



## Effectiveness of betaine, taurine, and myo-inositol in normalizing the antioxidant status of laying hens under heat stress

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**PDB:** Conceptualization; Methodology; Investigation; Data curation; Formal analysis; Validation; Visualization; Writing — original draft, review & editing.

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None to declare.

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All procedures with chickens were performed in compliance with the provisions of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, 2005), Council Directive 2010/63/EU, and the Law of Ukraine no. 3447-IV "On the Protection of Animals from Cruelty" with amendments 440-IX from 14.01.2020, following protocol no. 115a from 28.09.2022 of the Bioethics Commission for Scientific Research of the Institute of Animal Biology NAAS.

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Heat stress is a critical factor in the adaptation of animals to changing environmental temperature conditions and can significantly impact their health. Elevated ambient temperatures induce considerable stress, potentially leading to various adverse effects in poultry, including impairments in the antioxidant system. The imbalance between antioxidant and pro-oxidant processes can result in the excessive formation of free radicals, which harm cells and may contribute to the development of diseases. This study investigates the effects of artificially induced heat stress on the antioxidant system and lipid peroxidation products in the blood of laying hens. Laying hens, as commercial poultry lines selected for high egg productivity, are particularly vulnerable to high ambient temperatures due to their intensive metabolism, increased energy demand for egg production, and limited thermoregulatory capacity compared to other bird species. In intensive poultry farming, where bird density is high, these factors can exacerbate heat stress. The study aimed to identify changes in specific indicators of the antioxidant system and the content of lipid peroxidation products in the blood of chickens under the influence of betaine, taurine and myo-inositol. Analyzing parameters such as lipid hydroperoxides (LOOH), reduced glutathione (GSH), glutathione peroxidase (GSH-Px), glutathione reductase (GR), catalase (CAT), and superoxide dismutase (SOD) provides insights into the antioxidant defense system and oxidative stress levels under heat stress conditions. The study involved 15 laying hens housed in the vivarium of the Institute of Animal Biology NAAS, and was conducted in two phases. During the first phase, hens were kept at an ambient temperature of 20°C for three weeks. During the second phase, heat stress conditions were simulated by raising the temperature to 30°C for 6 hours daily over 7 days. Birds were divided into two groups: the control group (fed a standard diet) and the experimental group (supplemented with 0.5 g/kg betaine, 5 g/kg taurine, and 2 g/kg myo-inositol). Results showed that with increased ambient temperature in the control group, the content of LOOH decreased by 63% ( $P < 0.05$ ), while the activities of CAT, SOD, GSH-Px, and GR decreased by 28% ( $P < 0.001$ ), 49% ( $P < 0.01$ ), 15% ( $P < 0.01$ ), and 30% ( $P < 0.01$ ), respectively, compared to thermoneutral conditions. Conversely, GSH content increased by 37% ( $P < 0.01$ ). In the experimental group supplemented with betaine, taurine, and myo-inositol, CAT, GSH-Px, and GR activities decreased by 14% ( $P < 0.01$ ), 30% ( $P < 0.001$ ), and 23% ( $P < 0.05$ ), respectively, under thermoneutral conditions. Under heat stress conditions, LOOH content decreased by 59% ( $P < 0.05$ ), and GSH-Px activity decreased by 15% ( $P < 0.01$ ), while SOD and CAT activities increased by 55% ( $P < 0.001$ ) and 11% ( $P < 0.05$ ), respectively, compared to the control. The findings indicate the positive effects of betaine, taurine, and myo-inositol on the antioxidant system of laying hens under heat stress. The results highlight the potential of these supplements as effective strategies to maintain poultry health and productivity during heat stress.

**Key words:** laying hens, heat stress, oxidative stress, antioxidant defense system



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## Introduction

In recent years, global climate change has led to an increase in the frequency and duration of high-temperature periods, significantly impacting agriculture, particularly poultry farming [8]. Poultry are especially sensitive to heat stress (HS) due to their biological characteristics, such as high metabolic rates, elevated heat production, rapid growth, and high productivity. Commercial poultry species, particularly laying hens selected for high egg productivity, are even more vulnerable to HS due to their specific biological and physiological traits [11]. The optimal ambient temperature for their growth typically ranges between 18–24°C [15]. Temperatures exceeding this range, particularly above 30°C, can cause HS [10].

Elevated ambient temperatures can overload the thermoregulatory systems of animals, leading to increased heat production and, consequently, the activation of mechanisms that generate free radicals (FRs). These radicals are highly reactive molecules capable of damaging cells, proteins, lipids, and DNA. Under HS, the body attempts to counteract the adverse effects of high temperatures by activating antioxidant systems to neutralize FRs. These systems include both non-enzymatic low-molecular-weight antioxidants, such as reduced glutathione (GSH), and enzymatic high-molecular-weight antioxidants, such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and glutathione reductase (GR). These antioxidants limit the rate and progression of oxidation by detoxifying superoxide radicals, thereby protecting cells from oxidative damage [3]. However, intense HS can overwhelm the antioxidant system's capacity to manage these radicals, leading to increased oxidative stress (OS).

OS is characterized by an imbalance between the generation of reactive oxygen species (ROS) and their neutralization by antioxidant systems, resulting in damage to cells and tissues. ROS can react with unsaturated fats in cell membranes, forming lipid peroxides that compromise membrane integrity, increase permeability, and lead to cellular content leakage. OS also causes protein oxidation, which can result in protein denaturation and loss of biological activity. DNA oxidation may lead to mutations and strand breaks, affecting cellular division and function, increasing the risk of genetic alterations, and promoting the development of various diseases [12].

The antioxidant properties of compounds such as betaine, taurine, and myo-inositol are essential for maintaining poultry health, especially under stress conditions. Betaine, a natural methyl donor, can lower homocysteine levels in poultry blood, enhancing overall antioxidant activity and reducing OS [5]. Taurine, an amino acid, plays a critical role in antioxidant defense by neutralizing FRs and regulating intracellular calcium levels, thus mitigating cellular stress and maintaining

cellular function [1]. Myo-inositol reduces OS through multiple mechanisms, including lowering pro-oxidant molecule levels, activating antioxidant enzymes (SOD and CAT), and supporting cellular metabolism, such as phospholipid synthesis and signal transduction, which helps maintain membrane integrity and reduces oxidant accumulation [2]. Collectively, these compounds may significantly reduce OS and promote overall poultry health, making them valuable dietary supplements, particularly under HS conditions.

This study aimed to evaluate the effects of betaine, taurine, and myo-inositol on selected antioxidant system parameters (GSH levels, GPxs, GR, SOD, CAT activities) and the content of lipid hydroperoxides (LOOH) in the blood of chickens under HS conditions simulated in a controlled environment.

## Materials and Methods

The study involved 15 laying hens. The first, control group (C, n=7), consisted of birds fed a standard diet without additional components. The second, experimental group (E, n=8), received a compound feed supplemented with betaine (0.5 g/kg), taurine (5 g/kg), and myo-inositol (2 g/kg) based on dry matter. Experimental work was conducted in the vivarium of the Institute of Animal Biology NAAS. The hens were housed in metal cages equipped with automatic feeders and waterers. During the study, birds were fed a complete compound feed balanced for all necessary nutrients, vitamins, and microelements, with access to clean drinking water. The vivarium conditions were controlled to maintain specified temperature, humidity, and lighting according to the experimental plan.

The study consisted of two phases. In the first phase, birds were kept under thermoneutral conditions (TN) at a temperature of 20°C and relative humidity of 60% (temperature-humidity index = 66 [6]). Blood samples were collected on day 7 for biochemical analysis. Starting on day 8, the vivarium temperature was raised to 30°C for 6 hours daily, maintaining a relative humidity of 70% to simulate heat stress (temperature-humidity index = 81 [6]). On day 14, after one week of high-temperature exposure, the birds were decapitated, and biological material was collected for further studies.

All procedures with chickens were performed in compliance with the provisions of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, 2005), Council Directive 2010/63/EU, and the Law of Ukraine no. 3447-IV "On the Protection of Animals from Cruelty" with amendments 440-IX dated 14.01.2020, following protocol no. 115a dated 28.09.2022 of the Bioethics Commission for Scientific Research of the Institute of Animal Biology NAAS.

The lipid hydroperoxide (LOOH) content was determined using the method described in [19]. A mixture of

0.2 ml blood plasma, 0.05 ml 50% TCA, and 2.8 ml ethanol was shaken for 5–6 minutes, then centrifuged at 3500 rpm for 10 minutes. A 1.5 ml supernatant aliquot was mixed with 1.2 ml ethanol, 0.02 ml concentrated HCl, and 0.03 ml 1% ammonium thiocyanate solution, then left for 30 seconds. A 20% thiocyanate solution induced a crimson color, and optical density was measured at 480 nm for 10 minutes. LOOH concentration was calculated as the difference between control and test samples and expressed in anson unit/ml (AU/ml).

Reduced glutathione (GSH) content was measured colorimetrically using 5,5'-dithiobis-2-nitrobenzoic acid (DTNB). In the test sample, 2 ml blood hemolysate and 3 ml precipitating reagent were combined. A control used 2 ml distilled water. After 5 minutes of incubation and centrifugation, the supernatant was mixed with 0.3 M  $\text{Na}_2\text{HPO}_4$  and Ellman's reagent. Absorbance was measured at 412 nm. GSH content was calculated using a calibration graph and expressed in mmol/l [19].

Glutathione peroxidase (GPxs) activity was assessed by measuring GSH oxidation with tertiary butyl hydroperoxide. A mixture of 0.1 ml hemolysate, TRIS buffer with EDTA and sodium azide, and GSH solution was incubated at 37°C. Tertiary butyl hydroperoxide was added, followed by 10% TCA. After centrifugation, the supernatant was mixed with TRIS buffer and Ellman's reagent, and absorbance was measured at 412 nm. GPxs activity was expressed as nmol GSH/min  $\times$  mg protein [19].

Glutathione reductase (GR) activity was determined by measuring NADPH oxidation. The mixture contained  $\text{K}_2\text{HPO}_4$ , oxidized glutathione, and EDTA. The reaction was initiated with NADPH and hemolysate, and absorbance decrease at 340 nm was monitored for 1 min. Enzyme activity was expressed as  $\mu\text{mol NADPH/min} \times \text{mg protein}$  [19].

Superoxide dismutase (SOD) activity was measured based on nitroblue tetrazolium (NBT) reduction by superoxide anions. A reaction mixture included hemolysate, NBT, EDTA, and PMS in phosphate buffer, with NADH added. Samples were incubated at 20°C for 10 min, and absorbance was recorded at 540 nm. SOD activity was expressed in anson unit/mg protein (AU/ml), using a calibration curve [19].

Catalase (CAT) activity was determined using molybdenum salts reacting with  $\text{H}_2\text{O}_2$  to form a colored complex. The test sample mixed hemolysate with  $\text{H}_2\text{O}_2$ . Control samples used ammonium molybdate. After incubation and acid addition, absorbance was measured at 410 nm [19].

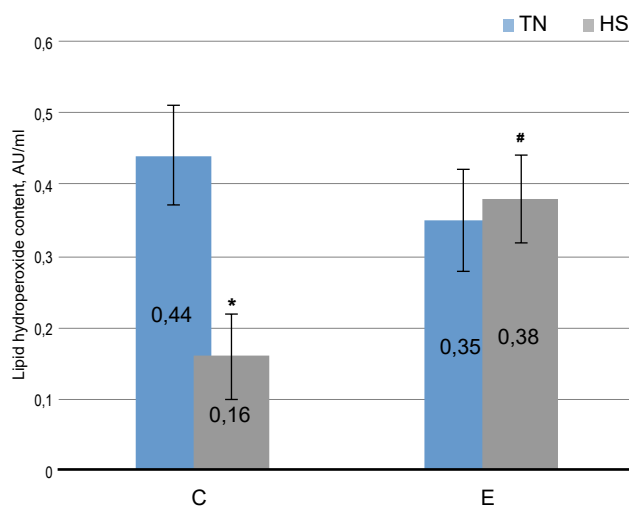
Statistical analysis was performed as described in [13]. Data are presented as mean  $\pm$  standard deviation. All data were analyzed using *Statistica 10* software. Statistical significance was determined using one-way analysis of variance (ANOVA). Student's *t*-test was used to evaluate differences between the two groups. Differences were considered statistically significant at  $P < 0.05$ .

## Results and Discussion

Lipid peroxidation is the process of oxidizing unsaturated fatty acids in cell membranes, leading to the formation of peroxides and other reactive products. This process can be triggered by free radicals and other oxidative agents [14]. Excessive activation of lipid peroxidation in biological systems increases the levels of lipid hydroperoxides and reactive aldehydes, which can be toxic to cells and tissues [16].

LOOH are the initial intermediates in cellular oxidation. They are formed when lipids react with free radicals and are unstable, quickly breaking down into other compounds such as aldehydes and ketones. Under stress, animals generally exhibit an increase in blood LOOH levels as an indicator of OS [7]. However, our study found that HS decreased LOOH levels in the blood serum of control group chickens by 2.7-fold ( $P < 0.05$ ) compared to chickens reared under thermoneutral conditions (fig.). This can be explained by HS reducing the availability of substrates for lipid peroxidation (e.g., polyunsaturated fatty acids), thereby decreasing LOOH formation. Significant GSH accumulation may also contribute to the reduction in LOOH levels [14].

The inclusion of dietary supplements under stressful conditions increased LOOH levels in chicken plasma by 2.4-fold ( $P < 0.05$ ) compared to the control group, reaching levels characteristic of TN conditions. Betaine and myo-inositol may stabilize cell membranes and maintain osmotic balance, but they also influence the metabolism of membrane lipids, particularly phosphatidylinositols containing polyunsaturated fatty acids [2, 5]. These acids are primary targets for lipid peroxidation, which could explain the increased LOOH levels.



**Fig.** Lipid hydroperoxide content in the plasma of laying hens under HS with the addition of betaine, taurine, and myo-inositol (M $\pm$ m, n=15)

*Note.* Here and further there is a statistically significant difference in data between HS and TN: \* —  $P < 0,05$ ; \*\* —  $P < 0,01$ ; \*\*\* —  $P < 0,001$ ; statistically significant difference in experimental data (E) compared to the control (C). TN — thermoneutral conditions; HS — heat stress.

SOD represents the first line of cellular defense against OS. It catalyzes the conversion of superoxide radicals ( $O_2^-$ ) into hydrogen peroxide ( $H_2O_2$ ) and oxygen ( $O_2$ ). This process is vital for the organism as superoxide radicals are highly reactive and can damage cellular components, including DNA, proteins, and lipids. By reducing superoxide levels, SOD helps prevent cellular damage and maintains homeostasis [4].

In our study, HS in the control group caused a 49% reduction in SOD activity in the blood ( $P < 0.01$ ) compared to TN conditions (table 1). In the experimental group, dietary supplementation under HS increased SOD activity in chicken erythrocytes by 55% compared to the control group, restoring levels to those characteristic of TN conditions. This suggests enhanced antioxidant protection through taurine, betaine, and myo-inositol supplementation, which activates SOD under HS, thereby providing better protection for erythrocytes against oxidative damage.

CAT is an enzyme that catalyzes the breakdown of hydrogen peroxide into water and oxygen, preventing the accumulation of this toxic compound in cells. Hydrogen peroxide is potentially harmful as it can initiate the formation of hydroxyl radicals, which damage cellular structures such as proteins, nucleic acids, and lipids [18]. In our study, CAT activity in the erythrocytes of laying hens under HS in the control group decreased significantly by 28% ( $P < 0.001$ ) compared to TN conditions. The addition of supplements under normal temperatures reduced CAT activity in animal erythrocytes by 14% ( $P < 0.01$ ). However, under elevated temperatures, CAT activity increased by 11% ( $P < 0.05$ ) compared to control values. This suggests a modest positive effect of supplements on the ability of erythrocytes to maintain catalase activity under stress, potentially linked

to increased cellular resistance to oxidative stress and an improved overall antioxidant status in laying hens [14].

The glutathione pathway of antioxidant defense is a key system for maintaining cellular redox homeostasis. It neutralizes ROS and prevents oxidative damage to biomolecules. The primary enzymes of this system operate in close coordination, regenerating antioxidants and maintaining a stable redox balance, which is critical for cellular function under oxidative stress [17]. This pathway includes GSH, GPxs, and GR, which we analyzed in the blood of laying hens in our study (table 2).

GSH is one of the most important non-enzymatic antioxidants, playing a crucial role in reducing LOOH through GPxs and neutralizing ROS and free radicals [16]. Under HS, we observed a 37% ( $P < 0.01$ ) increase in GSH levels in the control group and a 35% ( $P < 0.01$ ) increase in the experimental group compared to TN conditions. These findings indicate an increased need for antioxidant defense in response to heat stress.

GPxs is a key antioxidant enzyme that catalyzes the reduction of peroxides using GSH as a reducing agent [4]. As shown in table 2, HS in the control group significantly decreased GPxs activity in the blood of laying hens by 15% ( $P < 0.01$ ) compared to TN conditions, likely due to enzyme depletion under chronic oxidative stress. In the experimental group, dietary supplementation reduced GPxs activity in animal blood by 30% ( $P < 0.001$ ) under TN and by 15% ( $P < 0.01$ ) under HS compared to control values. This reduction may reflect decreased overall pro-oxidative stress due to membrane stabilization and increased GSH activity.

GR is an enzyme that catalyzes the conversion of oxidized glutathione (GSSG) into GSH using NADPH as a reducing agent [16]. GR activity analysis showed a 30% ( $P < 0.01$ ) decrease in erythrocytes of the control group under HS compared to TN. This indicates a decline in glutathione reductase functionality under HS due to prolonged oxidative stress, reducing the efficiency of the glutathione cycle. Dietary supplementation also reduced GR activity in birds' blood by 23% ( $P < 0.05$ ) compared to controls under normal temperature conditions, likely due to decreased need for GSSG reduction as peroxide and ROS levels declined. No significant changes in GR activity were observed under elevated temperatures in the experimental group, although there was a tendency for reduced activity.

Overall, the avian response to stress may involve increased production of certain antioxidants, such as GSH, to mitigate oxidative stress. However, this can deplete resources, negatively affecting other antioxidant enzymes like CAT, SOD, GPxs, and GR [3].

The reduced activity of some antioxidants in the experimental group may result from supplements such as betaine, taurine, and myo-inositol acting as direct

**Table 1.** Superoxide dismutase and catalase activities in the erythrocytes of laying hens under HS with the addition of betaine, taurine, and myo-inositol ( $M \pm m$ ,  $n=15$ )

Indicators	Condi-tions	Control	Experiment
SOD, AU/mg protein	TN	17,43±1,38	17,79±1,41
	HS	8,90±1,10**	19,57±0,87###
CAT, mmol $H_2O_2$ /min×mg of protein	TN	247,64 ± 3,35	212,16 ± 13,03##
	HS	177,10 ± 5,10***	198,04 ± 7,99#

**Table 2.** Indicators of the glutathione pathway of antioxidant defense in the erythrocytes of laying hens under HS with the addition of betaine, taurine, and myo-inositol ( $M \pm m$ ,  $n=15$ )

Indicators	Condi-tions	Control	Experiment
GSH, mmol/L	TN	0,50±0,06	0,58±0,09
	HS	0,79±0,04**	0,89±0,09**
GPxs, nmol GSH/min×mg protein	TN	145,65±7,73	101,36±6,70###
	HS	123,78±6,50**	104,58±5,41##
GR, $\mu$ mol NADPH/min×mg protein	TN	8,03±1,07	6,17±0,38#
	HS	5,61±0,69**	5,12±0,56

antioxidants. These compounds may interact with free radicals and oxidative molecules like hydrogen peroxide and superoxide anions, reducing their activity and converting them into less harmful compounds. Additionally, these compounds enhance cellular protective mechanisms, reduce inflammation, and support overall redox balance [1, 9]. Hence, antioxidant supplementation may trigger adaptive processes, reducing the need for endogenous antioxidant enzyme production due to the external antioxidant protection. This mechanism may contribute to stress mitigation and homeostasis restoration.

The study demonstrated that heat stress negatively affects the antioxidant status of laying hens, evidenced by reduced activity of key antioxidant enzymes (CAT, SOD, GPxs, GR) and LOOH levels. This indicates increased oxidative stress, disrupting the balance between pro- and antioxidant processes. Concurrently, the compensatory increase in GSH levels suggests the activation of non-enzymatic antioxidant defense pathways as a partial response to oxidative burden.

Dietary supplementation with taurine, betaine, and myo-inositol under HS normalized the antioxidant system of laying hens. Specifically, SOD activity was restored to TN levels, CAT activity moderately increased, and LOOH levels stabilized. The reduced GPxs and GR activity in the experimental group indicates a lower demand for their function due to enhanced alternative antioxidant pathways and reduced pro-oxidative stress.

Thus, the findings confirm that betaine, taurine, and myo-inositol supplements can mitigate oxidative stress, although their efficacy under HS remains limited. Further research is needed to optimize supplement composition and dosing to enhance antioxidant activity under stress conditions.

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## Ефективність бетаїну, таурину та міо-інозитулу у нормалізації антиоксидантного статусу курей при тепловому стресі

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Тепловий стрес є одним із ключових чинників, що впливають на адаптацію тварин до змін температури навколишнього середовища, і може значно позначитися на їхньому здоров'ї. Підвищена температура навколишнього середовища спричиняє значний стрес, що потенційно призводить до різних негативних наслідків у птиці, зокрема до порушень у роботі антиоксидантної системи. Дисбаланс між антиоксидантними та прооксидантними процесами може спричинити надмірне утворення вільних радикалів, які шкодять клітинам і можуть сприяти розвитку захворювань. У цьому дослідженні вивчено вплив штучно індукованого теплового стресу на антиоксидантну систему та продукти пероксидного окислення ліпідів у крові курей-несучок. Курей-несучки як комерційні лінійні птиці, селекціоновані для високої продуктивності яєць, є особливо вразливими до підвищених температур через інтенсивний обмін речовин, збільшений енергетичний попит на виробництво яєць та обмежену здатність до терморегуляції порівняно з іншими видами птахів. В умовах інтенсивного птахівництва, де щільність утримання висока, ці фактори можуть посилювати тепловий стрес. Метою дослідження було визначити зміни в окремих показниках антиоксидантної системи та вмісту продуктів пероксидного окислення ліпідів у крові курей під впливом бетаїну, таурину та міо-інозитулу. Аналіз таких параметрів, як ліпідні гідроперекиси (LOOH), відновлений глутатіон (GSH), глутатіонпероксидаза (GSH-Px), глутатіонредуктаза (GR), каталаза (CAT) та супероксиддисмутаза (SOD), дає змогу оцінити стан антиоксидантного захисту та рівень оксидативного стресу в умовах теплового стресу. Дослідження проводили на 15 курках-несучках, яких утримували у віварії Інституту біології тварин НААН. Воно складалося з двох етапів: протягом першого етапу курей утримували за температури 20°C протягом трьох тижнів. На другому етапі моделювали тепловий стрес, підвищуючи температуру до 30°C на 6 годин щодня протягом 7 днів. Птицю розділили на дві групи: контрольну (годували стандартним раціоном) та експериментальну (додатково згодовували 0,5 г/кг бетаїну, 5 г/кг таурину та 2 г/кг міо-інозитулу). Результати показали, що за підвищення температури в контрольній групі вміст LOOH знизився на 63% ( $P < 0,05$ ), а активність CAT, SOD, GSH-Px та GR зменшилася на 28% ( $P < 0,001$ ), 49% ( $P < 0,01$ ), 15% ( $P < 0,01$ ) та 30% ( $P < 0,01$ ) відповідно порівняно з термонеутральними умовами. Водночас вміст GSH зріс на 37% ( $P < 0,01$ ). В експериментальній групі, де застосовували добавки, активність CAT, GSH-Px та GR знизилася на 14% ( $P < 0,01$ ), 30% ( $P < 0,001$ ) та 23% ( $P < 0,05$ ) відповідно в термонеутральних умовах. За умов теплового стресу вміст LOOH знизився на 59% ( $P < 0,05$ ), активність GSH-Px — на 15% ( $P < 0,01$ ), тоді як активність SOD та CAT зросла на 55% ( $P < 0,001$ ) та 11% ( $P < 0,05$ ) відповідно порівняно з контрольною групою. Отримані результати свідчать про позитивний вплив бетаїну, таурину та міо-інозитулу на антиоксидантну систему курей-несучок за умов теплового стресу. Це підкреслює перспективність використання цих добавок для збереження здоров'я та продуктивності птиці в умовах високих температур.

**Ключові слова:** кури-несучки, тепловий стрес, оксидативний стрес, антиоксидантна система захисту D. B. Perederiy