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Serum clinical biochemical markers of Hy-Line W-36 laying hens under the influence of increased stocking densities in cages of multilevel batteries

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Today, the organism of hens is constantly exposed to numerous technological stressors in the conditions of industrial poultry farming, the least studied of which are long-term, which can cause the development of chronic stress. One of such stressors is the increased stocking density of hens, which is also a way of saving resources in egg poultry and is often used by producers to obtain more eggs per 1 m² of poultry area. The aim of this study was to examine the effect of overcrowding, as a factor of chronic stress development, on the body of hens of a modern high-performance cross, which is necessary to select the best ways to keep them. Four groups of hens were formed for this purpose, which were kept at different stocking densities, according to European standards, Ukrainian standards and with increasing overcrowding. In this way, the gradually increasing intensity of the technological stressor was modeled. Long-term keeping of laying hens at high stocking density did not affect the content in the serum of total protein, albumin, urea and cholesterol, which were within the physiological norm. It was found that the increase in the stocking density of hens to Ukrainian standards, compared to the European, was accompanied by an increase in the activity of lactate dehydrogenase in the serum of their blood. With an increase in stocking density above European and Ukrainian standards, namely to 25.3 birds/m², there was an increase in the activity of three enzymes – lactate dehydrogenase, aspartate aminotransferase and gamma-glutamyltransferase. It is proved that further overcompaction of hens to 26.7 birds/m² is accompanied by an increase in serum glucose, creatinine, as well as a decrease in the ratio of calcium and phosphorus, which was confirmed by an increase in alkaline phosphatase activity. Also, increased activity of aspartate aminotransferase, lactate dehydrogenase and gamma-glutamyltransferase was observed. Thus, the main effects of chronic stress caused by prolonged keeping of hens at high stocking densities are reflected in the biochemical parameters of their serum, namely in the increase of glucose, creatinine, enzyme activity, as well as the violation of the ratio of calcium and phosphorus.

Keywords: laying hens; stocking density; chronic stress; glucose; creatinine; enzyme activity.

Introduction

The body of hens is constantly exposed to numerous technological stressors in the conditions of industrial production of poultry products (Ericsson et al., 2016; Hedlund et al., 2019). The action of stressors begins with the incubator (Hedlund & Jensen, 2021) and accompanies the entire period of use of hens due to transportation, high stocking density, changes in the microclimate of production facilities and diet, vaccination, carrying out forced molting etc. (Bedanova et al., 2007; Kang et al., 2018; Gorelik et al., 2020). Technological stress reduces the level of immunological reactivity of the birds (Sloan et al., 2010; Hall et al., 2014), which leads to a decrease in their productivity (Lara & Rostagno, 2013; Stoianovskiy et al., 2018; Goel, 2021) and leads to significant economic losses (Sakhatsky et al., 2020). At the same time, the increased stocking density of hens is both a technological stressor and a method of resource conservation in egg poultry, which is often used by producers to obtain more eggs from each 1 m² of poultry area (Sakhatsky et al., 2020). Therefore, the study of the degree of physiological disorders in the body of hens caused by high stocking density is an urgent issue of poultry farming, and its solution will provide the opportunity to choose the best ways to keep laying hens.

It is known that during stress the activity of all the body systems of hens is strained, which is aimed at adaptation to new living conditions (Infante et al., 2017; Shevchuk et al., 2018). A prerequisite for the development of a stress response is the strengthening of the function of the endocrine glands and especially the hypothalamus – anterior pituitary – adrenal cortex axis (Olubodun et al., 2015). The main role in the develop-

ment of stress, according to Selye, is played by the adrenal cortex, which under the influence of the pituitary gland increases the secretion of steroid hormones involved in the process of adaptation (Selje, 1979). Therefore, it is believed that the main mechanisms in the implementation of stress in poultry are the sympathoadrenal and hypothalamic-pituitary-adrenocorticotrophic (GGAC) systems, namely the development of adaptive responses to various nonspecific environmental factors is a common mechanism: through the hypothalamic-pituitary-adrenal axis and sympathoadrenal system involving catecholamines (Infante et al., 2017). Catecholamines ensure the transition of the body from a state of rest to a state of excitement due to their biological effects and allow it to stay in this state for a long time. At the same time, the occurrence and course of physiological reactions in poultry due to hormones of the adrenal medulla and mediators of the sympathoadrenal system is accompanied by increased and qualitative changes in metabolic processes in immunocompetent tissues (Stayanovskiy et al., 2018), which is reflected in their blood. Leukocyte formula (El-Lethey et al., 2000; Jiang et al., 2017; Liew & Kubes, 2019) and the concentration of hormones in the blood (Scanen, 2016; Weimer et al., 2018) are usually used to diagnose stress, as well as the characteristics of hens' adaptation processes. Recently, some biochemical markers of blood serum have been actively used in poultry (Nwaigwe et al., 2020; Ruiz-Jimenez et al., 2021), which, in contrast to the leukocyte formula and hormonal status, allow us to describe the general physiological state of the body, adaptation processes (Kraus et al., 2021) and diagnose metabolic disorders of organs and tissues (Kudair & Al-hussary, 2010; Koronowicz et al., 2016). In general, serum biochemical markers are indicators of hens'

health and reflect any physiological or even pathological changes occurring in their body (Koronowicz et al., 2016). And any changes in their body affect not only the health of hens, but also inevitably affect their productivity (Pavlik et al., 2007). It is known that some changes in the values of various biochemical markers (glucose, total protein, albumin, enzyme activity) are associated with certain pathological processes in animals (Greene et al., 2013; Mollahosseini et al., 2017; Zhang et al., 2019). Therefore, the aim of this study was to examine the physiological changes in the body of laying hens due to increased stocking density based on the analysis of the markers of the clinical biochemistry of serum, as well as to determine the physiological limit of permissible overcrowding.

Materials and methods

Hy Line W-36 commercial egg layers were used as the object of research. Experiments were performed with experimental animals in accordance with the rules of the European Convention for the Protection of Vertebrate Animals (Official Journal of the European Union L276/33, 2010), as well as the Law of Ukraine "On protection of animals from cruelty" of 21.02.2006, No. 3447-IV.

Four groups of hens were formed in the conditions of a modern complex for production of food eggs, each of which was kept in a separate poultry house-analogue on the area (2640 m²) equipped with 12-tier cage batteries "Salmet" (Germany), consisting of 18144 cages with an area of 7506 cm² (120 × 62.55 cm).

Chronic stress was simulated by 34-week keeping of hens, namely from the beginning of laying (at 18 weeks of age) to 52 weeks of age, at different stocking densities. The stocking densities I group of hens met the European standards and requirements of the cross-country developer (Hy-Line W-36 Final Hybrid Content Guide, 2019) – in the range of 13–20 birds/m² (490–750 cm²/bird), the II group – Ukrainian standards (VNTP-APK-04.05.) – within 22–25 birds/m² (area – 400–450 cm²/bird), and hens of the III and IV groups were kept with increasing overcrowding (Table 1). In this way, the gradually increasing intensity of the technological stressor was modeled.

Table 1

The scheme of the experiment to determine the effect of increased stocking density on serum clinical biochemical markers in cages of multilevel batteries

Characteristic	Group of laying hens			
	I	II	III	IV
Number of hens in the cage	10	18	19	20
Number of hens in the group, thousand	181440	326592	344736	362880
Stocking density, birds/m ²	13.3	24.0	25.3	26.7
Provision of area, cm ² /bird	750.6	417.0	395.1	375.3
Feeding front, cm	12.0	6.7	6.3	6.0

Hens were provided with drinking water, complete feed (Table 2) during the experiment and kept in accordance with the requirements (VNTP-APK-04.05).

Thirty blood samples were taken from laying hens of each group at the age of 18 weeks (at the beginning of the study) and at 52 weeks. 1.0–1.5 mL of blood was taken from the axillary vein in an EDTA tube. Biochemical markers and activity of serum enzymes of laying hens were determined on a biochemical analyzer BioChem FC-360 (High Technology Inc., USA), namely the content of total protein, albumin, glucose, creatinine, urea, cholesterol, phosphorus, calcium, alanine aminotransferase activity, aspartate aminotransferase, alkaline phosphatase, lactate dehydrogenase and gamma-glutamyltransferase in the laboratory "Bald" (certificate No. LB/02/2016). Commercial diagnostic kits (High Technology Inc., USA) were used for determination of glucose – HT-G242 (method: oxidase, endpoint; wavelength – 500 nm), total proteins – HT-T251 (method: biuret, endpoint; wavelength – 540 nm), albumin – HT-A203 (method: bromocresol green, endpoint; wavelength – 630 nm), creatinine – HT-C225 (method: Jaffe, kinetics; wavelength – 510 nm), urea – HT-U254 (method: Trinder/uricase, endpoint; wavelength – 520 nm), cholesterol – HT-C218 (method: enzymatic, endpoint; wavelength – 500 nm), phosphorus – HT-P244 (method: ammonium molybdate, endpoint; wavelength – 340 nm), calcium – HT-C216 (method: OKF, end-

point; wavelength – 570 nm), alanine aminotransferase – HT-A206 (method: IFCC, kinetics; wavelength – 340 nm), aspartate aminotransferase – HT-A109 (method: IFCC, kinetics; wavelength – 340 nm), alkaline phosphatase – HT-A205 (method: kinetics; wavelength – 405 nm), lactate dehydrogenase – HT-L236 (method: Modified Wacker/Tris method, kinetics; wavelength – 340 nm), gamma-glutamyltransferase – (method: kinetics; wavelength – 405 nm). Concentration of the biochemical constituents was calculated according to the manufacture instruction. Reference values biochemical markers and activity of serum enzymes of laying hens according to Nasonov et al. (2014).

Table 2

The composition of feed for laying hens in the productive period (%)

Component	Egg-laying intensity, %			
	95–100	93	88	85
Wheat	20.418	19.336	12.000	10.566
Com	37.053	45.399	54.330	52.334
Sunflower meal	20.754	22.278	18.166	23.533
Soybean meal	7.000	0	3.000	0
Soybean oil	0.959	0.661	0	0.500
Shell 0–3 mm	10.701	9.922	10.25	11.088
Salt	0.210	0.200	0.200	0.210
Monocalcium phosphate	1.193	0.811	0.805	0.532
Sodium sulfate	0.160	0.117	0.120	0.095
Methionine	0.186	0.105	0.088	0.076
Lysine sulfate	0.637	0.585	0.516	0.579
Threonine	0.127	0.095	0.057	0.065
Loxidan TD 100	0	0.010	0	0
Millersheim	0.013	0.015	0.011	0
Globamax 1000	0.100	0	0	0
ProActive	0	0	0.150	0.150
Enteronormin Detox	0.150	0.150	0	0
Mastersorb	0.150	0.130	0.130	0
Mycocide Pro	0	0	0	0.090
Choline chloride	0.050	0.050	0.040	0.035
Cronozyme	0	0	0	0.011
Yellow carnation	0.003	0.003	0.003	0.003
Red carnation	0.003	0.003	0.003	0.003
Mineral complex	0.100	0.100	0.100	0.100
Vitamin complex	0.033	0.030	0.030	0.030
Together	100.000	100.000	100.000	100.000

Significance of group differences was assessed using one-way analysis of variance (ANOVA) and Tukey-Cramer multiple comparison test as a post-hoc test tool. The data in the tables are presented in the form of $\bar{x} \pm SD$ (mean $\pm$ standard deviation). Verification of the distribution of sample data for normality was performed according to the Kolmogorov-Smirnov test. The nonparametric Mann-Whitney U-test was used if the data distribution was significantly different from normal. Differences between groups were considered significant at $P < 0.05$.

Result

Biochemical markers of blood serum of hens of all experimental and control groups at the beginning of the study were within physiological norms for each parameter. No significant differences were found between the groups. At the same time, long-term keeping of laying hens at high stocking density also did not affect the content in the serum of total protein, albumin, urea and cholesterol (Table 3), which were within the physiological norm. The content of creatinine and glucose was within the physiological norm in the serum of I–III groups of hens, with their stocking density of 13.3 to 25.3 birds/m². The highest glucose content was found in the IV group of hens, with their stocking density of 26.7 birds/m², exceeding the upper limit of the physiological norm by 9.1%. The glucose content in the serum of hens of the IV group was higher by 20.0% compared to the I group, by 22.4% and 21.6% compared to the II and III groups. The content of creatinine in the serum of hens of the IV group exceeded the upper limit of physiological norm by 6.7% and was higher by 27.2% compared to the I group and by 23.1% compared to the II and III in groups.

The content of calcium in the serum of hens of all groups was within the physiological norm. In the IV group of hens its amount was lower by 5.3% compared to the I and III groups, and by 6.6% compared to the

II group. The content of phosphorus in the serum of the IV group exceeded the physiological norm by 11.8% and was higher by 74.5% compared to the I group and by 59.7% and 54.7% compared to groups II and III.

Table 3

Biochemical profile of blood serum of laying hens under the influence of increased stocking densities ($\bar{x} \pm \text{SD}$, $n = 30/\text{group}$)

Marker	Group of laying hens				Reference values
	I	II	III	IV	
Total protein, g/L	57.2 ± 4.4 ^a	55.4 ± 6.9 ^a	54.1 ± 2.5 ^a	57.1 ± 5.9 ^a	37.8–59.0
Albumin, g/L	18.7 ± 1.2 ^a	16.4 ± 1.1 ^a	19.4 ± 0.5 ^a	17.8 ± 0.8 ^a	15.0–25.0
Glucose, mmol/L	15.0 ± 1.5 ^a	14.7 ± 2.2 ^a	14.8 ± 0.6 ^a	18.0 ± 0.8 ^b	10.0–16.5
Creatinine, μmol/L	22.4 ± 1.8 ^a	23.4 ± 3.6 ^a	23.4 ± 2.7 ^a	28.8 ± 1.1 ^b	22.0–27.0
Urea, mmol/L	1.0 ± 0.2 ^a	0.9 ± 0.1 ^a	0.9 ± 0.3 ^a	1.0 ± 0.1 ^a	0.7–2.4
Cholesterol, mmol/L	3.5 ± 1.1 ^a	2.3 ± 0.3 ^a	3.9 ± 1.1 ^a	3.4 ± 0.3 ^a	2.0–4.0
Phosphorus, mmol/L	1.4 ± 0.3 ^a	1.5 ± 0.4 ^a	1.6 ± 0.4 ^a	2.5 ± 0.2 ^b	1.2–2.2
Calcium, mmol/L	4.5 ± 0.4 ^a	4.6 ± 0.9 ^a	4.5 ± 0.1 ^a	4.3 ± 0.3 ^b	2.8–4.6
Calcium/phosphorus	3.4 ± 1.1 ^a	3.0 ± 0.6 ^a	3.0 ± 0.8 ^a	1.8 ± 0.2 ^b	3.0–3.8:1

Note: ^a, ^b, ^c – indicate values that significant differed in one row of the Table $P < 0.05$.

Table 4

Activity of serum enzymes of laying hens under the influence of increased stocking densities (units/L, $\bar{x} \pm \text{SD}$, $n = 30$)

Marker	Group of laying hens				Reference values
	I	II	III	IV	
Alanine aminotransferase	1.0 ± 0.3 ^a	0.8 ± 0.4 ^a	0.6 ± 0.3 ^a	1.0 ± 0.1 ^a	13.0–26.5
Aspartate aminotransferase	208.6 ± 32.3 ^a	212.2 ± 30.7 ^a	248.4 ± 17.7 ^b	259.8 ± 12.7 ^c	125–210
Gamma-glutamyltransferase	22.2 ± 6.1 ^a	22.4 ± 5.7 ^{ab}	24.2 ± 4.1 ^b	30.1 ± 7.6 ^c	*
Alkaline phosphatase	684.8 ± 125.2 ^a	746.4 ± 128.3 ^b	777.6 ± 129.5 ^b	992.2 ± 133.9 ^c	350–830
Lactate dehydrogenase	1908.8 ± 160.3 ^a	2154.8 ± 199.2 ^b	2235.6 ± 202.9 ^b	2731.9 ± 271.3 ^c	636–1960

Note: ^a, ^b, ^c – indicate values that significant differed in one row of the table ($P < 0.05$); * – reference values are not available for laying hens.

Discussion

The increase in the stocking density of hens from 13.3 to 25.3 birds/m² did not affect the biochemical profile of their serum, while overcompaction to 26.7 birds/m² was accompanied by an increase in glucose levels of 22.4% with an excess of physiological norm by 9.1%. In our opinion, the increase of serum glucose occurs due to increased destructive processes in hens under conditions of overcompaction, as well as the action of hormones – glucocorticoids and catecholamines (Downing, 2012; Kraus et al., 2021). The findings are consistent with the results of other studies describing hyperglycemia as a reaction of the birds to chronic (González & Silva, 2006; Gupta et al., 2017; Kraus et al., 2021) and acute stress (Virden & Kidd, 2009; Mert & Yildirim, 2016), as well as the experimental introduction of adrenocorticotrophic hormone (Puvadolpirod & Thaxton, 2000; Odihambo Mumma et al., 2006). Research conducted by Kang et al. (2006, 2018) with co-authors confirms that increasing the stocking density from 5 to 10 birds/m² of floor and from 13 to 19 birds/m² of aviary does not cause changes in serum glucose levels in laying hens. At the same time, there are reports that an increase in serum glucose is not observed with cyclic heat stressors (Laganá et al., 2007; Bueno et al., 2017).

In addition, Guo et al. (2012) noted an increase in serum creatinine in laying hens with increasing stocking density, which is confirmed by the study data. Thus, the increase in the density of hens to 26.7 birds/m² was accompanied by an increase in serum creatinine by 27.2% in excess of the physiological norm by 6.7% (Goel, 2021). Bueno et al. (2017), who studied the response of broiler chickens to cyclic heat stress, as well as Abo Ghanima et al. (2020), who studied the response of ducks to increased stocking density, did not notice an increase in their creatinine levels.

The increase in the stocking density of hens was accompanied by an increase in serum levels of phosphorus by 74.5% and a violation of the ratio of calcium and phosphorus, which manifested itself in a decrease of 47.1% and was confirmed by changes in alkaline phosphatase activity, which at a density of 26.7 birds/m² was higher by 44.9% and exceeded the physiological norm by 19.5%. The data obtained confirm the results of Rajman et al. (2006), which described a stress-induced increase in alkaline

phosphatase levels in the serum of chickens caused by limited access to feed. Also, the increase in alkaline phosphatase activity may be due not only to high egg production and excessive intake of phosphates with food (Al-Bustany et al., 1998), but also to the constant exposure to stressors (Onbaşlılar et al., 2016).

Along with the increase, stocking density of hens was accompanied by a change in the activity in the serum of aspartate aminotransferase and lactate dehydrogenase, which reflects the violation of tissue integrity (activation of destructive processes) of poultry, especially liver (Mikami et al., 2004) and muscle (Lin et al., 2006; Sandercock et al., 2006). In particular, an increase in aspartate aminotransferase activity was observed already with an increase in stocking density to 25.3 birds/m² – by 24.5% in excess of physiological norms. Similar results have been obtained by many researchers (Abudabos et al., 2013; Park et al., 2018; Kraus et al., 2021), who describe the increase in the activity of aspartate aminotransferase as a reaction of the chickens to the action of technological stressors. According to Capitelli & Crosta (2013) and Campbell et al. (2004), an increase in aspartate aminotransferase activity above 275 units/L indicates liver and muscle dysfunction and only excess levels activity of 800 units/L is evidence of severe liver damage. Obviously, in this study, the increase in aspartate aminotransferase activity to 259.8 units/L under the influence of overcrowding of hens is caused by their being kept in a constant state of neuromuscular tension. According to Everds et al. (2013), constant stress leads to an increase in the activity of aspartate aminotransferase and at the same time to an increase in the concentration of glucose in the serum of hens kept in cages, which is confirmed by the study data.

Scientists explain the increase in lactate dehydrogenase activity due to muscle destruction during neuromuscular tension (Sandercock et al., 2006), which in these studies was observed starting with a retention density of 24.0 birds/m² – by 12.9% and overcompaction to 26.7 birds/m² – by 43.1% in excess of the physiological norm by 39.4%. The increase in lactate dehydrogenase as a result of acute heat stress has also been described by Xie et al. (2015).

The increase in the activity of gamma-glutamyltransferase in the serum of hens was observed when the stocking density increased to 25.3 birds/m² and further increases in proportion to the increasing over-

compaction by 9.0–35.6%. The obtained results confirm the data of other authors, which described the increase in gamma-glutamyltransferase as a response of broiler chickens to cyclic heat stress (Bueno et al., 2017). Gamma-glutamyltransferase in chickens is found mainly in the liver, pancreas and kidneys according to Radin (2003). The increase in gamma-glutamyltransferase activity in blood serum is due to its increase in the liver, because in the kidneys gamma-glutamyltransferase is localized on the luminal surface of proximal renal tubules and in the case of enzyme damage gets directly into the urine and is excreted with it at the same time, according to González & Silva (2006). The authors also emphasize that the function of gamma-glutamyltransferase is associated with the metabolism of glutathione, and its increase may indicate cholestasis and proliferation of bile ducts in the liver of birds.

Conclusions

Long-term keeping of laying hens at high stocking density did not affect the content in the serum of total protein, albumin, urea and cholesterol. The increase in stocking density of hens to 24.0 birds/m² was accompanied only by an increase in the activity of lactate dehydrogenase in their blood serum by 12.9%. A further increase in stocking density to 25.3 birds/m² led to an increase in the activity of three enzymes – lactate dehydrogenase – by 17.1%, aspartate aminotransferase – by 19.1% and gamma-glutamyltransferase – by 9.0%. Overcompaction of hens to 26.7 birds/m² was accompanied by an increase of glucose levels by 22.4%, creatinine – by 27.2%, phosphorus – 74.5%, as well as a decrease in the ratio of calcium and phosphorus by 47.1%, which is confirmed by an increase in alkaline phosphatase activity by 44.9%. Also, with overcompaction of hens to 26.7 birds/m², there was an increase in the activity of aspartate aminotransferase by 24.5%, lactate dehydrogenase – by 43.1% and gamma-glutamyltransferase – by 35.6%. Thus, the main effects of chronic stress caused by prolonged keeping of hens at high stocking densities are reflected in the biochemical markers of their serum, namely in the increase of glucose, creatinine, phosphorus, enzyme activity, as well as a violation of the ratio of calcium and phosphorus.

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