.1	0.828
Antioxidant balance (U)	82.2 ± 16.0	69.8 ± 6.7	80.7 ± 13.9	0.762

At 17.5 days of incubation, eggs were injected in ovo with or without 0.6 mL of GABA at 10%. Hatchlings obtained were raised until day 28 and subjected to cyclic heat stress (from 22 °C to 33 °C for 6 h daily) or kept at a thermoneutral temperature from days 28 to 31. Data were analyzed via one-way ANOVA (n = 5 birds/treatment). Means in the same row with different superscript letters are significantly different ($p < 0.05$). Abbreviations: DPPH-RSA, 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity assay; MDA, malondialdehydes.

Table 7 shows the effects of the in ovo feeding of GABA on plasma metabolites and corticosterone levels. No significant effects were detected for triglycerides, cholesterol, and total protein in plasma. However, HS significantly increased plasma glucose levels in both CON+HS and G10+HS groups. In addition, HS significantly increased plasma corticosterone levels in the CON+HS group compared to the CON group. Interestingly, plasma corticosterone levels in the G10+HS group were not statistically different from the CON group.

Table 7. Effects of the in ovo injection of γ-aminobutyric acid on plasma metabolites and corticosterone in broilers subjected to cyclic heat stress.

Parameters	Treatments			
	CON	CON+HS	G10+HS	*p*-Value
Cholesterol (mg/dL)	120 ± 11.4	126 ± 4.0	119 ± 5.4	0.825
Glucose (mg/dL)	245 ± 3.3 [a]	277 ± 6.8 [b]	280 ± 9.6 [b]	0.008
Total protein (g/dL)	3.20 ± 0.14	3.10 ± 0.11	3.10 ± 0.18	0.928
Triglycerides (g/dL)	17.8 ± 3.7	18.2 ± 3.9	18.6 ± 5.9	0.093
Corticosterone (ng/mL)	0.76 ± 0.04 [a]	4.22 ± 1.41 [b]	1.52 ± 0.24 [ab]	0.027

At 17.5 days of incubation, eggs were injected in ovo with or without 0.6 mL of GABA at 10%. Hatchlings obtained were raised until day 28 and subjected to cyclic heat stress (from 22 °C to 33 °C for 6 h daily) or kept at a thermoneutral temperature from days 28 to 31. Data were analyzed via one-way ANOVA (n = 5 birds/treatment). Means in the same row with different superscript letters are significantly different ($p < 0.05$).

3.4. Hepatic mRNA Relative Expression

Figures 3–6 present the effects of the in ovo injection of GABA on hepatic mRNA expression of CAT, GPx1, SOD, ACC, NOX1, NOX4, FAS, HSP70, and HSP90 in heat-exposed chickens.

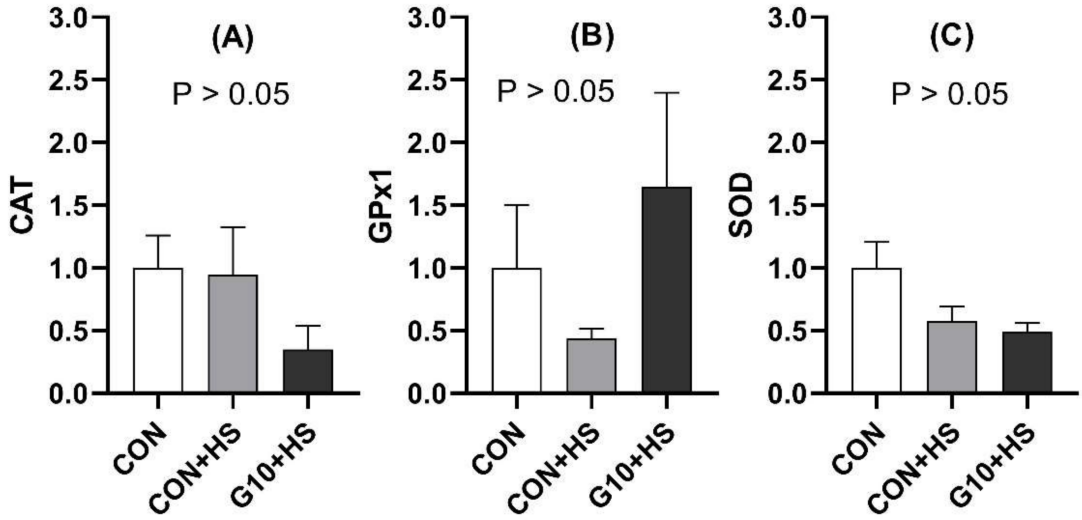

Figure 3. Effects of the in ovo injection of GABA on the relative mRNA expression of hepatic CAT (**A**), GPx1 (**B**), and SOD (**C**) in broilers exposed to cyclic heat stress. At 17.5 days of incubation, eggs were injected in ovo with or without 0.6 mL of GABA at 10%. Hatchlings obtained were raised until day 28 and subjected to cyclic heat stress (from 22 °C to 33 °C for 6 h daily) or kept at a thermoneutral temperature from days 28 to 31. Data were analyzed via one-way ANOVA (n = 5 birds/treatment). Abbreviations: CAT, catalase; GPx1, glutathione peroxidase 1; SOD, superoxide dismutase.

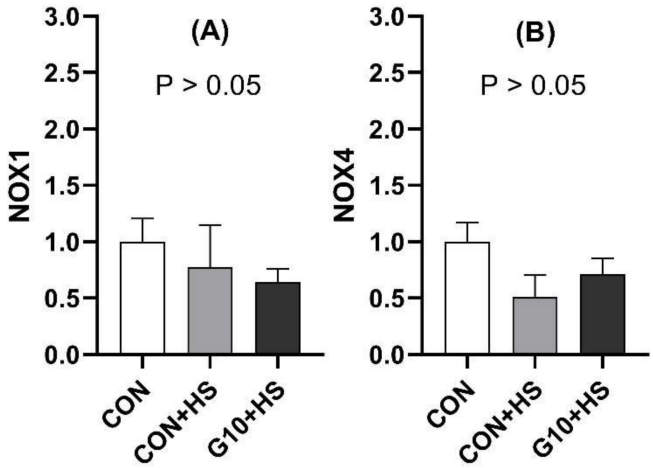

Figure 4. Effects of the in ovo injection of GABA on relative mRNA expression of hepatic NOX1 (**A**) and NOX4 (**B**) in broilers exposed to cyclic heat stress. At 17.5 days of incubation, eggs were injected in ovo with or without 0.6 mL of GABA at 10%. Hatchlings obtained were raised until day 28 and subjected to cyclic heat stress (from 22 °C to 33 °C for 6 h daily) or kept at a thermoneutral temperature from days 28 to 31. Data were analyzed via one-way ANOVA (n = 5 birds/treatment). Abbreviations: NOX1, nicotinamide adenine dinucleotide phosphate oxidase 1; NOX4, nicotinamide adenine dinucleotide phosphate oxidase 4.

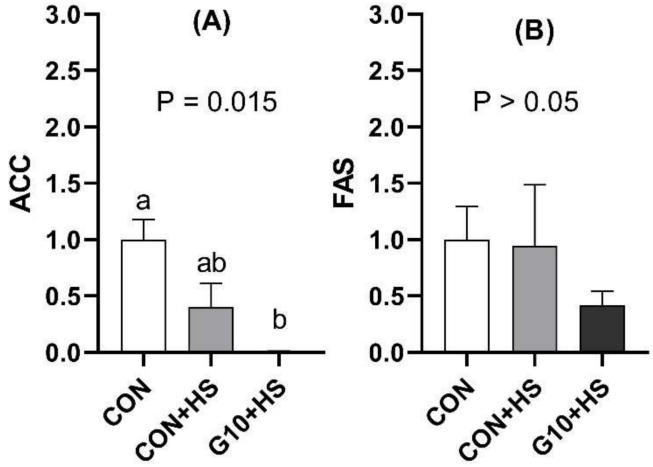

Figure 5. Effects of the in ovo injection of GABA on relative mRNA expression of hepatic ACC (**A**) and FAS (**B**) in broilers exposed to cyclic heat stress. At 17.5 days of incubation, eggs were injected in ovo with or without 0.6 mL of GABA at 10%. Hatchlings obtained were raised until day 28 and subjected to cyclic heat stress (from 22 °C to 33 °C for 6 h daily) or kept at a thermoneutral temperature from days 28 to 31. Abbreviations: ACC, acetyl-CoA carboxylase; FAS, fatty acid synthase. Data were analyzed via one-way ANOVA (n = 5 birds/treatment for the FAS gene). The ROUT test revealed outliers in the data set of ACC (n = 4 birds/treatment).

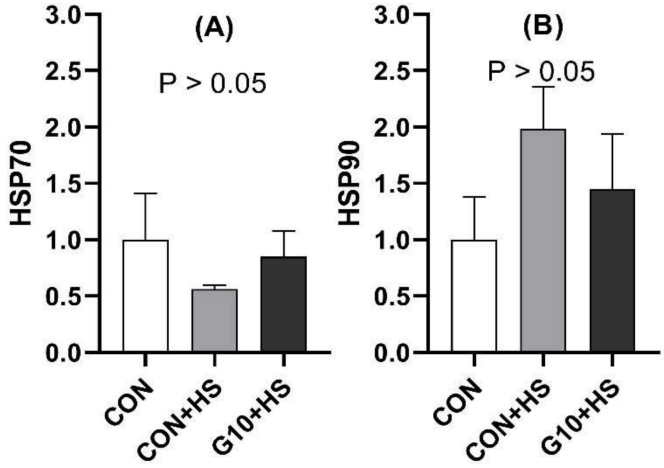

Figure 6. Effects of the in ovo injection of GABA on relative mRNA expression of hepatic HSP70 (**A**) and HSP90 (**B**) in broilers exposed to cyclic heat stress. At 17.5 days of incubation, eggs were injected in ovo with or without 0.6 mL of GABA at 10%. Hatchlings obtained were raised until day 28 and subjected to cyclic heat stress (from 22 °C to 33 °C for 6 h daily) or kept at a thermoneutral temperature from days 28 to 31. Data were analyzed via one-way ANOVA (n = 5 birds/treatment). Abbreviations: HSP70, heat shock protein 70; HSP90, heat shock protein 90.

There were no significant variations in the relative mRNA expression of CAT, SOD, and GPx1 in response to either cyclic HS or the in ovo feeding of GABA (Figure 3).

In addition, there were no differences between treatment groups concerning relative mRNA expression for the NOX family genes (NOX1 and NOX4), the HSP family genes (HSP70 and HSP90), and the FAS gene.

However, the in ovo feeding of GABA downregulated the expression of ACC in heat-stressed broilers compared to the CON group ($p < 0.05$) (Figure 5).

Table 8 shows the Pearson correlation r-values examined between the relative mRNA levels of genes pairwise. Positive correlations were found between FAS and NOX1 (r = 0.55, $p < 0.05$), NOX1 and NOX4 (r = 0.65, $p < 0.01$), and CAT and SOD (r = 0.62, $p < 0.05$).

Table 8. Pearson correlation values of the relative mRNA levels of the genes studied in the liver.

	HSP70	FAS	NOX1	NOX4	CAT	SOD	HSP90	GPx1	ACC
HSP70	1	0.38	0.15	0.25	−0.24	0.16	−0.04	−0.05	0.35
FAS		1	0.55 *	0.30	−0.28	−0.03	−0.17	−0.14	0.13
NOX1			1	0.65 **	−0.26	0.16	0.10	0.02	−0.20
NOX4				1	−0.24	−0.02	−0.22	−0.15	0.20
CAT					1	0.62 *	0.21	0.17	0.47
SOD						1	0.00	0.37	0.42
HSP90							1	−0.19	−0.09
GPx1								1	−0.23
ACC									1

At 17.5 days of incubation, eggs were injected in ovo with or without 0.6 mL of GABA at 10%. Hatchlings obtained were raised until day 28 and subjected to cyclic heat stress (from 22 °C to 33 °C for 6 h daily) or kept at a thermoneutral temperature from days 28 to 31. Data were analyzed via Pearson's correlation test. Abbreviations: ACC, acetyl-CoA carboxylase; CAT, catalase; FAS, fatty acid synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPx1, glutathione peroxidase 1; HSP70, heat shock protein 70; HSP90, heat shock protein 90; NOX1, nicotinamide adenine dinucleotide phosphate oxidase 1; NOX4, nicotinamide adenine dinucleotide phosphate oxidase 4; SOD, superoxide dismutase. * Correlation is significant at the 0.05 level. ** Correlation is significant at the 0.01 level.

4. Discussion

In poultry, HS is known for its adverse impacts on health, physiology, and efficiency [29,30]. Chronic HS exposure induces a reduction in broilers' feed intake and body weight while concomitantly increasing FCR [31]. Similarly, in our study, 4 days of cyclic HS led to a significant reduction in ADFI of the birds reared under HS compared to those under normal housing temperature. For regulating their body temperature under HS, broilers reduce their feed consumption to promote heat loss and decrease their metabolic heat production [32]. Therefore, even though reducing FI helps cope with high ambient temperature, it is naturally followed by severe growth impairment [33]. These findings were confirmed in the results of our study. The ADG of heat-stressed birds was statistically lower (by 47.4% for CON+HS vs. 29.1% for G10+HS) compared to the control birds.

Interestingly, the in ovo feeding of GABA could mitigate the weight loss (by 35%) occasioned by cyclic HS. This might be explained by the fact that birds hatched from eggs injected in ovo recorded a higher ADFI (15.8%) than control birds under the same high environmental conditions. Additionally, our results showed that the in ovo feeding of GABA numerically reduced the FCR (15.9%) compared to the control birds under HS. Previous results have already documented the orexigenic effect of dietary GABA supplementation [11,34]. These results might suggest that the mechanism behind in ovo feeding is closely linked to how dietary supplementation works. Indeed, in ovo feeding given during the late embryonic stages targeted the amnion [35]. The embryo, by consuming the content of the amnion, could access the injected nutrients before it started to hatch [14]. GABA's ability to improve the feed intake of HS-exposed birds was explained by the fact that it can lower the expression of anorexigenic neuropeptides and upregulate orexigenic neuropeptides [11].

The organ index is an important parameter reflecting the development status of organs. A recent study [36] reported an increase in the liver index when broilers were reared under chronic HS. There was a weakly significant (p = 0.099) tendency to increase the liver index of HS birds in the present study. In poultry, the liver is known as the main organ of fat synthesis. Consequently, higher liver indexes might be correlated with higher fat synthesis during HS [37]. Gastrointestinal length can be an indicator of digestive health. HS significantly decreased the absolute organ length of the duodenum, but its relative length was not affected. Therefore, no conclusive effect of either HS or the in ovo feeding of GABA on duodenum length could be ascertained. Rectal temperature is generally utilized as a marker for HS resistance in broilers [38]. Previous reports have recognized the HS-stimulating effect of rectal temperature in broilers [11,34]. As expected, birds belonging to both groups under cyclic HS had a higher rectal temperature in the current study.

DPPH-RSA has been widely used as a method to evaluate plasma total antioxidant capacity (TAC) [39]. In the present study, the in ovo feeding of GABA resulted in improved TAC in broilers subjected to cyclic HS. Researchers [40] previously reported that GABA supplementation increased TAC in chickens under HS. These findings indicate that similarly to dietary GABA, in ovo GABA injection could instigate the retrieval of antioxidant functions after cyclic HS exposure. In response to cyclic HS, broilers exhibited higher plasma levels of corticosterone. These results of the present study show consistency with those reported by earlier studies [41,42], mentioning that heat-challenged birds tend to have increased plasma corticosterone compared to those at thermoneutral temperature. This might be explained by the fact that heat stress activates the hypothalamic–pituitary–adrenal (HPA) axis, leading to increased corticosterone secretion [43]. Therefore, enhanced corticosterone release under HS may have reduced overall feed intake and body weight gain [44,45], as observed in the current study.

Interestingly, the in ovo feeding of GABA could reduce the plasma corticosterone levels of the birds exposed to HS. Earlier studies have revealed that dietary and GABA-enriched barley bran could lower adrenocorticotropic hormone (ACTH) and corticosterone levels in pigs and rats [46,47]. Indeed, under stressful conditions, the hypothalamus releases the corticotropin-releasing hormone, which stimulates the secretion of ACTH from the anterior pituitary gland [48]. Subsequently, ACTH provokes the synthesis of glucocorticoids such as corticosterone to combat stress [49]. Furthermore, GABA was shown to inhibit ACTH secretion by activating α1- and α2-adrenergic receptors [50]. Thus, we could hypothesize that the potential effect of GABA on corticosterone is linked to its inhibitory effect on ACTH.

In poultry, acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) are the key enzymes responsible for fat synthesis. ACC is involved in converting acetyl-CoA into malonyl-CoA and then into palmitate, whereas FAS determines the maximum capacity for fatty acid synthesis in tissues [51]. Our results showed that the hepatic mRNA levels of ACC were downregulated in heat-stressed broilers after the in ovo injection of GABA. In addition, a numerically lower value of relative mRNA levels of FAS was observed in the birds fed GABA in ovo. As previously mentioned, the liver is known to be the main organ of fat synthesis in poultry, and one of the deleterious effects of HS in broilers is lipid accumulation, engendered by the upregulation of FAS and ACC genes [36,52]. Therefore, lower mRNA hepatic levels of FAS and ACC indicate less fatty acid synthesis during HS in birds that were fed GABA in ovo.

Interestingly, we observed a positive correlation between FAS and NOX1 mRNA levels. The NOX gene family, responsible for ROS generation, was affected by heat stress [27], suggesting that the in ovo feeding of GABA may decrease ROS generation during cyclic HS. The mechanism by which GABA potentially reduces ROS generation could be related to its effect on glutathione peroxidase synthesis [53,54]. Significant positive correlations were detected between CAT and SOD mRNA levels. SOD and CAT are among the major antioxidant enzymes [55]. SOD is responsible for the dismutation of superoxide radicals to molecular oxygen and hydrogen peroxide (H_2O_2), whereas CAT is responsible for transforming H_2O_2 into water and oxygen [56]. The actions of both enzymes are synchronized, which may justify the observed positive correlation.

As the Materials and Methods section mentioned, a general blackout for 6 h led to a decrease in the temperature (from 36.8 to 20 °C) during the incubation. However, a potential effect of the incubation temperature drop can be neglected because all the treatment groups were kept in the same incubator. Even though a cold temperature during embryogenesis has been shown to enhance growth performance, such temperature manipulations were lower than 20 °C and repetitive to affect the thermoregulatory mechanism of chicks [57,58]. Furthermore, all the relevant data reported were cross-checked with the literature and showed no discrepancies in the current study. For example, the recorded growth performances of Arbor Acres in this study were concordant to recently published articles [59,60]. In addition, the average BWs obtained after hatch (45.1 g), 7 days (165.7 g), 21 days (981.1 g),

and 28 days (1624.1 g) were in line with the Arbor Acres broiler performances (as-hatched performance) objective, which recommends 43 g, 204 g, 978 g, and 1567 g for the same periods, respectively [61]. Similarly, plasma parameters [62] were within the same range of previously reported results.

5. Conclusions

The results from the current study suggested that the in ovo feeding of GABA at 0.1 g. mL^{-1} increased ADG and plasma TAC while reducing plasma corticosterone and ACC mRNA levels in heat-stressed broilers. However, further research is needed to elucidate whether the in ovo feeding of GABA should be considered as a viable technique for enhancing heat resistance in broilers.

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Institutional Review Board Statement: The study was approved by the Institutional Animal Care and Use Committee of Gyeongsang National University (GNU-200916-C0058) and was conducted according to the guidelines.

Informed Consent Statement: Not applicable.

Data Availability Statement: All the relevant data are available in the paper.

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