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The influence of heat stress on the antioxidant protection glutathione link and the content of lipid peroxidation products in chicken liver

D. B. Perederiy
peredina0310@gmail.com

Institute of Animal Biology NAAS, 38 V. Stusa str., Lviv 79034, Ukraine

ORCID:
D. B. Perederiy https://orcid.org/0000-0002-9759-3513

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Heat stress is one of the main reactions of the body's response to environmental factors, negatively affecting the welfare of various animal species. An increase in environmental temperature can cause stress, which, in turn, potentially has various negative consequences for animals including disruption of the antioxidant system functioning. Violating the antioxidant-prooxidant balance can lead to an increased free radicals formation in the body, which can damage cells and promote the development of various diseases. The article presents the study results of the artificially simulated heat stress effect on individual indicators of the antioxidant system and lipid peroxidation products in the chickens' liver. The choice of these animals for this study is due to the fact that the poultry is particularly sensitive to the elevated environmental temperatures and, accordingly, to heat stress. This is caused by the biological peculiarities of birds, in particular, they lack sweat glands, and their body surface is mostly covered with feathers, which, together with the high density of keeping in the industrial poultry farming, often leads to the heat stress. The purpose of this work was to find out the presence or absence of changes in individual indicators of the antioxidant system glutathione link and the lipid peroxidation products content in chickens' liver. The analysis of indicators such as lipid hydroperoxides (LOOH), TBA-active products, reduced glutathione (GSH), glutathione peroxidase (GSH-Px) and glutathione reductase (GR) will allow us to obtain information about the body's antioxidant defense system state and the oxidative stress level under heat stress conditions. In this study we used 18 hens of the white Leghorn breed. The research was conducted in the vivarium of the Institute of Animal Biology of the National Academy of Sciences in two stages. During the first stage, chickens were kept at an air temperature of 20°C for 7 days. During the second stage, we created heat stress conditions by increasing the temperature to 35°C 6 hours a day, also for 7 days. It has been found that as the ambient temperature increased, the content of LOOH, GSH, and activity of GSH-Px in the liver of chickens increased ($P<0.01$), while, on the contrary, the content of TBA-active products and activity of GR decreased ($P<0.01$). The obtained data prove the heat stress effect on the antioxidant protection glutathione link in chicken liver. Research results can be of practical value for improving the bird keeping conditions in the industrial poultry farming, developing strategies for protection against stress, and improving the conditions to ensure the animals' welfare and health.

Key words: chickens, liver, heat stress, oxidative stress, antioxidant defense system

Introduction

Recently, the influence of climatic anomalies, changes, and fluctuations has become a great challenge in many areas of human activity, especially agriculture and, in particular, animal husbandry. Today, most countries of the world, including Ukraine, suffer significant economic losses due to the problems of high temperatures in various technological processes of raising agricultural animals. Such temperature regime violations often lead to heat stress in animals, which, in turn, harms their health, reproductive ability, and productive indicators. Heat stress (HS) is a condition in which animals cannot maintain a balance between the heat production and release. HS in chickens occurs when their body temperature rises to a dangerous level. This can happen due to high air temperature, humidity, or the combination of both factors. Poultry is particularly susceptible to HS due to its biological peculiarities — intensive metabolism level, increased heat production, rapid growth, and high productivity level. This imbalance can be caused by changes in the environmental factors combination (e.g. sunlight, thermal radiation, air temperature, humidity, and motion) and animal characteristics (e.g. species, sex, and metabolic rate) [20]. Among these factors, high ambient temperature plays the major role in the HS occurrence [23]. For optimal chickens' growth the thermoneutral zone is 18–21°C [26], and for laying hens the perfect environmental temperature ranges from 21 to 28°C [7]. Although there are data that any ambient temperature higher than 25°C already causes HS in poultry [21].

When the bird's body tries to maintain its thermal homeostasis, it simultaneously increases the level of reactive oxygen species (ROS). Many radicals and metabolites are described as found to be toxic and start as "reactive oxygen species" [16]. ROS are partially reduced oxygen metabolites, such as superoxide anions, hydrogen peroxide, and hydroxyl radicals [11]. Oxidized molecules pull electrons away from other molecules, causing a chain reaction that, if left unchecked, can cause significant tissue damage. As a result, the body enters the oxidative stress (OS) stage — a disproportion between antioxidants and free radicals in the system of elements. Antioxidants (AO) are substances that, on the contrary, prevent the oxidation of other molecules [2], meanwhile the free radicals are molecules containing active oxygen and precisely they cause the cell molecules oxidation. Increased ROS release is harmful, it intensifies muscle aging and protein degradation. ROS are highly reactive and can modify biologically active cellular macromolecules such as proteins, lipids, and nucleic acids [8, 10].

Despite numerous diverse pathological changes caused by heat stress, at the physiological level, many of the observed effects are the result of OS [9, 22]. This was confirmed by previous studies on poultry [13, 37]. Oxidative phenomena lead to the development of many metabolic dysfunctions, including OS, which can cause cell death [16]. In addition, OS can alter the redox balance of several cellular redox couples — e.g. reduced glutathione (GSH), and glutathione disulfide (GSSG), leading to altered expression of key enzymes in detoxification, antioxidant defense, cellular transitions, inflammatory responses, etc.

It is known that in chickens, acute stress induces the ROS production, disrupting the mitochondria function, which leads to a decrease in the aerobic fat and glucose metabolism and an increase in glycolysis, which ultimately leads to poor quality of chicken meat [13]. The body protects itself from the adverse effects of ROS using two important mechanisms, which are: the regulation of membrane permeability and the antioxidant system (AS) [1, 27]. This system protects cells from oxidative damage to proteins, lipids, and DNA [3, 5]. AS biochemical elements include both non-enzymatic low-molecular components — e.g. vitamin C, reduced glutathione (GSH) and uric acid, and enzymatic high-molecular substances — e.g. superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), glutathione reductase (GR), catalase (CAT). The SOD enzyme absorbs the superoxide radical, which is vital for creating a defense mechanism against ROS [18]. It is responsible for the dismutation of free radicals to water peroxide; after that, GSH-Px and CAT split water peroxide into water and oxygen molecules, creating inactive radicals [27, 36]. Heat stress has been shown to decrease cellular ROS levels in several animal models [19]. They limit the oxidation rate and progression, thereby protecting cells from oxidative damage. And already after cell damage, the recovery mechanisms are activated [13].

According to recent scientific studies, it has been established that an increase in ambient temperature above the physiological optimum can cause oxidative damage to the liver tissues in birds, which further disrupts lipid metabolism [12]. When chickens are exposed to HS, the content of free radicals in their bodies increases, while the AO enzymes activity and their ability to absorb free radicals decrease [24]. Mitochondria are both a major generator of cellular ROS and a highly sensitive target for the damage they cause. Accordingly, the mitochondrial function disruption and the subsequent effect on systemic metabolism and energy balance are the main determinants of the HS effect on the birds' liver [25]. Therefore, HS, frequently occurring in chickens and other types of commercial poultry, creates a big problem for poultry farming, especially in conditions of global warming, because, unfortunately, in the upcoming decades they predict only the climatic anomalies frequency rising and increase in heat loads [35]. Due to the above, it is important to deepen research of the physiological and biochemical mechanisms in the heat stress influence on various metabolism links in chickens, to search for biochemical markers of heat stress, as well as ways to mitigate it and reduce its negative effects.

This study aimed to investigate the status of the glutathione system specific parameters — the content of reduced glutathione (GSH), the activity of glutathione peroxidase (GSH-Px), glutathione reductase (GR), as well

as the content of lipid peroxidation products — TBA-active products and lipid hydroperoxides (LOOH) in chicken liver homogenates under normal temperature conditions and heat stress simulated in vivarium conditions.

Materials and Methods

The research was conducted in the vivarium of the Institute of Animal Biology of the National Academy of Sciences on 18 White Leghorn chickens. The poultry was kept in metal cages with feeders and automatic drinkers. During the experiment, the birds consumed standardized complete feed and clean drinking water. In the vivarium room the required temperature, humidity, and light mode were monitored daily and maintained according to the experimental scheme. During the first seven days, the chickens were kept at a temperature of 20°C as the normal temperature conditions — control group (C), 9 chickens. On the seventh day, the biological material of the liver was collected for further biochemical studies. From the eighth day of the experimental period, the air temperature in the vivarium raised to 35°C for 6 hours every day — experimental group (E), 9 chickens. On the 14th day or after seven days of exposure of chickens to elevated temperature, the biological material was collected again. Therefore, liver samples were taken from each bird both before and after exposure to heat stress. These samples were used to determine the following indicators: the content of LOOH, TBA-active products, GSH, GSH-Px, and GR. The conducted studies were not repeated.

All the manipulations with chickens were carried out by the principles of the "European Convention for the Protection of Vertebrate Animals Used for Experiments and Other Scientific Purposes" (Strasbourg, 2005), Council of Europe Directive no. 2010/63/EU and Law of Ukraine no. 3447-IV "On the Protection animals from cruel treatment" with amendments 440-IX from 14.01.2020, according to protocol no. 115 from 28.09.2022 of the meeting of the Commission on bioethics of scientific research of the Institute of Animal Biology NAAS.

Glutathione peroxidase (GSH-Px) activity (KF 1.11.1.9) was determined by the rate of GSH oxidation before and after incubation with tertiary butyl hydroperoxide, as described [32]. The development of the reaction is based on the interaction of SH-groups with 5,5-dithiobis-2-nitrobenzoic acid, resulting in the formation of a colored product — thionitrophenyl anion. Its amount is directly proportional to the number of SH groups that have reacted with 5,5-dithiobis-2-nitrobenzoic acid. 0.2 ml of tissue homogenate was incubated in a water bath at 37°C for 10 minutes. with 0.83 ml Tris-HCl buffer pH 8.5, which contained 6 mM EDTA solution and 12 mM sodium azide solution. A 4.8 mM solution of GSH (reduced glutathione) was prepared on this buffer. Then 0.05 ml of 20 mM tertiary butyl hydroperoxide solution was added and incubated again for 5 min. at 37°C. The reaction was stopped by adding 0.4 ml of a 10% THO solution, after which 5 ml of 0.1 M Tris-HCl buffer (pH 8.5), 0.1 ml of Ellman's reagent was added to 0.1 ml of the supernatant, and the optical density was measured at λ = 412 nm. GSH-Px activity was expressed in nmol GSH/min. per 1 mg of protein.

Glutathione reductase (GR) activity (KF 1.6.4.2) was determined by the method [6]. The principle of the method is based on the catalytic NADPH-dependent reaction of the reduction of the oxidized form of glutathione, the intensity of which is estimated by the rate of decrease in extinction, at which the NADPH solution has a light absorption maximum (340 nm). For the reaction, the liver homogenate was diluted with 0.1 M potassium phosphate buffer (pH 7.4) in a ratio of 1:9. The reaction was carried out in a thermostatic cuvette at a temperature of 37°C. 1.8 ml of 0.1 M potassium phosphate buffer (pH 7.0) with the addition of 1 mM EDTA, 0.1 ml of a 20 mM aqueous solution of oxidized glutathione, and 100 µl of diluted homogenate were added to the cuvette. After 3 min. the reaction was started by adding 0.1 ml of a 2 mM NADPH solution dissolved in 10 mM Tris-HC1 buffer, pH 7.0. The optical density of the investigated solutions was measured at a wavelength of 340 nm. GR activity was calculated according to the Bouguer-Lambert-Behr law, using the molar light absorption coefficient for NADPH at a wavelength of 340 nm (ε = 6200 M^{-1} cm^{-1}). Enzyme activity was expressed in µmol of NADPH/min. per 1 mg of protein.

The content of reduced glutathione (GSH) was measured before and after the reaction colorimetrically according to the method [17]. The color reaction is based on the interaction of SH-groups with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) with the formation of a colored product — thionitrophenyl anion. The content of the latter is directly proportional to the number of SH-groups that have reacted with DTNB. 0.2 ml of 20% sulfosalicylic acid solution was added to 0.6 ml of liver homogenate for protein precipitation. The samples were centrifuged for 10 min. at 3,000 g. An aliquot (0.1 ml) was transferred to tubes containing 2.55 ml of 0.1 M Tris-HC1 buffer with 0.01% EDTA, pH 8.5. 25 µl of DTNB solution was added to the resulting mixture. After the formation of color, the extinction index was determined on a spectrophotometer at λ=412 nm. The GSH content was calculated according to the calibration graph and expressed in mmol/g of tissue.

The concentration of TBA-active products was determined using the reaction between MDA and thiobarbituric acid (TBA), as described [32]. During the determination, 4.5 ml of 20% phosphotungstic acid was added to 1 ml of liver homogenate and centrifuged for 15 minutes at 700 g. The supernatant liquid was drained, and 1.0 ml of 0.8% TBA solution was added to the precipitate and kept for 1 h in a water bath at a temperature of 100°C. After that, the tubes were cooled and centrifuged. In the resulting centrifuge, the optical density was measured at a wavelength of 535 and 580 nm to exclude the absorption of painted complexes by TBA substances of a non-lipid nature. The concentration of TBA-active products was expressed in nmol of MDA per 1 g of tissue.

The content of lipid hydroperoxides (LOOH) was determined as described [32], according to which 2.8 ml of ethanol and 0.05 ml of a 50% THO solution were added to 0.2 ml of liver homogenate and shaken for 5–6 min. 1.5 ml of the supernatant was taken and adjusted to 2.7 ml with ethanol, 0.02 ml of concentrated HCl, and 0.03 ml of a 1% solution of Mohr's salt in a 3% solution of HCl were added. The optical density was measured after adding ammonium thiocyanate at λ = 480 nm. The LOOH content in biological material was expressed in terms of optical density at 480 nm per 1 g of tissue.

Statistical analysis was performed as described [29]. Data are expressed as mean ± standard deviation. All the 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 examine differences between 2 groups. The difference was considered statistically significant at $P<0.05$.

Results and Discussion

It is known that GSH and a complex of enzymes — glutathione peroxidase, glutathione transferase, and glutathione reductase are an important system of free radicals inactivation. It is also known that GSH is synthesized in the liver, from where it is transported to various organs and tissues [41]. Therefore, the indicators of the glutathione link of the antioxidant system of the liver of chickens under the influence of heat stress are of obvious interest. As shown by the results of our research presented in fig. 1, the content of GSH in the liver of chickens increased after a week of TS compared to the control ($P<0.001$). When analyzing the available scientific literature, we can see that similar results were obtained in many works by different scientists and years [24, 40]. However, at the same time, there is quite a lot of data that indicates the opposite direction of changes in the content of GSH in body tissues, which indicates that heat stress leads to a decrease in glutathione levels. GSH depletion is associated with increased ROS generation and increased sensitivity to hyperthermia [34]. We also found a significant ($P<0.001$) increase in glutathione peroxidase activity in the liver homogenates of chickens subjected to heat stress compared to the control group (fig. 1B). This seems quite logical, based on the known fact that GSH-Px protects the body's cells from oxidative stress, inhibits inflammation and oxidant-induced regulated cell death, catalyzes the breakdown of hydrogen peroxide, and oxidizes glutathione. However, as in the case of GSH, it should be emphasized that the data regarding the direction of changes in glutathione peroxidase activity in related studies are not unambiguous. Thus, an increase in GSH-Px activity, similar to the data we obtained, was also reported by S. Pamok et al. [15], W. Habashy et al. [28], L. Yang et al. [40]. But in contrast, P. Seven et al. [33] found a decrease in GSH-Px activity in the blood, liver, kidney, and heart of broilers. Interestingly, K. Sahin et al. [30] found that quail suffering from chronic heat stress had reduced hepatic GSH-Px activity. This was also confirmed in other researches [22, 31].

Regarding glutathione reductase activity of the liver, in our study, we observed its significant ($P<0.001$) decrease in the group of chickens that were subjected to daily heat stress for 7 days compared to birds that were kept under physiologically comfortable temperature conditions (fig. 1C). It is known that GR is involved in the regulation, modulation, and maintenance of cellular redox homeostasis. Therefore, a possible explanation for the decline in GR activity in liver homogenates that we observed may be a parallel simultaneous increase in the content of GSH in the liver. One of the reasons for the decrease in glutathione reductase activity under conditions of increased heat load may be a simultaneous decrease in the content of NADH and NADPH in the cells since GR is an enzyme dependent on NADPH, the activity of which is inhibited in the event of accumulation of the oxidized form of the nucleotide (NADP). NADPH + H^+ is formed in the hexose monophosphate shunt (pentose cycle) and provides H^+ for the regeneration of GSH from GSSG by GR. The normal functioning of the NADPH-dependent GR in the cell is very important for preventing oxidative damage to mitochondria, which are unable to synthesize HV *de novo* and therefore depend on the intensity of GR recovery of oxidized glutathione and its entry from the cytosol through the outer mitochondrial membrane.

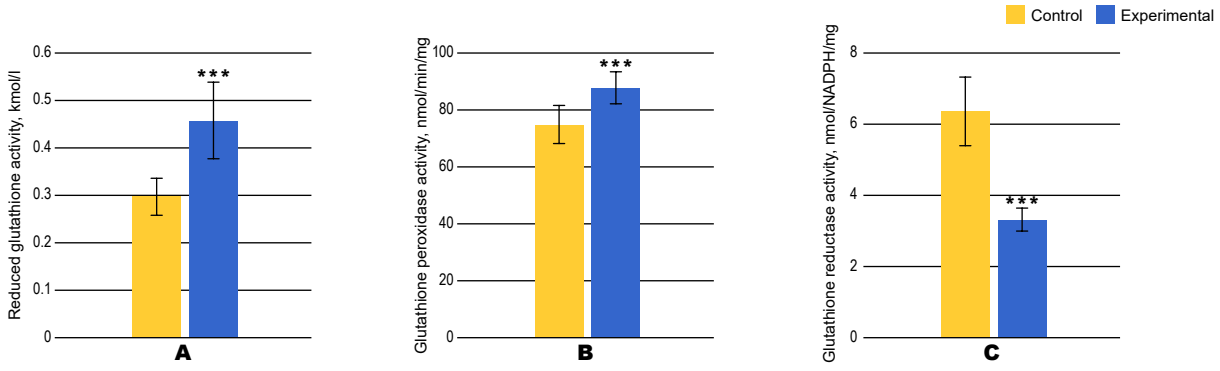

Fig. 1. Activity of antioxidant indicators in the liver of chickens: A — reduced glutathione, B — glutathione peroxidase, C — glutathione reductase
Note. Here and further ** — $P<0.01$, *** — $P<0.001$ compared to the control group.

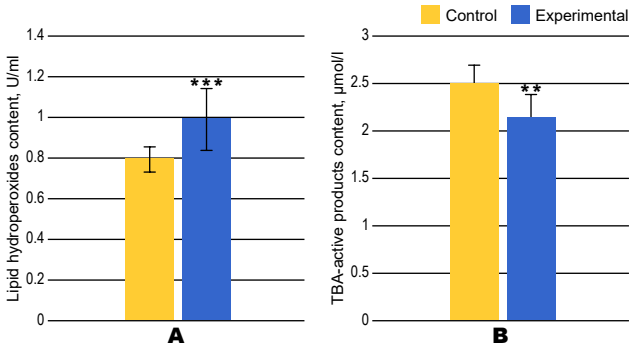

Fig. 2. The content of lipid peroxidation products in the chickens' liver: A — lipid hydroperoxides, B — TBA-active products

As in our case, according to the data obtained by W. Habashy et al. [15], 12 days after HS the GR level in the liver decreased (P<0.01) compared to the liver homogenate of the control groups. The similar studies were conducted by K. Sahin et al. on Japanese quail [30] and their results showed that there was a decrease in hepatic GR activity. But the results of Q. Miao et al. [24], on the contrary, showed that with the ambient temperature rise, the GR activity increased significantly (P<0.05).

As can be seen from the fig. 2, the LOOH content significantly increased (P<0.001) in the liver of chickens that were subjected to heat stress for seven days compared to the control group of birds. In general, LOOHs are reactive intermediates that occur during the peroxidation of unsaturated lipids in biological membranes. They are formed in the biological system in enzymatic and non-enzymatic ways. LOOHs have various harmful effects on cellular macromolecules and are also important regulators of cellular processes.

The fact that the content of LOOH, a byproduct of lipid peroxidation, increases in liver homogenates during thermal stress on chickens indicates the simultaneous occurrence of oxidative stress in the body. As for the content of TBA-active products, we found that their content decreased significantly (P<0.001) in the liver of chickens after exposure to heat stress for 7 days compared to the control. TBA-active products are also a marker of oxidative stress and in most cases their amount increases during HS. The research by M. Azad et al. [4] showed a 1.5-fold increase in TBA-active products. The results of the study on Japanese quail [30] showed an increase in the level of TBA-active products in the liver by 84.8%. Another experiment on one-day-old broiler chickens [31] confirmed an increase in the content of TBA-active products. L. Yang et al. [40], L. Tang et al. [38] and Y. Wang et al. [39] also showed that HS induced the formation of TBA-active products. However, at the same time, the results of Q. Miao et al. [14], W. Habashy et al. [24] showed that the content of TBA-active products did not change when the ambient temperature increased. In general, the increase in the level of LOOH, GSH, and GSH-Px indicates the activation of AS, because these AO neutralize free radicals in the animal's body to protect it from stress. As shown in the majority of modern studies, the content of TBA-active products mainly increases under the HS influence.

The data obtained in our study convincingly indicate that the activation of oxidation processes in poultry is a direct consequence of heat stress. The main redox pathways of the glutathione link of the antioxidant system, including GSH, GSH-Px, GR, and TBA-active products sometimes react differently in the liver, according to the tissue's need for ROS detoxification or tolerance to them.

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Вплив теплового стресу на глутатіонову ланку антиоксидантного захисту та вміст продуктів пероксидного окиснення ліпідів у печінці курей

Д. Б. Передерій
peredina0310@gmail.com

Інститут біології тварин НААН, вул. В. Стуса, 38, м. Львів, 79034, Україна

Тепловий стрес є однією з основних реакцій відповіді організму на фактори навколишнього середовища, який негативно впливає на добробут різних видів тварин. Підвищення температури навколишнього середовища може провокувати стрес, який, зі свого боку, загрожує різними негативними наслідками для тварин, зокрема збоєм у функціонуванні антиоксидантної системи. Порушення антиоксидантно-прооксидантної рівноваги може призвести до підвищеного утворення в організмі вільних

радикалів, здатних пошкоджувати клітини та сприяти розвитку різних захворювань. У статті наведено результати дослідження впливу штучно змодельованого теплового стресу на окремі показники антиоксидантної системи та продукти пероксидного окиснення ліпідів у печінці курей. Вибір цих тварин для дослідження зроблено з огляду на те, що птиця особливо чутлива до впливу підвищених температур довкілля і, відповідно, до теплового стресу. Це пов'язано із біологічними особливостями птахів, зокрема у них відсутні потові залози, поверхня тіла значною мірою вкрита пір'ям, що разом із високою щільністю посадки поголів'я в умовах промислового птахівництва нерідко стає причиною виникнення теплового стресу. Метою цієї роботи було з'ясувати наявність або відсутність змін окремих показників глутатіонової ланки антиоксидантної системи та вмісту продуктів пероксидного окиснення ліпідів у печінці курей. Аналіз таких показників, як гідропероксиди ліпідів (ГПЛ), ТБК-активних продуктів, відновлений глутатіон (ВГ), глутатіонпероксидаза (ГП) і глутатіонредуктаза (ГР), дозволить отримати інформацію про стан антиоксидантної захисної системи організму та рівень окислювального стресу за умов теплового стресу. У роботі було використано 18 курей породи Білий Леггорн. Дослідження проведено у віварії Інституту біології тварин НААН двома етапами: на першому курей утримували за температури повітря 20°С протягом 7 діб, на другому — створили умови теплового стресу підвищенням температури до 35°С на 6 год. в день також протягом 7 діб. Встановлено, що з підвищенням температури навколишнього середовища вміст ГПЛ, ВГ та активність ГП у печінці курей зростав ($P<0,001$), а вміст ТБК-активних продуктів та активність ГР — навпаки, знижувалися ($P<0,01$). Отримані дані засвідчують вплив теплового стресу на глутатіонову ланку антиоксидантного захисту печінки курей. Результати досліджень можуть мати практичне значення для вдосконалення умов утримання птахів у промисловому птахівництві, розробки стратегій захисту від стресу та покращення умов їх утримання з метою забезпечення добробуту та здоров'я тварин.

Ключові слова: кури, печінка, тепловий стрес, оксидативний стрес, антиоксидантна система захисту

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