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Effect of dietary glycine supplementation on productive performance, egg quality, stress response, and fatty liver incidence in laying hens raised under heat stress conditions

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ABSTRACT The current experiment aimed to investigate the effect of dietary glycine (**Gly**) supplementation on productive performance, egg quality, stress response, and fatty liver incidence in laying hens raised under heat stress (**HS**) conditions. A total of two hundred eighty 24-wk-old Lohmann Brown-Lite laying hens were randomly allotted to 1 of 4 dietary treatments with 7 replicates. The negative control (**NC**) diet was prepared to meet or exceed the nutrient and energy requirement for Lohmann Brown laying hens, whereas the positive control (**PC**) diet was formulated to increase AME_n by 100 kcal/kg compared with the NC diet. Two additional diets were prepared by supplementing 0.341% and 0.683% Gly to the NC diet. All hens were exposed to cyclic HS at 31.4 ± 1.17°C for 8 h/d and 26.7 ± 1.10°C for the remaining time for a 12-wk trial. Results indicated that increasing supplementation of Gly in diets tended (linear, $P = 0.088$) to decrease the FCR of laying hens. Increasing supplementation of Gly in diets increased (linear, $P < 0.05$) eggshell lightness and decreased (linear, $P < 0.05$) egg yolk color. Moreover, a tendency for a quadratic association ($P < 0.10$) of serum aspartate aminotransferase and alanine aminotransferase concentrations with increasing supplementation of Gly was observed. Increasing supplementation of Gly in diets decreased (linear, $P < 0.05$) blood heterophil:lymphocyte ratio of laying hens. Hens fed the NC diet showed higher fatty liver incidence ($P < 0.05$) than those fed the PC diet, but increasing supplementation of Gly decreased (linear, $P < 0.05$) fatty liver incidence of laying hens. In conclusion, increasing supplementation of Gly up to 0.683% in diets decreases FCR, stress response, and fatty liver incidence in laying hens raised under HS conditions.

Key words: fatty liver incidence, glycine, heat stress, laying hen, productive performance

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INTRODUCTION

Heat stress (**HS**) is a significant challenge in the current poultry production because of rapid global warming (Biswal et al., 2022). Several previous studies have reported deleterious effects of HS on productive performance (Balnave and Muheereza, 1997; Kirunda et al., 2001; Wasti et al., 2020) and egg quality of laying hens (Mahmoud et al., 1996; Balnave and Muheereza, 1997; Mashaly et al., 2004), which are highly associated with a concomitant decrease in the health and welfare of laying hens (Zhu et al., 2019; Abdel-Moneim et al., 2021). Therefore, several studies have been conducted to alleviate the adverse outcomes of HS in laying hens, but few promising methods have been identified.

Glycine (**Gly**) is the simplest amino acid (**AA**) and is typically considered nonessential for poultry (Corzo et al., 2004; Li and Wu, 2018). However, the recent research has suggested that dietary Gly should be considered a conditionally essential AA for poultry because Gly requirement can change with various internal or external factors such as improved genetic potential and stressful environment (Balnave, 2004; Awad et al., 2015). Moreover, increasing requirements of dietary Gly are likely due to elevated metabolic demands of Gly as a precursor molecule of purine, glutathione, bile salt, uric acid, and creatine synthesis in poultry (Rowe et al., 1978; Wyss and Kaddurah-Daouk, 2000; Te Braake et al., 2008). It was reported that additional supplementation of Gly was required in broiler diets containing low concentrations of CP (Awad et al., 2015). Han and Thacker (2011) also reported that additional supplementation of 0.05 or 0.10% Gly over its requirement

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improved productive performance and egg quality in laying hens, possibly due to increased energy and nutrient digestibility. However, limited information regarding the effect of additional supplementation of Gly in diets is available in laying hens. Moreover, the effect of dietary supplementation of Gly under HS conditions has not been investigated in laying hens. In particular, our previous study reported increased AA oxidation of broiler chickens exposed to HS (Kim et al., 2021), suggesting that laying hens exposed to HS may have increased Gly requirements because of increasing synthesis of uric acids. However, this hypothesis has not been tested. Finally, HS is known to accelerate fatty liver development in poultry by increasing de novo lipid synthesis in the liver (Molero, 2007; Emami et al., 2021; Kim et al., 2021), and previous studies have reported that dietary supplementation of betaine decreased fat accumulation and fatty liver incidence of poultry exposed to HS (Molero, 2007; Ratriyanto and Mosenthin, 2018). Dietary supplementation of Gly may also show an anti-fatty liver effect as our previous study indicated that dietary Gly had a HS-reducing role in broiler chickens, which was similar to the effect of dietary betaine (Won et al., 2023). However, there is a lack of information regarding the effect of dietary supplementation of Gly on fatty liver incidence in laying hens raised under HS conditions.

Therefore, the objective of the current study was to investigate the effect of dietary supplementation of Gly on productive performance, egg quality, stress response, and fatty liver incidence in laying hens raised under HS conditions.

MATERIALS AND METHODS

Animals, Diets, and Experimental Design

All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee (**IACUC**) at Chung-Ang University (approval number: 2020-00099). A total of two hundred eighty 24-wk-old Lohmann Brown-Lite laying hens were allotted to 1 of 4 dietary treatments with 7 replicates in a completely randomized design. Each replicate consisted of 10 consecutive cages (24 cm × 36 cm × 39 cm) with 1 hen per cage. The average BW and egg production rate of hens at the start of the experiment were 1.74 kg and 90.5%, respectively. The negative control (**NC**) diet was prepared mainly with corn, soybean meal, and corn gluten meal. The energy and nutrient concentrations of the NC diet were formulated to meet or exceed the nutrient and energy requirement estimates for Lohmann Brown-Lite laying hens (Table 1; Lohmann Breeders, 2021). The concentrations of AA in the NC diet were calculated from standardized ileal digestible (**SID**) AA of ingredients presented in the Brazilian Tables for Poultry and Swine (Rostagno et al., 2011). The positive control (**PC**) diet was formulated to contain higher AME_n by 100 kcal/kg than the NC diet by inclusion of 1.22 % of soybean oil in replace of celite. The PC diet was prepared to simulate the commercial high-energy diet typically used for laying hens during the hot season in South Korea (Kim et al., 2017). Two additional diets were prepared by adding 0.345% and 0.690% Gly (99.0%, Neo Cremar Co., Ltd., Seoul, Republic of Korea) to the NC diet in replace of celite in order to increase supplementation of Gly in diets by 0.341% and 0.683%, respectively. Increasing supplemental levels of Gly in diets (i.e., 0.341% and 0.683%) were set to increase SID Gly+serine (**Ser**) concentrations by 25% and 50% compared with those in the NC diet (i.e., calculated SID Gly + Ser in the NC diet = 1.365%; Won et al., 2023). Analyzed concentration of total Gly in the NC diet was 0.645%, whereas analyzed concentrations of total Gly in diets supplemented with 0.341% and 0.683% Gly were 1.003% and 1.313%, respectively (Table 1). The diets and water were provided ad libitum for 12 wks of the feeding trial. All hens were exposed to a cyclic HS condition at 31.4 ± 1.17°C for 8 h/d and 26.7 ± 1.10°C for the remaining time. The average relative humidity was 74 ± 13.1% during the whole experiment. Based on the HS index for laying hens, which was calculated from ambient temperature and relative humidity (Hy-Line, 2016), the values for HS index in the current environmental conditions were approximately 77 - 84, indicating that laying hens in this study were raised under severe HS conditions (Hy-Line, 2016). A 16-h lighting schedule (16 L:8 D) was maintained during the entire experiment.

Sample Collection and Analysis

The experimental diets were sampled and analyzed for CP (method 990.03; AOAC, 2007) using the Kjeldahl method (Kjeltec auto system 2300 Analyzer, FOSS, Hilleroed, Denmark). The diets were also analyzed for total Gly using L8900 amino acid analyzer (Hitachi, Tokyo, Japan) after hydrolysis in 6 N HCl at 110°C for 24 h (method 982.30; AOAC, 2007). The concentrations of total Ca and P in diets were also determined using an inductively coupled plasma spectrometer (Optima 5300 DV, Perkin Elmer Inc., Shelton, CT) as reported by Kim et al. (2016). These analyses were performed at the BT research facility center, Chung-Ang University, Republic of Korea.

At the conclusion of the experiment, 1 hen with a BW that was close to the average BW in each replicate was selected and euthanized by CO_2 asphyxiation. The samples for blood, liver, and feather were collected. The liver and feather samples were stored at -80°C for further analyses.

Productive Performance and Egg Quality

Productive performance including hen-day egg production, egg weight, egg mass, and broken and shell-less egg production rate was recorded daily. However, feed intake (**FI**) and feed conversion ratio (**FCR**) of laying hens were recorded weekly. Egg mass was calculated

Table 1. Composition and nutrient contents of experimental diets (as-fed basis).

Item	Dietary treatments[1]			
	PC	NC	0.341% Gly	0.683% Gly
Ingredients				
Corn	61.07	61.07	61.07	61.07
Soybean meal, 45% crude protein	19.20	19.20	19.20	19.20
Corn gluten meal	6.06	6.06	6.06	6.06
Soybean oil	1.22	0.00	0.00	0.00
Monodicalcium phosphate	1.45	1.45	1.45	1.45
Limestone	8.98	8.98	8.98	8.98
98.5% DL-Methionine	0.22	0.22	0.22	0.22
99% L-Lysine HCl	0.17	0.17	0.17	0.17
99% Threonine	0.01	0.01	0.01	0.01
99% L-Arginine	0.02	0.02	0.02	0.02
99% L-Isoleucine	0.01	0.01	0.01	0.01
98% L-Tryptophan	0.02	0.02	0.02	0.02
99% Glycine[2]	0.000	0.000	0.345	0.690
Celite	0.780	2.000	1.655	1.310
Salt	0.17	0.17	0.17	0.17
50% Choline	0.17	0.17	0.17	0.17
$NaHCO_3$	0.10	0.10	0.10	0.10
Antioxidant	0.05	0.05	0.05	0.05
Vitamin premix[3]	0.15	0.15	0.15	0.15
Mineral premix[4]	0.15	0.15	0.15	0.15
Total sum	100.00	100.00	100.00	100.00
Calculated nutrient and energy content				
AME_n, kcal/kg	2,825	2,725	2,731	2,736
Crude protein, %	16.82	16.82	17.23	17.64
Digestible lysine, %	0.88	0.88	0.88	0.88
Digestible methionine + cysteine, %	0.79	0.79	0.79	0.79
Digestible methionine, %	0.51	0.51	0.51	0.51
Digestible threonine, %	0.62	0.62	0.62	0.62
Digestible tryptophan, %	0.19	0.19	0.19	0.19
Digestible arginine, %	0.91	0.91	0.91	0.91
Digestible isoleucine, %	0.77	0.77	0.77	0.77
Digestible valine, %	0.81	0.81	0.81	0.81
Digestible glycine + serine, %	1.365	1.365	1.706	2.048
Total calcium, %	3.73	3.73	3.73	3.73
Available phosphorus, %	0.38	0.38	0.38	0.38
Analyzed crude protein, %	16.53	16.62	17.09	17.80
Analyzed total glycine, %	0.635	0.645	1.003	1.313
Analyzed total calcium, %	3.76	3.56	3.68	3.78
Analyzed total phosphorus, %	0.58	0.54	0.56	0.55

[1]Dietary treatments: PC, positive control diet (NC + 100 kcal/kg AME_n); NC, negative control diet; 0.341% glycine (Gly), NC + 0.341% Gly; 0.683% Gly, NC + 0.683% Gly.

[2]Inclusion levels of Gly were set to increase Gly supplementation by 0.341% and 0.683% with consideration of the purity of supplemental Gly (99%, Neo Cremar Co., Ltd., Seoul, Republic of Korea).

[3]Provided per kg of the complete diet: vitamin A, 11,700 IU (retinyl acetate); vitamin D_3, 3,600 IU (cholecalciferol); vitamin E, 27 IU (DL-α-tocopheryl acetate); vitamin K_3, 2.7 mg (menadione dimethylpyrimidine); vitamin B_1, 2.7 mg; vitamin B_2, 6.3 mg; vitamin B_6, 4.5 mg; vitamin B_{12}, 18 μg; folic acid, 1.35 mg; biotin, 135 μg; niacin, 45 mg; pantothenic acid, 10.8 mg.

[4]Provided per kg of the complete diet: copper, 7.35 mg; iron, 46.75 mg; manganese, 87.34 mg; zinc, 75.21mg; chromium, 100 μg; selenium, 235 μg.

multiplying egg weight by hen-day egg production. The data for productive performance were summarized for 12 wks of the feeding trial.

Egg quality including eggshell color (eggshell color fan and colorimeter), egg yolk color, eggshell strength, eggshell thickness, and Haugh unit was assessed using 10 eggs randomly collected per replicate with 5 eggs per day at the conclusion of the experiment. The detailed procedures for analyzing egg quality were demonstrated in our previous study (Han et al., 2023).

Serum Measurements

Blood samples were immediately obtained via a heart puncture into a 6-mL serum tube (BD, Franklin Lakes, NJ) using BD Vacutainer Eclipse Blood Collection Needle (21 gauge × 1.25 inches; BD, Franklin Lakes, NJ). The blood samples were immediately centrifuged at 3,000 × g and at 4°C for 20 min to separate the serum in a centrifuge machine (Labogene 1248R, Gyrozen, Daejeon, Republic of Korea). The collected serum was stored at −20°C before analysis. The concentrations of aspartate aminotransferase (**AST**), alanine aminotransferase (**ALT**), uric acid, and creatinine in the serum were analyzed using a HITACHI 7020 automatic analyzer (Hitachi, Tokyo, Japan).

Stress Indicators

The heterophil:lymphocyte ratio (**H:L ratio**) in the blood was measured as a stress biomarker. The H:L ratio in the blood was analyzed by the method of Lentfer et al. (2015) with a minor modification. Briefly, a standard microscope slide with approximately 7 μL of blood was

prepared and dried at room temperature. The slides were then prefixed for 5 min after immersion in methanol. Afterwards, the slides were stained with 0.2 mL wright stain solution (Wright stain solution, Muto pure chemicals, Tokyo, Japan) for 2 min at room temperature and then rinsed with distilled water until the edges of the slides turned pinkish red. The slides were again stained with 0.2 mL giemsa stain (Giemsa's staining solution, Duksan pure chemicals, Ansan, Korea) for 5 min at room temperature and then rinsed with distlled water until the edges of the slides turned pinkish red. After air drying, the hemocyte smears were inspected under a microscope. Blood H and L were counted by the same 2 people to 100 cells per individual smear and blood H:L was calculated based on the method of Cengiz et al. (2015) and Yu et al. (2021).

The corticosterone (**CORT**) concentrations in the feather were also analyzed as the other stress biomarker. The CORT extraction from the feather was undertaken using a procedure described by Bortolotti et al. (2008) and Häffelin et al. (2020) with a minor modification. The detailed procedure for analyzing feather CORT concentrations was described previously by Lee et al. (2022). In short, feathers were separated to vane and rachis with a scalpel, and then the vane was sliced with scissors into pieces of <5 mm^2. Vane pieces were placed in 50 mL tubes with 10 mL methanol (HPLC grade, Honeywell, Charlotte, NC). The tubes were sonicated in a water bath at room temperature for 30 min, and then incubated in a shaking water bath at 50°C overnight. Afterwards, the methanol was separated from the remaining feather using syringe filters and a filtration funnel (HyunDai Micro, Anseong, Republic of Korea). The feather remnant was washed twice again with 2 mL methanol and the washes were added back to the original extract. To evaporate the methanol, samples were placed in a water bath at 50°C until complete evaporation. Samples were re-suspended in 500 mL of Assay Buffer 15 (Tris-buffered saline) provided in the ELISA kit (Enzo Life Sciences Inc., New York, NY). Samples were frozen at -80°C before CORT analysis. The CORT concentrations in the feather were determined using the commercial Enzo Life Sciences Corticosterone ELISA Kit ADI-901-097 (Enzo Life Sciences Inc., New York, NY). The calculation of CORT concentrations in the tissues was performed based on the manual of Enzo Life Sciences Corticosterone ELISA Kit ADI-901-097 (Enzo Life Sciences Inc., New York, NY), whereby the standard curve was fitted using a 4-parameter logistic curve to interpolate CORT concentrations.

Fatty Liver Incidence

Fatty liver incidence was measured based on liver color score and total fat concentrations (Han et al., 2023). In short, the liver was pictured to measure the subjective fatty liver score using a scale from 1 to 5 (1 = dark red; 5 = yellowish red). The liver hemorrhage was also scored from 0 to 5, with 1 indicating normal liver and 5 indicating large and massive hemorrhages. Three investigators conducted the liver scoring in the blind test. In addition, the Commission Internationale de l'Eclairage (**CIE**) color scales for the lightness (**L***), redness (**a***), and yellowness (**b***) in the liver were also determined using a colorimeter (model CR-10, Konica Minolta Optics Inc., Tokyo, Japan). Finally, a portion of the middle part of the liver was sampled for analyzing total fat concentrations (method 996.01; AOAC, 2007).

Statistical Analysis

All data were analyzed by ANOVA as a completely randomized design using the PROC MIXED procedure (SAS Inst. Inc., Cary, NC, US). The replicate was considered as an experimental unit. Outlier data were identified based on a normal probability plot and residuals of the raw data, which were generated by the UNIVARIATE and BOXPLOT procedure of SAS, but no outliers were detected. The LSMEANS procedure was used to calculate treatment means. The PDIFF option of SAS was used to compare treatment means. In addition, the equal-spaced polynomial contrast test was conducted to determine linear and quadratic effects of increasing supplementation of Gly in diets without the data for PC treatment. A probability of $P < 0.05$ was considered significant and $0.05 \leq P \leq 0.10$ was considered a tendency.

RESULTS

Productive Performance

The hen-day egg production, egg weight, egg mass, and broken and shell-less eggs were not influenced by dietary treatments under HS conditions (Table 2). However, hens fed the NC diet had a greater ($P < 0.05$) FI than those fed the PC diet. Increasing supplementation of Gly in diets decreased (linear, $P < 0.05$) FI of laying hens. Increased FCR was observed for hens fed the NC diet than those fed the PC diet; however, significance was not identified. Increasing supplementation of Gly in diets tended (linear, $P = 0.088$) to decrease FCR of laying hens. Hens fed diets supplemented with 0.683% Gly had similar FCR to those fed the PC diet.

Egg Quality

Hens fed the NC diet had decreased ($P < 0.05$) eggshell a* values than those fed the PC diet; however, no differences in other egg quality were observed between PC and NC treatments (Table 3). Increasing supplementation of Gly in diets increased (linear, $P < 0.05$) eggshell L* values and decreased (linear, $P < 0.05$) egg yolk color. Eggshell strength, eggshell thickness, and Haugh unit were not affected by increasing supplementation of Gly in diets.

Table 2. Effect of dietary supplementation of glycine (Gly) on productive performance of laying hens raised under heat stress conditions.[1]

	Dietary treatments[2]					*P*-value		
Item	PC	NC	0.341% Gly	0.683% Gly	SEM	Total[3]	Gly (linear)[4]	Gly (quadratic)[4]
HD, %	93.4	95.1	93.6	95.4	0.85	0.210	0.817	0.130
EW, g	54.6	54.7	54.7	53.9	0.58	0.664	0.330	0.540
EM, g	51.0	52.1	51.2	51.4	0.88	0.818	0.623	0.658
FI, g/hen/d	91[c]	96[a]	95[a,b]	92[b,c]	1.3	0.026	0.043	0.486
FCR, g/g	1.79[b]	1.84[a,b]	1.86[a]	1.79[b]	0.021	0.042	0.088	0.146
BS, %	0.05	0.03	0.05	0.02	0.012	0.407	0.549	0.165

Abbreviations: BS, broken and shell-less egg production rate; EM, egg mass; EW, egg weight; FCR, feed conversion ratio; FI, feed intake; HD, hen-day egg production.

[a-c]Means within a variable with no common superscript differ significantly ($P < 0.05$).

[1]Data are least squares means of 7 observations per treatment.

[2]Dietary treatments: PC, positive control diet (NC + 100 kcal/kg AME_n); NC, negative control diet; 0.341% glycine (Gly), NC + 0.341% Gly; 0.683% Gly, NC + 0.683% Gly.

[3]Overall effects of treatments.

[4]Linear and quadratic effects of increasing supplementation of Gly in diets without the data for PC treatment.

Table 3. Effect of dietary supplementation of glycine (Gly) on egg quality of laying hens raised under heat stress conditions.[1]

		Dietary treatments[2]					*P*-value		
Item		PC	NC	0.341% Gly	0.683% Gly	SEM	Total[3]	Gly (linear)[4]	Gly (quadratic)[4]
Eggshell color (Shell color fan)		13.2	12.8	12.9	12.7	0.17	0.120	0.542	0.484
Eggshell color (CIE Lab value)	L*	50.2	50.1	50.6	51.3	0.40	0.128	0.035	0.829
	a*	21.8[a]	21.0[b]	21.6[ab]	21.3[ab]	0.21	0.046	0.403	0.132
	b*	29.3	29.1	29.2	29.5	0.21	0.522	0.158	0.560
Egg yolk color (Roche color fan)		8.4	8.5	8.5	8.3	0.07	0.164	0.025	0.230
Eggshell strength, kg/cm^2		4.68	4.70	4.66	4.67	0.122	0.994	0.848	0.843
Eggshell thickness, μm		399	395	397	397	3.2	0.858	0.601	0.810
Haugh unit		91.1	91.0	91.0	90.5	0.57	0.887	0.504	0.618

Abbreviations: a*, redness; b*, yellowness; CIE, Commission Internationale de l'Eclairage; L*, lightness.

[a,b]Means within a variable with no common superscript differ significantly ($P < 0.05$).

[1]Data are least squares means of 7 observations per treatment.

[2]Dietary treatments: PC, positive control diet (NC + 100 kcal/kg AME_n); NC, negative control diet; 0.341% glycine (Gly), NC + 0.341% Gly; 0.683% Gly, NC + 0.683% Gly.

[3]Overall effects of treatments.

[4]Linear and quadratic effects of increasing supplementation of Gly in diets without the data for PC treatment.

Serum Measurements

No differences in serum measurements including AST, ALT, uric acid, and creatinine concentrations were found between PC and NC treatments (Table 4). However, a tendency for quadratic association of serum AST ($P = 0.097$) and ALT ($P = 0.053$) concentrations with increasing supplementation of Gly in diets was found, with hens fed diets supplemented with 0.341% Gly showing the least serum AST and ALT concentrations. The serum uric acid and creatinine concentrations were not influenced by increasing supplementation of Gly in diets.

Stress Indicators

Hens fed the NC diet had a greater blood H:L ratio than those fed the PC diet although the difference was not significant (Table 5). Increasing supplementation of

Table 4. Effects of dietary supplementation of glycine (Gly) on serum measurements of laying hens raised under heat stress conditions.[1]

	Dietary treatments[2]					*P*-value		
Item	PC	NC	0.341% Gly	0.683% Gly	SEM	Total[3]	Gly (linear)[4]	Gly (quadratic)[4]
AST, U/L	232.1	306.0	200.7	269.3	37.70	0.196	0.540	0.097
ALT, U/L	2.40	4.00	1.87	3.65	0.783	0.158	0.757	0.053
Uric acid, mg/dL	3.67	5.30	3.92	5.96	0.905	0.216	0.589	0.107
Creatinine, mg/dL	0.25	0.25	0.23	0.27	0.016	0.418	0.359	0.183

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase.

[1]Data are least squares means of 7 observations per treatment.

[2]Dietary treatments: PC, positive control diet (NC + 100 kcal/kg AME_n); NC, negative control diet; 0.341% glycine (Gly), NC + 0.341% Gly; 0.683% Gly, NC + 0.683% Gly.

[3]Overall effects of treatments.

[4]Linear and quadratic effects of increasing supplementation of Gly in diets without the data for PC treatment.

Table 5. Effect of dietary supplementation of glycine (Gly) on stress indicators of laying hens raised under heat stress conditions.[1]

Item	Dietary treatments[2]				SEM	*P*-value		
	PC	NC	0.341% Gly	0.683% Gly		Total[3]	Gly (linear)[4]	Gly (quadratic)[4]
Feather CORT, ng/g	11.5	7.9	6.8	6.1	2.50	0.393	0.549	0.933
Blood H:L ratio	0.18[ab]	0.25[a]	0.25[a]	0.16[b]	0.027	0.039	0.042	0.236

Abbreviations: CORT, corticosterone; H:L ratio, heterophil to lymphocyte ratio.
[a,b]Means within a variable with no common superscript differ significantly ($P < 0.05$).
[1]Data are least squares means of 7 observations per treatment.
[2]Dietary treatments: PC, positive control diet (NC + 100 kcal/kg AME_n); NC, negative control diet; 0.341% glycine (Gly), NC + 0.341% Gly; 0.683% Gly, NC + 0.683% Gly.
[3]Overall effects of treatments.
[4]Linear and quadratic effects of increasing supplementation of Gly in diets without the data for PC treatment.

Gly in diets decreased (linear, $P < 0.05$) blood H:L ratio of laying hens. Hens fed diets supplemented with 0.683% Gly had similar blood H:L ratio to those fed the PC diet. However, feather CORT concentrations measured as the other stress indicator did not differ among treatments.

Fatty Liver Incidence

Hens fed the NC diet had greater ($P < 0.05$) subjective color score, the CIE values for L*, a*, and b*, hemorrhagic score, and total fat concentrations in the liver than those fed the PC diet (Table 6). However, increasing supplementation of Gly in diets decreased subjective color score (linear and quadratic, $P < 0.05$), the CIE values for L*, a*, and b*, hemorrhagic score, and total fat concentrations (linear, $P < 0.05$) in the liver. Hens fed diets supplemented with 0.683% Gly had similar values measured for fatty liver incidence to those fed the PC diet.

DISCUSSION

Decreased productive performance is frequently reported in laying hens exposed to HS conditions, which is typically caused by decreased FI with impaired dietary nutrient and energy utilization of birds (Balnave and Muheereza, 1997; Whitehead, 1998; Kirunda et al., 2001; Mashaly et al., 2004). Thus, as a practical approach to alleviate this adverse outcome, increasing energy and/or nutrient concentrations in diets is widely applied in poultry industry during hot seasons (Kim et al., 2017; Abdel-Moneim et al., 2021). In the current experiment, we also included the PC diet with increasing energy concentrations by 100 kcal/kg AME_n than the NC diet for the purpose of simulating a high-energy commercial diet for laying hens raised under HS conditions and its subsequent comparison of the effect of dietary supplementation of Gly. As expected, the PC diet resulted in improved productive performance including FCR and decreased stress response including blood H:L ratio compared with the NC diet, although significance was not detected. Similar improvement in productive performance by increasing energy concentrations with fat addition in diets was previously observed in broiler chickens raised under HS conditions (Daghir, 2008; Ghahremani et al., 2017; Olgun et al., 2021). However, increasing energy concentrations or fat addition in diets have shown inconsistent results in laying hens raised under HS conditions (Kim et al., 2017, 2019). In this experiment, moreover, the PC diet decreased the liver color score and hemorrhagic score as accompanied with reduced fat concentrations in the liver than the NC diet, which may show its potential preventive effects on fatty liver development in laying hens exposed to HS conditions. The possible reason may be related to increased fat concentrations in the PC diet because additional

Table 6. Effect of dietary supplementation of glycine (Gly) on fatty liver incidence of laying hens raised under heat stress conditions.[1]

Item		Dietary treatments[2]				SEM	*P*-value		
		PC	NC	0.341% Gly	0.683% Gly		Total[3]	Gly (linear)[4]	Gly (quadratic)[4]
Subjective color score		1.50[b]	3.04[a]	1.43[b]	1.33[b]	0.242	<0.001	<0.001	0.010
CIE color value	L*	25.5[c]	32.0[a]	28.6[b]	25.7[b,c]	1.05	<0.001	0.001	0.867
	a*	14.4[c]	20.7[a]	18.5[a,b]	16.2[b,c]	1.19	0.003	0.016	0.972
	b*	7.3[b]	14.8[a]	10.5[b]	7.4[b]	1.28	<0.001	0.001	0.713
Hemorrhagic score		0.68[b]	1.39[a]	1.04[a,b]	0.58[b]	0.218	0.043	0.017	0.856
Total fat concentration (% DM)		15.1[b]	29.5[a]	22.1[a,b]	19.3[b]	3.23	0.007	0.048	0.576

Abbreviations: a*, redness; b*, yellowness; CIE, Commission Internationale de l'Eclairage; L*, lightness.
[a-c]Means within a variable with no common superscript differ significantly ($P < 0.05$).
[1]Data are least squares means of 7 observations per treatment.
[2]Dietary treatments: PC, positive control diet (NC + 100 kcal/kg AME_n); NC, negative control diet; 0.341% glycine (Gly), NC + 0.341% Gly; 0.683% Gly, NC + 0.683% Gly.
[3]Overall effects of increasing supplementation of Gly in diets.
[4]Linear and quadratic effects of increasing supplementation of Gly in diets without the data for PC treatment.

supplementation of fat in diets has been reported to decrease hepatic fat retention in laying hens, possibly via feed-back mechanisms (Haghighi-Rad and Polin, 1982; Han et al., 2023).

Dietary Gly is considered semi-essential AA for poultry because of its various specific metabolic functions other than its general role of structural AA in proteins (Heger and Pack, 1996; Powell et al., 2011; Siegert and Rodehutscord, 2019). Dietary Gly is readily converted to Ser and is associated with the synthesis of nucleic acids, porphyrins, glutathione, uric acid, and creatine in animals (Wang et al., 2013). This functional role of Gly in synthesizing those biomolecules may become more important in animals exposed to HS conditions; therefore, it was hypothesized that increasing supplementation of Gly in diets may alleviate the negative outcomes of HS in laying hens raised under HS conditions. The current results indicated that increasing supplementation of Gly up to 0.683% in diets decreased FCR of laying hens, which was associated with decreased FI without affecting egg production rate. In addition, hens fed the diet supplemented with 0.683% Gly showed similar FI and FCR to those fed the PC diet, suggesting that dietary supplementation of 0.683% Gly may be used as an alternative to high-energy commercial diets for laying hens under HS conditions. This improvement in FCR by dietary supplementation of 0.683% Gly may be attributed to increased synthesis of essential biomolecules including nucleic acids, porphyrins, glutathione, uric acid, and creatine in laying hens exposed to HS although the metabolism related to the biomolecule synthesis was not investigated in this experiment. Moreover, additional supplementation of dietary Gly may aid in increasing bile salt formation, which is required for efficient fat digestion in poultry (Powell et al., 2009; Han and Thacker, 2011). However, these speculations should be verified in further studies.

Decreased eggshell quality is often observed in laying hens raised under HS conditions (Mahmoud et al., 1996; Balnave and Muheereza, 1997; Mashaly et al., 2004). This observation is likely attributed to decreased nutrient intake, particularly lowered Ca intake (Mashaly et al., 2004). Respiratory alkalosis and reduced blood flow to the shell gland have also been related to decreased eggshell quality of laying hens exposed to HS conditions (Mahmoud et al., 1996). However, no differences in eggshell thickness and strength, which are highly involved in Ca intake and metabolism, were found among dietary treatments. In this study, daily Ca intake calculated from daily FI and analyzed Ca concentrations in diets was similar among treatments. Therefore, our result may indicate that the current dietary treatments and HS conditions are unlikely to affect Ca metabolism and respiratory alkalosis, leading to a lack of the effect of dietary treatments on eggshell quality.

The Gly is required to synthesize the protoporphyrin IX, the major pigment in brown eggshells, through porphyrin synthesis in the uterine mitochondria with the condensation of Gly and succinyl-CoA (Ponka, 1999). Furthermore, Gly is required to support various biological functions such as protein synthesis and antioxidant actions, which may aid in egg formation in laying hens (Han and Thacker, 2011). Therefore, impaired eggshell color by HS was expected to be ameliorated by additional supplementation of Gly in diets; however, we found the decrease in brown eggshell color and egg yolk color by increasing supplementation of Gly in diets. The reason for this observation is unknown; nevertheless, the values for most of the egg quality measurements in the current experiment were placed within the normal quantitative range of egg quality in laying hens (Choi et al., 2021), indicating that additional supplementation of Gly at the current levels used in this experiment may have little impact on egg quality of laying hens raised under the current HS conditions.

Serum concentrations of AST and ALT are widely used as possible indicators of liver damage and diseases (Ma et al., 2014). A previous study has reported that HS increases serum concentrations of ALT and AST in broiler chickens (Hosseini-Vashan et al., 2012). The Gly is required for the synthesis of antioxidant molecules such as glutathione and creatinine to maintain cellular functions in the liver (McCarty et al., 2018). Moreover, Gly is an integral precursor of uric acid, which is the main excretory product derived from AA oxidation in poultry (Siegert and Rodehutscord, 2019). In the current experiment, no differences were observed in serum ALT and AST concentrations between PC and NC treatments. However, a tendency for a quadratic relationship was found between serum ALT and AST concentrations and increasing supplementation of Gly, with hens fed diets supplemented with 0.341% Gly showing the least ALT and AST concentrations. This result may indicate that dietary supplementation of 0.341% Gly ameliorates the possible liver damage due to HS conditions. On the other hand, dietary supplementation of 0.683% Gly did not show such a positive effect, and the reason for this observation is unclear. To our knowledge, no data regarding the effect of increasing supplementation of Gly in diets on the liver health in laying hens raised under HS conditions are currently available.

Blood H:L ratio and feather CORT concentrations are appreciated as stress indicators in poultry (Bortolotti et al., 2008; Lee et al., 2022). Previous studies have reported that HS induces increased blood H:L ratio due to decreasing lymphocytes and increasing heterophils in poultry (Akhavan-Salamat and Ghasemi, 2016; Gitoee et al., 2018). Moreover, feather CORT concentrations are considered an efficient stress indicator because circulating CORT in the blood is readily incorporated in the feather (Häffelin et al., 2020). Thus, both blood H:L ratio and feather CORT concentrations were accessed by dietary treatments in the current experiment. Increasing supplementation of Gly in diets decreased blood H:L ratio, indicating HS-relieving effects of additional supplementation of Gly in laying hens. Dietary supplementation of 0.683% Gly showed a similar effect on decreasing blood H:L ratio to the effect

of the PC diet. Likewise, feather CORT concentrations were also decreased by increasing supplementation of Gly in diets; however, significance was not detected. The clear reason for little effect on feather CORT concentrations is not identified, but it may be related to different responsiveness of stress indicators between blood H:L ratio and feather CORT concentrations (Lee et al., 2022).

Fatty liver hemorrhagic syndrome (**FLHS**) is frequently observed in laying hens, mostly due to increased fat accumulations with hens' age (Zaefarian et al., 2019; Han et al., 2023). The FLHS is considered the primary cause of increasing mortality in caged laying hens (Shini et al., 2019). Laying hens raised under HS conditions are likely more susceptible to FLHS development because of enhanced fatty acid synthesis in the liver (Molero, 2007; Emami et al., 2021). Therefore, potential nutritional strategies to decrease the fatty liver incidence should be developed to improve production and health in laying hens exposed to HS conditions. The current results revealed that increasing supplementation of Gly up to 0.683% in diets induced the clear reduction in all measurements related to fatty liver incidence, including liver color, hemorrhagic score, and total fat concentrations. The effect of dietary supplementation of 0.683% Gly on lowering fatty liver incidence was comparable to that of the PC diet. To our knowledge, this is the first experiment reporting anti-fatty liver effects of dietary supplementation of Gly in laying hens exposed to HS conditions. This effect may be related to the close metabolic relationship of Gly with methionine, choline, and betaine, of which these nutrients are known to prevent hepatic fat accumulations in poultry (Kidd et al., 1997; Garcia Neto et al., 2000). For instance, choline and betaine are the direct metabolic precursors of Gly in the body, and Gly can also be used for the synthesis of choline and betaine after the conversion of Ser (Wang et al., 2013; Siegert and Rodehutscord, 2019). As a consequence, it may be speculated that additional supplementation of Gly in diets increases both choline and betaine availability in the body, leading to an improvement in their potential role in preventing hepatic fat accumulations (Won et al., 2023). However, this speculation should be verified in further molecular studies on the metabolic relationship among these nutrients (i.e., methionine, choline, betaine, and Gly) in laying hens, especially under HS conditions.

CONCLUSIONS

Increasing supplementation of Gly up to 0.683% in diets improves feed efficiency and decreases stress response in laying hens raised under HS conditions. Dietary supplementation of Gly exerts an anti-fatty liver effect, and therefore, can be used to prevent fatty liver development in laying hens raised under HS conditions. Dietary supplementation of 0.683% Gly may be a possible alternative to high-energy commercial diets for laying hens under HS conditions.

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DISCLOSURES

The authors declare no conflict of interest for the data presented in this experiment.

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