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Original article

PRODUCTIVE, BIOCHEMICAL AND OXIDATIVE INDICES IN HENS WITH ZINC AND VITAMIN C SUPPLEMENTATION IN A HOT ENVIRONMENT

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Summary

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Heat stress is a critical factor that negatively affects poultry health and performances. Several nutritional strategies have been proposed to alleviate the adverse effects of heat stress, with vitamin and mineral supplementation being considered one of the most effective. This study aimed to investigate the impact of dietary supplementation of both zinc and vitamin C on various blood parameters – corticosterone, malondialdehyde (MDA), ferric reducing ability of plasma (FRAP), total cholesterol and total protein, as well as production performance indicators, including egg-laying capacity and live body weight. The research was conducted on laying hens housed under a semi-open rearing system within a hot environment. A total of 400 DeKalb Brown laying hens were divided into two groups: a control group and an experimental group, each consisting of 180 females and 20 males. The experimental group received a diet supplemented with 35 mg zinc oxide and 250 mg vitamin C per kilogram feed. In a high-temperature environment (average ambient temperature of 32.6 °C), hens supplemented with zinc and vitamin C showed significantly lower levels of corticosterone ($P<0.001$), MDA ($P<0.001$), oxidative stress index (OSI) ($P<0.05$) and cholesterol ($P<0.01$), compared to the control group. At the same time, the experimental group showed a significantly greater egg-laying capacity ($P<0.001$) and increased live body weight ($P<0.01$) compared to the control birds. Zinc and vitamin C may therefore act synergistically towards both heat and oxidative stress reduction, with beneficial effects on egg-laying capacity and body weight maintainance of hens in a hot environment.

Key words: heat stress, laying hens, oxidative stress, vitamin C, zinc

INTRODUCTION

High summer temperatures can provoke heat stress in laying hens kept in free-range, organic, and semi-open farming systems in temperate, transitional Mediterranean, and tropical regions. Under heat stress conditions, the laying hens respond with activation of the hypothalamic-pituitary-adrenal system and the sympathetic nervous system. A series of physiological and metabolic changes occur in the body, leading to elevated blood corticosterone levels, changes in the metabolic status of birds and impaired productivity (Vandana *et al.*, 2021).

Elevated ambient temperatures in the summer lead to oxidative stress, as evidenced by increased levels of protein oxidation (an increase in protein carbonyls) and lipid peroxidation, indicated by high concentrations of MDA (Emami *et al.*, 2020). At the same time, levels of blood metabolites like glucose, triglycerides, and cholesterol rise (Lu *et al.*, 2019). During heat stress, hens reduce heat production, resulting in a decrease in plasma T_3 levels, while T_4 concentrations rise (Beckford *et al.*, 2020). Simultaneously, feed intake in chickens decreases, leading to a reduction in their overall growth performance (Beckford *et al.*, 2020). High-temperature environments negatively affect egg-laying capacity, egg production and quality in poultry species (Xing *et al.*, 2019; Kim *et al.*, 2024).

Scientific studies suggest that using suitable supplements, such as micronutrients, vitamins, and minerals with strong antioxidant properties is crucial for mitigating the impact of heat stress (Alagawany *et al.*, 2021; Cornescu *et al.*, 2023). Under high temperature stress conditions, many authors recommend vitamin C supplementation to alleviate the heat stress (Reyes *et al.*, 2021; Pečjak *et al.*, 2022; Van Hieu *et al.*, 2022). Other authors recommend supplementation of zinc compounds to the diet due to their antioxidant properties and ability to mitigate stress (Jin *et al.*, 2023; Adam *et al.*, 2024; Hu *et al.*, 2024). Zinc is an essential trace mineral necessary for growth and optimal function of the avian immune system. It contributes to the growth of poultry, increases feed efficiency, boosts immune response and prevents infectious diseases (Hu *et al.*, 2024). Onderci *et al.* (2003) provided evidence that the addition of zinc supplements led to an increase in serum vitamin C levels in hens.

Currently, there are few comprehensive scientific studies regarding the combined impact of zinc supplements and vitamin C on reducing heat and oxidative stress in laying hens and thus improving egg production and preventing body weight loss during the hot summer periods.

The aim of the current study was to examine the effect of the dietary combination of zinc and vitamin C on various blood parameters, such as corticosterone, malondialdehyde (MDA), ferric reducing ability of plasma (FRAP), total cholesterol and total protein and overall production performance, including egg production and live body weight in laying hens housed in a semi-open rearing system in a hot environment.

MATERIALS AND METHODS

The experiment took place at the Poultry farm of the Agricultural Institute, Stara Zagora, Bulgaria. The farm is situated in a transitional Mediterranean climate and housed a flock of 400 DeKalb Brown laying hens, aged up to 42 weeks. The study was conducted from April to July 2023. The hens were divided into two groups, consisting of 180 females and 20 males, housed in a semi-open compartment measuring 7 by 7 m (49 m^2 in total), with a stocking density of approximately 4.08 hens per m^2. Each compartment was lined with a 20 cm layer of soft bedding material, composed of chopped straw and cobs. Additionally, it was fitted with 7 round feed dispensers and 5 drinking stations, providing access widths of 4 cm for feeding and 2.8 cm for drinking. All procedures involving birds were conducted in accordance with the European Council Directive 2010/63/EU and Bulgarian Regulation No. 20 (01 November 2012) on the protection of animals used for experimental and scientific purposes.

The hens from the first group served as control (control group) and were fed a standard commercial basal diet, as detailed in Table 1. The hens in the second group (experimental group) received the same basal diet, supplemented with 100 mg/kg Zinteral 35 (Lohmann Animal Health, Cuxhaven, Germany), containing 35 mg of zinc oxide per kilogram feed and 250 mg vitamin C per kilogram feed (L-acidum ascorbicum, CSPC Weisheng Pharmaceutical, Shijiazhuang Co. Ltd, China). The products were thoroughly mixed into the basal diet to achieve the desired concentrations according to the experimental design. Feed was prepared weekly to ensure uniform distribution and nutrient stability. The supplemented diets were offered *ad libitum* throughout the experimental period, and birds had free access to fresh water.

Between April 26 and May 26, 2023, which marked the thermoneutral subperiod, and from June 26 to July 26, 2023, corresponding to the hot subperiod, all groups were provided with the same type of compound feed.

Blood samples for analysis were collected from 18 female hens per group on May 26 and on July 26 from *v. subcutanea ulnaris* using sterile vacutainers (Vacutainer® Plus plastic plasma tube 13 x 75 mm, 4.0 mL BD), that contained 75 USP units of sodium heparin. The blood collection procedures lasted no longer than 2 min. The collected blood samples were then transported to the laboratory in a cooled container to ensure their integrity.

Table 1. Analysis of ingredients and nutritional composition of the basal experimental diet for DeKalb Brown laying hens

Ingredients	g/kg
Yellow maize	356.2
Wheat	200.0
Toasted whole soybeans	170.0
Sunflower expeller	180.0
Limestone	80.0
Dicalcium phosphate	9.0
Sodium chloride	2.8
Vitamin and mineral premix[1]	2.0
Nutrient analysis:	
ME, kcal/kg	2842.0
Protein (N × 6.25), g/kg	171.0
Fat, g/kg	40.0
Lysine, g/kg	7.4
Methionine + cysteine, g/kg	6.4
Threonine, g/kg	6.2
Tryptophan, g/kg	1.9
Calcium, g/kg	32.1
Available phosphorus, g/kg	3.0

[1]The vitamin and mineral premix Rovimix 15-C Layer provided per kilogram feed: vitamin A, 12,000 IU; vitamin D_3, 3,000 IU; vitamin E, 30 mg; vitamin K3, 3.0 mg; vitamin B_1, 2.0 mg; vitamin B_2, 5.0 mg; vitamin B_6, 5.0 mg; vitamin B_{12}, 0.016 mg; niacin, 30 mg; pantothenic acid, 12.0 mg; folic acid 1.0 mg; biotin, 0.05 mg; Co, 0.15 mg; I, 1 mg; Fe, 50 mg; Zn, 80 mg; Mn, 100 mg; Cu, 8 mg; Se, 0.2 mg; antioxidant, 25 mg.

Corticosterone concentrations in blood plasma were measured using a commercially available ELISA kit (Corticosterone ELISA RE52211, IBL GmbH, Hamburg, Germany).

TBARS determination serves as an indicator of lipid peroxidation. This method relies on the chemical interaction between MDA and thiobarbituric acid within a glacial acetic acid medium. The reaction results in a red-coloured adduct, which can be quantified by measuring its absorbance at 532 nm. The intensity of this colorimetric change correlates directly with the concentration of MDA, reflecting the extent of lipid peroxidation occurring in the sample (Rizzo, 2024). 1,1,3,3 tetraethoxypropane (Sigma-Aldrich Chemie GmbH, Munich, Germany) served as the standard for measuring MDA levels in our assays.

The FRAP (ferric reducing ability of plasma) assay was performed using the method described by Benzie & Strain (1996) for measuring the antioxidant capacity of plasma. The oxidative stress index (OSI) was assessed using the methods developed by Armstrong & Browne (1994) and Benzie & Strain (1996).

Measurements of total cholesterol, total protein and creatinine levels were performed using the BA-88 biochemical analyzer at the Research Biochemical Laboratory at the Faculty of Veterinary Medicine, Trakia University, Stara Zagora.

Egg-laying capacity (%) was determined as a ratio between the total number of eggs produced and the number of hens present, multiplied by 100. To determine the live body weight of each bird, they were individually weighed using balances with a sensitivity of 0.001 kg.

Microclimate conditions were assessed using a portable environmental meter specifically designed to measure temperature, humidity, air velocity, and light intensity (CO_2 Data Logger by AeroQual S200 Monitor).

The data were analyzed using GraphPad InStat 3 software (GraphPad Software, San Diego, CA). One-way ANOVA was conducted to compare the differences between the control and experimental groups during both the thermoneutral and hot subperiods. Results were considered statistically significant at P<0.05.

RESULTS

During the hot summer subperiod, the microclimatic conditions within the hens' living area under a semi-open rearing system were as followed: ambient tempera-

Table 2. Blood corticosterone levels, oxidative stress index (OSI) and biochemical parameters (mean ± SEM) in DeKalb Brown laying hens supplemented with zinc and vitamin C during the thermoneutral and hot subperiods under semi-opening rearing system

	Thermoneutral subperiod		Hot subperiod	
	Control group (n=18)	Experimental group (n=18)	Control group (n=18)	Experimental group (n=18)
Corticosterone, nmol/L	86.17 ± 7.29	60.82 ± 3.02 **	142.67 ± 13.40 $^{\#\#}$	73.67 ± 8.74 ***
FRAP, mmol/L	1.00 ± 0.17	1.29 ± 0.19	1.14 ± 0.15	1.34 ± 0.03
MDA, µmol/L	19.80 ± 2.57	11.58 ± 0.71 **	24.08 ± 3.06 $^{\#\#}$	12.29 ± 0.90 ***
OSI	2.51 ± 0.75	1.11 ± 0.33	2.56 ± 0.71	0.92 ± 0.08 *
Total cholesterol, mmol/L	2.28 ± 0.04	2.13 ± 0.02 **	3.33 ± 0.09 $^{\#\#}$	2.77 ± 0.16 **
Total protein, g/L	72.65 ± 6.17	77.11 ± 3.30	70.16 ± 5.17	75.13 ± 3.42
Creatinine, µmol/L	74.32 ± 6.67	70.48 ± 3.16	75.17 ± 6.67	72.48 ± 4.16

* statistically significant difference between control and experimental groups (* P<0.05, ** P<0.01, *** P<0.001); # statistically significant difference between control groups during thermoneutral and hot sub-periods ($^{\#\#}$ P<0.01).

Table 3. Productive indicators (mean ± SEM) in DeKalb Brown laying hens supplemented with zinc and vitamin C during the thermoneutral and hot subperiods under semi-opening rearing system

	Thermoneutral subperiod		Hot subperiod	
	Control group (n=180)	Experimental group (n=180)	Control group (n=180)	Experimental group (n=180)
Daily feed intake per hen, g/hen	88.34 ± 2.09	88.33 ± 1.82	81.32 ± 2.25 $^{\#}$	83.68±2.60
Average egg-laying capacity, %	95.28 ± 0.23	95.93 ± 0.30*	54.34 ± 3.17$^{\#\#\#}$	68.91±2.76 $^{***\ \#\#\#}$
Live body weight, kg	2.80 ± 0.03	2.81 ± 0.03	2.63 ± 0.03 $^{\#\#\#}$	2.73±0.03$^{**\ \#}$

* statistically significant difference between control and experimental groups (* P<0.05, ** P<0.01, *** P<0.001); # statistically significant difference between thermoneutral and hot sub-periods ($^{\#}$ P<0.05, $^{\#\#\#}$ P<0.001).

ture 32.6 ± 0.88 °C, relative humidity 65.75 ± 1.12%, air velocity 1.12 ± 0.42 m/s, and light intensity 250.33 ± 26.00 lx. During that subperiod, the control group showed significantly high levels of corticosterone (P<0.01), MDA (P<0.01) and total cholesterol (P<0.01) when compared to the thermoneutral subperiod (Table 2).

After a four-month supplementation with zinc and vitamin C, the levels of blood corticosterone (P<0.001), MDA (P<0.001) and total cholesterol (P<0.01) in the experimental DeKalb Brown laying hens were significantly reduced compared to the control group. After supplementation with zinc and vitamin C during the hot period, the experimental group showed a significantly higher average egg-laying capacity (68.91±2.76%) compared to the control group (P<0.001, Table 3). Meanwhile, the hens in the experimental group exhibited a significantly higher average live body weight compared to those in the control group (P<0.01).

DISCUSSION

The average ambient temperature in the hens' living area (32.6±0.88 °C) was significantly higher than the permitted range of 15‒25 °C established for this category of poultry under Bulgarian Regulation No. 25 on minimum welfare requirements for laying hens (Anonymous, 2009). High temperatures induce changes in neural and hormonal activity, starting with the stimulation of the hypothalamus and leading to the release of corticotropin-releasing factor. It stimulates the anterior pituitary gland to produce ACTH which subsequently acts on the production and release of corticosteroid by the adrenal glands (Smith & Vale, 2006). High ambient temperatures induce significant neuroendocrine, biochemical and antioxidant changes in DeKalb Brown laying hens (Table 2). In addition, heat stress in poultry leads to increased production of free radicals and a decrease in the activity of antioxidant enzymes (Miao *et al.*, 2020).

During the hot subperiod, the control group showed significantly higher blood levels of corticosterone (P<0.01), MDA (P<0.01) and total cholesterol (P<0.01) vs the thermoneutral subperiod. Other researchers also reported similar findings, highlighting increased corticosterone levels in birds along with high MDA concentrations caused by heat stress (Jing *et al.*, 2023; Liu *et al.*, 2023).

Following supplementation with zinc and vitamin C, the levels of blood corticosterone (P<0.001), MDA (P<0.001) and total cholesterol (P<0.01) in the experimental DeKalb Brown laying hens were significantly decreased compared to the control group. The lower levels of oxidative and biochemical markers observed could be attributed to the antioxidant and anti-stress effects of zinc and vitamin C supplements (Adam *et al.*, 2024; Aryal *et al.*, 2025). Zinc plays a role in regulating corticosterone levels as a crucial cofactor for antioxidant enzymes such as Cu/Zn superoxide dismutase (SOD_1) (Lee, 2018). Additionally, it plays a role in inhibition of NADPH-dependent lipid peroxidation, protecting cells from oxidative damage (Prasad & Kucuk, 2002). Sahin & Kucuk (2003) found that additional zinc intake can lead to an increase in serum vitamin C levels.

Total cholesterol levels were effectively reduced when the hens' diet was supplemented with vitamin C and zinc, which act as natural anti-stress agents. In supplemented hens under heat stress, total cholesterol levels were significantly lower compared to the control group (P<0.01). On the other hand, ascorbic acid acts as a powerful antioxidant that reduces anxiety and stress by influencing corticosterone levels (Julian, 1998). According to Sahin *et al.* (2003), supplementing the diet with vitamin C reduced the levels of MDA, homocysteine, and adrenocorticotropic hormone in the blood of heat-stressed Japanese quails. Our research indicates that dietary supplementation of zinc and vitamin C effectively decreased serum corticosterone and cholesterol levels. This combined approach helps reducing the negative impacts of heat stress, supporting the birds' overall health, maintaining their production performance and physical condition.

Kucuk *et al*. (2003) reported that dietary zinc supplementation increased total serum protein levels, suggesting that the observed slight elevation in total protein concentrations may represent a physiological adaptation to heat stress, potentially associated with enhanced synthesis of transport and antioxidant-related proteins stimulated by zinc and vitamin C supplementation.

Following supplementation with zinc and vitamin C during the hot period, the experimental group showed a significantly higher average egg-laying capacity (68.91 ± 2.76%) compared to the control group (P<0.001) and a significantly higher average live body weight than untreated controls (P<0.01). The observed differences can be attributed to the anti-stress and antioxidant properties of zinc and vitamin C combination. Heat stress significantly impairs poultry growth and productivity, leading to reduced feed efficiency, lower body weight gain, and decreased egg production (Vandana *et al.*, 2021; Apalowo *et al.*, 2024; Tesakul *et al.*, 2025). Zinc may diminish the negative impact of corticosterone on luteinising hormone levels, which is crucial for stimulating egg-laying in poultry (Yang *et al.*, 1998; Naz *et al.*, 2016). Emami *et al.* (2020) confirmed that heat stress can disrupt the production of yolk precursors in the liver, especially vitellogenin – a key protein supplying nutrients to the developing eggs. Studies on heat-stressed quails have shown that supplementing their feed with 30 mg/kg zinc can improve growth rate and increase feed consumption (Kucuk, 2008). Supplementing the diet of Japanese quails with 60 mg/kg zinc demonstrated several positive effects: a reduction in MDA concentration, increase in serum vitamin C and vitamin E levels, and improved egg production (Sahin & Kucuk, 2003). Vitamin C supplementation is a highly effective approach to counteract the negative impact of heat stress in poultry. According to Onagbesan *et al*. (2023) supplementing 250 mg vitamin C per kilogram of feed improved growth performance, nutrient absorption, egg production and quality, enhance immunity and antioxidant system in birds exposed to high temperatures.

During the thermoneutral subperiod, blood corticosterone, MDA, and total cholesterol were significantly lower ($P<0.01$) in the experimental group, indicating reduced physiological stress, decreased oxidative damage, and potentially improved lipid metabolism. FRAP was higher in the experimental group (1.29±0.19 mmoL/L), indicating enhanced antioxidant capacity. Creatinine concentrations remained comparable in both groups, indicating that kidney function was not affected by the treatment. Overall, during the thermoneutral sub-period, the experimental group demonstrated decreased stress hormone levels, reduced oxidative damage and improved antioxidant capacity. These findings suggested that zinc and vitamin C treatment had a health-promoting effect on the physiological condition of the birds at normal temperatures.

Our results clearly demonstrate the relationship between the heat stress and poultry productivity. Thus, the development and implementation of strategies and management practices are essential for effectively reducing heat stress.

CONCLUSIONS

Dietary supplementation of DeKalb Brown laying hens exposed to high summer temperatures with 35 mg zinc oxide and 250 mg vitamin C per kilogram feed exerted a synergistic effect that effectively mitigated responses to heat and oxidative stress. The supplements significantly reduced blood plasma corticosterone, cholesterol, and MDA concentrations, indicating enhanced antioxidant capacity and improved physiological adaptation to heat stress. Furthermore, it helped maintain live body weight and increased egg production during the hot summer months. The findings indicate that the combined use of zinc and vitamin C is a convenient nutritional strategy to alleviate the adverse effects of heat stress to support productivity in laying hens under semi-open rearing conditions.

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