

**THE INFLUENCE OF LONG TERM SOUND STRESS ON THE BLOOD LEUKOCYTE COUNT, HETEROPHIL/LYMPHOCYTE RATIO AND CUTANEOUS BASOPHIL HYPERSENSITIVE REACTION TO PHYTOHEMAGGLUTININ IN BROILER CHICKENS**

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*We investigated the influence of long term sound stress on some functions of the immune system in "HYBRO" broiler chickens. It was documented that this type of stress leads to a decreased blood leukocyte count, increased heterophyl/lymphocyte ratio and decreased cutaneous hyper-sensitivity to phytohemagglutinin. All observed phenomena indicated the existence of immunosuppression due to the long term sound stress. The age of the birds and duration of the treatment were important factors in the stress reaction.*

*Key words: stress, immune system, chickens*

**INTRODUCTION**

Stress can be defined as the set of responses to external demands upon animals to adapt to a new or abnormal situation. All stimuli that have a tendency to affect physiological balance i.e. homeostasis are called stressors and they generally can be divided into physical, chemical and psychophysical agents (Khasari et al. 1990). Freeman (1987) suggested a more detailed classification of common stress sources. He grouped stress factors into the following categories: 1. Climatic stress (extreme heat and cold, high humidity), 2. Environmental stress (bright light, wet litter, poor ventilation) 3. Nutritional stress (shortages of nutrients, feed intake problems), 4. Physiological stress (rapid growth, sexual maturation), 5. Physical stress (catching, immobilization, injections, transport) 6. Social stress (overcrowding, poor body weight, uniformity) and 7. Psychological stress (fear, harsh caretakers). According to this classification, sound stress is at the same time psychological and environmental. A strong need for better understanding of stressor influence in domestic animals lies in the fact that acute, and more often, long-term stress can affect productivity as well as state of health. There is a voluminous scientific literature about the influence of stress factors on immune system functions both in experimental (Batuman et al. 1990, Moynihan et al. 1990, Jain et al. 1991, Bonneau et al. 1991, Dimitrijević et al. 1994, Theoharides et al. 1995, Dhabhar et al. 1995, Dhabhar and McEwen 1996) and domestic animals

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(Regnier and Kelley 1981, Kelley et al. 1982, Golemboski et al. 1994, Zulkifli and Siegel 1995, Eckel et al. 1995, Hicks et al. 1998). It is well known that chronic or long-term stress depresses the activity of body defense mechanisms in poultry, including antibody synthesis, leading to an increased susceptibility to infection (Siegel 1985).

Recently, sound stress has attracted much scientific attention because noise is a stressor that sometimes is extremely difficult to avoid under farm conditions. McFarlane et al. (1989a) investigated the influence of continuous noise, in combination with other stressors, in poultry and concluded that in nearly all cases (combinations), stress lowered body weight gain and increased food consumption. On the other hand, continuous noise alone did not influence hematological parameters. The same group of authors (McFarlane et al. 1989b) reported that in poultry, multiple stress influenced hematological values and changed the biochemical composition of meat. Interestingly, Gross and Siegel (1983) as well as McFarlane and Curtis (1989) showed that heterophil/lymphocyte ratio is a better indicator of stress than plasma corticosterone concentration. Gross (1990) investigated the influence of acute sound stress on chickens and found that heterophil/lymphocyte ratio was increased 18 hrs after the stress but returned to basic values 30 hrs later. The cutaneous hypersensitivity reaction (CBHR) is a widely applied test indicator of changes in the cellular immune response following exposure to various stressors. The degree of reaction depends of the applied stressor and the duration on exposure (Eckel et al. 1995).

One of the basic mechanisms that are activated during stress is increased secretion of CRH (Corticotiberin) from the hypothalamic neurons and consequently increased secretion of ACTH (Corticotropin) from the adenohypophysis. This leads to stimulation of the adrenal cortex and increases glucocorticosteroid levels in plasma. Glucocorticosteroid hormones influence numerous functions of the immune system and also have detrimental effects on body weight gain, reproduction and health status (Beck 1991). Gross and Siegel (1981) reported that in broiler chickens long term social stress resulted in decreased body weight, together with atrophy of bursa Fabricii, spleen and thymus. Exogenous application of corticosterone had similar effects (Gross et al 1979). Isobe and Lillehoj (1992) reported that exogenous corticosterone decreased T - lymphocyte count, NK cell count, lymphokine and interferon production in chickens.

The aim of our investigation was to estimate the influence of long term sound stress on the blood leukocyte count, heterophil/lymphocyte ratio and cutaneous hypersensitivity reaction (CBHR) to phytohemagglutinin (PHA) in HYBRO broiler chickens.

#### MATERIALS AND METHODS

The experiments were conducted on 90 HYBRO broiler chickens, divided into nine groups (each group consisting of 10 birds) as follows:

E 1-15 chickens exposed to the sound stress from 1<sup>st</sup> to 15<sup>th</sup> day of life

C 1-15 control (non exposed) group

- E 1-30 chickens exposed to the sound stress from 1<sup>st</sup> to 30<sup>th</sup> day of life
- E 15-30 chickens exposed to the sound stress from 15<sup>th</sup> to 30<sup>th</sup> day of life
- C 1-30 control group
  
- E 1-45 chickens exposed to the sound stress from 1<sup>st</sup> to 45<sup>th</sup> day of life
- E 15-45 chickens exposed to the sound stress from 15<sup>th</sup> to 45<sup>th</sup> day of life
- E 30-45 chickens exposed to the sound stress from 30<sup>th</sup> to 45<sup>th</sup> day of life
- C 1-45 control group

The chickens were kept in two different buildings under the same conditions of temperature, light, humidity and number of animals per m<sup>2</sup>. They were fed *ad libitum* and had free access to water. According to the experimental schedule chickens were moved from the building without noise to the building where they were subjected to sound stress. The animals were exposed to sound stress in the sound attenuated building using a fire alarm bell (95 dB) and stress sessions lasted 120 min. every day. The bell was programmed to ring for 5 sec in a variable interval schedule (5 to 115 s) through signals generated by PC software.

Every 15 days, blood samples were taken by cardiac puncture for analysis and the test of cutaneous basophil hypersensitivity to PHA was performed on the same day. Leukocyte count was estimated by diluting blood samples (20 x) with Shaw's solution (crystal violet 10 mg, Na - citrate 3.8 g, formaldehyde 35% 0.4 ml, H<sub>2</sub>O to 100 ml) and using a Spencer type hemocytometer. The heterophil/lymphocyte ratio was calculated from the differential white blood cell count following examination of blood smears stained by the May-Grunwald Giemsa method. The test for cutaneous basophil hypersensitivity reaction was performed by intradermal inoculation of 100 µg PHA (INEP, Zemun) dissolved in 0.1 ml of phosphate buffered saline (PBS), pH = 7.2, between 3<sup>rd</sup> and 4<sup>th</sup> finger of the left foot (Corrier and De Loach 1990, Miljković et al. 1993). The same amount of PBS was inoculated into the right foot as a control and 12 hrs later the thickness was measured of the both legs using an adapted cutinometer. The difference between the thickness of the left and right leg was calculated and taken as the CBHR in mm.

Statistical analyses were performed using analyses of variance and Student's t test.

## RESULTS

### Blood leukocyte counts

Mean values for the blood leukocyte counts in the samples from the chickens subjected to long term sound stress, at different ages are presented in Table 1 and Figure 1.

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Table 1. Blood leukocyte counts ( $\bar{x} \pm \text{SD}$ ) of the sound stressed chickens at different ages ( $10^9/\text{L}$ )

Day of sampling	C	E <sub>1</sub>	E <sub>15</sub>	E <sub>30</sub>
15	25.3 $\pm$ 5.47	28.95 $\pm$ 8.97		
30	25.4 $\pm$ 5.88	22.99 $\pm$ 6.48	27.24 $\pm$ 11.33	
45	21.25 $\pm$ 6.92	15.6 $\pm$ 6.04	13.91 $\pm$ 2.73	12.35 $\pm$ 1.92

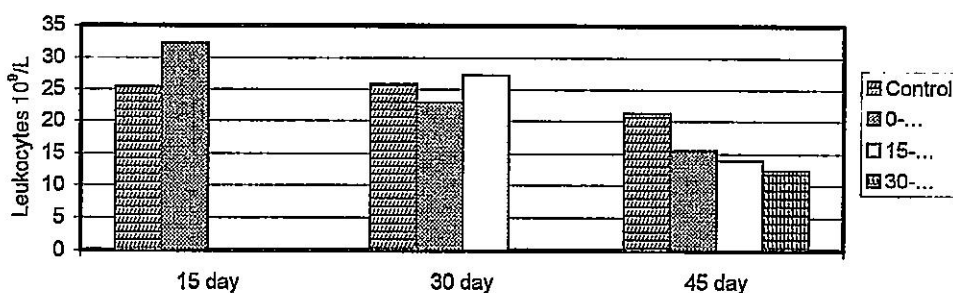


Figure 1. Mean number of leukocytes in the blood of chickens exposed to the sound stress

At the age of 15 days chickens subjected to the long term sound stress had moderately elevated blood leukocyte counts, compared to the control group, but the difference was not statistically significant ( $p > 0.05$ ). The differences in the blood leukocyte counts at the age of 30 days were not significant either. At the age of 45 days the highest leukocyte count was observed in the control group and the lowest in the group of chickens exposed to the sound stress from 30<sup>th</sup> to 45<sup>th</sup> day (Table 1 and Figure 1). We also noticed a decreased blood leukocyte count in the group of chickens exposed to the sound stress from 15<sup>th</sup> to 30<sup>th</sup> days of age. These differences were significant ( $p < 0.01$ ) when compared to the control group of the same age (Table 2).

Table 2. Statistical significance of the differences in the blood leukocyte counts in 45 day old chickens

	E <sub>1-45</sub>	E <sub>15-45</sub>	E <sub>30-45</sub>
C <sub>1-45</sub>	n.s.	$p < 0.01$	$p < 0.01$
E <sub>1-45</sub>		n.s.	n.s.
E <sub>15-45</sub>			n.s.

n.s. - non significant

The analyses of variance (data not shown here) did not indicate significant influence of age on the blood leukocyte count, so we have compared differences between groups of chickens of different ages.

Chickens exposed to the sound stress from the 1<sup>st</sup> to the 15<sup>th</sup> day of life had slightly higher blood leukocyte counts than those in the group E<sub>1-30</sub> (Table 1) but that difference was insignificant (Table 3). At the same time, chickens exposed to the sound stress from the 1<sup>st</sup> to the 15<sup>th</sup> day of life had significantly higher blood leukocyte counts than those in group E<sub>1-45</sub> (Tables 1 and 3;  $p < 0.01$ ). Similar differences were obtained between groups E<sub>1-30</sub> and E<sub>1-45</sub> ( $p = 0.05$ ) as shown in Table 3.

Table 3. Statistical significance of the differences in the blood leukocyte counts between groups of chickens exposed to long term sound stress from the first day of life

	E <sub>1-15</sub>	E <sub>1-30</sub>
E <sub>1-30</sub>	n.s.	
E <sub>1-45</sub>	0.01	0.05.

n.s. - non significant

The chickens from group E<sub>15-30</sub> also had higher blood leukocyte counts compared to group E<sub>15-45</sub> ( $p < 0.01$ ).

We may, thus conclude, that the duration of sound stress influenced the blood leukocyte counts by lowering their values.

The highest mean blood leukocyte count, in the chickens exposed to sound stress for 15 days, was observed in group E<sub>1-15</sub> (Table 1 and Figure 1). In group E<sub>15-30</sub> that number was slightly decreased but the difference was insignificant (Table 4). On the contrary, differences in the blood leukocyte counts between groups E<sub>1-15</sub> and E<sub>30-45</sub> were statistically significant, as well as those between groups E<sub>15-30</sub> and E<sub>30-45</sub> (Table 4).

Table 4. Statistical analyses of the differences in the blood leukocyte counts between groups of chickens exposed to the long term sound stress for 15 days

	E <sub>1-15</sub>	E <sub>15-30</sub>
E <sub>15-30</sub>	n.s.	
E <sub>30-45</sub>	$p < 0.01$	$p < 0.01$

n.s. - non significant

Thus, we may conclude, that the older chickens were more susceptible to the sound stress than the younger ones because they had lower values of blood leukocyte counts.

Moreover, the chickens subjected to the sound stress during 30 days had higher blood leukocyte counts in group E 1-30 than in group E 15-45 ( $p < 0.01$ ), again indicating that older chickens were more susceptible to the sound stress than younger ones.

#### Heterophil-lymphocyte index (H/L)

As mentioned earlier, H/L index is a relevant indicator of stress in poultry. The H/L index values of the chickens exposed to the sound stress and control birds are summarized in Table 5 and Figure 2.

Table 5. H/L Index ( $\pm$  S.D.) in the sound stressed and control groups of chickens at different ages

Day of sampling	C	E 1-...	E 15-...	E 30-...
15	$0.39 \pm 0.05$	$0.79 \pm 0.07$		
30	$0.43 \pm 0.03$	$1.04 \pm 0.09$	$2.46 \pm 0.43$	
45	$0.56 \pm 0.08$	$0.88 \pm 0.08$	$1.7 \pm 0.17$	$2.63 \pm 0.22$

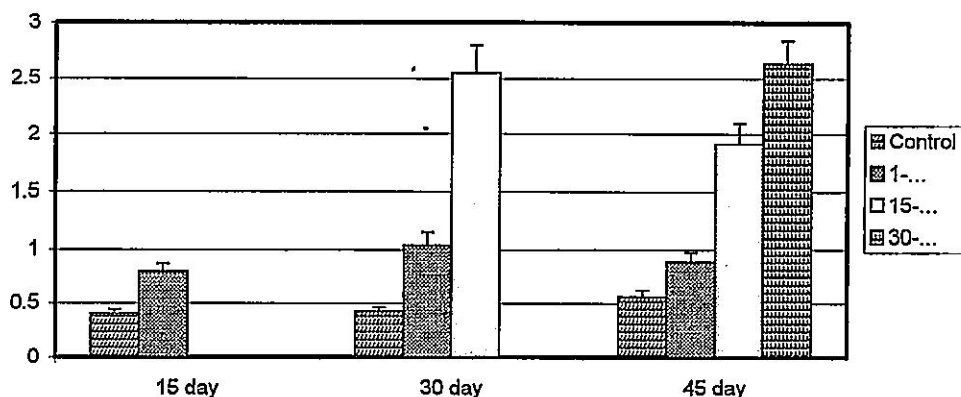


Figure 2. Heterophil/lymphocyte index in chickens exposed to the sound stress

At the age of 15 days chickens subjected to the long term sound stress had a higher mean H/L value than control birds and that difference was significant ( $p < 0.01$ ). At the age of 30 days we also noticed significant differences ( $p < 0.01$ ) in H/L index values between control and both experimental groups as well as between groups E 1-30 and E 15-30 (Table 6.).

Table 6. Statistical significance of the obtained differences in mean H/L values (30 days)

	E <sub>1-30</sub>	E <sub>15-30</sub>
C <sub>1-30</sub>	p < 0.01	p < 0.01
E <sub>1-30</sub>		p < 0.01

The lowest and the highest mean H/L values at the age of 45 days were obtained in the control group and in group E<sub>35-45</sub> respectively (Table 5). Statistical analyses revealed the presence of significant differences between all tested groups (p < 0.01; Table 7)

Table 7. Statistical significance of the obtained differences in mean H/L values (45 days)

	E <sub>1-45</sub>	E <sub>15-45</sub>	E <sub>30-45</sub>
C <sub>1-45</sub>	p < 0.01	p < 0.01	p < 0.01
E <sub>1-45</sub>		p < 0.01	p < 0.01
E <sub>15-45</sub>			p < 0.01

Analyses of the variance (data not shown here), indicated a significant influence of age on H/L values and for that reason, comparison between experimental and control groups at different ages was not possible.

#### Cutaneous basophil hypersensitivity reaction (CBHR)

The cutaneous basophil hypersensitivity reaction (CBHR) is an indicator of the cellular immune response both in mammals and birds. The CBHR values of control and long term sound stress exposed chickens (differences in the degree of skin thickness measured between 3rd and 4th finger, in mm, after PHA application) are summarised in Table 8 and Figure 3.

Table 8. Differences in the degree of skin thickness between 3rd and 4th finger after PHA application ( $\bar{x} \pm \text{S.D.}$  in mm)

Age	C	E <sub>1-...</sub>	E <sub>15-...</sub>	E <sub>30-...</sub>
15	0.43 ± 0.15	0.24 ± 0.1		
30	0.79 ± 0.32	0.58 ± 0.19	0.47 ± 0.2	
45	0.95 ± 0.34	0.6 ± 0.2	0.48 ± 0.17	0.73 ± 0.24

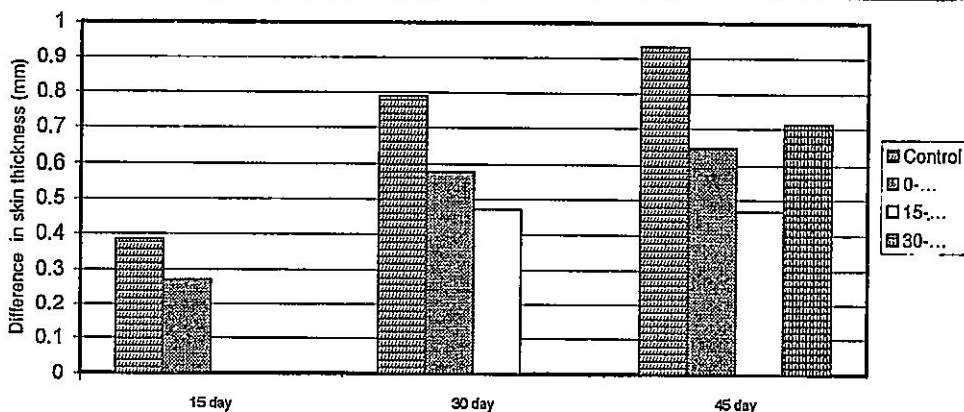


Figure 3. Differences in the degree of skin thickness after PHA application in chickens exposed to the sound stress

At the age of 15 days chickens subjected to the long term sound stress had lower CBHR values than those in the control group and that difference was significant ( $p < 0.05$ ). Differences in CBHR values between control and experimental groups were also found but these differences were significant ( $p < 0.05$ ) only between groups C and E 15-30 (Table 9).

Table 9. Statistical significance of the obtained differences in mean CBHR values (30 days)

	E 1-30	E 15-30
C 1-30	n.s.	$p < 0.05$
E 1-30		n.s.

n.s. - non significant

At the age of 45 days, the highest CBHR values were observed in the control group and the lowest in group E 15-45 (Table 8). The significances of the obtained differences in CBHR values between the groups are summarized in Table 10.

Table 10. Statistical significance of the obtained differences in mean CBHR values (45 days)

	E 1-45	E 15-45	E 30-45
C 1-45	$p < 0.05$	$p < 0.01$	n.s.
E 1-45		n.s.	n.s.
E 15-45			$p < 0.05$

n.s. - non significant



Analyses of variance (data not shown here), revealed significant influence of age on CBHR values and for that reason, comparison between different age groups receiving different treatments was not possible.

Finally, the relative differences in CBHR values between control and experimental groups of chickens are presented in Figure 4. Values obtained for each control group were taken as 100 %.

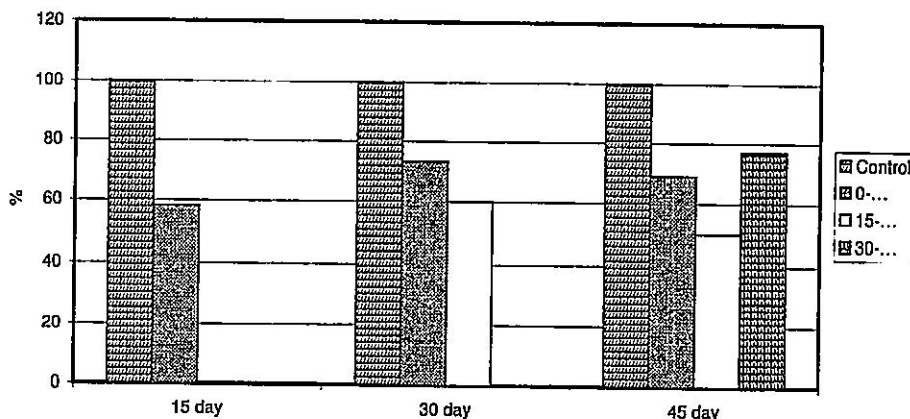


Figure 4. Relative proportions in CBHR between the groups of chickens exposed to the sound stress

It is evident that there was a significant relative decrease in the CBHR values in all age groups. However, this phenomenon was more markedly expressed in groups E 1-15, E 15-30 and E 15-45

#### DISCUSSION

In this study we were able to demonstrate that the applied model was effective for stress induction in broiler chickens. It was documented earlier (McFarlane et al. 1989a) that continuous noise did not affect hematological values in broiler chickens because of the adaptive reaction. On the contrary, in our experiment, blood leukocyte counts were changed during the sound stress especially at the end of the experiment. Our data are in agreement with the results of Batuman et al. (1990) who demonstrated that repeated stress treatments (transportation, immobilization and electrical shock) decrease the number of leukocytes and especially T and B lymphocytes in rats. On the other hand, Regnier and Kelley (1981) were not able to show that temperature stress for five days affected the blood leukocyte counts. Furthermore, social stress in chickens led to the elevation of leukocyte number in blood but not in the spleen (Cunnick et al. 1994). Dhabhar et al. (1995) found that in animals exposed to a stress of low intensity (short term immobilization) leukocyte counts were lowered in blood but

elevated in skin. The authors conclude that stress has an influence on leukocyte distribution in order to enhance body defence mechanisms.

It was also stated that acute sound stress led to changes in the H/L ratio but the values returned to the basic level after 18<sup>hrs</sup> (Gross 1990). In our model these changes remained constant even after cessation of the stress. As presented in Figure 2. H/L ratio was elevated in all experimental groups reaching the maximum value at the end of the experiment. It can be also noted that there is an adaptation phenomenon in H/L values because birds that were exposed to the sound stress longer, exhibited lower values (these differences were still statistically significant). At the end of our experiment H/L index values were 4 times higher in group E 30-45 than in the control group. Differences between all groups were significant at the level of 99 % probability. McFarlane et al. (1989) stated that elevated concentrations of ammonia, electrical shocks and high temperature also elevated the H/L index. Similar results in rats exposed to low intensity stress (2 hours immobilization) were reported by Dhabhar and McEwen (1996). In this model the authors were able to demonstrate a decrease in total leukocyte number, lymphocytes, Ts lymphocytes, Th lymphocytes, B lymphocytes, monocytes and NK cells accompanied with increased neutrophil number. They also noted that corticosterone concentration and leukocyte number returned to physiological values 3 hours after stress cessation. Interestingly in adrenalectomized animals similar changes in leukocyte distribution were observed but not to the same extent indicating that corticosterone is not the only relevant factor in cell redistribution. These findings were confirmed in experiments where an inhibitor of corticosterone synthesis (cyanketone) was administered to animals exposed to the same stress (Dhabhar et al. 1996a). Exogenous ACTH or temperature stress in rats did not influence B lymphocyte number in blood and spleen but the number of CD4+ and CD8+ cells was reduced in blood and elevated in the spleen of treated animals (Trout and Mashaly, 1994). Apart from the influence on leukocyte distribution, stress induce hormonal apoptosis of lymphocytes in Bursa Fabricii (Compton et al 1990).

We have also observed changes in CBHR values leading to the conclusion that the cellular immune reaction is also affected when animals are exposed to long term sound stress. In all experimental groups CBHR values were lower than in the respective control groups. These differences are more prominent when values for the control groups are considered as 100 % (Figure 4.). The degree of changes correlated with the duration of stress period. Regnier and Kelley (1981) reported that heat stress in chickens resulted in lower CBHR. This test has been applied successfully as a stress indicator in some other animal species. Pigs exposed to stress by moving from one building to another had a weaker response to PHA (Ekkel et al. 1995). Immobilization stress in pigs had the same consequences in PHA response (Westly and Kelley, 1984). Thermal stress of bulls, if longer than two weeks, also altered (decreased) the response of animals to PHA (Kelley et al. 1982).

The changes correlated with the duration of the stress stimuli and with the age of the animals. Generally, older chickens were more susceptible to long term sound stress as judged by leukocytopenia, lymphocytopenia and decreased

CBHR values than younger ones. Older animals were more susceptible because of their decreased ability to adapt to noise as a stress factor.

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**UTICAJ DUGOTRAJNOG ZVUČNOG STRESA NA BROJ LEUKOCITA,  
HETEROFILNO/LIMFOCITNI INDEKS I REAKCIJU PREOSETLJIVOSTI NA  
FITOHEMAGLUTININ KOD BROJLERSKIH PILIĆA**

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**SADRŽAJ**

U ogledu je ispitivan uticaj dugotrajnog zvučnog stresa na neke parametre aktivnosti imunološkog sistema pilića hibrida HYBRO. Dokazano je da ova vrsta stresa dovodi do smanjenja broja leukocita, povećanja heterofilno/limfocitnog indeksa i smanjenja stepena ko'ne preosetljivosti na fitohemaglutinin. Svi uočeni fenomeni ukazuju na postojanje imunosupresije koja nastaje delovanjem hroničnog zvučnog stresa. Dokazano je da su starost životinja i trajanje tretmana značajni faktori za stepen reakcije na delovanje stresora.