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VITAMIN C AND ZINC EFFECT ON SOME PRODUCTIVITY AND BLOOD INDICES IN LAYING HENS DURING A COLD PERIOD

Nadya Bozakova[1*]

[1]Faculty of Veterinary Medicine, Trakia University - Stara Zagora, Hristina Morphova street 2, 6000 Stara Zagora, Bulgaria

[*]e-mail: nadiab@abv.bg

Abstract

One appropriate way for cold stress reduction in poultry in transient Mediterranean climate areas is the dietary vitamin C and Zn supplementation. These additives have a strong anti-stress and antioxidant effect, are economically viable and the this combination helped to improve the laying capacity and preserve the body weight of the hens due to the synergistic action of the two components.

The purpose of the present study was to study the effect of dietary combination - 250 mg/kg vitamin C and 35 mg/kg zinc oxide on some productive performances (egg production and live body weight) and blood indices (corticosterone, malondialdehyde (MDA), ferric reducing ability of plasma (FRAP), total cholesterol, glucose, total protein, and creatinine) in DeKalb Brown laying hens (n = 200) under semi-open rearing system during a cold period. Laying capacity was determined as a ratio between the number of eggs per day and the number of hens, expressed as a percentage. The live body weight of hens was determined by the weighing method with precision up to 0.001 kg. The blood corticosterone levels were assayed by means of commercial ELISA kit. The biochemical parameters were determined by automatic biochemical analyzer. The statistical processing of the results was performed by one-way ANOVA (GraphPad InStat 3.06) at level of significance $P < 0.05$.

Low ambient temperatures during the experiment (average temperature was 6.65 ^{0}C) caused a significant cold stress in the hens. Higher daily egg laying capacity ($P < 0.001$) and higher live body weight ($P < 0.05$) were determined in experimental group two months after the onset of dietary supplementation. Simultaneously the exanimated combination helped reduce blood corticosterone levels, ($P < 0.01$), MDA ($P < 0.001$), FRAP ($P < 0.01$), cholesterol ($P < 0.001$) and glucose ($P < 0.001$) concentrations in experimental hens, compared to the controls.

The tested combination of vitamin C and zinc is an appropriate way for cold and oxidative stress reduction in laying hens under semi-open rearing system during the cold period due to the synergistic action of the two components. This way the combination helped to improve the daily laying capacity and preserve the live weight of the hens during the cold period.

***Key words**: Cold stress, Vitamin C and Zn- supplementation, Egg production, Live body weight.*

1. Introduction

Free-range, semi-open and organic poultry farming systems create conditions for cold stress in winter period in transient Mediterranean climate areas. Ambient temperature discomfort negatively affects laying capacity, weight and quality of eggs in various bird species (Wolfenson *et al.,* [1], Özbey *et al*., [2], Kucuk *et al.,* [3]).

The free-range and semi-open rearing of hens in winter is characterized with low ambient temperatures, which have an adverse influence on birds. The cold temperatures provoke thermal stress in hens and compromise their welfare. According to Lin *et al.,* [4]), the response of laying hens to stress is mediated by the activation of the hypothalamo-pituitary-adrenal system and is accompanied by a series of physiological and metabolic changes, which result in the reduction of daily egg production and live weight of birds (Sahin *et al.,* [5], Sahin *et al.,* [6]). Nutritional strategies are important with regard to maintaining egg productivity and limiting temperature stress by using appropriate supplements (micronutrients, vitamins, minerals) in rations (Heinzen, [7], Rodenburg *et al.,* [8], Lin *et al.,*[4], Sahin *et al*., [9]).

In ambient temperature stress conditions, many authors recommend vitamin C addition to the diet to alleviate cold stress during winter periods (McDowell, [10], Sahin *et al.,* [5]). Other authors advise a supplementation of zinc compounds in the diet because of its antioxidant and anti-stress effect (Sahin *et al*., [6], Nollet *et al.*, [11], Kucuk, [12], Kucuk *et al.,* [3], Jankowski *et al.,* [13]). Furthermore, the addition of this element is justified by its reduced retention in the body during stress (Bartlett and Smith, [14]).

The use of dietary supplements in these regions during the cold months is economically profitable. McDowell, [10], included vitamin C in the diet to alleviate cold stress. To reduce the adverse effect of cold stress, Nollet *et al.,* [11], and Jankowski *et al.,* [13] recommend feed supplementation with zinc (Zn) because of its antioxidant and anti-stress effect. Similarly, Sahin *et al.,* [5]) report that the addition of 30 mg Zn/kg to the feed of laying hens submitted to cold stress (6.8 ^{0}C) decreases blood corticosterone, glucose and cholesterol and improved egg laying performance. These additives - vitamin C and Zn, which have a strong anti-stress and antioxidant effect, are economically viable. So far, we have not found enough information about the synergistic effect of the combination of vitamin C and zinc to reducing both cold and oxidative stress during a cold period, nor a possibility for improving egg production and live body weight in DeKalb Brown laying hens under semi-open rearing system.

The aim of present study is to investigate the effect of dietary combination - 250 mg/kg vitamin C and 35 mg/kg zinc oxide on some productive performances and blood indices in DeKalb Brown laying hens (n = 200) under semi-open rearing system during a cold period. Egg production, live body weight and daily consumption per hen have been included in the performance indicators and: corticosterone levels, malondialdehyde (MDA) concentrations, ferric reducing ability of plasma (FRAP), total cholesterol, glucose, total protein, and creatinine concentrations which have been tested during the research.

2. Materials and Methods

2.1 Materials

The experiments were performed at a private poultry farm situated at 10 km east of Stara Zagora, Bulgaria, in transient Mediterranean climate with (n = 200) DeKalb Brown laying hens at the age of 38 weeks. The study was implemented from February 1 to March 31, 2016.

The hens were divided into 2 groups (n = 90 ♀ and 10 ♂) and were placed in a 21 m long and 7 m wide semi-open building. Each group was located in a square compartment with a side of 7 m (area = 49 m^2), with a density of accommodation of 2.04 hens/m^2 (the norm is 8 birds/m^2 as per Regulation 44, [15]). The laying hens from the first group were used as control (control group) and they were fed with a commercial basic diet presented in Table 1. The hens of the second group were fed with the same feed, supplemented with 250 mg vitamin C (L-acidum ascorbicum - CSPC Weisheng Pharmaceutical, Shijiazhuang Co. Ltd) per 1 kg diet and 100 mg/kg Zinteral 35 (Lohmann Animal Health, Cuxhaven, Germany) containing 35 mg/kg diet zinc oxide (Vit. C + Zn - group).

Table 1. Ingredients and nutrient analysis of experimental basal diet for DeKalb Brown laying hens

Ingredients	g/kg
Corn yellow	356.2
Wheat	200
Soybeans, toasted whole	170
Sunflower expeller	180
Limestone	80
Dicalcium phosphate	9
Sodium chloride	2.8
Vitamin and mineral premix[1]	2
Nutrient analysis:	
ME, kcal/kg	2,842
Protein (N x 6.25), g	171
Fat, g	40
Lysine, g	7.4
Methionine + cysteine, g	6.4
Threonine, g	6.2
Tryptophan, g	1.9
Arginine, g	10.1
Calcium, g	32.1
Phosphorus available, g	3

Legend: [1]The vitamin and mineral premix Rovimix 15-C Layer provided per kilogram of diet: vitamin A, 12.000 IU; vitamin D3, 3.000 IU; vitamin E, 30 mg; vitamin K3, 3 mg; vitamin B1, 2 mg; vitamin B2, 5 mg; vitamin B6, 5 mg; vitamin B12, 0.016 mg; niacin, 30 mg; pantothenic acid, 12 mg; folic acid 1 mg; biotin, 0.05 mg; Co, 0.15; I, 1 mg; Fe, 50 mg; Zn, 80 mg; Mn, 100 mg; Cu, 8 mg; Se, 0.2 mg; antioxidant, 25 mg.

2.2 Methods

2.2.1 Laying capacity

It was determined as a ratio between the number of eggs per day and the number of hens, expressed as a percentage, or L egg = N egg/N hens x 100%, where N_{egg} was the number of eggs produced per week, and N_{hens} was the number of laying hens.

2.2.2 Hens live body weight

It was determined by the weighing method with precision of up to 0.001 kg.

2.2.3 Microclimatic conditions

They were determined by routine methods - by portable composite device for measuring temperature, humidity, air velocity, and luminance intensity - Gas CO_2/ Humidity/Temp. DataLogger. Ammonia concentration - by Monitor AeroQual S200.

2.2.4 Blood samples

Blood samples for corticosterone determination were taken from nine (three per replicate) female hens on March 31 from *v. subcutanea ulnaris* in sterile vacutainers (Vacutainer® Plus plastic plasma tube 13 x 75 mm x 4.0 mL BD), containing 75 USP units of sodium heparin. The blood manipulations did not exceed 2 min, which is considered by Lagadic *et al.,* [16] as a non-menacing duration for blood glucose levels in birds. Blood samples were carried to the lab in a cooling bag.

2.2.5 The plasma corticosterone levels

They were assayed with immunoenzymatic ELISA kit (Corticosterone ELISA RE52211, IBL Gesellschaft fur Immunchemie und Immunbiologie MBH, Hamburg, Germany) in the Laboratory of Innate Resistance Investigation in Faculty of Veterinary Medicine.

2.2.6 TBARS determination

Lipid peroxidation assay is based on the formation of a 1:2 red adduct between malondialdehyde (MDA) and 2-thiobarbituric acid in acid medium that is quantitated at 532 nm after extraction with n-butanol (Uchiyama and Michara, [17]; Andreeva *et al*. [18]). 1,1,3,3 tetraethoxypropane (Sigma Aldrich Chemie GmbH, Munich, Germany) was used as an MDA standard.

2.2.7 Ferric reducing ability of plasma (FRAP) assay

At low pH, reduction of a ferric tripyridyltriazine (FeI - TPTZ) complex to a ferrous form results in the production of an intense blue color. The change in absorption, therefore, is directly related to the amount of electron-donating antioxidants present in the reaction mixture. FRAP values in mmoL /L are obtained by comparing the absorbance change at 593 nm in test reaction mixtures with those containing ferrous ions in known concentration. Seven concentrations of aqueous solutions of $FeSO_4 \times 7H_2O$ in the range of 100 - 1000 mmol/L were used for calibration (Benzie and Strain, [19]).

2.2.8 Blood total cholesterol, glucose, total protein and creatinine

They were determined with the biochemical analyzer "BA-88" in the innate resistance research lab at the Veterinary Genetics and Breeding Unit, Department of General Animal Breeding, Faculty of Veterinary Medicine, Trakia University - Stara Zagora.

2.2.9 Statistical analysis

Results were processed by one-way ANOVA (GraphPad InStat 3.06) at a level of significance $P < 0.05$.

3. Results and Discussion

During cold winter period, the microclimatic parameters in hens' living area under the semi-opening rearing were as following: ambient temperature - 5.65 ± 0.52 ^{0}C; relative humidity - 69.67 ± 2.12%; air velocity -0.92 ± 0.02 m/s, and light intensity - 68 ± 5 lx. The average ambient temperature in the hens' living area was substantially lower than the permitted 18 - 25 ^{0}C for this category of birds by Regulation 44, [15]. Low temperatures induced neural and hormonal changes beginning with hypothalamic stimulation and the production of corticotrophin releasing factor. It stimulates the anterior pituitary to produce adrenocorticotropic hormone (ACTH) and causes adrenal cortical tissue to increase the production of corticosterone in laying hens (Puvadolpirod and Thaxton, [20], Puvadolpirod and Thaxton, [21], Puvadolpirod and Thaxton, [22], Sahin *et al.,* [23]). Increased corticosterone levels are a reliable indicator of stress provoked by low temperatures in poultry farming (Sahin *et al.,* [23], Sahin *et al.,* [24]). According to Donkoh, [25], and Sahin *et al.,* [23] higher cholesterol have been found under such stress conditions. Low ambient temperature provokes significant blood hormonal, biochemical and antioxidant changes in DeKalb Brown laying hens (Table 2). In addition, the formation of excessive amount of free radicals and oxidative stress in hens is triggered as a result of temperature stress (Halliwell and Gutteridge, [26]). Similarly, corticosteronee levels, biochemical blood parameters and malondialdehyde data in DeKalb Brown hens are also available (Table 2).

After a two-month supplementation with vitamin C and zinc, blood corticosterone ($P < 0.01$), MDA ($P < 0.001$), total cholesterol ($P < 0.001$) in supplemented DeKalb Brown hens were significantly lower than in the control group, simultaneously the concentration of FRAP was higher than in the control one ($P < 0.01$), (Table 2).

Table 2. Biochemical parameters, corticosterone levels and oxidative stress parameters in DeKalb Brown hens supplemented with Zn and vitamin C during the cold period (mean ± SEM, n = 9 ♀)

Parameters	Control group	Vit. C + Zn - group
Corticosterone, nmol/L	117.78 ± 3.64	99.44 ± 4.03**
Total cholesterol, mmol/L	2.66 ± 0.09	2.18 ± 0.01***
Glucose, mmol/L	7.48 ± 0.34	7.89 ± 0.10
Total protein, g/L	72.98 ± 6.17	79.12 ± 4.30
Creatinine, μmol/L	75.34 ± 6.67	68.59 ± 3.17
FRAP, mmol/L	0.52 ± 0.02	1.30 ± 0.25**
MDA, μmol/L	26.19 ± 1.27	16.17 ± 1.75***

Legend: "*" indicates statistically significant difference between control and experimental group. **P < 0.01; ***P < 0.01: statistically significant difference between control and experimental group during the cold period.

Table 3. Productive indicators in DeKalb Brown laying hens supplemented with vitamin C and Zn during the cold period (mean ± SEM) under semi-opening rearing during the cold period

Productive indicators	Cold period	
	Control group	Vit. C + Zn - group
Daily consumption per hen, g/hen	162 ± 5.62	169.33 ± 3.17
Average laying capacity, %	71.66 ± 0.53	79.53 ± 0.96***
Live body weight, kg	1.983 ± 0.034	2.063 ± 0.027*

Legend: "*" indicates statistically significant difference between control and experimental group; * - level of statistical significance P < 0.05; *** - level of statistical significance P < 0.001.

These lower levels could be explained by the antioxidant and anti-stress effect of vitamin C and Zn supplements (Sahin *et al.*, [5], Sahin *et al.*, [23], Sahin *et al.*, [24], Yanchev *et al.* [27], Sahin *et al.*, [9], Jankowski *et al.*, [13]). Concerning Prasad [28], and Prasad, and Kucuk [29], zinc limits the excessive secretion of corticosterone as a co-factor of essential antioxidant enzymes - Cu/Zn superoxide dismutase and inhibiting NADPH-dependent lipid peroxidation. In addition, Onderci *et al.*, [30] report that supplemental Zn increases serum vitamin C.

The significantly elevated total cholesterol in experimental hens can be explained with the anti-stress effect of vitamin C + Zn hens subjected to cold ambient stress. On the other hand, ascorbic acid has a strong antioxidant, corticosterone and anxiety-reducing effects (Satterlee *et al.*, [31], Jones *et al.*, [32]). According to Kutlu and Forbes, [33], vitamin C reduces the synthesis of corticosteroid hormones in poultry. By decreasing the corticosteroids synthesis and secretion, vitamin C alleviates the negative effects of stress (McDowell, [10]).

Zinc and vitamin C, applied as a dietary supplement to compound feed, promote the reduction of serum corticosterone and cholesterol concentrations and thus contribute to the lower adverse effect of ambient temperature-related stress in layers, helping to sustain their production potential and to preserve body condition (Table 3).

After a two-month supplementation with the combination of vitamin C and Zn during a cold period, the average daily laying capacity in the experimental group was significantly higher (79.53 ± 0.96) than the control one, (P < 0.001) (Table 3). Similarly, the average live body weight of the hens from the second group was significantly heavier vs the control group, (P < 0.05). These statistically significant differences can be explained by the anti-stress and anti-oxidative effects of the tested combination - 250 mg/kg vitamin C and 35 mg/kg zinc oxide. According to Sahin *et al.*, [5], Star *et al.*, [34] and Yardibi and Turkay, [35], temperature-induced stress in hens is punctuated by negative effects on their growth, productivity and poorer economic results due to the bigger and more inefficient energy expenditure.

On the other hand, Bollengier-Lee *et al.*, [36], and Kirunda *et al.*, [37] claim that the negative influence of stress temperatures on the laying capacity is mainly due to blocking the yolk proteins' synthesis. Under the influence of vitamin C and Zn-supplementation during the cold period, a significant increase in daily laying capacity in experimental hens, compared to the control (P < 0.001) was found. This can be ascribed to the capacity of Zn to curb the suppressive effect of corticosterone on luteinizing hormone which is known to stimulate laying activity in poultry (Yang *et al.*, [38], Wiesinger, [39], and Heinzen, [7]). Sahin and Kucuk, [40], reported a feed utilization improvement in supplemented with $ZnSO_4$ (at doses of 30 and 60 mg/kg of feed) quail, under temperature stress conditions. In a similar experiment, Kucuk *et al.*, [3] reported that Zn (30 mg/kg) and pyridoxine (8 mg/kg) had improved both feed utilization and poultry intake.

As for the influence of vitamin C, a number of authors have reported that vitamin C supplementation (150 mg/kg feed) has positively influenced growth in broiler chickens exposed to multifocal stress (McKee and Harrison, [41]). There are many data on the positive effect of vitamin C supplementation on laying capacity, growth rate in laying hens as well as on the shell thickness of the eggs under cold stress conditions (McDonald *et al.*, [42], Bashir *et al.*, [43], and Ahmadu *et al.*, [44]).

The negative influence of temperature stress also affected the body weight of DeKalb Brown hens during a cold period (Table 3). There was a significant body weight increase in the experimental group compared to the control ($P < 0.05$). According to Donkoh, [25], Sahin *et al.,* [24], Star *et al.,* [34], and Yardibi and Turkay, [35], temperature stress negatively affects poultry weight and productivity. This could be explained by the Zn reducing effect of negative stress temperature on feed consumption, absorption and metabolic processes in the hens under ambient temperature stress (MacDonald, [42], Sahin and Kucuk, [40], Sahin *et al.,* [24], and Ahmadu *et al.,* [44]).

4. Conclusions

- Prolonged low temperatures (5.65 ^{0}C) in winter were a significant stress factor for laying hens and provoked high corticosterone levels, malondialdehyde and cholesterol concentrations, lowering the average daily laying capacity and live body weight in DeKalb Brown hens under semi-open rearing system.

- The supplementation of 250 mg of vitamin C together with 35 mg/kg of zinc oxide in the feed, saved the hens from both cold and oxidative stress, decreasing the plasma concentration of corticosterone, cholesterol, malondialdehyde. Thus, the tested combination supported the increase of daily laying capacity and the maintenance of live body weight in hens during a cold period.

- The combination of 35 mg/kg zinc and 250 mg vitamin C reduced both cold and oxidative stress in DeKalb Brown laying hens in the winter period due to the synergistic effect of both components.

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