

ETHOLOGICAL STUDY OF FREE-RANGE HENS WITH ZINC AND VITAMIN C SUPPLEMENTED DIET

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Abstract

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The study was designed to examine the effects of a dietary supplementation with zinc (35 mg/kg) and vitamin C (250 mg/kg) on the behaviour and plasma corticosterone in New Hampshire NG-line hens reared under free range conditions (sleeping pens and outdoor walking yards) during cold (7°C), thermoneutral and hot (31°C) subperiods.

Hens' behaviour was recorded by video cameras. Blood plasma corticosterone was assayed by ELISA.

In all birds, both low and high ambient temperatures, combined with high light intensity, induced a significant increase in plasma corticosterone compared to the thermoneutral period ($P < 0.01$). During those periods hens were more aggressive which indicated poorer welfare. Hens supplemented with either zinc or zinc + vitamin C had lower plasma corticosterone than controls. Dietary zinc, either alone or co-administered with vitamin C, reduced plasma corticosterone and increased the number of egg-laying, dust bathing and preening birds during the cold and hot periods. The aggressive behaviour was reduced ($P < 0.01$) indicating a higher welfare level.

The group supplemented with zinc + vitamin C showed more intensive preening and resting, as well as less aggression and movement than the zinc-supplemented group ($P < 0.05$), suggesting a synergistic action of both supplements towards alleviating stress and therefore, a possibility for improving hen welfare.

Key words: poultry welfare, behaviour, corticosterone, zinc and vitamin C, stress

Introduction

One of the ways to improve the welfare of free-range hens in temperate climate zones is to reduce environmental stress during the cold and hot periods by appropriate dietary supplements. According to Jones et al. (2005) and Moura et al. (2006), the welfare of birds is strongly associated with the environment in these zones. That is why both low and high ambient temperatures commonly provoke worse poultry welfare in free range systems.

In domestic poultry, the thermal comfort zone ranges between 18 and 22°C (Sahin et al., 2009), and an adequate level of welfare is believed to exist between 10-27°C (Duncan and

Hawkins, 2010). The thermoregulation mechanisms are triggered in both cold (7°C) and hot (31°C) weather conditions, which provoke cold or hot stress, respectively (Ensminger et al., 1990; Sahin et al., 2009).

Poultry possess numerous thermoregulatory mechanisms to counteract thermal stress. However, free-range farming may challenge their ability to adapt to changed environment.

Under low ambient temperatures, body heat loss is reduced, whereas the heat gain rate is enhanced. The feed consumption and locomotion levels are increased, water drinking is lower as is the behaviour linked to heat loss – wing spreading, feather cleaning, dust bathing etc. (Ensminger et al., 1990, Sahin and Kucuk, 2003).

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During hot summer days, the rate of heat loss is enhanced while heat production is decreased. Thermoregulation in hens is difficult because of their high body temperature (41.5°C) and limited heat exchange mechanisms in hot weather. That is why the primary mechanism for heat loss is evaporation from mucous membranes during accelerated respiration (Ensminger et al., 1990; Sahin et al., 2009). Other behaviour traits include frequent drinking, wandering for shady hiding places etc. which lead to a greater body heat loss (Bozakova, 2010; Bozakova et al., 2012).

One of the existing ways of reducing environmental stress in poultry is the supplementation of their diet with zinc or vitamin C. According to Sahin and Kucuk (2003) and Sahin et al. (2005), this is justified by the fact that following thermal stress, the concentrations of antioxidant minerals and vitamins in the organism are reduced. Zinc supplements are especially important for poultry stress alleviation (Sahin et al., 2002; Sahin et al., 2009). Stress triggers an excessive production of free radicals namely oxidative stress (Sahin et al., 2002; Sahin et al., 2005). Zinc is known to play a key role in the endogenous antioxidant protection system. In support of this hypothesis, Onderci et al. (2003) provided evidence that supplemental zinc increased serum vitamin C in hens. Kutlu and Forbes (1993) reported that ascorbic acid reduces the synthesis of corticosteroid hormones in poultry. Additionally Bains (1996) reported a corticosterone-modulating effect of vitamin C via its involvement in the gluconeogenesis to enhance energy supply during stress.

According to Sossidou and Elson (2009) and Nääs et al. (2010) poultry welfare is associated with behaviour, health, mortality, physiology etc. Graves (1982) and Popova–Ralcheva et al. (2002) confirmed that behaviour is an indicator for the better and more precise estimation of poultry welfare. The most important indices of poultry welfare are the changes in their behaviour together with alterations in blood corticosterone concentrations.

Poultry welfare is particularly well characterized by two types of behaviour – comfort behaviour, and fear and aggression behaviour. A number of authors – Sherwin and Kelland (1998), Olsson and Keeling (2005) and Dixon et al. (2008) outline that comfort behaviour comprises frequent dust bathing, stretching, and preening. Olsson and Keeling (2005) and Dixon et al. (2008) pointed that the behaviour of taking dust baths is an important indicator of social welfare of poultry. According to Sherwin and Kelland (1998), the better welfare in turkeys increased the time spent on stretching, feather pecking, and dust bathing. Similarly, Stoyanchev et al. (2006) and Bozakova et al. (2012) affirm that dust bathing is the uppermost demonstration of comfort in turkey broilers and hens.

The more frequent episodes of aggression in birds are signs of worse welfare and are associated with stress. Aggression, along with increased blood corticosterone concentrations are believed to be important parameters for welfare evaluation in poultry (Puvadolpirod and Thaxton, 2000 a,b,c; Popova–Ralcheva et al., 2002; Sahin et al., 2005; Sahin et al., 2009; Bozakova et al., 2012).

The purpose of the study was to find out the effect of dietary supplementation with 35 mg zinc/kg feed, used either independently or combined with 250 mg vitamin C/kg feed upon the different types of behaviour (ingestive, gregarious, agonistic and mating) and plasma corticosterone concentrations in hens reared in a free range system during cold (7°C), thermoneutral and hot (31°C) periods.

Materials and Methods

Experimental design and diets

The experiment was conducted in the Experimental Farm of the Agricultural University – Plovdiv between March 1 and June 21, 2010. The experimental poultry were 78 female New Hampshire NG-line hens at the age of 36 weeks. The New Hampshire NG-line is a dual purpose line – for meat production and egg-laying. The sire line of the bilinear hybrids was a general purpose one (Lalev et al., 2012). The origin of all chickens was the same – same line, simultaneously hatched, purchased from the Institute of Agriculture – Stara Zagora. Up to 8 weeks of age, they had been reared on permanent litter indoor, under controlled microclimatic conditions. After the 8th week the birds' weight was averaged between the groups and they were placed in sleeping houses and walking yards.

The hens were divided into three groups, (n = 26, females) with body weight averaged among the groups. The non-supplemented hens were used as a control group (Control-group). The diet of the second group hens was supplemented with 100 mg/kg Zinteral 35 (Lohmann Animal Health, Cuxhaven, Germany) containing 35 mg/kg zinc oxide (Zn-only-group). The diet of the third group hens was supplemented with the same amount of Zinteral 35 together with 250 mg vitamin C (L-acidum ascorbicum, CSPC Weisheng Pharmaceutical, Shijiazhuang Co. Ltd) per 1 kg diet (Zn + Vit. C – group). The hens from the three groups were fed ad libitum with the same compound feed according to the age category. The feed nutrient composition, obtained from the analysis in an accredited laboratory is presented in Table 1.

The whole experimental period (from March 1 to June 21, 2010) included three observation sub-periods – cold sub-period (from March 1 to March 14, 2010), thermoneu-

Table 1
Composition of hens' diets

Ingredients	(g/kg)	
Corn yellow	356.2	
Wheat	200.0	
Soybeans, toasted, whole	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:		Source of reference ²
Metabolizable E, MJ/kg	11.89	10.88-11.71
Protein (N x 6.25), g	171	min.150
Crude fat, g	40	min. 30
Crude fiber, g	40.57	max.60
Lysine, g	7.40	6.00-6.50
Methionine, g	6.00	5.80-6.00
Threonine, g	6.20	6.00
Tryptophan, g	1.90	1.90
Calcium, g	32.10	30 – 38
Phosphorus available, g	3.00	2.60 – 4.00
Zinc, g	0.080	180 mg·kg-1
Vitamin C, g	0	

¹The 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, 3.0 mg; vitamin B1, 2.0 mg; vitamin B2, 5.0 mg; vitamin B6, 5.0 mg; vitamin B12, 0.016 mg; niacin, 30 mg; pantothenic acid, 12.0 mg; folic acid 1.0 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

²Marinov, B., 2004

tral sub-period (from May 1 to May 14, 2010) and hot sub-period (from June 7 to June 20, 2010). According to the European Commission issued Regulation No. 1334/2003, the maximum tolerable zinc levels in feed mixtures for livestock should be restricted to 150 mg/kg and in animal breeding at 250 mg/kg.

Under the free range conditions, hens from each group were housed in sleeping pen and outdoor walking yard. The sleeping pens were identical (size 3.50/2.50/2.75 m) equipped with 3 perches, 2 m in length, and 8 two-floor wooden nests of 30/30/40 cm each. The housing density of hens in sleeping houses was 2.97 birds per 1 m² area (norm 3.5 hens/ m², Regulation 44/ 2006). The light intensity coefficient (ratio of window area to floor area) in sleeping pens was 1:10 (about 55 lx).

On the bottom of the southern wall of pens, there was a 30/40 cm rectangular opening for outdoor access. Each yard was 9.20/24 m of size with perennial broadleaf trees in the middle. The housing density of birds in walking yards was

0.12 hens/m² (significantly more than the area according to Regulation 44/ 2006).

Under experimental conditions, all birds spent the night indoors in sleeping houses from 7.00 PM to 7.00 AM. The door was opened in the morning at 7.01 AM and birds were allowed to go out. In the evening, all hens returned back to the houses and the door was closed (7.00 PM). Therefore, birds were able to spend 12 hours outdoors (from 7 AM to 7 PM). Video recordings of their behaviour were made during that period.

Yards were provided with two rows of tubular feeders, placed under the eaves of sleeping houses and with watering troughs ensuring feeding and drinking widths of 10 and 3 cm, respectively as required by zoohygienic norms for this category of birds (Regulation 44/2006). During the winter, watering troughs were supplied with anti-freezing devices. The intake of a sufficient amount of feed was also continuously controlled.

Microclimatic conditions

The microclimatic rearing conditions were determined at 2 sites (in sleeping houses – until 7.00 AM (for the night), and in walking yards – from 7.01 AM to 19.00 (during the day), where the major part of birds reside.

The repeated observations on hens' behaviour showed that all preferred to go out in yards. Very few birds returned to the house to lay eggs and to sleep. That is why we registered the ambient temperatures and humidity in the living space of birds – in sleeping houses – until 7.00 AM (for the night), and in walking yards – from 7.01 AM to 7.00 PM (during the day) to register the microclimatic parameters of the place, where the major part of birds resided. The temperature and the relative air humidity were measured by 2 weekly records of thermohygrographs (ERZG, GDR, Type 405) for each group.

They were measured during the three observation sub-periods – cold sub-period (from March 1 to March 14, 2010), thermoneutral sub-period (from May 1 to May 14, 2010) and hot sub-periods (from June 7 to June 21, 2010) by routine methods.

The air motion speed was determined by a catathermometer, on a weekly basis, over three consecutive days at 7:00 AM, 2:00 PM and 9:00 PM at 5 micro zones within each compartment at the bird level. The light intensity was measured by a digital luxmeter (Taschen-luxmeter LM37, Germany) at 5 micro zones within each compartment at the bird level. The concentration of ammonia was determined by indicator tubes (Ammonia 0.2-A, Hygitest Association, Bulgaria) and a Dräger ammonia sensor at 5 micro zones within each compartment at bird level (Table 2).

Table 2
Microclimatic parameters in the hen's living area determined on the base of the measured indicators in the walk yards and in the sleep houses during the cold, thermoneutral and hot periods

Periods	Ambient temperature, (°C)	Air humidity, (%)	Air velocity, (m/s)	NH ₃ (ppm)	Light intensity, (lx)	Light length, (h:min:s)
Cold period	7.39±0.54	68.10±3.43	0.50±0.06	traces	69.67±3.88	11:56:28
Thermoneutral period	19.44±2.5	66.07±1.59	0.50±0.04	traces	248.33±26.96	14:37:48
Hot summer period	31.24±0.88	54.75±1.25	0.41±0.03	traces	410.00±15.34	15:17:54
Minimum-maximum parameters	3.50-40.50	52.75-71.53	0.38-0.56	traces	65.79 – 425.34	11:56-14:37
Reference values*	18–25	50–70	0.2–0.5	< 15	30-60	12-16

*Reference values as per Regulation 44/ 2006

Behavioural studies

The behaviour of hens was recorded during the three observation sub-periods with two video cameras, located within the sleeping pen and walking yard for 12 hours over 4 consecutive days during each sub-period: March 11-14; May 11-14 and June 18-21, 2010 for each group.

The recordings served as a source to prepare ethogrammes as per Wojcik and Filus (1997). During the ethological study we counted the number of birds engaged in specific forms of behaviour: feed and water intake, gregarious behaviour (resting, moving, egg-laying, dust bathing and preening), mating and agonistic behaviour. The personnel charged with registering birds' behaviour was not familiar with the type of dietary supplement given to the group. Egg-laying behaviour was registered on the basis of average number of hens, lying down to lay eggs.

Laboratory analyses

The blood samples for corticosterone determination were collected from *v. subcutanea ulnaris* during the three observation sub-periods, once for each of the three periods on March 15, May 15 and June 21, 2010 in sterile heparinised vacutainers (Vacutainer® Plus plastic plasma tube 13×75 mm × 4.0 mL BD). They were consistently collected between 13:00 and 14:00 h to avoid the influence of the circadian rhythm of corticosterone. Blood samples were collected from nine (three per replicate) hens randomly chosen from each treated group. The manipulations related to blood collection did not exceed 2 min, which is considered by Lagadic et al. (1990) as a duration having no effect on blood corticosterone levels in birds. Samples were transported to the lab in a cooling bag.

Plasma samples were harvested after centrifugation (886 × g for 20 min) and stored deep frozen (- 40°C) until all blood samples had been collected. Blood plasma corticos-

terone was assayed with a commercial ELISA kit (Corticosterone ELISA RE52211, IBL Gesellschaft für Immunchemie und Immunbiologie MBH, Hamburg, Germany) 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.

Statistical analyses

The obtained data were statistically analysed by one-factorial ANOVA using StatSoft Statistica® Software (2009). All results are presented as mean±SEM. The differences were considered as significant when *P* values were less than 0.05.

Results

The average ambient temperature during the cold period was 7.39±0.54°C and 31.24±0.88°C during the hot sub-period (Table 2). The average light intensity also exceeded the reference values during the three sub-periods, especially during the thermoneutral (248.33±26.96 lx) and the hot summer periods (410.00±15.34 lx).

The other microclimatic parameters were within the reference range for this category of poultry.

High and low ambient temperatures, combined with high light intensity in free range hens had a significant effect on their behaviour during the three observation sub-periods: cold (from March 1 to March 14, 2010), thermoneutral (from May 1 to May 14, 2010) and hot (from June 7 to June 21, 2010).

During our study period, there were no dead birds in the groups. There were no cases of impaired feather cover, foot pad dermatitis or any other injuries. No deviations from the normal behaviour were noted.

Table 3, Table 4 and Table 5 present the averaged results for the different types of behaviour on the basis of ethogrammes for each of the 3 groups within one temperature period. During

Table 3

Number of hens from the control group and experimental groups supplemented with zinc (Zn-only-group) or zinc and vitamin C (Zn+vit. C-group) exhibiting a specific type of behavior during the cold period. Data are presented as mean±SEM, (n=26)

Behavior	Cold period					
	Control group	%	Zn-only-group	%	Zn + Vit.C- group	%
Feeding	8.70±0.63	33.46	8.55±0.54	32.88	8.55±0.54	32.88
Drinking	5.00±0.26	19.23	4.50±0.17	17.31	4.50±0.17	17.31
Egg-laying	0.57±0.10	2.19	0.75±0.13	2.88	1.11±0.16**#	4.27
Moving	5.84±0.39	22.46	5.16±0.25	19.85	4.14±0.37**#	15.92
Resting	1.50±0.21	5.77	1.91±0.20	7.35	2.45±0.22**#	9.42
Preening	0.59±0.12	2.27	0.66±0.09	2.54	0.95±0.15**#	3.65
Dust bathing	0.93±0.17	3.58	1.36±0.11*	5.23	1.50±0.13**	5.77
Aggression	1.66±0.16	6.38	1.20±0.14*	4.62	1.00±0.13**	3.85
Mating behaviour	1.16±0.15	4.46	1.45±0.11	5.58	1.77±0.15**#	6.81

The symbol “**” indicates statistically significant difference for a given type of behavior between control and treated groups. The symbol “#” indicates statistically significant difference for a given type of behavior between the two treated groups (Zn –only or Zn + Vitamin C)

*, # – level of statistical significance $P < 0.05$; **, ## – level of statistical significance $P < 0.01$.

Table 4

Number of hens from the control group and experimental groups supplemented with zinc (Zn-only-group) or zinc and vitamin C (Zn+vit. C-group) exhibiting a specific type of behavior during the thermoneutral period. Data are presented as mean±SEM, (n=26)

Behavior	Thermoneutral period					
	Control group	%	Zn- only-group	%	Zn + Vit.C- group	%
Feeding	7.59±0.69	29.19	7.20±0.70	27.69	6.48±0.72	24.92
Drinking	4.18±0.31	16.08	3.80±0.29	14.62	3.95±0.29	15.19
Egg-laying	2.09±0.27	8.04	2.36±0.29	9.08	2.86±0.35*	11.00
Moving	2.75±0.32	10.58	2.11±0.30	8.12	1.43±0.23***#	5.50
Resting	1.11±0.18	4.27	1.5±0.18	5.77	1.48±0.28	5.69
Preening	1.27±0.13	4.88	1.23±0.14	4.73	1.71±0.17*#	6.58
Dust bathing	2.89±0.26	11.12	3.55±0.34	13.65	3.82±0.36*	14.69
Aggression	2.20±0.12	8.46	1.91±0.15	7.35	1.75±0.13**	6.73
Mating behaviour	1.89±0.16	7.27	2.30±0.19	8.85	2.55±0.17**	9.81

The symbol “**” indicates statistically significant difference for a given type of behavior between control and treated groups. The symbol “#” indicates statistically significant difference for a given type of behavior between the two treated groups (Zn –only or Zn + Vitamin C)

*, # – level of statistical significance $P < 0.05$; **, ## – level of statistical significance $P < 0.01$; *** – level of statistical significance $P < 0.001$

the cold sub-period, the number of egg-laying, preening, dust bathing, aggressive birds and those exhibiting mating behaviour from the control group was lower compared to the thermoneutral period ($P < 0.001$; $P < 0.01$). At the same time, there were significantly more moving birds ($P < 0.001$), Table 3.

The supplementation with zinc only and zinc with vitamin C increased considerably the number of egg-laying ($P < 0.01$ for Zn + Vit. C- group), resting ($P < 0.01$ for group for Zn + Vit. C- group), preening ($P < 0.05$ for Zn + Vit. C- group), dust bathing birds ($P < 0.05$ for Zn-only- group and $P < 0.01$ for Zn + Vit. C- group) and bird mating behaviour

($P < 0.01$ for Zn + Vit. C- group) vs. controls, respectively. The number of moving ($P < 0.01$ for Zn + Vit. C- group) and aggressive hens ($P < 0.01$ for Zn + Vit. C- group) decreased substantially compared to controls.

The number of egg-laying, resting, preening birds and mating behaviour in the group supplemented with Zn + vitamin C was statistically higher than in Zn-only group ($P < 0.05$). Simultaneously the moving hens in Zn + vitamin C-group were less than those in the Zn-only group.

During the thermoneutral sub-period, the dietary supplementation had also influenced the behaviour of birds com-

Table 5

Number of hens from the control group and experimental groups supplemented with zinc (Zn-only-group) or zinc and vitamin C (Zn + vit.C-group) exhibiting a specific type of behavior during the hot period. Data are presented as mean±SEM, (n=26)

Behavior	Hot period					
	Control group	%	Zn-only group	%	Zn + Vit.C group	%
Feeding	5.86±0.59	22.54	4.57±0.55	17.58	4.70±0.60	18.08
Drinking	6.16±0.29	23.69	6.05±0.29	23.27	6.16±0.29	23.69
Egg-laying	0.77±0.17	2.96	1.18±0.22	4.54	1.34±0.25*	5.15
Moving	3.45±0.13	13.27	3.63±0.28	13.96	2.00±0.21***###	7.69
Resting	4.21±0.52	16.19	4.45±0.54	17.12	4.80±0.55	18.46
Preening	1.11±0.10	4.27	1.34±0.13	5.15	1.48±0.14*	5.69
Dust bathing	1.14±0.17	4.38	1.61±0.17a	6.19	1.95±0.16***	7.50
Aggression	1.91±0.17	7.35	1.66±0.16	6.38	1.48±0.14*	5.69
Mating behaviour	1.50±0.12	5.77	1.52±0.14	5.85	2.05±0.19**#	7.88

The symbol “*” indicates statistically significant difference for a given type of behavior between control and treated groups. The symbol “#” indicates statistically significant difference for a given type of behavior between the two treated groups (Zn -only or Zn + Vitamin C)

*, # – level of statistical significance $P < 0.05$; **, ## – level of statistical significance $P < 0.01$; ***, ### – level of statistical significance $P < 0.001$

pared to the control group (Table 4). The moving and aggressive birds from the Zn + vitamin C-group were considerably less ($P < 0.001$; $P < 0.01$), whereas egg-laying ($P < 0.05$), preening ($P < 0.05$), dust bathing ($P < 0.05$) birds and birds with mating behaviour ($P < 0.01$) were more than the respective numbers from controls.

The frequency of preening ($P < 0.05$) was statistically higher in birds supplemented with zinc and vitamin C compared to the birds receiving zinc. At the same time, moving hens were less in the group with two supplements compared to the group with a single supplement ($P < 0.05$).

Similar changes were observed in the bird's behaviour during the hot sub-period. In the control birds there were less feeding ($P < 0.05$), egg-laying ($P < 0.001$), dust bathing ($P < 0.001$), mating ($P < 0.05$) and more drinking and resting hens ($P < 0.001$) compared to the thermoneutral period, Table 5.

Water drinking was more intensive in the hens from the three groups, compared to the thermoneutral period ($P < 0.001$). In Zn + Vit. C-group there were less moving and aggressive birds, ($P < 0.01$), but more egg-laying ($P < 0.05$), preening ($P < 0.05$) and dust bathing birds ($P < 0.001$), compared to the controls. The supplementation of zinc + vitamin C during the hot period increased mating behaviour frequency ($P < 0.05$) and decreased the number of moving birds ($P < 0.001$) compared to those supplemented with zinc only.

Control birds exhibited significantly higher average blood corticosterone levels during both the cold and the hot sub-periods compared to the thermoneutral sub-period ($P < 0.001$), Figure 1. Plasma corticosterone concentrations

during the cold sub-period (Figure 1) were statistically significantly lower in experimental groups than in controls ($P < 0.05$ for the Zn-only and $P < 0.001$ for the Zn + Vit. C groups). The differences between both supplemented groups were also significant ($P < 0.05$ during the cold sub-period and $P < 0.01$ during the hot sub-period).

Discussion

In free range system the average ambient temperatures during the cold ($7.39 \pm 0.54^\circ\text{C}$) and hot ($31.24 \pm 0.88^\circ\text{C}$) sub-periods were significantly deviating from the thermal comfort range for the hens and induced environmental stress.

The stress response in birds is mainly mediated through activation of hypothalamic-pituitary-adrenal axis and the sympathetic autonomic nervous system (Puvadolpirod and Thaxton, 2000 a,b,c; Popova-Ralcheva et al., 2002; Hai et al., 2006). The hypothalamus is activated by stressors and activates the adrenal cortex. The latter reacts with enhanced secretion of glucocorticosteroids (corticosterone being the major member of this group in birds) which trigger a chain of biochemical, behavioural, immune and production changes indicating a worsen welfare.

Our results about blood corticosterone in experimental birds also confirmed that extreme ambient temperatures resulted in statistically significantly higher corticosterone concentrations ($P < 0.01$). The dietary supplementation of birds with zinc or zinc plus vitamin C during the three sub-periods resulted in reduction of blood corticosterone compared to the

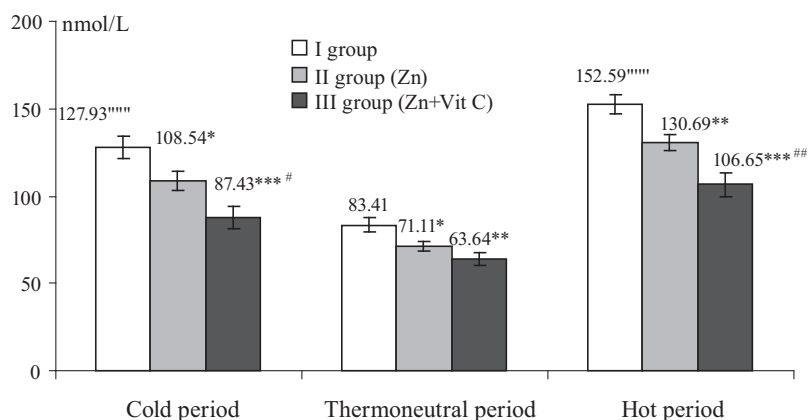


Fig. 1. Plasma corticosterone levels (nmol/L) during the cold, thermoneutral and hot period in hens from the control group and experimental groups supplemented with zinc (Zn-only-group) or vitamin C+zinc (Zn+vit. C-group). Data are presented as mean \pm SEM (n = 9, ♀).

^{*}P < 0.05, ^{**}P < 0.01, ^{***}P < 0.001 statistically significant difference between control-group (I) and experimental groups (Zn-group and Zn+vit. C-group) during each period; [#]P < 0.05, ^{##}P < 0.01- statistically significant difference between supplemented groups (Zn-group and Zn+vit. C-group) during each period. ^{****}P < 0.001 statistically significant difference between thermoneutral and either cold or hot periods.

control group. Similarly, Sahin et al. (2002) reported that the addition of 30 mg Zn/kg feed in breeder hens submitted to cold stress (6.8°C) decreased blood corticosterone, glucose and cholesterol. Under heat stress, the addition of 30 or 60 mg Zn to kg feed in quails contributed to maintaining lower corticosterone levels (Sahin and Kucuk, 2003; Sahin et al., 2005).

The observed effect on corticosterone concentrations could be attributed to the antioxidant and anti-stress effect of zinc. Environmental stress provokes the formation of excessive amount of free radicals (Halliwell and Gutteridge, 1989). Being a co-factor of essential antioxidant enzymes Cu/Zn superoxide dismutase and inhibiting NADPH-dependent lipid peroxidation (Prasad, 1997; Prasad and Kucuk, 2002), zinc limits the excessive secretion of corticosterone tightly linked to stress and anxiety in birds (Popova-Ralcheva et al., 2002).

The reduction of elevated corticosterone levels was more significant in the group which received both supplements (Zn+vitamin C) than in the group supplemented only with zinc. These findings correspond to the data of Satterlee et al. (1993) establishing a reduction of blood corticosterone in Japanese quails supplemented with vitamin C. Vitamin C plays a major role in the biosynthesis of corticosterone, a primary glucocorticoid hormone involved in gluconeogenesis to enhance energy supply during stress (McDowell, 1989; Sahin et al. (2002). It was also demonstrated that supplemental zinc increased serum vitamin C concentrations in hens. Thus, both supplements act synergically in the reduction of

environmental stress-induced high blood corticosterone and contribute to the better welfare of birds.

No deviations from the normal behaviour have been noted in our study. According to the results, two types of behaviour are indicative for evaluating the change in welfare level of birds: comfort behaviour (dust bathing, preening, mating, improved egg-laying) and fear and aggression behaviour. Both tested supplements (zinc, vitamin C) could alter these behaviours and through them, the change in poultry welfare under different ambient temperatures could be evaluated.

The changes in the behaviour of poultry supplemented with both zinc and vitamin C during the three sub-periods of the study were interpreted as beneficial for their welfare. During the cold sub-period, there was a higher number of egg-laying ($P < 0.01$ for Zn + Vit. C group), resting ($P < 0.01$ for Zn + Vit. C – group), preening ($P < 0.05$ for Zn + Vit. C group), dust bathing birds ($P < 0.05$ for Zn-only-group and $P < 0.01$ for Zn + Vit. C – group) and birds exhibiting mating behaviour ($P < 0.01$ for Zn + Vit. C – group) vs. controls, respectively. The number of moving ($P < 0.01$ for Zn + Vit. C – group) and aggressive hens ($P < 0.01$ for Zn + Vit. C – group) was substantially decreased vs controls.

This could be attributed to the antioxidant and stress-alleviating effect of zinc and vitamin C, acting to reduce the negative effect of corticosterone on luteinizing hormone, stimulating egg-laying in birds (Heinzen, 2003).

During the hot period, there were more egg-laying ($P < 0.05$), preening ($P < 0.05$) and dust bathing birds ($P < 0.001$),

together with less moving and aggressive hens, ($P < 0.01$) in the zinc + vitamin C supplemented group than in the control one. The supplementation of zinc + vitamin C during the hot sub-period increased mating behaviour frequency ($P < 0.05$) and decreased the moving bird number ($P < 0.001$) compared to those supplemented with zinc only. This could be related to the synergistic effect of zinc (Prasad and Kucuk, 2002) and vitamin C (Jones et al., 1996) to attenuate the gonad-suppressing effect of corticosterone (Yang et al., 1998).

The dust bathing behaviour was considerably more frequent in birds supplemented with zinc or zinc + vitamin C ($P < 0.01$). It could also be explained by the combined stress-reducing effect of additives for welfare improvement during the hot summer sub-period. Additionally Olsson and Keeling (2005) and Dixon et al. (2008) found that the behaviour of taking a dust bath is an important indicator of social welfare of the bird group. In turkeys, improved welfare is manifested with increased time spent in stretching, preening and dust bathing (Sherwin and Kelland, 1998).

The improved behaviour and welfare of experimental birds was also evidenced by the lower ($P < 0.01$) number of aggressive hens vs controls, as also stated by Popova-Ralcheva et al. (2002). Moreover, the birds receiving both supplements were calmer and more relaxed during the three sub-periods compared to birds receiving zinc only. Our data confirm the report of Jones et al. (1996) that the preliminary addition of vitamin C (24 h before the stressors) was accompanied with less panic and calmer behaviour in Japanese quails. This effect was attributed to the corticosterone and fearfulness reducing effects of vitamin C in hens (Satterlee et al., 1993; Jones et al., 1996). All these facts provided evidence for the synergistic activity of zinc and vitamin C towards environmental stress reduction and poultry welfare improvement.

Conclusions

The extremely low or high environmental temperatures in free-range reared hens induced an environmental stress, manifested with excessive blood corticosterone concentrations.

After supplementation with zinc or zinc + vitamin C, blood corticosterone levels were substantially reduced during the cold and the hot sub-periods. The reduction was more pronounced in the group receiving both supplements, supporting the hypothesis about the synergistic stress-alleviating effect of zinc and vitamin C.

The number of egg-laying, resting, preening, dust bathing birds, as well as those exhibiting mating behaviour from the groups supplemented with zinc and zinc + vitamin C was

higher during the cold and the hot sub-periods, indicating a better hen's welfare than controls.

The supplementation with zinc and vitamin C in free-range reared birds stimulated dust bathing, mating behaviour and reduced the movements compared to the hens receiving zinc alone indicating an improved welfare. This confirmed the synergistic effect of both substances in reducing stress and therefore, in improving the welfare of hens.

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