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EFFECT OF BETAINE AND AIR AMMONIA CONCENTRATION ON BROILER PERFORMANCE, PLASMA CORTICOSTERONE LEVEL, LYMPHOID ORGAN WEIGHTS AND SOME HAEMATOLOGICAL INDICES

D. Gudev[1], S. Popova-Ralcheva[2], I. Ianchev[1], P. Moneva[1]

[1]Institute of Animal Science, 2232 Kostinbrod, Bulgaria
[2]Institute for Information Serving of the System, Sofia, Bulgaria
Corresponding author: pv_moneva@abv.bg
Original scientific paper

Abstract This study was initiated to examine the effect of betaine supplementation (1g/kg) on hematocrit level, erythrocyte and leukocyte number, heterophil/lymphocyte ratio, plasma corticosterone, rectal temperature, relative lymphoid organ weights and average daily gain in broiler chickens reared in a poultry house under natural ambient conditions and high air ammonia level. A total of 100 broiler chickens at the age of 45 d were allocated into 2 groups: control and betaine supplemented. The broilers were kept on deep litter in windowless poultry house. The results of this study indicated no significant changes in terms of hematocrit, erythrocyte number, leukocyte number, lymphoid organ weights and average daily gain.Betaine supplemented broilers had lower heterophil/lymphocytes ratio at d8 ($P<0.05$) relative to control broilers inspite of the similar values of plasma corticosterone between the groups. Plasma corticosterone concentration in betaine supplemented broilers increased ($P<0.05$) at d 15, relative to d8. The observed increase in plasma corticosterone was not accompanied by increase in heterophil to lymphocyte ratio. These results are interpreted to suggest that the decreased heterophil/lymphocyte ratio in experimental broilers was due to the modulatory effects of ammonia on NO synthesis rather than to the modulatory effect of corticosterone.

Key words: betaine, ammonia, hematocrit, erythrocyte, heterophil/lymphocyte ratio, relative lymphoid organ weights, rectal temperature

Introduction

Dietary betaine supplementation has been found to have beneficial effect in broiler chickens exposed to osmotic disturbance *(Honarbakhsh et al., 2007a,b)*.

Betaine is involved in protein and energy metabolism due to its methyl group donor function *(Eklund et al., 2005)*. Observations on the biological effect of betaine revealed that betaine may have the potential to improve the digestability of specific nutrients *(Eklund et al., 2006a,b)*. It is involved in the osmoregulation of duodenal epithelium of broiler chicks and affects the movement of water across the small intestinal epithelium in vitro *(Kettunen et al., 2001)*. Furthermore, betaine has been shown to increase nitric oxide (NO) and tissue factor pathway inhibitor *(Iqbal et al., 2006; Messadek, 2010)*. Supplemental dietary betaine improved weight gain and feed conversion in some poultry studies *(Mthews and Southern, 2000; Hassan et al., 2005)*, whereas other studies showed minimal or no effect of betaine on animal performance *(Zulkifi et al., 2004; Feng et al., 2006)*.

These controversial results are attributed to the occurrence of osmotic stress and the concentration of methyl group donor in the diet. Atmospheric ammonia has been reported to have adverse effect on poultry health and performance *(Kristensen and Wathes, 2000)*. Furthermore, airborne ammonia was found to affect peritoneal macrophages, spleen, lymphocytes, heat shock proteins and NO production *(Parfenyuk et al., 2010)*.

The objective of this study was to investigate the combined effect of supplemental dietary betaine and air ammonia concentration on hematocrit, erythrocyte number, leukocyte number, plasma corticosterone, white blood cell differential count, rectal temperature, some internal organ weight and average daily gain in commercially raised chickens under summer temperature and air ammonia fluctuation.

Materials and Methods

The experiment comprised 100 broiler chickens at 45 d of age randomly allocated into two groups consisting of 50 broilers each. All birds were fed commercial broiler feed throughout the trial which lasted 17 days. The experimental group unlike the control group, was given supplemental dietary betaine (1g/kg). Fresh water was supplied ad libitum. The birds were reared under summer conditions and variable natural temperatures. The indoor temperature readings were taken in the morning (8.30 am) and afternoon (14.00 h) throughout the experimental period. Air ammonia was measured by Aeroqual ammonia monitor model S-200, relative humidity was measured by psychrometer. Both indices were registered at d 1, 3, 8, and 15 of the experimental period at about 0.3 m above the litter. The following data were collected during the experimental period: body weight- measured at the start and again at the end of the trial. Rectal temperature was measured at d 3, 8 and 15, by digital thermometer. Venous blood samples were collected by brachial vein puncture at d 8 and 15 between 10 and 12 am. Hematocrit, leukocyte and erythrocyte count were determined at d 8 and 15.

Total erythrocyte and leukocyte count was determined by manual haemocytometer chamber count. Hematocrit was determined by centrifuging heparinized blood in a capillary tube. Peripheral blood leukocytes were counted on smears. Two drops of blood were taken via brachial vein puncture, 1 drop being smeared on each of two glass slides. The smears were stained using May-Grunvald and Gimsa stains *(Lucas and Jambros, 1961)*. Two hundred leukocytes including heterophils, eosinophils, basophils, lymphocytes and monocytes were counted microscopically on 1 slide of each bird.

Plasma corticosterone was determined using enzyme immunoassay kit (IBL (IBL gesellchaft fur immunchemie und immunbiologie, MBH, D 22335 Hamburg, Germany).

A total of 14 broilers (seven birds from each group) were randomly selected and weighted at d 17. Then the birds were slaughtered. Lymphoid organs (thymus, spleen, bursa of Fabricius) as well as liver, testicals and adrenal glands were separated and weighted. Organ-to-body ratios were calculated. Average daily gain was calculated at the end of the experimental period by substracting initial weight from final weight. The results are expressed as means ± SEM and were analyzed by ANOVA.

Results and Discussion

Hematocrit levels at d 8 were similar in both groups of broilers. This is not surprising because ammonia level at that time was within the permitted range (Table 1) and it is widely accepted that the positive effect of betaine on animal performance is more pronounced in animals, reared under osmotic stress *(Klassing et al., 2002; Honarbanhsh et al., 2007)*. Besides air temperature (Figure 4) was within the normal range. Hematocrit level in betaine supplemented birds tended to be higher ($P>0.05$) at d 15 relative to d 8 (Figure 1) despite the unchanged erythrocyte number (Figure 2). The observed ammonia level at that day was 10 times higher than the level at d 8 (Table 1). It has been reported that exposure to large doses of ammonia increases erythrocyte membrane permeability. The authors suggested that because of the large erythrocyte mass these cells carry a significant fraction of the total blood ammonia *(Labotka et al., 1995)*. Furthermore, ammonia increases intracellular osmolarity evoked by the elevation of glutamine as a consequence of the enchanced metabolic activity associated with ammonia detoxification in glioma cells *(Zwingmann et al., 2000)*. Also it increases nitric oxide production which in turn reduces the capacity of glutamine synthetase, thus decreases the rate of ammonia elimination *(Monfort et al., 2002)*. Increases in osmolarity stimulate betaine uptake in rat kidney medulla via betaine transporters *(Lohr et al., 1991)*. Consequently, supplementation with betaine might have assisted erythrocyte to tolerate the high osmolarity, by increasing erythrocyte

volume. It has been demonstrated that betaine increases the cytoplasmic volume and free water content of the cells at high osmolarity and thus permit cell proliferation under stress conditions *(Csonka, 1989)*. Also, betaine serves as a stabilizer of cell organelles against the denaturing effects of high ionic strength *(Kempf and Bremer, 1998)*. In our previous experiment, which had the same experimental design and dynamics of air ammonia we found lower erythrocyte number accompanied by increased hematocrit level in laying hens given supplemental betaine (1.5 g/kg) for 22 days (in press) which is in agreement with the current results and suggest that betaine modulates erythrocyte volume in birds reared under high ammonia level. Higher hematocrit and hemoglobin levels have also been reported in broilers chickens reared up to 35 and 21 d of age under graded concentrations of anhydrous NH3 *(Olanrevaju et al., 2008)*. Our data concerning hematocrit values in the control chickens showed slightly higher level of hematocrit on d 15 ($P<0.05$) when ammonia concentration was within 27.8-84.2 ppm. The lack of significance could be explained with the fact that the experiment was initiated when the chickens reached 48 d of age and probably were at least partly adapted to the increased air ammonia. It has been reported that different aged birds respond differently to NH3 concentrations of 75 ppm- younger birds being more responsive to the NH3 than older birds *(Olanrevaju, 2009)*. However, erythrocyte number in control birds tended to be lower at d 15 (Figure 2) relative to that at d 8, despite the observed trend of hematocrit increase and indicate a possible increase of erythrocyte volume. It is worth to note that the observed trend of erythrocyte volume enhancement occurred against the background of higher hematocrit ($P>0.05$) and erythrocyte number in betaine supplemented chickens at d 8 and 15 relative to their control counterparts. Therefore it could be assumed that betaine improved oxygen binding capacity which has been reported to be lower in broiler chickens reared under high air ammonia levels *(Olanrevaju et al., 2009)*.

Table 1. Indoor air ammonia and relative humidity measured four times per day between 11.00 and 15.00 h

	Days through the experiment			
	1 day	3 day	8 day	15 day
Air ammonia range (ppm)	9.2 – 23.8	23.6 – 58.0	5.2 – 7.1	27.8 - 84.2
Relative humidity (%)	61 – 62	56 -59	70 – 76	69 – 71

Table 2. Relative weight of liver, adrenal glands, testicles and some lymphoid organs (n=7)

Items	Groups			
	Control		Betaine supplemented	
	Mean	SEM	Mean	SEM
Adrenal gland, %	0.012	0.0008	0.012	0.002
Spleen, %	0.22	0.02	0.19	0.03
Bursa of Fabricius, %	0.1	0.02	0.12	0.02
Thymus, %	0.2	0.03	0.22	0.04
Testes, %	0.02	0.0023	0.035	0.007
Liver, %	2.12	0.1	2.15	0.23

Values are means ± SEM

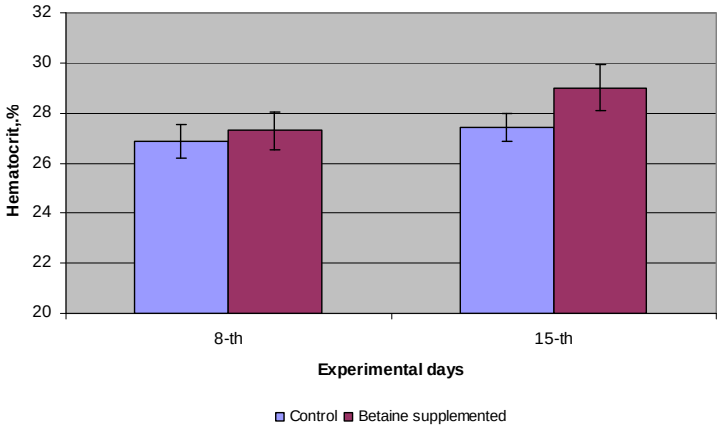

Figure 1. Hematocrit in broilers fed betaine supplemented diet

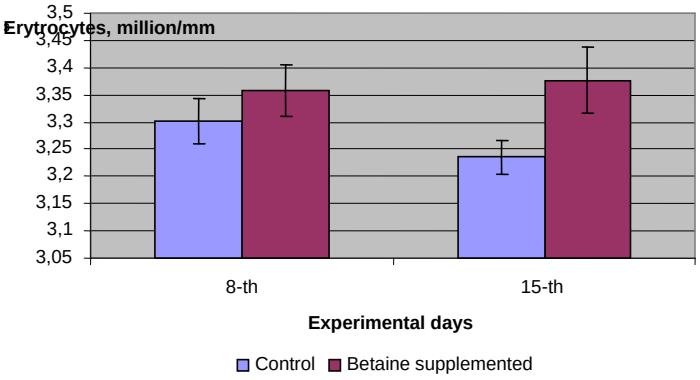

Figure 2. Erythrocyte number in broilers fed betaine supplemented diet

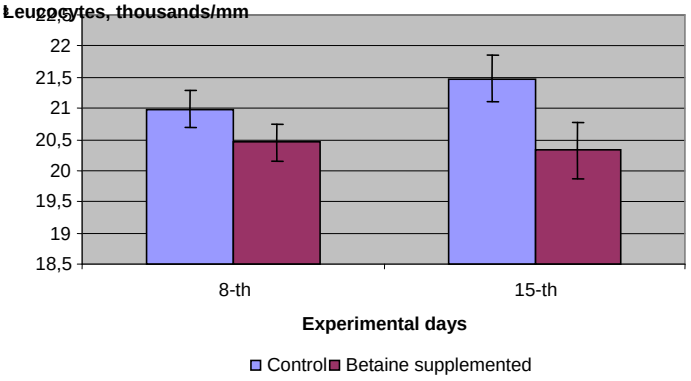

Figure 3. Leukocyte number in broilers fed betaine supplemented diet

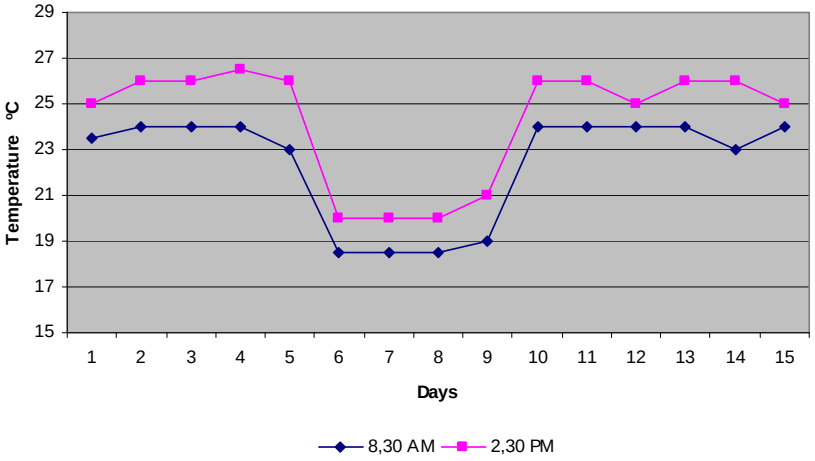

Figure 4. Indoor temperature fluctuation measured throughout the experimental period

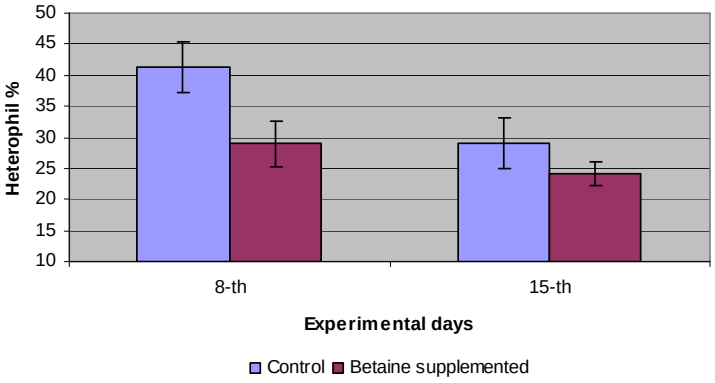

Figure 5. Effect of betaine on blood heterophil percentage in broilers kept indoor under natural summer temperatures

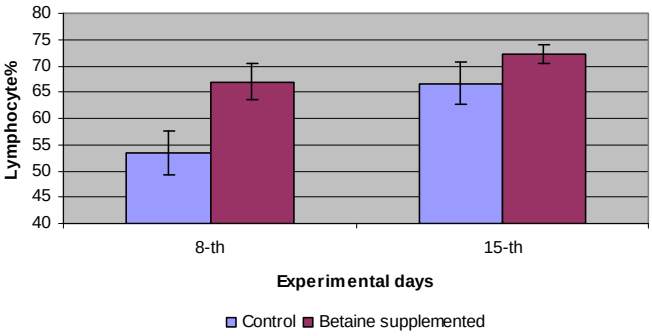

Figure 6. Effect of betaine on blood lymphocyte percentage in broilers kept indoor under natural summer temperatures

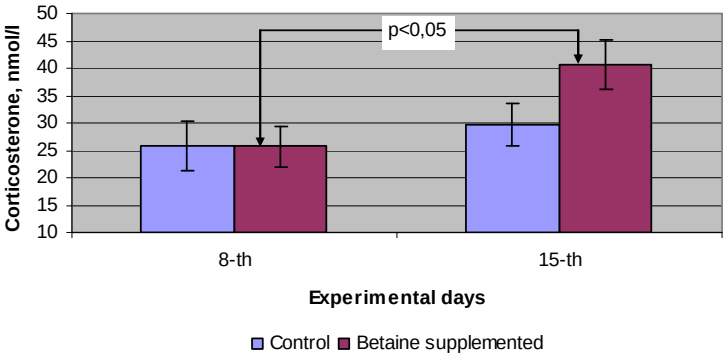

Figure 7. Effect of betaine on plasma corticosterone level in broilers kept indoor under natural summer temperatures

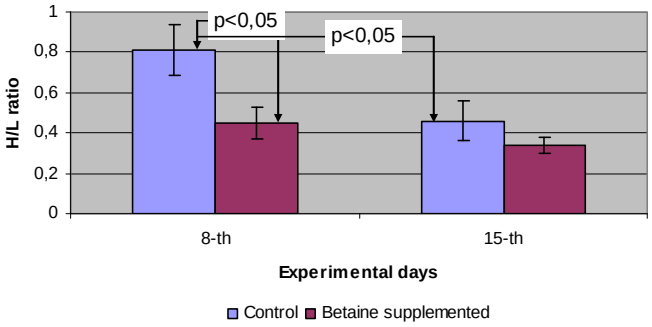

Figure 8. Effect of betaine on heterophil to lymphocyte ratio in broilers kept indoor under natural summer temperatures

There were no significant differences in the percentage of heterophils and lymphocytes at d 8 and 15. (Figure 5, Figure 6). However the percentage of heterophils in the control birds were higher than that in betaine supplemented broilers at d 8 ($P>0.05$), when ammonia concentration was relatively low as compared to that at d 15. The observed difference in the percentage of heterophils in control broilers is further supported by the registered higher heterophil/lymphocyte ratio ($P<0.05$) in the control broilers relative to betaine supplemented broilers at d 8 (Figure 8). Heterophil to lymphocyte ratio (H/L) has long been recognized as a good indicator of stress *(Gross and Siegel, 1983; Maxwell, 1993)*. Also it is widely accepted that hormones released by the adrenal glands in response to stress increase H/L ratio *(Dhabhar et al., 1995)*. However, plasma corticosterone levels in the control and experimental broilers at d 8 had similar values (Figure 7). On the contrary, betaine supplemented broilers had higher ($P<0.05$) corticosterone level at d 15 relative to d 8, but the registered H/L ratio at d 15 tended to be lower than that at d 8. The observed discrepancy between plasma corticosterone level and H/L ratio in both groups suggest that corticosterone in our case is not the only underlying cause of change in H/L ratio. Therefore, we should consider another candidate mediator of the observed change in H/L ratio. The most probable candidate for mediator of H/L ratio seems to be Nitric oxide (NO). It has been reported that nitric oxide synthase (NOS) isoforms are expressed in eosinophils, lymphocytes, monocytes and neutrophils *(Weinberg, 1998; Dambaeva et al., 2003)*. Also it was found that exposure of mice to NOS inhibitors increases the number of neutrophils and leukocytes in the peripheral blood and decreases the fractions of lymphocytes and monocytes *(Geffner et al., 1995; Frutos et al., 2001)*.

The very fact that adrenal hormones exert the same effect as that of NO on leukocyte subpopulations distribution suggests that corticosterone- induced enhancement of H/L ratio could be mediated by NO. This hypothesis is supported by the reported suppression of NO production by the ceca of adrenocorticotropic hormone treated broilers *(Monticha, 2007)*. Our view is further supported by the established inhibitory effect of dexamethasone on NO production by cultured astrocytes *(Llanos and Roldán, 1999)*. Furthermore, hydrocortisone and dexamethasone were found to block nitric oxide production by microglia *(Chang and Liu, 2000)*. It is important to note that dexamethasone exerted its effect on one particular isoform of NOS known as inducible NOS *(Katsuyama et al., 1999; Korhonen et al., 2002)*. This finding coincides with the fact that phagocytes possess the same isoform of NOS. According to *Katsuyama et al. (1999)* glucocorticoids act on multiple levels to regulate inducible NO expression depending on cell types. Betaine, like corticosterone acts as a modulator of nitric oxide synthesis *(Messadek, 2010)*. Ammonia has also been implicated in the control of NO synthase activity (Swamy et al., 2005). Gaseous ammonia was found to increase neutrophil percentage in stress during transport by ship *(Phillips et al., 2010)*. Moreover, NO synthase activity was reported to increase in all regions of brain in acute ammonia toxicity *(Swamy et al., 2005)*. Therefore, the decreased H/L ratio and leukocyte number in the experimental relative to control broilers was probably related to betaine accumulation and its specific effect on nitric oxide isoforms exerted at multiple levels. Furthermore, nitric oxide inhibits glucocorticoid synthesis *(Monau et al., 2010; Cymerang et al., 1999)*. Consequently, it could be assumed that the high air ammonia concentration might have exerted inhibitory effect on adrenal function. The observed discrepancy between plasma corticosterone levels and H/L ratio in control and experimental broilers at d 8 could also be attributed to the specific effect of betaine on NO production in glucocorticoid synthesizing cells. This assumption is consistent with the reported unchanged levels of corticosterone in broiler chickens reared under graded levels of ammonia *(Olanrevaju et al., 2008; Olanrevaju et al., 2009)*. Ammonia level in poorly ventilated poultry houses has been shown to reduce plasma total triiodthyronin *(Fidanci et al., 2010)* and hypothyroid Japanese quails exposed to confinement stress had blunted adrenal response *(Weigel, 2007)*. Similarly, stress induced increases in corticosterone were dampened in neural NOS knockout mice *(Bilbo et al., 2003)*. The possible involvement of neural NOS in the regulation of the neuroendocrine stress response is substantiated by the finding that neural NOS is highly expressed by cells of the hypothalamic-pituitary-adrenal axis *(Hori et al., 2005; Orlando et al., 2008)*. Also increased expression of NO mRNA and neural NOS- positive neurone has also been reported in the rat hypothalamus after 30 or 60 min of restrain *(Hori et al., 2005)*. Moreover, in our previous study with laying hens reared under similar dynamics of air ammonia concentration we found significantly lower H/L ratio in the hens fed 1.5 g/kg supplemental dietary

betaine, despite of the unchanged corticosterone level at that time (in press). The lack of relation between plasma corticosterone and H/L ratio gives further support to our hypothesis, regarding the possible mediatory role of NO on betaine and/or ammonia-induced change in H/L ratio. Adrenal weights in both groups were similar (Table 2) and support once more our assumption that the increased H/L ratio in control birds at d 8 was not due to increased adrenal activity. It is well known that glucocorticoids induce growth retardation of lymphoid organs which ultimately leads to reduction of their relative weights *(Post et al., 2003; Shini et al., 2008)*. Several studies reported that relative lymphoid organ weight decrease with increasing ammonia concentration *(Kling and Quarles, 1974; Wang et al., 2010)*. Supplemental betaine increased insignificantly testes weight. There was no difference in the relative weight of lymphoid organs between the groups. However, it is important to note that betaine supplemented broilers had higher testes weight, which approached the lowest level of significance. It is known that sexual hormones induce involution of lymphoid organs *(Ansar et al., 1985)*. Consequently, it could be assumed that betaine supplemented broilers might have produced higher level of testosterone which is known to contribute to the involution of lymphoid organs. The similarity between lymphoid organ weights in both groups despite the expected lower weight in betaine supplemented birds could be attributed to the pronounced inhibitory effect of ammonia on the growth of lymphoid organs *(Kling and Quarles, 1974; Wang et al., 2010)*.

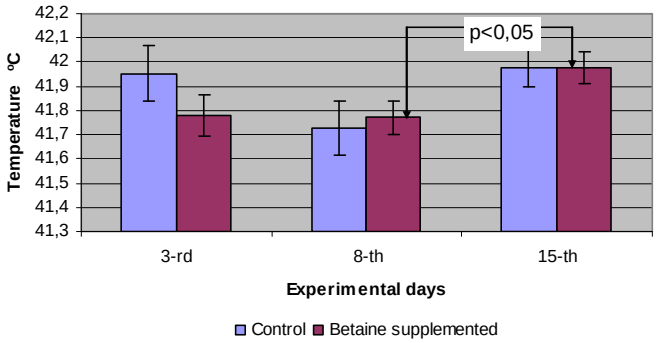

Figure 9. Rectal temperature in control and betaine supplemented broilers kept indoor under natural summer temperatures

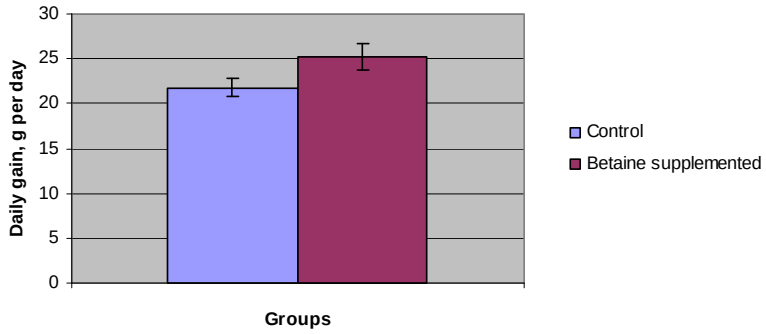

Figure 10. Average daily gain in broilers fed betaine supplemented diet for 15 days

Rectal temperature tended to be lower in betaine supplemented chickens at d 3 when ambient temperature was 25,5 ^{0} C (Figure 9) . It declined in both groups at d 8, when the temperature was within the thermoneutral zone and increased significantly ($P<0.05$) at d 15 in comparison with that at d 8. Betaine has been reported to decrease body temperature in rabbits (Hassan et al., 2011) and slow growing chickens *(Attia et al., 2008)* reared under high ambient temperature.

Nitric oxide has also been reported to be effective as a central modulator of temperature regulation *(Gerstberger, 1999)*. Bearing in mind that both ammonia and betaine are modulators of nitric oxide production we assume that the pattern of rectal temperature response to ambient temperature was modulated by the interactive effects of ammonia and betaine mediated by NO.

Supplemental dietary betaine increased the average daily gain which approached significance at the 5% level (Figure 10). Lower growth rate has been reported in broilers chickens reared under graded levels, of air ammonia *(Wang et al., 2011)*. Our data suggest that supplemental betaine reduces the negative effect of ammonia on growth rate.

Conclusion

Dietary betaine supplementation (1g/kg) decresed H/L ratio at d 8 ($P<0.05$) and d 15 ($P>0.05$) in broiler chickens reared under high air ammonia. Supplemented betaine increased plasma corticosterone level ($P<0.05$), testes weight ($p>0.05$) and average daily gain ($P>0.05$).

Uticaj betaina i koncentracije amonijaka u vazduhu na proizvodne rezultate brojlera, nivo kortikosterona u plazmi, teržinu limfoidnih organa i neke hematološke indikatore

D. Gudev, S. Popova-Ralcheva, I. Ianchev, P. Moneva

Rezime

Studija je obavljena da bi se ispitao uticaj suplementacije betaina (1g/kg) na vrednost hematokrita, broj eritrocita i leukocita, odnos heterofila prema limfocitima, nivo kortikosterona u plazmi, rektalnu temperaturu, relativnu težinu limfoidnih organa i prosečan dnevni prirast kod brojlerskih pilića gajenih u živinarnicima u prirodnom ambijentu sa visokim nivoom amonijaka u vazduhu. Ukupno 100 brojlerskih pilića uzrasta 45 dana podeljeno je u dve grupe: kontrolnu i betain sumplementiranu. Brojleri su držani na dubokoj prostirci u živinarniku bez prozora. Rezultati studije pokazuju da nije bilo značajnih promena u vrednostima hematokrita, broju eritrocita i leukocita, relativnoj težinu limfoidnih organa i prosečnom dnevnom prirastu.

Betain suplementirani brojleri imali su niži odnos heterofila prema limfocitima 8. dana ($P<0.05$) u odnosu na kontrolnu grupu brojlera uprkos sličnim vrednostima plazma kortikosterona između grupa. Koncentracija plazma kortikosterona kod betain suplementiranih brojlera se povećala ($P<0.05$) 15. dana u odnosu na 8. dan. Utvrđeni porast plazma kortikosterona nije bio praćen povećanjem odnosa heterofili – limfociti. Ovi rezultati ukazuju da je smanjenje odnosa heterofili – limfociti kod eksperimentalnih brojlera posledica modulatornog efekta amonijaka na NO sintezu pre nego modulatornog efekta kortikosterona.

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