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Effect of Dietary Supplementation of Arginine, Tryptophan, and Taurine on Productive Performance, Egg Quality, and Health Status of Laying Hens Raised Under Heat Stress Conditions

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ABSTRACT

The objective of this experiment was to investigate the effect of dietary supplementation of arginine (Arg), tryptophan (Trp), and taurine (Tau) on productive performance, egg quality, liver visual characteristics, antioxidant status, immune response, and stress indicator of laying hens raised under heat stress conditions. A total of two hundred eighty 47-wk-old Hy-Line Brown laying hens were randomly allotted to 1 of 4 dietary treatments with 7 replicates consisting of 10 cages per replicate. A basal diet (BD) was prepared to meet or exceed nutrient requirement estimates. Two additional diets were formulated to increase either digestible Arg or Trp by 50% greater than the BD. Finally, one more diet was prepared by adding 0.5% Tau to the BD. The experimental diets were fed to hens on an *ad libitum* basis for 8 wk. Average room temperature and relative humidity were maintained at 30.7±1.41°C and 72.5±11.61%, respectively. Results indicated that laying hens in Arg and Trp treatments tended (p= 0.06) to have a higher egg yolk color (Roche color fan) than those in the Tau treatment. Likewise, there was a tendency (p= 0.05) for a lower liver color score in the Tau treatment than Arg and Trp treatments. In conclusion, dietary supplementation of Arg, Trp, and Tau at the current levels (0.37% SID Arg, 0.075% SID Trp, and 0.5% Tau) in diets has no positive effects on productive performance, egg quality, liver visual characteristics, antioxidant status, immune response, and stress indicators of laying hens raised under the current heat stress conditions.

Keywords: arginine; heat stress; laying hen; taurine; tryptophan

INTRODUCTION

Heat stress is one of the major environmental stressors in the poultry industry because of the current global warming situation (Habibian *et al.,* 2015). In particular, poultry is considered highly sensitive to heat stress conditions because of a lack of sweat glands and high feather coverage (Brugaletta *et al.,* 2022). Poultry exposed to heat stress, therefore, is well-known to show various physiological malfunctions, which leads to an impairment in productive performance, product quality, and health status (Lara & Rostagno, 2013; Sugiharto, 2020). Thus, the development of effective strategies to decrease the negative outcomes from heat stress is crucial for the current and future poultry industry.

Dietary managements by supplementation of functional nutrients have been widely practiced to reduce heat stress in the current poultry industry. Various amino acids (AA), including arginine (Arg), tryptophan (Trp), and taurine (Tau) have gained increasing attention as functional nutrients to mitigate the heat stress of poultry. Dietary Arg is an essential amino acid (EAA) for poultry, and it can act as a precursor molecule of polyamines, creatine, and nitric oxide, which are well-known to have a variety of functions, especially for improving productive performance, product quality, and health status in poultry (Khajali & Wideman, 2010). Similarly, dietary Trp is also an EAA for poultry. In particular, Trp is a precursor of serotonin, which is a potential stress-relieving hormonal molecule in animals exposed to stressful environments (Woodger *et al.,* 1979; Martin *et al.,* 2000; Shen *et al.,* 2012). Likewise, dietary Tau is considered one of the semi-EAA for animals, reporting to show stress-alleviating effects, possibly due to its role in osmoregulation, anti-inflammation, cell membrane stabilization, antioxidation, and neuromodulation in animals (Cassol *et al.,* 2010). Accordingly, several previous experiments reported the beneficial effects of additional supplementation of Arg, Trp, and Tau in the diets of broiler chickens (Chamruspollert *et al.,* 2004; Yue *et al.,* 2017; Hafeez *et al.,* 2021). However, there is still limited information regarding the effect of this functional AA in laying hens exposed to heat stress. Moreover, comparative effects of these 3 functional AA have not been reported previously in laying hens exposed to heat stress.

Therefore, the objective of the present study was to compare the efficacy of dietary supplementation of Arg, Trp, and Tau on productive performance, egg quality, liver visual characteristics, antioxidant status, immune response, and stress indicator in laying hens raised under heat stress conditions.

MATERIALS AND METHODS

Animals, Diets, and Experimental Design

All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee at Chung-Ang University (IACUC No. 2020-00099). A total of two hundred eighty 47-wk-old Hy-Line Brown 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 (24cm × 36cm × 39cm) with 1 hen per cage. Initial body weight (BW) and egg production rate of hens were similar among treatments. A basal diet (BD) was prepared to meet or exceed nutrient requirement estimates for Hy-Line Brown laying hens (Table 1; Hy-Line Brown International, 2018). The concentrations of AA in the diet were based on standardized ileal digestible (SID) AA. The concentrations of Arg and Trp in the BD were 0.74% SID Arg and 0.15% SID Trp, respectively. Two additional diets were formulated to increase either SID Arg or Trp by approximately 50% greater than the BD with supplementation of 0.37% Arg or 0.075% Trp. Thus, one diet (T-Arg) contained 1.11% SID Arg, whereas the other diet (T-Trp) contained 0.225% SID Trp. Finally, one more diet (T-Tau) was prepared by adding 0.5% Tau to the BD. The continuous heat stress conditions were maintained throughout the experiment. The averages of ambient temperature and relative humidity were 30.7±1.41°C and 72.5±11.61%, respectively. Based on the heat stress index calculated from the ambient temperature and relative humidity (Hy-Line Brown International, 2016), the heat stress index in our environmental conditions was 83, indicating that laying hens in the present experiment were raised under severe heat stress conditions (Hy-Line Brown International, 2016). The experimental diets were fed to hens on an *ad libitum* basis for 8 wk. A 16-h lighting schedule (16 light:8 dark) was used during the entire experiment.

Sample Collection and Analysis

At the conclusion of the experiment, 3 birds with a BW that was close to the average BW in each replicate were selected. One bird was euthanized by CO_2 asphyxiation, and then immediately dissected. One of the remaining 2 birds was used for analyzing the blood heterophil to lymphocyte ratio (H:L ratio) as a stress indicator, whereas the other bird was used for analyzing immune responses.

Productive Performance

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

Egg Quality

Egg quality was assessed using randomly collected samples of 10 eggs per replicate with 5 eggs per day during the last 2 days of 4 and 8 wk. The detailed procedures for egg quality were demonstrated in our previous study (Kim *et al*., 2017).

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

Items	Value %
Ingredients	
Corn	61.50
Soybean meal, 45% Crude protein	16.90
Corn gluten meal	1.64
DDGS[1]	5.00
Tallow	0.79
Monodicalcium phosphate	1.26
Limestone	10.26
54% Lysine H_2SO_4	0.34
98.5% Threonine	0.06
Liquid Methionine	0.28
98.5% L-Tryptophan	0.11
NaCl	0.27
50% Choline	0.10
$NaHCO_3$	0.10
Vitamin premix[2]	0.09
Mineral premix[3]	0.10
Celite	1.20
Total	100.00
Nutrient and energy content[4]	
AME_n[5] kcal/kg	2,740
Crude protein %	14.61
Digestible lysine %	0.71
Digestible methionine + cysteine %	0.65
Digestible methionine %	0.44
Digestible threonine %	0.50
Digestible tryptophan %	0.15
Digestible arginine %	0.74
Digestible isoleucine %	0.51
Digestible valine %	0.65
Total calcium %	4.18
Available phosphorus %	0.36

Note: [1]DDGS, corn distillers dried grains with solubles.
[2]Provided per kg of the complete diet: vitamin A 11,700 IU (retinyl acetate); vitamin D3 3,600 IU; vitamin E 27 IU (DL-α-tocopheryl acetate); vitamin K3 2.7 mg (menadione dimethylpyrimidinol); vitamin B1 2.7 mg; vitamin B2 6.3 mg; vitamin B6 4.5 mg; vitamin B12 18 μg; folic acid 1.35 mg; biotin 135 μg; niacin 45 mg; pantothenic acid 10.8 mg.
[3]Provided per kg of the complete diet: copper 7.35 mg; iron 46.75 mg; manganese 87.34 mg; zinc 75.21 mg; chromium 100 μg; selenium 235 μg.
[4]Calculated values from Hy-Line (2018).
[5]AMEn, nitrogen-corrected apparent metabolizable energy.

Liver Visual Characteristics

For a measure of the occurrence of fatty liver, the liver attached to the body was pictured to measure the subjective fatty liver score using a scale from 1 to 5 (1= dark red; 5= yellowish red; Choi *et al.,* 2012). In addition, the objective CIE color scale for the lightness (L*), redness (a*), and yellowness (b*) was also determined using a colorimeter (model CR-10, Konica Minolta Optics Inc., Tokyo, Japan). The liver hemorrhage was scored from 0 to 5, 0 indicating normal liver and 5 indicating large and massive hemorrhages (Diaz *et al.,* 1999).

Antioxidant Status

Antioxidant statuses in the liver, such as malondialdehyde (MDA), total antioxidant capacity (TAC), and reactive oxygen species (ROS), were determined using a commercially available OxiSelectTM TBARS Assay Kit (MDA Quantitation; STA-330, Cell Biolabs, USA), OxiSelectTM Total Antioxidant Capacity (TAC) Assay Kit (STA-360, Cell Biolabs, USA), and OxiSelectTM In Vitro ROS/RNS Assay Kit (STA-347, Cell Biolabs, USA), respectively, according to the manufacturer's protocol. Protein concentrations were also analyzed using a commercial kit (Thermo Fisher Scientific Inc.; Shen *et al.,* 2012). The detailed procedure was reported in our previous experiment (Yu *et al.,* 2021).

Immune Responses

Cutaneous basophil hypersensitivity (CBH), a measure of cell-mediated immune responses, was determined based on the method of Kean & Lamont (1994). The detailed procedure was reported in our previous experiment (Kim *et al.,* 2021).

Stress Indicators

The H:L ratio 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. The detailed procedure was reported in our previous experiment (Yu *et al.,* 2021).

Statistical Analysis

All data were analyzed by one-way ANOVA as a completely randomized design using the PROC MIXED procedure in SAS (SAS Institute Inc., Cary, NC, USA). The replicate was considered as an experimental unit. Data were checked for outliers using the UNIVARIATE procedure of SAS, but no outliers were identified. The LSMEANS procedure was used to calculate treatment means. Means were separated by the PDIFF option in SAS. A probability of $p<0.05$ was considered significant and $0.05 \le p \le 0.10$ was considered a tendency.

RESULTS

Productive Performance

During the overall experimental period, the productive performance, including final BW, BWG, hen-day egg production, EW, egg mass, FI, FCR, and broken and shell-less egg production in laying hens, was not influenced by dietary treatments (Table 2).

Egg Quality

The egg quality of laying hens was not influenced by dietary treatments (Table 3). However, laying hens fed diets supplemented with additional Arg and Trp tended (p= 0.06) to have a higher egg yolk color (Roche color fan) than those fed diets supplemented with Tau.

Liver Visual Characteristics and Antioxidant Status

The liver color and hemorrhagic score of laying hens were not influenced by dietary treatments (Table 4). However, laying hens fed diets supplemented with Tau tended (p= 0.05) to show the least liver color score among dietary treatments. Similarly, the antioxidant

Table 2. Effects of dietary supplementation of Arg, Trp, and Tau on productive performance of laying hens raised under heat stress conditions[1]

Variables[3]	Dietary treatments[2]				SEM	p-value
	CON	T-Arg	T-Trp	T-Tau		
Initial BW, g	1,817	1,820	1,818	1,819	47.9	1.00
Final BW, g	1,806	1,832	1,787	1,814	39.6	0.88
BWG, g	-11	12	-31	-5	16.6	0.35
Hen-day egg production, %	89.4	90.3	88.7	91.8	1.51	0.31
EW, g	56.3	56.7	56.8	55.9	0.44	0.48
Egg mass, g	50.3	51.2	50.3	51.5	0.88	0.71
FI, g/hen/d	99	99	97	99	1.6	0.57
FCR, g/g	1.96	1.94	1.92	1.93	0.023	0.60
Broken and shell-less eggs, %	0.15	0.16	0.14	0.13	0.077	0.60

Note: [1]Data are least squares means of 7 replicates per treatment.
[2]CON: control (0.74% SID arginine + 0.15% SID tryptophan); T-Arg: arginine treatment (CON + 0.37% SID arginine); T-Trp: tryptophan treatment (CON + 0.075% SID tryptophan); T-Tau: taurine treatment (CON + 0.5% taurine).
[3]BW: body weight; BWG: body weight gain; EW: egg weight; FCR: feed conversion ratio; FI: feed intake.

status in the liver of laying hens was not influenced by dietary treatments (Table 5).

Immune Responses and Stress Indicators

No significant effect of dietary treatments on the CBH response was found (Table 6). Likewise, H:L ratio of hens was not affected by dietary treatments (Table 6).

DISCUSSION

Productive Performance

Heat stress is reported to decrease egg production, EW, and FI of laying hens (Barrett *et al.*, 2019; Xing *et al.*, 2019). This decrease in the productive performance of laying hens was most likely due to the decrease in FI, which is directly involved in decreasing the supply of available energy and nutrients used for the productive performance of laying hens (Mashaly *et al.*, 2004). In line with this observation, Zhou *et al.* (1998) and Mahmoud *et al.* (1996) reported that heat stress decreased plasma Ca and protein concentration, which are essential for egg formation in laying hens. Similar results for decreased nutrient and energy utilization with decreased FI were observed in broiler chickens exposed to heat stress (Bonnet *et al.*, 1997).

As a practical approach to reducing heat stress, nutritional manipulation is widely practiced in the current poultry production system (Khan *et al.*, 2012).

Table 3. Effects of dietary supplementation of Arg, Trp, and Tau on egg quality of laying hens raised under heat stress conditions[1]

Variables		Dietary treatments[2]				SEM	p-value
		CON	T-Arg	T-Trp	T-Tau		
Eggshell thickness, μm		396	390	399	398	4.6	0.50
Eggshell strength, kg/cm²		3.84	3.81	3.90	4.03	0.129	0.64
Haugh unit		88.4	88.0	88.8	87.7	0.79	0.74
Egg yolk color (Roche color fan)		7.0	7.1	7.1	6.9	0.06	0.06
Eggshell color (Shell color fan)		12.7	12.7	12.8	12.4	0.25	0.67
Eggshell color (CIE Lab value)[3]	L*	53.7	53.5	53.6	54.1	0.68	0.83
	a*	20.3	20.4	20.4	20.5	0.32	0.98
	b*	30.5	30.2	30.1	30.4	0.20	0.62

Note: [1]Data are least squares means of 7 replicates per treatment.
[2]CON: control (0.74% SID arginine + 0.15% SID tryptophan); T-Arg: arginine treatment (CON + 0.37% SID arginine); T-Trp: tryptophan treatment (CON + 0.075% SID tryptophan); T-Tau: taurine treatment (CON + 0.5% taurine).
[3]L*: lightness; a*: redness; b*: yellowness.

Table 4. Effects of dietary supplementation of Arg, Trp, and Tau on liver visual characteristics of laying hens raised under heat stress conditions[1]

Variables		Dietary treatments[2]				SEM	p-value
		CON	T-Arg	T-Trp	T-Tau		
Liver color (CIE Lab value)[3]	L*	31.7	30.4	30.9	28.8	1.17	0.35
	a*	19.8	19.2	19.9	18.3	0.77	0.47
	b*	10.6	10.3	10.3	9.2	0.93	0.71
Liver color score		2.96	2.64	2.68	1.82	0.286	0.05
Liver hemorrhagic score		0.82	1.11	1.00	0.82	0.289	0.87

Note: [1]Data are least squares means of 7 replicates per treatment.
[2]CON: control (0.74% SID arginine + 0.15% SID tryptophan); T-Arg: arginine treatment (CON + 0.37% SID arginine); T-Trp: tryptophan treatment (CON + 0.075% SID tryptophan); T-Tau: taurine treatment (CON + 0.5% taurine).
[3]L*: lightness; a*: redness; b*: yellowness.

Table 5. Effects of dietary supplementation of Arg, Trp, and Tau on antioxidant capacity of laying hens raised under heat stress conditions[1]

Variables[3]	Dietary treatments[2]				SEM	p-value
	CON	T-Arg	T-Trp	T-Tau		
MDA, μmol/mg protein	2.94	2.60	2.93	3.00	0.379	0.87
TAC, μmol/mg protein	728	751	769	738	30.8	0.81
ROS, mM	65.8	53.7	54.6	57.7	7.28	0.64

Note: [1]Data are least squares means of 7 replicates per treatment.
[2]CON: control (0.74% SID arginine + 0.15% SID tryptophan); T-Arg: arginine treatment (CON + 0.37% SID arginine); T-Trp: tryptophan treatment (CON + 0.075% SID tryptophan); T-Tau: taurine treatment (CON + 0.5% taurine).
[3]MDA: malondialdehyde; TAC: total antioxidant capacity; ROS: reactive oxygen species.

Table 6. Effects of dietary supplementation of Arg, Trp, and Tau on cutaneous basophil hypersensitivity (CBH) test and stress indicator of laying hens raised under heat stress conditions[1]

Dietary treatments[2]	CBH test mm				H:L ratio[3]
	Time				
	0 h	6 h	12 h	24 h	
CON	0.00	0.41	0.29	0.23	0.32
T-Arg	0.00	0.34	0.20	0.13	0.25
T-Trp	0.00	0.32	0.21	0.09	0.30
T-Tau	0.00	0.41	0.33	0.19	0.29
SEM		0.056	0.051	0.054	0.033
p-value		0.58	0.29	0.32	0.59

Note: [1]Data are least squares means of 7 replicates per treatment.
[2]CON: control (0.74% SID arginine + 0.15% SID tryptophan); T-Arg: arginine treatment (CON + 0.37% SID arginine); T-Trp: tryptophan treatment (CON + 0.075% SID tryptophan); T-Tau: taurine treatment (CON + 0.5% taurine).
[3]H:L ratio: heterophil to lymphocyte ratio.

Dietary Trp is an essential amino acid for animals and is also known to decrease the stress response in animals (Koopmans *et al.,* 2012). Dietary Trp is reported to ameliorate abnormal behavior and stress responses of animals by promoting serotonin synthesis (Shen *et al.,* 2012). It is appreciated that serotonin is associated with regulating the central nervous system for inhibiting aggressive behavior and controlling stress responses in animals (Shen *et al.,* 2012). However, we did not find any beneficial effects of T-Trp treatments (i.e., 50% higher than the requirement of SID Trp) in the current experiment. A similar result was also observed by Dong *et al.* (2012), who reported that dietary supplementation of 0.2 g/kg Trp and 0.4 g/kg Trp had no effect on the productive performance of laying hens raised under heat stress conditions. The reason for these results may be related to the fact that the current concentrations of Trp (0.225% SID Trp) in T-Trp treatments by 0.075% Trp supplementation in diets may not be sufficient to show the beneficial effects. In addition, based on the ideal protein concept, the balance of amino acid concentrations is important for the normal productive performance of laying hens. The concentration of other amino acids should be concomitantly increased with increasing levels of Trp from 0.15% SID Trp to 0.225% SID Trp when laying hens was raised under heat stress conditions (Dong *et al.,* 2012), which was not applied in this experiment.

Dietary Arg is an essential amino acid for poultry, and it can be metabolized to various functional molecules such as polyamines, creatine, and nitric oxides, which are likely related to an amelioration in heat stress (Wu *et al.,* 2010). Several previous studies have reported that dietary supplementation of Arg positively affected the productive performance of animals exposed to heat stress (Chamruspollert *et al.,* 2004; Zhu *et al.,* 2014; Yun *et al.,* 2020). Similarly, dietary Tau has been reported to show several important biological functions in the animal body, and thus, it is now considered one of the semi-essential amino acids for animals (Ripps & Shen, 2012; Surai *et al.,* 2020). In particular, dietary Tau plays an important role in stress alleviation by osmoregulation, anti-inflammation, and anti-oxidation (Cassol *et al.,* 2010). Accordingly, many previous studies reported that dietary supplementation of additional Tau may alleviate oxidative damage in broiler chickens exposed to heat stress. However, the results have been controversial (Shim *et al.,* 2006; He *et al.,* 2019; Lu *et al.,* 2019b; Hafeez *et al.,* 2021). In the current situation, however, limited information regarding the effects of dietary supplementation of Arg and Tau is available for laying hens exposed to heat stress. Therefore, we hypothesized that dietary supplementation of Arg and Tau might ameliorate the negative effect of heat stress conditions on laying hens. However, our results indicated that during 8 wk of the feeding trial, the productive performance of laying hens was not affected by T-Arg treatments (i.e., 50% higher than the requirement of SID Arg). Likewise, T-Tau treatments (i.e., adding 0.5% Tau to the BD) had no beneficial effect on productive performance in laying hens. The reason is unclear; however, it may be related to the increased requirements of Arg and Tau due to heat stress. Although we added additional Arg and Tau in the diet, it is unlikely that those supplemental levels fulfill their increased requirements of laying hens raised under the current heat stress conditions.

Egg Quality

Heat stress is known to decrease the egg quality of laying hens, such as eggshell thickness, eggshell strength, Haugh unit, egg yolk color, and eggshell color (Mahmoud *et al.,* 1996; Balnave & Muheereza, 1997; Mashaly *et al.,* 2004). The decrease in egg quality of laying hens was most likely due to a reduction in Ca intake by decreased FI (Mashaly *et al.,* 2004; Franco-Jimenez *et al.,* 2007). In addition, it was also reported that Ca utilization (Odom *et al.,* 1986) and Ca uptake by duodenal epithelial cells (Mahmoud *et al.,* 1996) were decreased by heat stress in laying hens (Mashaly *et al.,* 2004). Mahmoud *et al.* (1996) reported that plasma Ca level was significantly decreased in laying hens when hens were exposed to heat stress. In addition, when hens are exposed to heat stress conditions, they show increased respiration rates to reduce body temperature through evaporative cooling (El Hadi & Sykes, 1982). The increased respiration rate of laying hens leads to a reduction in blood partial pressure of CO_2, HCO_3^-, and an increase in blood pH, which is often associated with respiratory alkalosis (Koelkebeck & Odom, 1994; Franco-Jimenez *et al.*, 2007). The higher blood pH reduces the amount of ionized Ca in the blood of laying hens (Odom *et al.*, 1986), which is the form of Ca utilized by the shell gland. Moreover, in laying hens, blood HCO_3^- plays an important role in the formation of the $CaCO_3$ required for eggshell formation (Franco-Jimenez & Beck, 2007).

A nutritional strategy has been suggested to relieve the negative effect of heat stress on egg quality, such as dietary supplementation of Trp (Dong *et al.*, 2012). The positive effect of dietary supplementation of Trp has been reported by Dong *et al.* (2012), who observed that dietary supplementation of 0.2 g/kg Trp and 0.4 g/kg Trp improved eggshell strength of laying hens compared with those fed on the control diet. Dietary Trp is known to increase FI in animals (Woodger *et al.*, 1979) and to improve feed utilization (Wu, 2009). Therefore, dietary Trp may improve the egg quality of laying hens raised under heat-stress conditions. On the contrary, to our knowledge, there have been no previous studies regarding the effect of dietary Arg and Tau on egg quality in laying hens raised under heat-stress conditions. However, dietary Arg should be sufficiently presented in poultry diets to support normal biological functions such as protein synthesis and growth (Khajali & Wideman, 2010). Likewise, dietary Tau is known to have several biological functions in laying hens, and it is considered one of the semi-essential amino acids in poultry diets raised under heat stress (Ripps & Shen, 2012; Surai *et al.*, 2020). Therefore, we hypothesized that T-Arg, T-Trp, or T-Tau treatments might ameliorate the negative effect of heat stress conditions on egg quality in laying hens. However, our results indicated that T-Arg, T-Trp, or T-Tau treatments did not affect the egg quality of laying hens raised under heat-stress conditions. The reason is not clear; however, it may be attributed to the variations in animals and environmental conditions among the experiments because the extent of heat stress on poultry is influenced by both animal (e.g., age and genetics) and environmental factors (e.g., stocking density, ambient temperature and humidity, duration and the extent of heat stress, and rearing facility (Whitehead & Keller, 2003; Wasti *et al.*, 2020).

Liver Visual Characteristics and Antioxidant Status

Several previous studies have reported that heat stress can increase fat deposition in poultry (Lu *et al.*, 2007; Emami *et al.*, 2021). The reason for increased fat deposition in poultry is caused by increased hepatic de novo lipogenesis (Lu *et al.*, 2019a). Different from the mammal, the adipose tissues of birds serve only as a fat storage site, and the liver is the main site of de novo lipogenesis in poultry (Emami *et al.*, 2021). Very low-density lipoprotein (VLDL) is the important lipoprotein transporting triglycerides from the liver to extrahepatic tissues in animals (Cryer, 1981). However, when the transportation of VLDL is impaired, the excessive lipids are retained in the liver, which causes hepatic steatoses such as fatty liver syndrome (FLS) and fatty liver hemorrhagic syndrome (FLHS) in poultry (Zhang *et al.*, 2008b). The FLS and FLHS are frequently observed in laying hens and increasing economic losses in laying hens are often followed by abnormal lipid accumulation in the liver (Navarro-Villa *et al.*, 2019). As a result, in heat stress conditions, the liver color and the hemorrhagic score of laying hens are reported to increase. We hypothesized that T-Arg, T-Trp, or T-Tau treatments might ameliorate the negative effect of heat stress conditions on the liver status in laying hens. However, our results indicated that T-Arg, T-Trp, or T-Tau treatments had no beneficial effect on the liver lipid status of laying hens raised under heat-stress conditions. This result may indicate that T-Arg, T-Trp, or T-Tau treatments have no effects on lipid synthesis and transportation in the liver of laying hens. Moreover, in this experiment, we used relatively younger laying hens, and therefore, abnormal lipid status is not frequently observed in this age of hens, such that those functional supplements are unlikely to show beneficial effects on the hepatic lipid status of laying hens exposed to heat stress.

Antioxidant status in animals can be measured by TAC, MDA, and ROS concentration in the body (Young, 2001; Zhang *et al.*, 2008a; Rubio *et al.*, 2016). The TAC is a measurement of assessing the antioxidant potentials of various biological samples (Rubio *et al.*, 2016), with low TAC values representing increased oxidative stress (Young, 2001). The MDA is one of the final products of lipid peroxidation, with increasing MDA concentrations reflecting the increasing extent of lipid peroxidation (Zhang *et al.*, 2008a). The ROS are free radicals and peroxides produced within the cells by increasing oxidative stress (Wasti *et al.*, 2020). Many previous poultry experiments have consistently reported that heat stress increases oxidative stress in the whole body (Estévez, 2015; Surai *et al.*, 2019). Abdel-Moneim *et al.* (2021) reported that poultry exposed to heat stress conditions elevated the body temperature and accelerated the metabolic rates, leading to an increase in ROS production. Accordingly, an increase in ROS concentrations over antioxidant capacity facilitates free radical-mediated oxidative chain reactions in cell components such as proteins (Stadtman & Levine, 2000), lipids (Rubbo *et al.*, 1994), polysaccharides (Kaur & Halliwell, 1994), and deoxyribonucleic acid (LeDoux *et al.*, 1999), which ultimately results in a decrease in cellular functions and eventually cell death.

Dietary Arg can be metabolized to polyamines, which are considered important biomolecules against oxidative stress in animals (Seiler, 1996; Wu *et al.*, 2010; Miller-Fleming *et al.*, 2015). Similarly, dietary Tau plays a role in stress alleviation by osmoregulation, anti-inflammation, cell membrane stabilization, and anti-oxidation in animals (Cassol *et al.*, 2010). Accordingly, many previous studies reported that dietary supplementation of additional Tau may alleviate oxidative damage induced by stressors in animals (Yang *et al.*, 2015; Zhang *et al.*, 2017). Dietary Trp is known to protect tissues from oxidative damage in animals (Christen *et al.*, 1990; Reyes-Gonzales *et al.*, 2009; Del Angel-Meza *et al.*, 2011). However, our results indicated that T-Arg, T-Trp, or T-Tau treatments have no beneficial effects on the antioxidant status of laying hens raised under heat-stress conditions. The reason is unclear; however, it may be related to the age of the hens used in this experiment because antioxidant capacity, changes with age (Gu *et al.*, 2021). We used the relatively younger laying hens with high antioxidant capacity such that dietary supplementation of Trp, Arg, and Tau may show little beneficial effects on antioxidant status in laying hens exposed to heat stress.

Immune Responses and Stress Indicators

Heat stress is well-known to impair the immune system of poultry, and therefore, heat stress may increase the susceptibility of birds to pathogenic infections or diseases (Habibian *et al.*, 2014; Akhavan-Salamat & Ghasemi, 2016; Hosseini-Vashan *et al.*, 2016). Moreover, the levels of antibodies and white blood cells were reported to be reduced in heat-stressed birds (Bartlett & Smith, 2003; Mashaly *et al.*, 2004). In the current experiment, the CBH responses were measured as cell-mediated immune responses in laying hens exposed to heat stress (Kim *et al.*, 2021). The CBH responses were determined by the level of basophil infiltration at the phytohaemagglutinin P injected site of the poultry' skin (Kim *et al.*, 2021; Yu *et al.*, 2021).

Dietary Arg is a precursor of nitric oxide, which plays a role in the activity of the autonomic and central neural systems (Moncada *et al.*, 1991; Malyshev *et al.*, 1999), thereby affecting immune responses in animals (Moncada *et al.*, 1991; Deroee *et al.*, 2009). Dietary Tau is reported to improve immune functions in poultry (Lee *et al.*, 2004; Koven *et al.*, 2016). Similarly, dietary Trp can be used as a precursor molecule for serotonin and melatonin synthesis, which shows promoting effects on animal immune responses (Reiter, 1998; Li *et al.*, 2011). However, limited information regarding the effects of dietary supplementation of Arg, Trp, or Tau on immune responses is available for laying hens raised at heat stress conditions. Therefore, we hypothesized that T-Arg, T-Trp, or T-Tau treatments might improve the immune response of laying hens raised under heat-stress conditions. However, our results indicated that T-Arg, T-Trp, or T-Tau treatments had no beneficial effect on the immune response of laying hens raised under heat-stress conditions.

The blood H:L ratio has been widely measured as a stress biomarker in poultry (Gross & Siegel, 1983). Poultry exposed to heat stress shows an increased H:L ratio in the blood (Shini *et al.*, 2008; Akhavan-Salamat & Ghasemi, 2016). The reason for the increased H:L ratio due to heat stress is related to increasing corticosterone concentrations in the blood because circulating corticosterone affects the characteristics and function of immune cells, such as heterophils and lymphocytes (Shini *et al.*, 2008; Akhavan-Salamat & Ghasemi, 2016).

Dietary Trp is the precursor of serotonin that has many key functions in the nerve system of poultry to reduce aggressive activity, such as feather-pecking behavior, and modulate stress response (Laycock & Ball, 1990; Shea *et al.*, 1990). Similarly, dietary Arg synthesizes nitric oxide, which regulates the production of serotonin in broiler chickens (Wideman *et al.*, 2013). Oxidative stress is considered to be the major reason for negative outcomes for heat-stressed poultry (Akbarian *et al.*, 2016; Farag & Alagawany, 2018). Many previous studies reported that dietary supplementation of additional Tau alleviated oxidative damages induced by stressors in broiler chickens raised under heat stress conditions (Yang *et al.*, 2015; Zhang *et al.*, 2017). It is suggested, moreover, that the requirement of Arg, Trp, and Tau may be increased during heat stress exposure in poultry (Surai *et al.*, 2019). Therefore, three functional AAs were expected to exert heat stress-relieving effects on laying hens with decreasing stress indicators such as blood H:L ratio. However, in the current experiment, we found no anti-stress responses in laying hens by feeding diets supplemented with 0.37% Arg, 0.075% Trp, or 0.5% Tau. To our best knowledge, there are no data pertaining to the effects of dietary supplementation of Arg, Trp, and Tau on stress indicators such as blood H:L ratio in laying hens exposed to heat stress conditions, and therefore, it may be speculated that the current supplemental levels and its final concentrations of Arg, Trp, and Tau in diets may not be adequate to exert the beneficial effects on stress responses of laying hens as observed in other measurements.

CONCLUSION

Dietary supplementation of Arg, Trp, and Tau at the current supplemental levels (0.37% SID Arg, 0.075% SID Trp, and 0.5% Tau) has no positive effects on productive performance, egg quality, liver visual characteristics, antioxidant status, immune response, and stress indicator of laying hens raised under the current heat stress conditions.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial, personal, or other relationships with other people or organizations related to the material discussed in the manuscript.

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