Influence of Immunobeta® Dietary Supplementation on Egg Production and Some Parameters of Oxidative Stress in Laying Hens

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ABSTRACT

The aim of this study was to evaluate the effects of the immunostimulant Immunobeta® on egg production and oxidative stress parameters, influenced by the environmental conditions in free range laying hens. A total of 81 laying hens and 9 cocks (Tetra Super Harco) were divided into three groups: group 1 (control) without supplementation; group 2 with 0.2%, and group 3 with 0.4% Immunobeta® as a dietary supplement. The laying period (19-55 weeks of age) was divided into three sub-periods depending on the ambient temperature – cold (from November to March 2015, from 19 to 37 weeks of age), thermoneutral (April and May 2016, from 38 to 46 weeks of age) and hot period (June and July 2016, from 47 to 55 weeks of age). Immunobeta® supplementation dose dependently improved the average egg production and mean egg weight, and in 0.4% dose treatment significantly reduced the feed conversion ratio for the entire laying period. Immunobeta® supplementation influenced the blood oxidative stress parameters, decreasing significantly the lipid peroxidation level during the cold period, increasing the glutathione level in hens supplemented with 0.4% dose in all periods, regulating the catalase activity during the hot period, and increasing glutathione peroxidase activity during the thermoneutral and hot periods. In conclusion, addition of Immunobeta® to the diet reduced the oxidative stress induced by thermal stress and enhanced the performance of free range laying hens.

Keywords: Egg production, Feed conversion ratio, Free-range, Oxidative stress.

INTRODUCTION

As a result of the changing consumer preferences, there is a tendency to increase production of table eggs from free or organically reared hens. Some of the leading poultry companies have recently changed their selective and management policy, focusing their efforts on creation and marketing of dual-purpose rural hens adapted for free-range rearing.

The free-range system provide open areas for walks and pastures, giving birds the opportunity to perform their natural behavioral instincts (rooting, dust bath), pecking grains or pebbles, have direct contact with the sun's rays and the fresh air from the environment. One of the biggest problems of the well-being of outdoor reared birds is the influence of high and low seasonal temperatures. It has been found that egg production, egg weight, shell weight,

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shell thickness, and specific gravity were significantly reduced among hens subjected to heat and cold stress (Lara and Rostagno, 2013; Durmus and Kamanli, 2015). It is widely accepted that poultry thermal stress is associated with induction of Oxidative Stress (OS) (Lara and Rostagno, 2013; Zhao *et al*., 2014; Akbarian *et al*., 2016). Thus, antioxidants such as vitamin C, vitamin E, selenium and herbals with antioxidant properties are used to prevent the negative impact of thermal stress on the well-being of poultry and their productivity (Ghazi Harsini *et al*., 2012; Akbari *et al*., 2016).

In our experiment, Immunobeta®, a substance containing *β*-glucans from *Saccharomyces cerevisiae*, was applied. Many in vitro as well as in vivo studies in animals and humans show that especially *β*-glucans derived from fungi and yeasts (as in this case) have pronounced immune modulating properties (Volman *et al*., 2008). The effects of *β*-glucans appeared to be mainly related to the activation of free radical processes and Reactive Oxygen Species (ROS) generation that modulate the cytokine release (Bonfim-Mendonça *et al*., 2014).

It is well known that excess ROS generation may induce OS and tissue injury. However, the moderate amounts of ROS play an important role in the regulation of gene expression since many transcription factors are redox sensitive. Numerous products associated with oxidant-modulated genes have been identified that include antioxidant enzymes, stress proteins, DNA repair proteins, and mitochondrial electron transport proteins (Turpaev, 2002). In vitro treatment of cell cultures with the prooxidant agents resulted in time- and dose-dependent increases of transcript levels of the three primary enzymes involved in ROS detoxification: Catalase (CAT), SuperOxide Dismutase (SOD) and Glutathione Peroxidase (GPX) (Franco *et al*., 1999). This transcriptional amplification led to increasing of the enzymes activities, although the magnitudes of the increases were less pronounced than those of the

respective transcript levels. In pathological conditions of OS etiology, a rise in antioxidant enzyme gene expression along with increase of antioxidant enzyme activities was also observed (Limaye *et al*., 2003). In this way, OS appears to be an important factor driving the adaptive responses of the organisms.

Thus, the aim of the present study was to investigate the impact of Immunobeta® on egg production and some blood OS parameters in free range laying hens during the cold, thermoneutral, and hot periods.

MATERIALS AND METHODS

Experimental Design

The experiment was carried out in the Poultry Division at Agricultural University – Plovdiv (Bulgaria) with onset of egg production from November 2015 (19 weeks old birds) to July 2016 (55 weeks old birds) i.e. the duration of the laying period was 37 weeks. For the purpose of the study, the laying period was divided into three sub-periods depending on ambient temperature – cold (from November to March 2015, from 19 to 37 weeks of age), thermoneutral (April and May 2016, from 38 to 46 weeks of age) and hot period (June and July 2016, from 47 to 55 weeks of age).

In the experiment, a total of 81 laying hens and 9 cocks Tetra Super Harco (originating in Babolna Tetra Kft, Hungary) were divided randomly into three groups. Each group included 27 hens and 3 cocks with average body weight ♀ 2150±40 and ♂ 3200±45 g at 19 weeks of age. The fowls from each group were reared in equal conditions in a sleeping pen and outdoor walking yard, i.e. in a free-range system. The sleeping pens (size 3.50/2.50/2.75 m) were equipped with 3 perches, 2.50 m in length, and 8 two-floor wooden nest boxes of 30/30/40 cm each. The housing density of poultry in sleeping houses was 3.43birds per m^2. The light intensity coefficient in sleeping pens was about 55 lx (ratio of

window area to floor area was 1:10). 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 0.14 birds m^{-2} i.e. 7.36 m^2 per bird.

Each group was provided with two tube feeders, placed under the eaves of sleeping houses and with watering troughs ensuring feeding and drinking widths of 10 and 3 cm per bird. The fowl were watered and fed ad libitum. The compound feed was according to the laying period (Table 1). The first group (control) received basal diet without additive; the second group– basal diet with 0.2% Immunobeta® and the third group–

basal diet with 0.4% Immunobeta® (Chemifarma S.p.A. – Italy) as a supplement. According to the manufacturer's data, Immunobeta® contains active ingredients: 30% *β*-glucans, 25% mannanoligosaccharides, and 5% nucleotides.

Egg Production and Egg Quality Traits

Eggs from each group were collected daily. Hen-week egg production (%) was determined as: *Total number of eggs produced by a flock per week×100/Total number of hens housed×7*

For each egg, individual measurements were performed once a week (on Fridays):

Table 1. Composition of the basal diets.

Components	Laying period in week			
	19-20	21-28	29-40	41-55
Feed ingredients (g kg^{-1})				
Corn yellow	336.7	308.2	308.2	320.0
Wheat	336.7	32.8.2	308.2	320.8
Soybeans toasted (440 g crude protein)	120.0	125.0	120.0	100.0
Sunflower expeller (340 g crude protein)	150.0	150.0	150.0	150.0
Sunflower oil	-	12.0	50.0	-
L-lysine	1.1	0.7	0.1	0.5
DL-Methionine	0.7	0.9	0.7	0.7
Sodium chloride	2.6	2.8	2.3	2.0
Limestone	38.0	82.0	97.0	100.0
Dicalcium phosphate	12.0	7.0	5.3	3.8
Premix TB 301 Layers[a]	2.0	2.0	2.0	2.0
Synergen[b]	0.2	0.2	0.2	0.2
Calculated composition (per kg)				
Metabolizable energy (MJ kg^{-1})	12.1	11.8	11.5	11.5
Crude protein (g)	178.0	174.0	171.0	165.0
Crude fiber (g)	45.0	44.0	44.0	43.0
Crude fats (g)	41.0	51.0	44.0	40.0
Calcium (g)	20.0	36.0	38.1	39.2
Phosphorus available (g)	6.6	5.6	5.3	3.0
Lysine (g)	8.5	8.1	7.5	7.3
Methionine+cysteine (g)	7.3	7.3	7.0	6.9

[a] Premix TB 301 Layers (made in De Heus Koudijs Animal Nutrition, The Netherlands) in 1 kg contains: Vitamin A– 5000000 IU; vitamin D$_3$– 1500000 IU; vitamin E– 4000 mg; vitamin K$_3$– 500 mg; vitamin B$_1$– 250 mg; vitamin B$_2$– 1500 mg; calcium-D-pantothenate– 3000 mg; vitamin PP– 10000 mg; vitamin B$_6$ – 500 mg; vitamin B$_9$ folic acid– 250 mg; vitamin B$_{12}$– 10000 mg; vitamin B$_4$– 50000 mg; Fe– 20000 mg; I– 400 mg; Cu– 2300 mg; Mn– 32500 mg; Zn– 25000 mg; Se– 125 mg; Antioxidants: Propyl gallate– 41.7 mg; BHT – 41.7 mg; ethoxycuine– 41.7 mg; Preserving agent: Citric acid– 0.1 g. [b] Synergen– A product of the solid state fermentation of *Aspergillus niger* (made in Alltech®, USA).

Egg Weight (EW) – With accuracy of ±0.01 g, with electronic scale OHAUS (Japan);

Shape Index (SI) – In % by the formula

$$I = \frac{B}{L}.100$$

, where *B* is maximum Breadth (mm) and *L* is egg Length (mm), with a caliper and accuracy of ±0.1 mm;

Weight of egg Shell (SW), egg Yolk (YW) and egg Albumen (AW) – With accuracy ±0.01 g, with electronic scale OHAUS;

Shell Thickness (ST) – With accuracy of ±1 μm, with a micrometer screw gauge at the pointed end (sharp), at the blunt end (air cell) and at the equatorial (the widest) part;

Color of egg Yolk (YC) – Determined with a Roche yolk color fan scale;

Yolk Index (YI) – Formulated as *(Yolk height/Yolk diameter)×100*;

Albumen Index (AI) – Formulated as *(Albumen height/(Albumen length+Albumen width)/2)×100*;

Haugh Unit value (HU) – By the formula of Haugh (1937):

HU= 100 log (H−1.7W$^{0.37}$+7.6), where, *H* is Height of albumen (mm) and W is egg Weight (g);

Egg Volume (EV), in cm³ by the formula of Narushin (2005): *EV= (0.6057−0.0018B)×LB²*

Surface Area (SA), in cm² by the formula

of Narushin (2005):

SA= (3.155−0.0136L+0.0115B)×LB, as *B* is maximum Breadth (cm) and *L* is egg Length (cm)

Albumen to yolk ratio (g/g).

Erythrocyte Pro/Antioxidant Status

To determine the pro/antioxidant indices, blood samples were collected from *v. subcutanea ulnaris* in sterile heparinised vacutainers (Vacutainer® Plus) plastic plasma tube 13×75 mm×4.0 mL BD) from six hens randomly chosen from each group during:

The cold period (36-week-old hens, the month of March; Weekly average ambient temperature: 6.39°C; Min. t: -2.8°C; Max. t: 18.0°C);

The thermoneutral period (44-week-old hens, the month of May; Weekly average ambient temperature: 17.24°C; Min. t: 8.2°C; Max. t: 26.0°C);

The hot period (53-week-old hens, the month of July; Weekly average ambient temperature: 25.6°C; Min. t: 16.0°C; Max. t: 35.8°C).

The daily data of average, maximum, and minimum ambient temperatures, relative humidity and precipitation were taken from
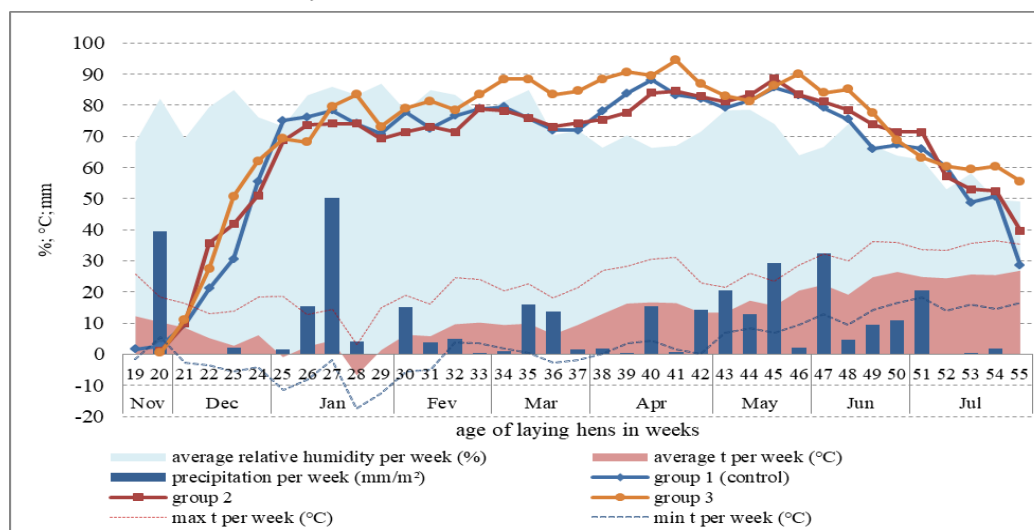

Figure 1. Egg production, weekly ambient temperatures, and relative humidity.

Agrometeorology Station (Plovdiv) at the National Institute of Meteorology and Hydrology. The weekly values of weather are provided in Figure 1.

Blood collection did not exceed 2 minutes (Lagadic *et al*., 1990). The samples were transported to the laboratory in a cooling bag. The blood was centrifuged at 4°C at 600×g for 10 minutes and the obtained erythrocytes were washed twice with 0.9% NaCl. A 5% erythrocyte suspension in 0.15 M NaCl – 10 mM sodium phosphate buffer, pH 7.2 was prepared and used for measuring the OS parameters (Petrov *et al*., 2010). Each sample was run in triplicate and the mean value from the three measurements was taken into consideration.

Reagents

The reagents: 2-ThioBarbituric Acid (TBA), trichloroacetic acid, HCl, H_2O_2, oxidized glutathione, nitro blue tetrazolium, reduced Nicotinamide Adenine Dinucleotide Phosphate (NADPH), Drabkin's Reagent (product code D5941) as well as the Glutathione Assay Kit (CS0260-Sigma) were obtained from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany).

Analytical Procedures

Haemoglobin (Hb) amount was determined using Drabkin's reagent (Samour, 2005). Ten μL 5% erythrocyte suspension was added into 990 μL Drabkin reagent and, after 20 minutes incubation at room temperature, the absorbance at 540 nm was read against Drabkin reagent. The total Hb concentration (g L^{-1}) was determined using a calibration curve.

Lipid Peroxidation (LPO) was estimated by the amount of ThioBarbituric Acid Reactive Substances (TBARS) (Gilbert *et al*., 1984). One mL of 5% erythrocyte suspension was incubated for 60 minutes at 37°C in presence of 2 mM NaN_3 and 10 mM H_2O_2. The reaction was stopped by addition of 0.5 mL 28% trichloroacetic acid (w/v) in 0.1M $NaAsO_2$. The samples were centrifuged for 10 minutes at 3,000 rpm and 1 mL of the resulting supernatant was added to 0.5 mL TBA (1% in 0.05N NaOH). The samples were heated at 100°C for 15 minutes. After cooling, the absorbance were read at 532 and 453 nm against appropriate blanks. The amount of TBARS was expressed in ng MalonDiAldehyde (MDA) per mg Hb, using a molar extinction coefficient of $1.56×10^5 M^{-1} cm^{-1}$.

The total Glutathione (tGSH) content was determined using a Glutathione Assay Kit according to the manufacturers' instructions. Glutathione caused reduction of 5,5′-DiThiobis-(2-NitroBenzoic) acid (DTNB) to TNB, which was assayed colorimetrically at 412 nm. The values were expressed in ng mg^{-1} Hb.

SuperOxide Dismutase (SOD) activity was determined according to Beauchamp and Fridovich (1971). The samples were illuminated for 4 min with a 250W Hg lamp and the O_2^--provoked nitro blue tetrazolium photoreduction was measured at 560 nm. The results were expressed as U mg^{-1} Hb. A unit of SOD activity was the amount of the enzyme producing 50% inhibition of NitroBlue Tetrazolium (NBT) reduction.

Catalase (CAT) activity was determined by the decrease in absorbance of the samples at 240 nm due to the enzymatic decomposition of H_2O_2 (13.2 mM H_2O_2 in 50 mM phosphate buffer, pH 7.0) (Aebi, 1984). The activity of CAT was expressed as A_{240} min^{-1} mg^{-1} Hb.

Glutathione Peroxidase (GPX) activity was determined according to Günzler *et al*. (1972) by the coupled reaction with glutathione reductase in presence of GSSG and NADPH. The decrease of the absorbance at 340 nm due to the oxidation of NADPH to $NADP^+$ was directly proportional to the GPX activity in the sample. The GPX activity was expressed in U/mg Hb, using a molar extinction coefficient of $6.22×10^6 M^{-1} cm^{-1}$.

Statistical Analysis

The results were expressed as mean±SEM. Statistical analyses were performed using SPSS statistical software (SPSS for Windows, version 16.0, 2008, SPSS Inc., Chicago, USA). Data were analyzed by Analysis Of Variance (ANOVA) and comparisons between treatment groups were performed with the paired Student's t-test. For all statistical procedures performed, a *P*-value< 0.05 was considered significant.

Ethical Rules

All experimental procedures were approved by Animal Ethic Commission at the Agricultural University–Plovdiv.

RESULTS

Egg Production and Egg Quality Traits

The egg laying in the control group started on 128 days (19 weeks) of age (16 November 2015), whereas in the two experimental groups later – on 134 days (20 weeks) of age (21 November 2015). For the entire laying period (up to 55 weeks of age) the average egg-laying rate was 64.90% (163.5 eggs hen^{-1}) for the first group, 67.61% (168.9 eggs hen^{-1}) for the second group and 72.49% (182.2 eggs hen^{-1}) for the third group – the differences between groups were significant at P< 0.05 (Figure 1). Up to 25 weeks of age, egg production increased rapidly regardless of the decrease in average daily ambient temperatures from 12.2 to -0.91ºC. From 25 to 33 weeks of age, egg production exhibited variations with a tendency towards increase, although the average daily ambient temperatures were low (from -6.76 to 9.65ºC). It is acknowledged that modern egg-laying strains attain peak egg production at that age under optimum microclimatic conditions in rearing premises. In free-range conditions

with walking yards, as in this experiment, low or high ambient temperatures do not stimulate, but inhibit, increase in egg production. At 28-29 weeks of age (end of January), the three groups demonstrated a marked reduction in egg production caused by very low ambient temperatures: -6.76 and 1.38ºC on the average for the two weeks. Then followed a period of slow gradual increase in daily temperatures (February, 30-33 weeks of age), which was beneficial for egg production. The decline in average weekly temperatures, especially minimum values, as well as precipitations in March (34-37 weeks of age) resulted in another decrease in egg production in the three groups.

Egg production was optimate during the thermoneutral period (April-May; from 38 to 46 weeks of age), when ambient temperatures started to increase slowly and average weekly values at the end of the period attained 20.49ºC. Slow fluctuations with lower egg production were detected by 42-43 weeks of age, which was associated with more intensive rainfall and colder weather. As age advanced, egg production decreased, which was genetically determined. In our experiment, high temperatures in June and July had a substantial effect on decreasing egg production, as the registered maximum temperatures were over 32ºC. Ambient temperature and precipitations are very powerful environmental factors in the free-range system without maintenance of optimum microclimatic conditions.

For the three periods, egg production in birds from group 1 was 57.68, 82.76 and 63.59% respectively; from group 2– 60.54, 82.34 and 67.30%; and from group 3– 65.92, 87.85 and 70.00%. The results showed clearly that dietary supplementation of Immunobeta® increased egg production, especially during the cold and hot months (P< 0.05 *vs.* control group). The effect of addition of Immunobeta® at a concentration of 0.4% was superior– egg production was higher throughout the entire experimental period, including the thermoneutral period.

The feed consumption per egg was 242.44 g in group 1, 246.80 g in group 2, and 239.57 g in group 3.

The investigation of egg morphometry demonstrated that eggs produced by group 3 were the heaviest, with statistically significant differences *vs*. group 1 (P< 0.05), but not *vs*. group 2 (P> 0.05) (Table 2). The higher egg weight was attributed mainly to the higher albumen weight– with statistically significant differences (P< 0.05) at groups 2 and 3 *vs*. group 1 (control), as well as to shell weight– at group 3 *vs*. both groups 1 and 2 (P< 0.05). There were no significant differences in YW among the groups, although the percentage ratio YW/EW was the highest in eggs produced by group 1 with considerable differences (P< 0.05) *vs*. both groups 2 and 3. The AW/YW ratio was the highest in the eggs of group 3, followed by group 2 and group 1 due to the higher albumen content and lack of significant differences in yolk weight. YC intensity was the highest in group 1 and the lowest in group 3, but the difference was below one unit of the Roche yolk color fan scale. Regarding the EW/EV, SA/EW, SA/EV ratios, there were no statistically significant differences among groups (P> 0.05).

Analysis of data showed that supplementation of the diet with 0.4% Immunobeta® (group 3) increased egg production (with 7.59% *vs*. group 1 and with 4.88% *vs*. group 2) and egg weight (with 1.57 g *vs*. group 1 and with 1.15 g *vs*. group 2) and reduced feed consumption per egg during the production cycle (with 2.87 g *vs*. group 1 and with 7.23 g *vs*. group 2). For all studied periods, egg production in layers from group 3 was higher than that of the other two groups.

Oxidative Stress Parameters

All oxidative status indicators of the animals in the control group were affected by the seasonal variations in ambient temperature.

Table 2. Morphological characteristic of eggs.[a]

Indices			First group (Control)	Second group	Third group
Egg Weight (EW)		g	58.64±0.46 [a]	59.06±0.40 [a]	60.21±0.39 [b]
Shape Index (SI)		%	76.18±0.17 [a]	77.26±0.19 [b]	76.86±0.22 [ab]
Shell Weight (SW)		g	5.66±0.04 [a]	5.67±0.04 [a]	5.96±0.05 [b]
		%	9.71±0.07 [a]	9.63±0.06 [a]	9.91±0.07 [b]
Shell	- Pointed end	µm	326±1.30	325±1.20	328±1.50
Thickness	- Equatorial end	µm	314±1.50 [a]	315±1.30 [a]	320±1.60 [b]
(ST)	- Blunt end	µm	306±1.50 [a]	309±1.50 [a]	315±1.60 [b]
Yolk Weight (YW)		g	16.73±0.21	16.34±0.21	16.30±0.20
		%	28.41±0.24 [a]	27.57±0.26 [b]	26.97±0.22 [b]
Yolk Index (YI)			45.64±0.29	45.57±0.34	45.02±0.33
Yolk Color (YC)			9.60±0.10 [a]	9.26±0.10 [b]	8.91±0.08 [c]
Albumen Weight (AW)		g	36.25±0.29 [a]	37.06±0.27 [b]	37.95±0.24 [c]
		%	61.88±0.22 [a]	62.79±0.26 [a]	63.12±0.21 [b]
Albumen Index (AI)			9.23±0.15	9.48±0.13 [a]	9.00±0.13 [b]
Haugh Unit (HU)			83.56±0.46	84.55±0.36	84.68±0.35
Egg Volume (EV)		cm³	55.10±0.43 [a]	55.33±0.36	56.31±0.38 [b]
Surface Area (SA)		cm²	69.82±0.37 [a]	69.98±0.31	70.82±0.33 [b]
	Ratios				
AW: YW			2.22±0.03 [a]	2.33±0.03 [b]	2.38±0.03 [b]
EW: EV			1.06±0.00	1.07±0.00	1.07±0.00
SA: EW			1.20±0.00	1.19±0.00	1.18±0.00
SA: EV			1.27±0.00	1.27±0.00	1.26±0.00

[a] Values with different superscript within a line differ significantly (P< 0.05).

LPO levels in the thermoneutral period were low and reflected the basal level of free radicals generation in the body (Figure 2). Administration of the immunomodulator did not significantly affect the LPO, although there was a trend towards reduction after application of the higher dose: 0.36±0.05 (group 1) *vs*. 0.31±0.04 (group 3) nmoles MDA mg^{-1} Hb. In the cold period, LPO increased dramatically in group 1 compared to the thermoneutral period. The supplementation of animal feed with the Immunobeta$^®$ led to reduction of LPO: 1.97±0.19 (group 1) *vs*. 1.18±0.18 (group 2) and 0.45±0.06 nmol MDA mg^{-1} Hb (P< 0.05) (group 3), as the higher dose decreased the values close to those reported in the thermoneutral period.

In the hot period, LPO also increased significantly compared to the thermoneutral period. Application of Immunobeta$^®$ did not influence significantly the LPO levels.

With regard to tGSH, the lowest levels were measured during the cold period (Figure 3). Application of the immunomodulator in this period resulted in an increase of the tGSH levels in comparison to the control group: 82.89±2.81 (group 1) *vs*. 97.88±6.65 (group 2) and 122.88±10.48 ng mg^{-1} Hb (P< 0.05) (group 3). In the thermoneutral period, the levels of tGSH rose significantly compared to the cold period. Addition of Immunobeta$^®$ to the feed at the higher dose significantly increased the concentration of tGSH, whereas the low dose had not a significant effect on the levels of tGSH compared to the controls.

In the warm period, the tGSH levels were high and application of the Immunobeta$^®$ further increased them: 631.32±69.93 (group 1) *vs*. 834.04±76.64 (group 2) and
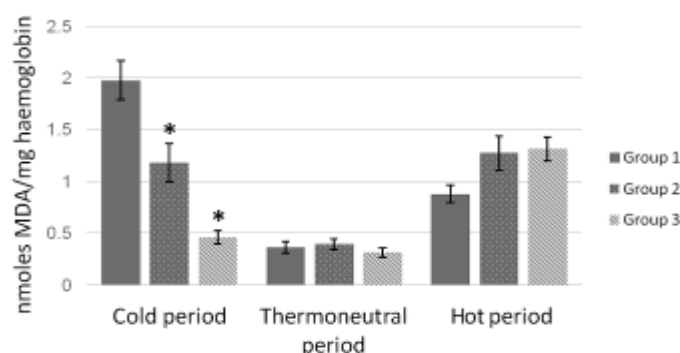

Figure 2. Effect of Immunobeta$^®$ on erythrocyte lipid peroxidation; * *P*< 0.05 *vs*. group 1.
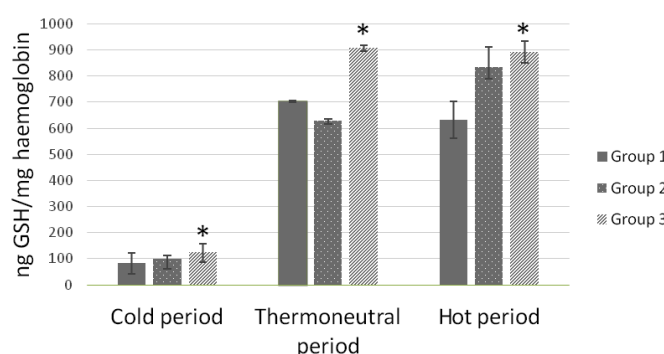

Figure 3. Effect of Immunobeta® on erythrocyte glutathione levels; * P< 0.05 vs. group 1.

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891.92±43.00 ng mg^{-1} Hb (group 3). Significantly higher levels of tGSH were observed when the higher dose was supplied.

The CAT activity in the control group increased along with increase in ambient temperature: 0.39±0.06 (cold period) *vs.* 0.46±0.03 (thermoneutral period) *vs.* 0.58±0.03 (hot period) U mg^{-1} Hb (Figure 4). In the thermoneutral period, only the higher dose of immunomodulator led to reduced activity of the enzyme (0.46±0.039 *vs.* 0.34±0.041 U mg^{-1} Hb). During the cold period, CAT activity did not change significantly with the application of Immunobeta® compared to the control group. In the hot period, supplementation of both doses led to significant reduction of CAT activity: 0.58±0.03 (group 1) *vs.* 0.41±0.03 (group 2) and 0.46±0.033 ΔA_{240} min^{-1} mg^{-1} Hb (P< 0.05) (group 3). The

activity of SOD in the control group increased during both cold and hot periods, compared to that in the thermoneutral period: 2.54±0.23 (cold period) *vs.* 1.75±0.18 (thermoneutral period) *vs.* 3.26±0.27 U mg^{-1} Hb (hot period) (P< 0.05). Immunobeta® supplementation did not significantly affect these changes (Figure 5). Administration of the immunomodulator during the cold period resulted in a tendency to reduction of SOD activity in a dose-dependent manner compared to the controls (2.54±0.23 in group 1 *vs.* 2.24±0.15 in group 2 and 2.01±0.12 in group 3 U mg^{-1} Hb). And during the hot period, only the higher dose application led to a decrease in SOD activity, however, this change was statistically insignificant. The activity of GPX in the control group showed a trend similar to that of SOD activity − an increase in cold and hot periods compared to the
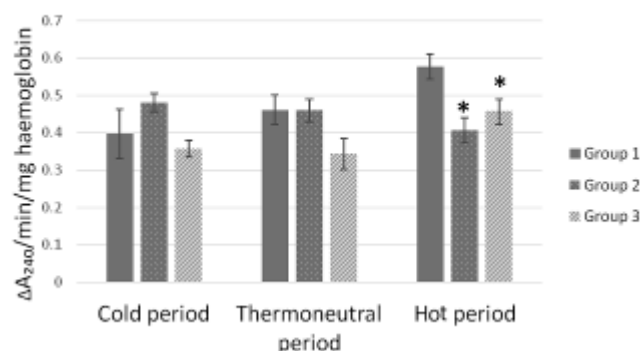

Figure 4. Effect of Immunobeta® on erythrocyte catalase activity; * *P*< 0.05 *vs.* group 1.
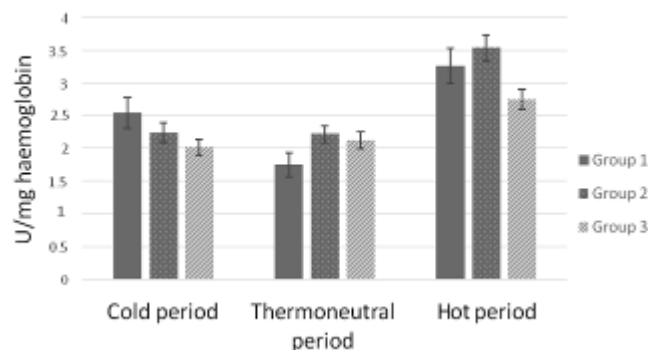

Figure 5. Effect of Immunobeta® on erythrocyte superoxide dismutase activity.

thermoneutral period (Figure 6). In cold period, application of both doses of immunomodulator did not affect significantly the GPX activity compared to that of the control group. During the thermoneutral period, administration of Immunobeta® increased the GPX activity in a dose-dependent manner: 13.50 ± 0.41 (group 1) _vs._ 20.47 ± 0.67 (group 2) and 29.20 ± 0.85 U mg^{-1} Hb (group 3). This trend was preserved in the hot period as compared to thermoneutral period, with much higher values: For the control group, values doubled, for the low dose of Immunobeta®, GPX activity increased by 30% and for the higher dose -10%.

DISCUSSION

Heat stress has been widely recognized as an inducer of OS in birds (Tan _et al._, 2010; Ghazi Harsini _et al._ 2012; Akbarian _et al._, 2016). Our results regarding laying hens, kept in a free-range system, are in line with these observations. We established increased LPO, decreased GSH levels, and rise in antioxidant enzymes activities in hot period, compared to thermoneutral period. In broiler chickens, elevated LPO in muscles (Ghazi Harsini _et al._, 2012), as well as elevated LPO and protein oxidation in liver and serum (Tan _et al._, 2010) has been reported after acute exposure to different high temperatures in experimental conditions. Ramnath _et al._ (2008) reported decreased

serum GSH concentration in heat stressed chickens (for both 5 and 10 days) in comparison to non-stressed birds. Depletion of the GSH is a typical consequence of OS, because of its high reducing power, ability to react directly with ROS, and function as a cofactor of the glutathione enzymes (Manjula, 2015). In regards to antioxidant enzymes, Ghazi Harsini _et al._ (2012) reported enhanced SOD activity in the pectoralis muscle of broiler chickens after exposure to heat stress and Tan _et al._ (2010) found increased activities of SOD and GPX in serum and liver after 3 h of exposure of broilers to high ambient temperature. Such observations allowed Akbarian _et al._ (2016) to conclude that, after acute heat stress, the activities of antioxidant enzymes (CAT, GPX and SOD) were typically increased to protect cells against excess superoxide formation.

According to our results, the low ambient temperature could induce OS in the birds even to a stronger extent than heat stress did: in control group, LPO increased fivefold as compared to the thermoneutral period and twice _vs._ the hot period and tGSH level was ten times lower compared to those measured in the thermoneutral and hot periods. Our findings are in line with literature data, which showed elevated MDA concentrations induced by low ambient temperatures (Akşit _et al._, 2008; Ramnath and Rekha, 2009; Yang _et al._, 2014) and a significant decrease in blood GSH in cold stressed chickens when compared to unstressed groups
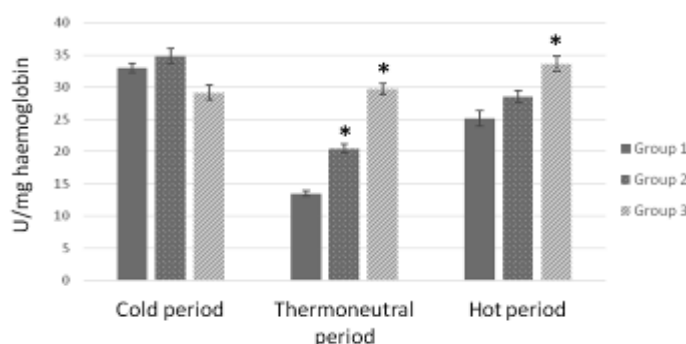

Figure 6. Effect of Immunobeta® on erythrocyte glutathione peroxidase activity; * _P_< 0.05.

(Ramnath and Rekha, 2009). Similar to the hot period, we observed higher antioxidant enzymes during the cold period.

Oxidative stress at the subcellular and cellular levels in turn affects the physiology of the organism. Researchers have shown that prevention of OS by antioxidants could improve bird status and performance (Akşit *et al*., 2008; Ghazi Harsini *et al*., 2012). Application of Immunobeta® in this study reduced OS, probably due to the activation of antioxidant enzymes. There are data that *β*-glucans increase SOD and CAT activities and prevent decrease of GPX activity in different models of pathological conditions with OS ethology (Ceyhan *et al*., 2012; Zeng *et al*., 2018). Furthermore, a direct scavenger effect of *β*-glucan against hydroxyl radicals has been established in in vitro experiments (Kofuji *et al*., 2012; Giese *et al*., 2015).

The physiological consequences of temperature stress are numerous and along with many other alterations include a depressed immunity (Akbarian *et al*., 2016; Kamel *et al*., 2017). Therefore, application of an immunomodulator could improve the avian welfare. Cox *et al*. (2010) suggested that *β*-glucans may enhance avian immunity altering the cytokine-chemokine balance. *β*-glucans by themselves did not cause a strong immune response and, therefore, did not affect the performance negatively. Further, it has been found that the primary and secondary lymphoid organs in broiler chicken enlarged under the action of *β*-glucan supplementation, so, it could improve several baseline immune responses (Guo *et al*., 2003).

The Immunobeta® supplement used by us along with its immunostimulatory effect is probiotic. It has been shown that the inclusion of probiotics in the diet improved significantly the egg quality (Inatomi, 2016). The improved intestinal environment, immune function and antioxidant potential were also found (Inatomi, 2016).

CONCLUSIONS

The dietary supplementation with Immunobeta® affected the erythrocyte OS, induced by changes in ambient temperature. The immunomodulator supplementation resulted in decrease of LPO during the cold period and increase of glutathione level during the cold and especially the hot period. The modulation of antioxidant enzyme activities reflected on OS level and the adaptation of the organism. The alterations in the pro-/antioxidant balance are an important regulator of animal response to stress conditions. The inclusion of 0.4% Immunobeta® in diet increased egg production, egg weight, and the respective albumin weight, shell weight, and feed conversion ratios. Overall, from the results of this study, it could be concluded that supplementation with appropriate dose of Immunobeta® exerted a beneficial effect on the welfare of layer hens reared freely in yards.

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اثرمکمل رژیم غذایی ®Immunobeta روی تولید تخم مرغ و برخی پارامترهای تنش اکسیداتیو در مرغ های تخمگذار

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چکیده

هدف این پژوهش بررسی ارزیابی اثر یک محرک ایمن سازی به نام ®Immunobeta روی تولید تخم مرغ و برخی پارامترهای تنش اکسیداتیو در مرغان تخمگذار در محوطه باز (free range laying hens) تحت تاثیر شرایط محیطی بود. در مجموع، ۸۱ مرغ تخمگذار و ۹ خروس (Tetra Super Harco) به سه گروه دسته بندی شد: گروه ۱(شاهد) بدون مکمل غذایی، گروه ۲ با ۰/۲٪ و

گروه۳ با ۰/۴٪ مکمل رژیم غذایی ®Immunobeta. دوره تخمگذاری (سن ۵۵-۱۹ هفتگی) به سه دوره بر اساس درجه حرارت محیط تقسیم شد: دوره سرد(از نوامبر تا مارچ ۲۰۱۵ در سن ۱۹ تا ۳۷ هفتگی)، دوره ترمونوترال (آوریل و می ۲۰۱۶، در سن ۳۸ تا ۴۶ هفتگی)، و دوره گرم(ژون تا ژوئیه، از ۴۷ تا۵۵ هفتگی). دُز ®Immunobeta به نسبت باعث بهبود میانگین تولید تخم مرغ و میانگین وزن تخم مرغ شد و در دُز ۰/۴٪ به طور معناداری برخه تبدیل غذایی را در همه دوره های تخمگذاری کاهش داد. مکمل رژیم غذایی ®Immunobeta پارامترهای تنش اکسیداتیو خون را تحت تاثیر قرار داد و سطح پراکسیداسیون چربی را در دوره سرد به طور معناداری کم کرد، سطح گلوتاتیون را در مرغ هایی که دُز ۰/۴٪ مکمل را دریافت کرده بودند افزایش داد، فعالیت کاتالاز را در دوره گرم تنظیم کرد، و فعالیت گلوتاتیون پراکسیداز را در دوره ترمونوترال و گرم افزایش داد. نتیجه اینکه افزودن ®Immunobeta به رژیم غذایی باعث کاهش تنش اکسیداتیو ناشی از تنش گرمایی شد وعملکرد مرغان تخمگذار در محوطه باز را ارتقا داد.