

## Effects of Dietary Betaine Supplementation on Production Performance, Egg Quality, and Serum Biochemical Parameters in Heat-Stressed Laying Hens: Postprint

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### Abstract

This experiment aimed to investigate the effects of dietary betaine supplementation on production performance, egg quality, and serum biochemical indices in heat-stressed laying hens. A total of 600 healthy 22-week-old commercial Roman Brown laying hens were selected and randomly divided into 5 groups with 8 replicates per group and 15 hens per replicate. Group I served as the positive control group, fed a basal diet under normal thermoneutral conditions with temperature-humidity index (THI) between 64.9-68.9; Group II served as the negative control group, fed a basal diet under heat stress conditions with  $\text{THI} > 72$ ; Groups III-V were supplemented with 200, 400, and 600 mg/kg betaine in the basal diet, respectively, all under heat stress conditions with  $\text{THI} > 72$ . The experimental period lasted for 14 weeks. The results showed that there were no significant differences in average daily feed intake, feed-to-egg ratio, and broken egg rate among all groups ( $P > 0.05$ ). Compared with Group I, Group II significantly decreased hen-housed egg production rate, hen-housed egg weight, and serum total protein (TP) content and alkaline phosphatase (AKP) activity ( $P < 0.05$ ), and significantly increased serum glutamic-oxaloacetic transaminase (GOT), creatine kinase (CK), and glutamic-pyruvic transaminase (GPT) activities ( $P < 0.05$ ). Compared with Group II, Group IV significantly increased hen-housed egg production rate, hen-housed egg production, and serum TP content, while Group V significantly increased hen-housed egg production and serum TP and albumin (ALB) contents ( $P < 0.05$ ); however, serum CK and GPT activities in Groups IV and V were significantly decreased, and serum triglyceride (TG) content in Group V was also significantly decreased ( $P < 0.05$ ). In conclusion, heat stress can alter metabolism and physiological functions in laying hens, leading to decreased production performance, whereas dietary betaine

supplementation can improve hen-housed egg production rate and hen-housed egg weight and ameliorate heat stress-induced damage in laying hens, with an appropriate supplementation level of 400 mg/kg in the diet.

## Full Text

### Effects of Dietary Betaine on Production Performance, Egg Quality, and Serum Biochemical Indices of Laying Hens under Heat Stress

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#### Abstract

This experiment was conducted to investigate the effects of dietary betaine supplementation on production performance, egg quality, and serum biochemical indices of laying hens under heat stress conditions. Six hundred healthy 22-week-old commercial Roman Brown laying hens were randomly allocated into five groups with eight replicates per group and fifteen hens per replicate. Group I served as the positive control, fed a basal diet under normal thermal conditions with temperature-humidity index (THI) between 64.9 and 68.9. Group II was the negative control, fed a basal diet under heat stress conditions (THI > 72). Groups III, IV, and V were fed the basal diet supplemented with 200, 400, and 600 mg/kg betaine, respectively, all under heat stress conditions (THI > 72). The experiment lasted for 14 weeks. The results showed no significant differences among groups in average daily feed intake, feed-to-egg ratio, or broken egg rate ( $P > 0.05$ ). Compared with Group I, Group II exhibited significantly lower hen-housed egg production, hen-housed egg yield, serum total protein (TP) content, and alkaline phosphatase (AKP) activity ( $P < 0.05$ ), while showing significantly elevated serum activities of glutamic-oxaloacetic transaminase (GOT), creatine kinase (CK), and glutamic-pyruvic transaminase (GPT) ( $P < 0.05$ ). Compared with Group II, Group IV showed significantly increased hen-housed egg production, hen-housed egg yield, and serum TP content, while Group V demonstrated significantly increased hen-housed egg yield and serum TP and albumin (ALB) contents ( $P < 0.05$ ). Additionally, Groups IV and V exhibited significantly reduced serum CK and GPT activities, with Group V also showing significantly decreased serum triglyceride (TG) content ( $P < 0.05$ ). In conclusion, heat stress alters the metabolism and physiological functions of laying hens, leading to decreased production performance. Dietary betaine supplementation can improve hen-housed egg production and egg yield while mitigating heat stress-induced damage, with an optimal supplementation level of 400 mg/kg.

**Keywords:** betaine; heat stress; laying hens; production performance; egg quality; serum biochemical indices

## Introduction

Environmental temperature and humidity are critical factors affecting poultry production performance. The temperature-humidity index (THI) reflects the comfort level of the animal production environment; when THI ranges between 55 and 72, animals experience minimal temperature-humidity stress and remain comfortable, whereas THI values exceeding 72 induce heat stress responses that intensify with increasing THI values [?]. Laying hens have extended production cycles, and high summer temperatures readily induce heat stress, resulting in reduced feed intake, egg production, feed conversion efficiency, and hatchability, exacerbated oxidative damage, and disrupted intestinal microbiota, potentially causing complete production cessation and substantial economic losses to the poultry industry [?]. Furthermore, heat stress can cause degeneration of small intestinal villi and increased intestinal cell membrane permeability, heightening susceptibility to pathogenic microorganisms and disease risk [?].

Betaine, or trimethylglycine, serves as a methyl donor in metabolic processes upon entering the body. Methyl groups are essential for the nervous, immune, renal, and cardiovascular systems [?]. Research indicates that betaine participates in protein and lipid metabolism, improving diet palatability, reducing body fat deposition, maintaining cellular osmotic pressure, and regulating electrolyte balance [?]. Klasing et al. [?] reported that coccidia-infected broilers exhibited significantly reduced duodenal villus height, cellular osmotic pressure, and body weight gain, all of which were ameliorated by dietary betaine supplementation. Similar findings have been widely reported in broilers, demonstrating that betaine helps alleviate heat stress damage and mitigate negative effects of dehydration [?]. However, limited research exists on betaine's effects on production performance and serum biochemical indices in heat-stressed laying hens. Therefore, this study investigated betaine's impact on production performance, egg quality, and serum biochemical parameters in heat-stressed laying hens to provide reference data for betaine application in layer diets.

## 1. Materials and Methods

**1.1 Experimental Animals and Diets** Six hundred healthy 22-week-old commercial Roman Brown laying hens with similar body condition and egg production were randomly divided into five groups with eight replicates per group and fifteen hens per replicate. Group I served as the positive control, fed a basal diet under normal thermal conditions (THI = 64.9-68.9). Group II was the negative control, fed a basal diet under heat stress conditions (THI > 72). Groups III, IV, and V were fed the basal diet supplemented with 200, 400, and 600 mg/kg betaine, respectively, all under heat stress conditions (THI > 72). The experimental period lasted 14 weeks, including a 2-week pre-trial period and a 12-week formal experimental period.

The basal diet was a corn-soybean meal-miscellaneous meal type formulated according to the nutrient recommendations for laying hens in the Chinese agri-

cultural industry standard “Feeding Standard of Chicken” (NY/T 33–2004). Dry matter, crude protein, calcium, and phosphorus contents of feed ingredients were measured values, while other indices referenced the “Feed Composition and Nutritional Value Table in China” (26th edition). Experimental diets were in powder form; the composition and nutrient levels of the basal diet are presented in Table 1. Commercial betaine with 98% purity was used in this experiment.

**1.2 Management** The experiment was conducted at Hongyan Poultry Farm in Yuzhong County, Lanzhou City, Gansu Province. Two poultry houses were used during the trial. During the pre-trial period, all 600 experimental hens were housed in House 1. For the formal experimental period, 480 hens from the negative control and treatment groups were transferred to House 2 and raised in two-tier cages, with five adjacent small cages per tier constituting one replicate (15 hens). Position effects were considered when allocating groups. All hens had ad libitum access to feed and water, with natural lighting supplemented by artificial lighting (16 h/d). Manual feeding occurred three times daily (06:30, 14:00, and 17:30). Manure was removed once daily, with weekly whole-house disinfection and routine vaccination and immunization protocols.

The experimental period ran from June 2 to September 7, 2015. Dry-bulb temperature and wet-bulb humidity in both houses were recorded at 08:00, 14:00, and 22:00 daily using a dry-wet bulb thermometer. THI was calculated using the formula:  $THI = 0.72(T_d + T_w) + 40.6$ , where  $T_d$  represents dry-bulb temperature and  $T_w$  represents wet-bulb humidity. The results are shown in Table 2. A  $THI > 72$  indicated that experimental hens were under chronic heat stress conditions.

**1.3 Production Performance Measurement** During the experimental period, daily records were maintained for each replicate, including feed intake, egg number and weight, discarded eggs (broken, misshapen, cracked, soft-shelled, shell-less), culled and mortality numbers, death time, and body (carcass) weight. Hen-housed egg production, hen-housed egg yield, average daily feed intake, feed-to-egg ratio, and broken egg rate were calculated.

**1.4 Egg Quality Measurement** On day 7 of weeks 28 and 33, six eggs were randomly selected from each replicate for egg quality measurement (completed within 24 h). Egg shape index (length/width) was calculated after measuring longitudinal and transverse diameters using an egg shape index meter (Shimadzu, Japan). Eggshell strength was measured using an eggshell strength meter (Shimadzu, Japan), and eggshell thickness was determined using an eggshell thickness gauge (Shimadzu, Japan). Albumen height was measured using an albumen height gauge (Shimadzu, Japan), and Haugh units were calculated using the formula:  $HU = 100\log(H - 1.7W^{0.37} + 7.57)$ , where HU, H, and W represent Haugh units, albumen height (mm), and egg weight (g), respectively.

**1.5 Serum Biochemical Indices Measurement** At the end of the feeding trial, one hen from each replicate was selected for blood collection from the jugular vein after fasting. Serum was separated by centrifugation at 4,000 rpm for 15 min and stored at -20 °C for detection of routine serum biochemical indices, including total protein (TP), albumin (ALB), globulin (GLO), creatine kinase (CK), alkaline phosphatase (AKP), glutamic-oxaloacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT), triglycerides (TG), total cholesterol (TC), phosphorus (P), and calcium (Ca). Detection kits were purchased from Nanjing Jiancheng Bioengineering Institute.

**1.6 Statistical Analysis** All data were organized using Excel 2013 software and analyzed using one-way ANOVA in SPSS 19.0. Tukey' s test was used for multiple comparisons when significant differences were detected. Results are expressed as means  $\pm$  standard deviation, with significance level set at  $P < 0.05$  and trends considered at  $0.05 < P < 0.10$ .

## 2. Results

**2.1 Effects of Dietary Betaine on Production Performance of Heat-Stressed Laying Hens** Only two experimental hens died during the trial, and these were not attributable to treatment effects; therefore, this data was not included in Table 3 . As shown in Table 3, hen-housed egg production and hen-housed egg yield in Group I were significantly higher than those in Group II ( $P < 0.05$ ). Compared with Group II, Group IV showed significantly increased hen-housed egg production and hen-housed egg yield ( $P < 0.05$ ), while Group V also demonstrated significantly increased hen-housed egg yield ( $P < 0.05$ ). No significant differences were observed among groups in average daily feed intake, feed-to-egg ratio, or broken egg rate ( $P > 0.05$ ), though Group II showed a tendency for increased feed-to-egg ratio ( $P = 0.084$ ).

Figure 1 [Figure 1: see original paper] illustrates that hen-housed egg production declined with increasing age, with a more pronounced decline in Group II. Significant differences in hen-housed egg production between Groups I and II were observed from weeks 32 to 35 ( $P < 0.05$ ), while betaine-supplemented groups (III-V) showed a more gradual decline, demonstrating betaine' s positive effect in alleviating heat stress. Figure 2 [Figure 2: see original paper] shows that average daily feed intake was comparable across all groups, with no reduction in Group II. Figure 3 [Figure 3: see original paper] reveals that feed-to-egg ratio increased with age, with a more marked increase in Group II compared to other groups, while betaine supplementation improved feed-to-egg ratio, with the greatest improvement observed in Group V.

**2.2 Effects of Dietary Betaine on Egg Quality of Heat-Stressed Laying Hens** The effects of dietary betaine on egg quality at weeks 28 and 33 are presented in Tables 4 and 5 , respectively. No significant differences were detected among groups in average egg weight, egg shape index, Haugh units,

eggshell strength, eggshell thickness, eggshell weight per unit area, or eggshell percentage at either 28 or 33 weeks of age ( $P > 0.05$ ), with all indices remaining within normal ranges.

### **2.3 Effects of Dietary Betaine on Serum Biochemical Indices of Heat-Stressed Laying Hens**

As shown in Table 6, heat stress significantly reduced serum TP content and AKP activity in Group II compared with Group I ( $P < 0.05$ ), while significantly increasing serum GOT, CK, and GPT activities ( $P < 0.05$ ). Betaine supplementation significantly increased serum TP content and AKP activity (except in Group IV) compared with Group II ( $P < 0.05$ ), while significantly decreasing serum CK and GPT activities ( $P < 0.05$ ). Additionally, serum ALB content in Group V was significantly higher than in Group II ( $P < 0.05$ ), while serum TG content in Group V was significantly lower ( $P < 0.05$ ).

## **3. Discussion**

### **3.1 Effects of Dietary Betaine on Production Performance of Heat-Stressed Laying Hens**

Laying hens possess dense feather coverage and lack sweat glands, relying on respiratory and evaporative cooling due to their high metabolic rate and elevated body temperature; consequently, high temperature represents a primary environmental factor affecting their production performance [?]. Early research on heat stress impacts in poultry primarily utilized broilers as model animals, with documented effects on production performance, physiological indices, and gene expression [?]. Quinteiro-Filho et al. [?] reported that when ambient temperature exceeded 31 °C (corresponding to THI = 89.3), experimental birds showed significantly reduced feed intake and body weight gain. Heat-stressed poultry consistently exhibit behavioral changes, including reduced feeding and walking time and increased drinking and resting time, leading to decreased feed intake and body weight gain [?]. In the present study, average daily feed intake in heat-stressed hens did not significantly decrease compared with the control group, a finding inconsistent with Quinteiro-Filho et al. [?] and Mack et al. [?]. The reason remains unclear but may be related to substantial diurnal temperature-humidity fluctuations at the experimental site, where reduced feed intake during high THI periods might be compensated by increased consumption during low THI periods, warranting further investigation. The decline in egg production was primarily attributed to reduced feed conversion efficiency. Heat stress impairs antioxidant function, generating free radicals that damage organs and intestinal villi, subsequently affecting nutrient digestion and utilization [?].

Betaine regulates osmotic pressure within the body [?]. When supplemented in the diet, betaine is absorbed by cells, preventing water loss and salt entry while regulating cellular osmotic pressure and ionic balance, thereby improving production performance [?]. In this study, dietary supplementation with 400 mg/kg betaine significantly increased hen-housed egg yield and production compared with the negative control, confirming its anti-stress effects. Betaine

can mitigate or eliminate decreases in DNA replication, protein synthesis, and cell proliferation rates induced by hyperosmotic conditions and suppress hyperosmotic medium-induced heat shock protein (HSP)-70 gene expression [?]. Additionally, under acute heat stress conditions, betaine supplementation can enhance energy utilization efficiency [?]. These findings demonstrate that betaine supplementation represents an effective measure for alleviating heat stress in laying hens.

**3.2 Effects of Dietary Betaine on Egg Quality of Heat-Stressed Laying Hens** Under heat stress conditions, compensatory mechanisms regulating acid-base balance in body fluids act synergistically to maintain stability. Although these responses are effective for survival, they cannot prevent heat stress impacts such as increased blood flow to peripheral tissues and reduced blood flow to internal organs including the oviduct, leading to decreased egg quality [?]. In this study, heat-stressed hens showed varying degrees of reduction in average egg weight, egg shape index, Haugh units, eggshell strength, eggshell thickness, eggshell weight per unit area, and eggshell percentage, though these differences were not significant, consistent with previous reports [?]. Betaine supplementation improved these indices in a dose-dependent manner, primarily because betaine provides active methyl groups essential for animal metabolism, particularly protein and lipid metabolism. Numerous metabolic reactions, including synthesis of adrenaline, carnitine, and creatine, as well as DNA and RNA methylation, require methyl groups, ultimately improving cellular metabolic capacity [?].

**3.3 Effects of Dietary Betaine on Serum Biochemical Indices of Heat-Stressed Laying Hens** Under chronic heat stress conditions, reduced basal metabolism and feed intake affect normal metabolic processes, decreasing nutrient concentrations in the body. The energy required for cellular metabolism must be obtained through nutrient catabolism, consequently increasing the activity of various catabolic enzymes [?]. Most serum enzymes originate from various tissues and organs, with their activity directly reflecting the metabolic level and functional status of corresponding tissues. The body's regulatory and adaptive capacities largely depend on the functional status of these organs [?]. Under heat stress, increased cell membrane permeability accelerates the release of intracellular enzymes into the blood, elevating serum GOT, CK, and GPT activities. This promotes a shift from aerobic oxidation to anaerobic glycolysis in glucose metabolism pathways to generate substantial energy, regulated by the sympathetic-adrenal medullary system and glucocorticoids, thereby increasing blood glucose concentration [?]. Simultaneously, heat stress reduces feed intake and inhibits thyroid hormone secretion, decreasing protein synthesis and consequently reducing serum TP and ALB contents [?], consistent with our findings.

In this study, dietary supplementation with 400 and 600 mg/kg betaine significantly increased serum TP and ALB contents and AKP activity compared with

the negative control group, while significantly decreasing serum CK and GPT activities and TG content, reaching levels comparable to the positive control group. The significant increase in serum TP and ALB contents indicates that betaine improves both humoral and cellular immunity, attributable to normalized protein metabolism through methyl donation [?, ?]. The restoration of AKP and CK activities in heat-stressed hens following betaine supplementation results from enhanced thyroid hormone activity and stabilized ionic balance [?]. The reduction in serum GPT activity and TG content confirms betaine's hepatoprotective effects, as it decreases hepatic lipogenic enzyme activity, promotes apolipoprotein synthesis and fat mobilization, thereby reducing hepatic TG content [?], and effectively suppresses hepatic inflammation [?].

### Conclusion

High summer temperatures alter the metabolism and physiological functions of laying hens, resulting in decreased production performance. Dietary betaine supplementation effectively regulates body fluid balance, increases egg yield and production, and mitigates heat stress-induced damage. Based on comprehensive evaluation, the optimal dietary betaine supplementation level is 400 mg/kg.

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