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Running head: DIETARY SYNTHETIC AND PHYTOGENIC ANTIOXIDANTS IN HEAT-STRESSED BREEDER HENS

Synthetic and phytogenic antioxidants improve productive performance, antioxidant activity, gene expression, and offspring quality in breeder hens subjected to heat stress

Phocharapon Pasri,* Sitthipong Rakngam,* Nadine Gérard,† Pascal Mermillod,† and Sutisa Khempaka*

*School of Animal Technology and Innovation, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima, 30000, Thailand; and †UMR de Physiologie de la Reproduction et des Comportements, National Research Institute for Agronomy and Environment (INRAe), 37380, Nouzilly, France

[1]Corresponding author

Phone: (66)44-22-4572

Email: khampaka@sut.ac.th

Scientific section: Metabolism and Nutrition

ABSTRACT

This study aimed to investigate the efficacy of a synthetic source (a combination of vitamin E, vitamin C, Selenium, and L-carnitine) and phytogenic sources (a combination of clove, green tea pomace, and Vietnamese coriander) in overcoming heat stress (**HS**) damage in female breeder hens on production, sperm survival in the oviduct, antioxidant properties, gene expression, and quality of offspring. One hundred SUT female breeder hens were housed in individual cages and divided into four treatment groups: T1) basal diets under thermoneutral temperature (**TN**); T2) basal diets under HS; 3) basal diets with synthetic antioxidants under HS; and T4) basal diets with phytochemical antioxidants under HS. The result revealed that HS condition had a negative effect on reducing final body weight, FCR, egg weight, and 1-day old chick weight while increasing water intake and altered blood chemicals in breeder hens compared to TN breeder hens ($P < 0.01$). However, both synthetic and phytogenic antioxidants resulted in increased egg production and hatchability, while decreasing the number of late stages of embryo death during the incubation ($P < 0.05$). Furthermore, the synthetic antioxidants also improved the uniformity of chicks and the late stage of embryo death compared with the phytogenic antioxidants ($P < 0.05$). HS breeder hens fed with both antioxidant sources exhibited higher antioxidant capacity in terms of DPPH and ABTS radical scavenging (in yolk, liver, and breast meat) and FRAP radical scavenging (in yolk and liver) and lower liver malondialdehyde than HS breeder hens fed with the control diet ($P < 0.05$). Additionally, the gene expression of antioxidant enzymes (superoxide dismutase, catalase, and glutathione peroxidase) in liver was significantly upregulated, whereas the expression of pro-inflammatory cytokines (nuclear factor-κB) and heat shock proteins **(**HSP70 and HSP90) was downregulated in breeder hens that received both antioxidant sources ($P < 0.05$). This indicates the potential use of various bioactive antioxidant compounds as a combination of synthetic and phytogenic antioxidants against the deleterious effects of HS in breeder hens.

Key words: dietary antioxidant, breeder hen, heat stress, antioxidant activity, hatchability

INTRODUCTION

In the context of global climate change, poultry production faces increasingly challenging conditions, particularly during heat stress (**HS**) episodes. High environmental temperature of 32 to 38°C coupled with high humidity could induce large amounts of reactive oxygen species (**ROS**) that lead to oxidative stress (**OS**) which negatively affects the integrity of sperm membrane and DNA in uterovaginal sperm storage tubules, consequently, influencing egg production, maturity, fertility, embryo development, and causing economic losses (Fouad et al., 2016). Female breeder hens are sensitive to HS because their physiology is covered with feathers and there are no sweat glands that are difficult to thermoregulate. Previous studies have focused more on HS mitigation in housing management and genetic improvement against HS than on the body's capacity for ROS scavenging (Gharaghani et al., 2015). HS has been reported to significantly reduce various key parameters in female breeder hens, including live weight, feed efficiency, egg production, egg quality, eggshell quality, fertility, hatchability, and the overall livability of hatchlings (Ajakaiye et al., 2011). Methods to overcome heat stress damage include supplementation with antioxidant substances, such as vitamin C, vitamin E, selenium (**Se**), manganese (**Mn**), zinc (**Zn**), or phytogenic substances (Hu et al., 2019). In addition, the resolution of HS using a mixture of antioxidant substances from synthetic or natural sources that function at all levels of the antioxidant defense network can potentially alleviate the negative impact of HS. Unfortunately, little information is available regarding the use of mixtures from synthetic or phytogenic sources in breeder hens.

The high potential of dietary antioxidants are expected to cover three major levels of antioxidant defense networks: organelles, subcellular compartments, and the extracellular space (Horváth and Babinszky, 2018). Antioxidant substances play a key role in activating

antioxidant enzymes, such as superoxide dismutase (**SOD**), catalase (**CAT**), and glutathione peroxidase (**GSH-Px**), during the early stages of ROS formation, necessitating optimal levels of Mn, Cu, Zn, and Se for effective detoxification. However, some ROS, particularly transition metal ions, may remain active, causing lipid peroxidation and damage to DNA and proteins. To address this issue, second-level antioxidants are required to break the chain by scavenging peroxyl radical intermediates to prevent the propagation of lipid peroxidation. These functions are performed by substances such as vitamins A, E, and C; carotenoids; coenzyme Q10; and L-carnitine. The third level of the antioxidant defense network works continuously from the second level, aiming to repair and remove damaged molecules (lipids, proteins, and DNA) affected by ROS. This process involves the action of heat shock proteins (**HSPs**), methionine sulfoxide reductases, DNA repair enzymes, and phospholipases (Surai and Kochish, 2019; Surai et al., 2019). Based on the antioxidant functions mentioned above, the challenge for nutritionists and feed formulators is to find suitable antioxidants that can effectively support hens in coping with HS conditions (Surai et al., 2016).

Previous studies have reported a synergistic effect of dietary vitamins E and C in poultry during HS on antioxidant status (reduced lipid peroxidation and enhanced SOD and CAT activities) (Jena et al., 2013) and productive performance (improved fertile eggs, hatchability, and embryo mortality) (Ipek and Dikmen, 2014). The combination of vitamin E and Se in diets provides highly effective protection against OS and improves the production and reproduction of poultry in comparison to the use of individual antioxidants (Harsini et al., 2012; Horváth and Babinszky, 2018). In addition, vitamin E and Se combinations in laying hen diets have been shown to transfer to the tissue and egg yolk, leading to decreased lipid peroxidation, increased GSH-Px enzyme levels, and enhanced hatchability and chick quality (Urso et al., 2015; Celebi, 2019). The interaction between vitamins C and Se has also been reported to improve egg production and feed efficiency compared with individual

supplementation (Attia et al., 2016). The combination of vitamins E, C, and Se in the diet can act synergistically as antioxidants to reduce HS and lipid peroxidation in poultry meat (Leskovec et al., 2019). Furthermore, the use of L-carnitine has gained significant attention due to its crucial role as a novel antioxidant via preventing DNA damage induced by ROS and stimulating antioxidant enzyme activities, transferring long- chain fatty acids to maintain β-oxidation in the matrix compartment of mitochondria and energy production by stabilization acetyl Co-A/Co-A ratio for adequate acetyl storage for laying hens (Surai, 2015; Agarwal et al., 2018). Dietary L-carnitine supplementation in poultry decreases malondialdehyde (**MDA**) levels and increases SOD, CAT, and GSH-Px activities under high stocking density stress (Çetin and Güçlü, 2019). In addition, some studies have reported improved egg production, hatchability, antioxidant activity, and offspring quality in laying hens and duck breeders fed L-carnitine (Salmanzadeh, 2011; Wang et al., 2013; Awad et al., 2017).

Phytogenic compounds consist of a variety of polyphenols derived from plant materials that can activate the expression of stress response proteins, such as HSPs and antioxidant enzymes, which can repress ROS and interfere with negative inducers in the HS response (Hu et al., 2019; Saracila et al., 2021). The most bioactive compound in cloves (*Syzygium aromaticum*) is eugenol, which is a potent natural antioxidant (Hemalatha et al., 2016). Supplementation of poultry diets with clove oil or powder can improve feed efficiency, egg production, immunity, and antioxidant activity (Sehitoglu and Kaya, 2021; Mahrous et al., 2017). Green tea (*Camellia sinensis*) is rich in polyphenols, particularly catechins, that exhibit antioxidant, antimicrobial, antifungal, and anticarcinogenic properties (Pinto et al., 2020). It has been shown that supplementation of green tea in breeder poultry diets (powder or extraction form) can reduce MDA egg yolk concentrations, while increasing fertility, hatchability, and sperm quality (Kara et al., 2016; Chen et al., 2021; Wang et al., 2021). Vietnamese coriander (*Persicaria odorata*) has a high phytochemical composition, especially gallic acid, quercetin,

ferulic acid, apigenin, and essential oils, which contribute to its antioxidant and biological activities (Pawłowska et al., 2020). Supplementing broiler or laying hen diets with Vietnamese coriander leaf meal has shown beneficial effects on growth performance, digestibility, egg production, egg weight, and lipid peroxidation in meat (Basit et al., 2020; Ooi et al., 2018; Glinubon et al., 2022). In our previous *in vitro* study, we examined 17 edible plant materials; clove, green tea pomace, and Vietnamese coriander showed notably high levels of phenolic and total flavonoid content as well as strong antioxidant activity in terms of DPPH, ABTS, and FRAP. The combination of these plants in equal parts (1:1:1 ratio, v:v:v) produced synergistic antioxidant properties and improved cell safety, making them suitable candidates for use as phytogenic antioxidant feed additives (Pasri et al., 2023).

Unfortunately, no information is available on the combined effects of vitamins E, C, Se, L-carnitine, and phytogenic on the productive performance and antioxidant activities of breeder hens under HS that would be applicable to the development of a potential group of feed additives to alleviate the adverse effects of HS. Therefore, the aim of this study was to investigate the efficacy of two sources of antioxidant substances, synthetic (combination of vitamin E, vitamin C, Se, and L-carnitine) and phytogenic (combination of clove, green tea pomace, and Vietnamese coriander), to overcome HS damage in breeder hens and prolong sperm survival in the oviduct, antioxidant properties, gene expression, and quality of offspring.

MATERIALS AND METHODS

Ethics Statement

All animal experiments were approved by the Animal Care and Use Committee of Suranaree University of Technology (SUT-IACUC-012/2020).

Housing, Birds and Experimental diets

This study maintained controlled temperature (°C) and humidity (%) levels according to the temperature and humidity stress indices for laying hens (Hy-line, 2016). Thermoneutral (**TN**) and chronic HS were implemented according to the methodology described by Duangjinda et al. (2017). The TN was set up at 23±1°C with the humidity of 40–70% by using an air conditioner. The HS room was kept at a temperature of 36±1°C for 4 h daily (from 1 pm to 5 pm) using a gas heater with thermostat-controlled equipment, while during the rest of time, the temperature was maintained at the same conditions in the thermoneutral zone.

A total of 100 female SUT breeder hens (33 weeks old) were housed in individual cages with dimensions of 40 × 45 × 40 cm^3 (length × width × height) and divided into four treatment groups, each consisting of 25 females, using a Completely Randomized Design. Group 1 was raised in a TN room, whereas groups 2, 3, and 4 were subjected to HS for 4 h daily.

In this study, two sources of antioxidants (synthetic and phytogenic) were evaluated in female SUT breeder hens under HS conditions. The experimental diets consisted of four treatments as follows: T1) basal diets under thermoneutral temperature, T2) basal diets under HS; 3) basal diets with combined synthetic antioxidants (200 mg of vitamin C/kg, 150 mg of vitamin E/kg, 0.30 mg of Se yeast/kg, and 150 mg of carnitine/kg) under HS, and T4) basal diets with 1% phytochemical antioxidants (a mixture of clove, green tea pomace, and Vietnamese coriander powders, 1:1:1 ratio/w:w:w) under HS. Diets were formulated to meet the nutrient requirements according to the NRC (1994) and Ross 308 parent stock standard recommendations (Aviagen. 2021) (15% CP, 2800 kcal ME/kg), as shown in Table 1. All breeder hens were provided 16 h of light per day, received 140 g of feed daily, and had unrestricted access to water throughout the experimental period. Each experimental diet was fed to breeder hens for approximately one month prior to starting the trial.

Blood Chemical Analysis

After the hens were subjected to HS for 4 h, 12 breeder hens from each treatment group were randomly selected for blood chemical analysis using an Abbott i-STAT 1 handheld blood gas analyzer (Abbott Point of Care Inc. IL, USA) equipped with a CG8+ cartridge (Abaxis item number 600−9001). This CG8+ cartridge performed various parameters such as partial pressure of carbon dioxide (**PCO_2**) and oxygen (**PO_2**), pH, saturation of oxygen (**sO_2**), concentration of bicarbonate ions (**HCO_3^-**), total concentration carbon dioxide (**TCO_2**), concentration ionized calcium (**iCa**), sodium (**Na**), potassium (**K**), glucose (**Glu**), hematocrit (**Hct**), hemoglobin (**Hgb**), and base excess (**BE**). Blood from each breeder hen was collected and placed in a lithium heparin tube quickly, which should be used for analysis within 3 min. One hundred μL of blood was dropped into the CG8+ cartridge, which was subsequently inserted into the Abbott i-STAT 1 handheld blood gas analyzer (Barrett et al., 2019).

Growth Performance and Productive Performance Measurements

Productive performance parameters were measured over a 7-week period from 38 to 44 weeks of age. Body weight was recorded at the beginning and end of the experiment. Daily records were made for the number of eggs, egg weight, feed intake, and water intake in each treatment throughout the experimental period and were used to calculate egg production, feed conversion ratio, average egg weight, average daily feed, and water intake.

The fertile period length of sperm was determined from eggs collected over 21 days (38−41 weeks of age). Semen samples were collected from 60 Lueng Hang Khao breeder males by pooling and diluting with Beltsville poultry semen extender II (1:1/v:v) prior to artificial insemination. The breeder hens were artificial inseminated two days continually (0.1 mL of pooled semen/time) and then the hens in groups 2, 3, and 4 were exposed to HS at 36ºC for 4 h/day. On day 3, eggs from all treatments were collected over a 21-day period and stored in a cool room at 15ºC. Every 7 days, these eggs were then placed in automatic incubator (Model

192, Petersime Incubation Equipment Co., Ltd., Zulte, Belgium) with optimal conditions at 37.67±0.20°C and 62−65% relative humidity at the hatchery of university farm. The fertile period of the sperm was candled on day 7 of incubation. When the infertile eggs broke, the germinal disc region was monitored for embryonic development. The number of days for the fertile period of sperm was counted from the last day of the fertile egg prior to a sequence of three consecutive days with detected infertile eggs (Biswas et al., 2010; Ahammad et al., 2013).

Fertility, hatchability, and embryonic mortality rate were performed over a period of 41−44 weeks of age. Each breeder hen was artificially inseminated twice per week with pooled semen in the afternoon, and induced daily with heat stress at 36°C for 4 h. Then eggs in each treatment were collected daily and stored in a cool room at 15°C. Each week, the eggs were incubated in an automatic incubator for 21 days. Fertility and early and final embryonic mortality rates were detected by candling on days 10 and 18, whereas hatchability was measured on day 21 of incubation by counting the number of 1-day old chicks. Productive performances were calculated using formulas: fertility (%) = [(number of fertilized eggs/total eggs set) × 100]; early embryonic mortality rate (%) = [(number of fertilized eggs before day 10/total eggs set) × 100]; late embryonic mortality rate (%) = [(number of fertilized eggs after day 10/total eggs set) × 100]; hatchability (%) = [(number of day old chicks/ fertilized eggs) × 100], and total hatchability (%) = [(number of 1-day old chicks/total eggs set) × 100] (Salmanzadeh, 2011; Urso et al., 2015).

Sample Collection and Sample Extraction for Antioxidant Activity

At the end of the experiments, 25 breeder hens were randomly selected and slaughtered after heating at 36 ºC for 4 h. Liver and breast tissues were collected, immediately frozen in liquid nitrogen, and stored at −80°C until further gene expression and antioxidant activity analyses. Two grams each of egg yolk, liver, and breast tissue were extracted with 2 mL of

99% ethanol in a centrifugal tube. The samples were grinded for 20 s by ultra-homogenizer after that centrifuged at 12,000 x g at 4°C for 10 min. The supernatants were used to estimate the antioxidant activity.

2,2-Diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) Scavenging Activity Assay

DPPH$^{\bullet}$ scavenging activity was determined according to the method described by Nuengchamnong et al. (2009). For each sample extraction (100 μL), 100 μL of 0.6 mM DPPH in ethanol was added to a 96 well microplate. The mixture was gently shaken and incubated in dark for 30 min. The absorbance of the reaction mixture was measured at 517 nm using a microplate spectrophotometer (Thermo Scientific^{TM}, Multidkan^{TM} GO, Japan). Ethanol was used as a reagent blank instead of the sample. The DPPH$^{\bullet}$-scavenging activity was calculated as follows: inhibition (%) = [(absorbance of blank-absorbance of sample)/(absorbance of blank)] × 100. All measurements were performed in triplicate.

Scavenging Activity Assay of 2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS$^{\bullet+}$) Radical

The ABTS$^{\bullet+}$ cation radical assay was conducted as described by Re et al. (1999). The ABTS$^{\bullet+}$ stock solution was prepared by mixing 7.4 mM ABTS with 2.6 mM of potassium persulfate in a 10 mM phosphate buffer solution at pH 7.4. The mixture was then left to react overnight (12−16 h) in the dark at 4°C. Before starting the reaction, the freshly prepared ABTS$^{\bullet+}$ stock solution was adjusted to an absorbance value of 0.70±0.02 at 734 nm by dilution with a 10 mM cooled phosphate buffer. For the assay, 180 µL of ABTS$^{\bullet+}$ working solution was added to 20 µL of the extracted sample solvents in a 96 well microplate.

After 6 min of inoculation at room temperature, absorbance at 734 nm was measured using a microplate spectrophotometer. The absorbance of the blank was measured using

ethanol used in the reaction. The $ABTS^{\bullet+}$ cation radical was calculated using the following equation: inhibition (%) = [(absorbance of blank-absorbance of sample)/(absorbance of blank)] × 100. The results were compared to the standard curve and reported in mM equivalent trolox/g sample weight (mM TE/g sample).

The Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP assay was conducted according to the method described by Benzie and Strain (1996). The working FRAP reagent required fresh preparation before use, consisting of 0.3 M acetate buffer (pH 3.6), 10 mM 2,4,6-Tris (2-pyridyl)-s-triazine in 40 mM hydrochloric acid, and 20 mM iron chloride, mixed at a ratio of 10:1:1/v:v:v. The reagent was incubated at 37°C for 15 min. The 200 uL of the FRAP working reagent was added to 20 µL of sample extraction solvents in a 96 well microplate and incubated for 30 min, and the absorbance was then measured using a microplate spectrophotometer at 593 nm. The FRAP value was calculated using a calibration curve of trolox (25–100 mM/mL) and the results were reported as mM Trolox equivalents per gram of sample weight (mM TE/g sample).

Thiobarbituric Acid Reactive Substances (TBARs)

Egg yolk, liver, and breast tissue (2 g) were homogenized with 6 mL of DI water and 34 μL of 7.2% butylated hydroxytoluene (**BHT**) in ethanol using ultra-homogenizer for 40 s. Subsequently, 2 mL of the homogenized sample was mixed with 4 mL of TBA-TCA solution (20 mM TBA in 15% TCA) in a 15 mL tube and boiled at 95°C for 20 min in an ultrasonic bath (Ultrasonic cleaner 3200 EP S3, Soltec, Italy, 40 KHz and 180 W). After cooling, the mixture was centrifuged at 5,000 x g for 10 min at room temperature. The supernatant (200 μL) was then transferred to a 96 well microplate, and absorbance readings were taken at 532 nm. To quantify lipid peroxidation, MDA was used as a standard at concentrations ranging 5–

40 μM. The TBARs value was expressed as MDA equivalents per gram of sample weight (μM MDA/g sample) and determined based on the calibration curve of MDA (Grotto et al., 2009).

Hepatic Gene Expression

Total RNA was extracted from liver tissue using QIAamp® DNA Stool Mini kits (Qiagen, Hilden, Germany) and purified using a QIAamp spin column (Qiagen, Hilden, Germany). RNA purity and quantification were assessed using a Nanodrop spectrophotometer at 260 nm/280 nm. Subsequently, 1 μg of high quality RNA sample was applied for complementary DNA (**cDNA**) synthesis using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). For real time PCR, each reaction's master mix (8 μL) contained 5 μL of SYBR Green, 0.2 μL of forward primer, 0.2 μL reverse primer, and 2 μL of cDNA samples in a 96 well microplate. The real time PCR was performed using the QuantiNova™ SYBR Green PCR kit (Qiagen, Hilden, Germany) and analyzed in triplicate as described by Humam et al., (2019). The primer sequences for SOD, CAT, GSH-Px, nuclear factor-κB (**NF-κB**), heat shock protein 70 (**HSP70**), heat shock protein 90 (**HSP90**), and β-actin are presented in Table 2. Reverse transcription-quantitative real-time PCR (**RT-qPCR**) was accomplished using the CFX96 real-time PCR system (BioRad, Hercules, California, USA). The RT-qPCR reactions were conducted as follows: initial heat activation at 94°C for 10 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing for 30 s, and final extension at 72°C for 30 s. Relative quantification of the target gene expressions was normalized using β-actin as the reference gene and calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Statistical Analysis

Statistical analysis of the data was performed using ANOVA in a Completely Randomized Design (CRD) with SPSS software (version 16.0). Tukey's test was used to assess

significant differences among treatments. Additionally, orthogonal contrasts were used to compare the following conditions: 1, thermoneutral vs. heat stress; 2, supplementation vs. non-supplementation; and 3, synthetic vs. phytogenic conditions. A significance level at $P < 0.05$ was used (SPSS Inc., Chicago, IL).

RESULTS

Productive Performances

The effects of antioxidant supplementation in the form of synthetic (vitamins C, E, Se, and L-carnitine) and phytogenic (clove, green tea pomace, and Vietnamese coriander) substances on production performance are shown in Table 3. In the orthogonal contrast test, HS breeder hens had lower final body weight (**FBW**), FCR, egg weight (**EW**), and day-old chick weight, but higher hatchability and water intake (**WI**) than TN hens ($P < 0.01$). Both the synthetic and phytogenic antioxidant supplementation groups showed increased egg production (**EP**) and hatchability as well as reduced feed intake (**FI**), WI, EW, early dead and last dead embryos, and old chick weight compared to the non-supplementation group ($P < 0.05$). However, no significant differences were observed in any of the measured parameters between the synthetic and phytogenic antioxidants ($P > 0.05$). Interestingly, based on the Tukey analysis, it was found that the supplementation of antioxidants in the HS group could increase EP, similar to that in the TN group ($P > 0.05$). In addition, both forms of antioxidant supplementation resulted in significantly higher hatchability compared than in the TN group ($P = 0.0001$). However, a reduction in FBW, FI, EW, and day-old chicks was observed in HS breeder hens that received phytogenic antioxidants compared to TN hens, whereas no such significant differences were observed for synthetic antioxidants ($P > 0.05$). Additionally, supplementation with synthetic antioxidants demonstrated an improvement in the last stage of

325 embryo development during incubation and chick uniformity compared with phytogenic
326 antioxidants ($P < 0.05$).
327
328 ***Blood Chemistry Parameters***
329 Based on orthogonal contrasts, breeder hens subjected to HS had lower blood values of
330 PCO_2, PO_2, BE, HCO_3^-, TCO_2, and iCa, while pH and sO_2 were higher than the TN breeder
331 hens ($P < 0.01$) (Table 4). In addition, HS led to decreased blood Na, K, Hct, and Hb and
332 increased blood Glu compared to TN ($P < 0.01$). However, under HS conditions,
333 supplementation with both synthetic and phytogenic antioxidant substances did not
334 significantly alter any of the blood parameters compared to the non-supplementation group (P
335 > 0.05). In particular, Tukey's test revealed that HS breeder hens supplemented with
336 phytogenic antioxidants had lower blood Na and K levels than the TN group ($P < 0.05$).
337
338 ***Antioxidant Activities in Liver, Breast, and Yolk***
339 The effects of antioxidant supplementation in the form of synthetic and phytogenic
340 substances in breeder hen diets on antioxidant activity are shown in Table 5. According to the
341 orthogonal contrast test, the HS breeder hen groups had significantly higher antioxidant
342 activities in terms of DPPH radical scavenging in egg yolk and liver, and FRAP radical
343 scavenging in the liver, but had lower levels of MAD in egg yolk than breeder hens in the TN
344 group ($P < 0.05$). Both antioxidant supplementation groups exhibited significantly highest
345 antioxidant activities in DPPH, FRAP, and ABTS radical scavenging in all samples (except
346 FRAP in breast tissue), as well as a decrease in MDA in egg yolk compared to the non-
347 supplementation group during HS ($P < 0.05$). The breeder hens fed with synthetic antioxidants
348 exhibited the highest DPPH and FRAP radical scavenging activity and the lowest MDA levels
349 in egg yolk compared to those fed with phytogenic antioxidants ($P < 0.01$). Furthermore,

supplementation with synthetic antioxidants in breeder hen diets also improved the values of DPPH, FRAP, and MDA in the liver and yolk compared to non-supplementation under both HS and TN conditions ($P < 0.05$).

Moreover, DPPH (in egg yolk and breast tissue), FRAP (in egg yolk and liver), and ABTS radical scavenging (in egg yolk, liver, and breast tissue) were elevated, whereas MDA levels (in egg yolk) were reduced in breeder hens fed phytogenic dietary supplementation compared to breeder hens without supplementation under HS ($P > 0.05$).

Gene Expression in Liver

The gene expression levels related to antioxidant enzymes, pro-inflammatory cytokines, and heat shock proteins are presented in Table 6. In orthogonal contrasts, upregulation of SOD and GHS-Px genes and downregulation of HSP70 and HSP90 genes were observed in HS breeder hens compared to those in TN breeder hens ($P < 0.05$). However, no significant differences in the expression of the CAT and NF-κB genes were found between the HS and TN groups ($P > 0.05$). During HS, it was found that both synthetic and phytogenic antioxidants can alter gene expression in the liver by upregulating SOD, CAT, and GSH-Px and down regulating NF-κB, HSP70, and HSP90 compared to non-supplementation ($P < 0.01$). It is interesting to note that phytogenic antioxidants were found to induce the expression of the GSH-Px gene in a similar manner to synthetic antioxidants ($P < 0.05$). In addition, both synthetic and phytogenic antioxidants showed similar effects on the expression of SOD, CAT, NF-kB, HSP70, and HSP90 under HS conditions ($P > 0.05$).

DISCUSSION

Exogenous dietary antioxidants are widely accepted as effective substances for mitigating the adverse effects of HS on breeder hens in terms of favorable productive and

reproductive performance, immunity, embryonic development, and antioxidant activity (Ibtisham et al., 2019; Amevor et al., 2021; Darmawan et al., 2022). Our results revealed the beneficial effects of synthetic (vitamins C, E, Se, and L-carnitine) and phytogenic (clove, green tea pomace, and Vietnamese coriander) antioxidants on egg production, hatchability, embryo development, and antioxidant activity of breeder hens under HS.

In this study, both synthetic and phytogenic antioxidant sources were found to improve egg production and hatchability in breeder hens subjected to HS. However, in the orthogonal contrast test, the FI in the HS hen groups supplemented with antioxidants were lower than that in the HS hen group without supplementation or the TN hen group. This led to insufficient nutrient uptake. However, these groups of hens were still able to maintain egg production. This is probably because birds anabolize fat storage in the body to conserve the nutrients needed for maintenance and production, which can be confirmed from the reduced BW of HS hens. In general, HS poultry require higher maintenance energy than usual for thermoregulation. However, the decrease in FI during HS results in sufficient nutrient intake, as chickens compensate by breaking down glycogen or fat stored through gluconeogenesis (Nawaz et al., 2021; Jastreski et al., 2017). In addition, Xie et al. (2015) reported that elevated plasma glucose levels in HS broiler breeders indicate changes in carbohydrate and lipid metabolism for maintaining the metabolic rate, which is consistent with our results, which revealed increased blood glucose in HS breeder hens. In addition, this study found reductions in PCO_2, PO_2, BE, HCO_3^-, TCO_2, and iCa, along with an increase in pH and sO_2 in the blood of HS breeder hens.

The current study indicates that both antioxidant sources play interconnected roles in metabolic pathways, which effectively assists breeder hens in combating the negative effects of HS. During HS, vitamin C serves as a co-factor for dopamine beta-hydroxylase, converting dopamine into norepinephrine in neural tissues during the HS, promoting gluconeogenesis, which indirectly causes an increase in heart rate, blood pressure, blood glucose, and skeletal

muscle blood flow (Shakeri et al., 2020). Vitamin E prevents liver damage and maintains vitellogenin synthesis, which is important for yolk formation and consequences of egg production (Khan et al., 2011). L-carnitine is crucial for transferring long-chain fatty acids from the cytoplasm to the mitochondrial matrix for β- oxidation and energy production, facilitating the anabolism of stored fat in bird bodies, which ultimately provides energy for follicle development and egg production (Zhai et al., 2008; Awad et al., 2017). In addition, Se is essential for optimal poultry performance during HS, indirectly regulating triiodothyronine (T3) and thyroxine (T4) hormones, which affect the metabolic rate, protein synthesis, and nutrient metabolism (Shakeri et al., 2020).

The improved hatchability and antioxidant capacity observed in breeder hens that received dietary phytogenic antioxidants in this study can be attributed to the deposition of bioactive antioxidant compounds. In phytogenic sources (clove, green tea pomace, and Vietnamese coriander), several bioactive compounds are associated with antioxidant properties, such as eugenol (from clove), catechin (from green tea pomace), and catechin, quercetin, kaempferol, and ellagic acid (from Vietnamese coriander). These polyphenols are potent antioxidants because their chemical structure contains more than two hydroxyl groups (PhenOH), which allow them to break bonds and release hydrogen and electrons. This effectively eliminates excess ROS (such as O_2, H_2O_2, $OH^{\bullet}$, $RO^{\bullet}$, and $RO_2^{\bullet}$) and transforms them into a phenoxyl radical (PhenO), which is more stable and less likely to initiate chain reactions than the initial radicals. Polyphenols can also transfer electrons to bind to metal-ion free radicals (such as Fe^{2+}, Cu^{2+}, or Cu^{+}) (Saracila et al., 2021). Dietary catechins or tea polyphenols have been shown to be transferred from the blood to the ovaries, magnum, and other organs, providing antioxidant capacity and stability for polyunsaturated fatty acids (**PUFA**) in eggs (Ariana et al., 2011). Chen et al. (2021) observed catechin deposition in chickens fed with green tea, which led to increased DPPH, ABST, and OH radical scavenging activities. Additionally,

clove oil or buds with high eugenol levels showed the potential to decrease MDA in eggs and increase the enzyme activity of reduced glutathione (GSH), SOD, and glutathione S-transferase (**GST**) in the breast muscle (Mahrous et al., 2017; Sehitoglu and Kaya, 2021). Feeding broilers with a mixture of herbal extracts (mulberry leaf, Japanese honeysuckle, and Goldthread) resulted in the accumulation of phenolic compounds in breast tissue and demonstrated the potential to increase DPPH and ABST and reduce TBARs (Jang et al., 2008). In this study, various bioactive polyphenol compounds, such as eugenol, catechin, quercetin, kaempferol, and ellagic acid, were detected, all of which could complement the mechanisms of action to eliminate ROS. However, there are limitations to the metabolism and bioavailability of polyphenols, as some are poorly absorbed in the small intestine and require enzymatic hydrolysis by gut microbes (Abd El- Hack et al., 2022). The rapid absorption and elimination of polyphenols is a major factor leading to their low accumulation in tissues compared to that of synthetic antioxidants; thus, frequent supplementation could potentially improve their biological activity (Hidalgo et al., 2012).

Although both the antioxidant sources maintained egg production, a reduction in egg weight was observed. HS is known to reduce FI in poultry, resulting in insufficient nutrient intake, particularly protein, which leads to a decrease in egg weight. Khatibi et al. (2021) revealed that laying hen diets with protein levels of 15.0−15.5% CP under a subtropical climate can improve production, egg weight, and egg mass compared to diets with 14.2−14.7% CP. Although both antioxidants in this study reduced egg weight compared to the control and HS without supplementation, the weight was still in the range of 50−70 g, which was suitable for hatching following the Cobb 500 or Ross 308 breeder guidelines. In general, egg weight is associated with chick weight, indicating that both antioxidant sources can help maintain egg weight, resulting in a normal chick weight, which benefits the poultry industry.

Neither the HS conditions nor antioxidant supplementation affected the fertility rate or fertile period length of the sperm. However, this phenomenon remains unclear, and related research on this subject is limited. HS can adversely affect the reproductive performance of both male and female poultry; however, its effects on male fertility, specifically spermatogenesis, have been extensively studied (Fouad et al., 2016). In this study, we attempted to minimize the sperm factor error by using artificial insemination techniques and pooling sperm. Based on our results, the fertility and fertile period length of sperm in breeder hens aged 47–50 weeks were 98–99% and 15 d, respectively. Notably, this fertility rate was higher than that of the Cobb 500 breeder hens (95%) (Cobb-Vantress Inc. 2018). This is consistent with previous reports in which the combination of vitamins C, E, and Se in ISA brown laying hen diets did not affect the fertility rate (77–80%) or fertile period length of sperm (17 days), possibly because the hens reached their maximum productivity aligned with their genetic potential (Pasri et al., 2018).

Notably, the hatchability rate in HS hens supplemented with both antioxidant sources was higher than that in TN and HS hens without supplementation. This is consistent with the results of increased antioxidant activities (DPPH, ABTS, and FRAP radical scavenging) and decreased lipid peroxidation (MDA) in the egg yolk, liver, and breast tissues of breeder hens. Both sources of dietary antioxidants were observed to enhance hatchability, probably because of the function of antioxidants that are deposited in the egg yolk and act on the elimination of ROS that occurs during embryonic metabolism. Embryonic tissues typically contain a high proportion of PUFA, rendering them vulnerable to lipid peroxidation (Zhai et al., 2008). ROS can also destroy other biological molecules, such as DNA, proteins, and carbohydrates, which are the leading causes of infertility and embryonic mortality (Surai et al., 2016). Various types of antioxidants have been observed in the egg yolk and tissues of chick embryos, which contribute to the achievement of high-quality and viable chicks, including antioxidant enzymes

such as SOD, GSH-Px, and CAT, water-soluble antioxidants such as vitamin C, taurine, L-carnitine, and glutathione, and fat-soluble antioxidants such as vitamin E, carotenoids, coenzyme Q, and Se (Urso et al., 2015; Surai et al., 2019).

The synergistic effects of a combination of various antioxidant substances, especially vitamin C, vitamin E, Se, and L-carnitine, have been reported (Abdel-Azeem et al., 2016; Leskovec et al., 2019; Shakeri et al., 2020). This revealed that a combination of these substances could function more effectively in the antioxidant defense network. Vitamin E plays an antioxidant role in cells and prevents the oxidation of low-density lipoproteins in cell membranes by donating electrons to lipid peroxyl radicals to stop chain-breaking antioxidant reactions before interacting with other lipids (Ebeid, 2012). Vitamin C acts as a potent reducing agent in ROS scavenging by interacting with the tocopheroxyl radical and regenerating reduced tocopherol (Akbari et al., 2016). Se plays a crucial role in antioxidant activity as an essential coenzyme of GSH-Px, which facilitates the disposal of hydrogen peroxide generated after the superoxide is catalyzed by SOD in the cellular antioxidant defense network, contributing to the detoxification of lipid peroxides (Lykkesfeldt and Svendsen, 2007; Mishra and Jha, 2019). Vitamins C and E supplementation in broiler breeder hen diets during the summer has been shown to improve FRAP activity and reduce MDA levels in erythrocytes compared with the control (Jena et al., 2013). Generally, MDA serves as a marker of oxidative stress and represents cell/tissue damage caused by lipid peroxidation, and its reduction is linked to the antioxidant defense system through antioxidant supplementation (Shakeri et al., 2020; Tang et al., 2022). It has also been reported that L-carnitine can prevent the formation of ROS in the mitochondria, such as xanthine oxidase, cytochrome p450, cyclooxygenase, lipoxygenase, nitric oxide synthase (NOS), and NADPH oxidases (NOXs), to maintain a normal electron transport chain during the elevated metabolic rates of rapid embryo development or HS exposure (Surai et al., 2016). The use of L-carnitine in female breeder hen diets has been shown

499 to increase carnitine deposition in egg yolks and promotes the utilization of yolk lipids for
500 energy production during embryogenesis (Zhai et al., 2008; Awad et al., 2017).

501 We also found that synthetic and phytogenic sources upregulated the expression of
502 gene-related antioxidant enzymes, such as SOD, CAT, and GSH-Px, in the liver. This result is
503 consistent with the finding that vitamin E, vitamin C, and L-carnitine have the ability to activate
504 the transcriptional factor activity of activator protein-1 (AP-1), nuclear factor erythroid 2
505 related factor 2 (**Nrf2**), and NF-κB DNA binding site; this activation helps in regulating the
506 expression of adhesive molecules, cytokines, and antioxidant enzyme genes, ultimately
507 providing additional protection in HS condition (Surai, 2015; Min et al., 2018). Se mainly
508 regulates GSH-Px mRNA through the biological processes of selenoproteins, requiring a
509 selenocysteine insertion sequence that incorporates a Se-specific elongation factor,
510 selenocysteinyl-tRNA, and a selenocysteine insertion sequence mRNA stem-loop structure
511 into the Se-insertion complex during translation. This complex subsequently modulates a
512 unique endonucleolytic cleavage site, resulting in increased GSH-Px mRNA expression (Weiss
513 and Sunde, 1997; Puangmalee et al., 2020). Studies have reported that feeding chickens
514 vitamins C, E, Se, and L-carnitine leads to increased expression of SOD, CAT, and GSH-Px
515 mRNA in the liver, which is considered the initial step in the antioxidant defense against free
516 radicals and superoxide (Elgendey et al., 2022). Polyphenols exert their influence on gene
517 expression in the liver of breeder hens through an indirect mechanism involving the synthesis
518 of ROS-removing enzymes, which can stimulate the Keap1-Nrf2 complex by modifying
519 cysteine residues in Kelch-like ECH-associated protein 1, leading to the translocation of Nrf2
520 into the nucleus. After that, Nrf2 binds to the antioxidant electrophile/antioxidant response
521 element (EpRE/ARE) sequence (Lee et al., 2017; Saracila et al., 2021), resulting in the
522 upregulation of cellular antioxidant enzymes, such as SOD, CAT, GSH-Px, and GST
523 (Hosseini-Vashan et al., 2016; Bernetoniene and Kopustinskinen 2018). Interestingly, our

study revealed that phytogenic antioxidants have a greater capacity to upregulate GSH-Px gene expression than synthetic antioxidants do. This difference is likely attributable to the fact that synthetic antioxidants contain only one type of Se, which is the main precursor for GSH-Px synthesis, whereas the phytogenic antioxidants found in cloves, green tea pomace, and Vietnamese coriander consist of a variety of polyphenols with antioxidant properties, including eugenol, gallic acid, catechin, ellagic acid, quercetin, and kaempferol. The characteristic chemical structures of certain polyphenols can also activate antioxidant enzymes via modification of the transcription pathway (Saracila et al., 2021). Epigallocatechin gallate from green tea exhibits outstanding antioxidant activity in poultry, surpassing that of vitamin E by 25 times and vitamin C by 100 times (Abd El-Hack et al., 2020). In vitro tests have demonstrated that clove oil exhibits pronounced positive effects on antioxidant properties. including radical scavenging of DPPH, ABTS, H_2O_2, superoxide anion radical, and chelating activities, compared to Butylated hydroxyanisole (BHA), BHT, tocopherol, and trolox (Gulcin et al., 2012). This can be attributed to the presence of natural antioxidants, essential fatty acids and lipid-soluble bioactive molecules typically found in clove oil (Sehitoglu and Kaya, 2021). While the bioactive compounds present in *Polygonum odoratum* L such as flavonoids, alkaloids, phenolic compound, and tannin showed moderately potent antioxidant activity (50.25 ± 0.61 mg/mL) compared to vitamin E (14.79 ± 0.78) and BHT (19.71 ± 0.79 mg/mL) (Somparn et al., 20.14).

We found that NF-κB, HSP70, and HSP90 mRNA expression was down-regulated in HS hens receiving synthetic and phytogenic antioxidants compared to HS hens without supplementation. In general, when animals are exposed to HS, two possible mechanisms are involved in homeostasis that make the body tolerant to HS. First, in response to HSPs, cells promote the expression of HSPs genes to protect against cell damage, particularly HSP70, which plays an important role in the HS response in chickens (Soleimani et al., 2011). HSP70

549 in cells also helps maintain protein refolding, promotes the degradation of misfolded proteins,
550 and reduces cell inflammation (Varasteh et al., 2015; Xu et al., 2018). Second, the oxidative
551 stress response is a defense system against HS, and its gene plays a key role in the regulation
552 is Nrf2 (Lian et al., 2020; Surai et al., 2021). Both response systems have been reported to
553 improve the antioxidant capacity, reduce lipid oxidation, and increase digestive enzyme
554 activity in the gastrointestinal tract (Shehata et al., 2020). Hence, dietary antioxidants (e.g.,
555 vitamins C, E, Se, catechin from green tea, quercetin from Vietnamese coriander, and other
556 phytochemicals) may inhibit NF-κB binding to inflammation-related genes. This in turn, can
557 lower the expression of pro-inflammatory cytokines and decrease the levels of HSPs (HSP60,
558 HSP70, and HSP90), which serve as mediators for inducing NF-κB expression (Jang et al.,
559 2014; Akbarian et al., 2016; Bernatoniene and Kopustinskien, 2018; Kumbhar et al., 2018;
560 Chansiw et al., 2019; Manaig et al., 2022).

561 This study revealed the benefits of a combination of synthetic antioxidants (vitamins C
562 and E, Se, and L-carnitine) and phytogenic antioxidants (clove, green tea pomace, and
563 Vietnamese coriander) on breeder hens exposed to HS. Both sources of antioxidants
564 demonstrated significant improvements in egg production and hatchability, and reduced
565 embryo mortality. In addition, both antioxidant sources also alleviate the adverse effects of HS
566 through antioxidant defenses and inflammation by reducing lipid peroxidation in yolk and
567 tissues, up-regulating the relative expression of SOD, CAT, GSH-Px mRNA, and down-
568 regulating of NF-κB, HSP70, and HSP90 mRNA expressions in liver. This finding suggests
569 the promising application of a combination of synthetic and phytogenic antioxidant bioactive
570 compounds to combat the deleterious effects of HS in breeder hens. This knowledge may lead
571 to the development of innovative strategies for HS management by integrating dietary
572 supplements with antioxidants.
573

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DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Table 1. Ingredients and calculated nutrient composition of experimental diets (as-fed basis).

	25-50 weeks of age	After 50 weeks of age
Ingredients (%)		
Corn	64.60	63.50
Soybean meal, 44 %CP	18.20	16.52
Full fat soybean meal	6.70	9.00
Calcium carbonate	8.50	8.90
Monocalcium phosphate	0.94	1.00
Salt	0.41	0.44
DL-methionine	0.135	0.134
Copper	0.00040	-
Iodine	0.00022	0.00022
Vitamin B2	0.00043	0.00043
Vitamin D3	0.00030	0.00030
Manganese	0.00490	-
Biotin	0.00140	0.00140
Zine	0.01230	0.00390
Premix[1]	0.50	0.50
Calculated compositions (%)		
Metabolizable energy (kcal/kg)	2,800	2,800
Dry matter	90.22	90.34
Crude protein	15.49	15.44
Crude fiber	3.09	3.07
Ether extract	3.84	4.19
Calcium	3.51	3.71
Total Phosphorus	0.53	0.54
Available phosphorus	0.31	0.32
Digestible Lysine	0.70	0.70
Digestible Methionine	0.35	0.35
Digestible Methionine + Cystine	0.57	0.57
Digestible Threonine	0.50	0.50

[1]Premix (0.5%) provided the following (per kg of diet) ; vitamin A, 15,000 IU; vitamin D3, 3,000 IU; vitamin E, 25 IU; vitamin K3, 5 mg; vitamin B1, 2 mg; vitamin B2, 7 mg; vitamin B6, 4 mg; vitamin B12, 25 mg; pantothenic acid, 11.04 mg; nicotinic acid, 35 mg; folic acid, 1

mg; biotin, 15 µg; choline chloride, 250 mg; Cu, 1.6 mg; Mn, 60 mg; Zn, 45 mg; Fe, 80 mg; I, 0.4 mg; Se, 0.15 mg.

Table 2. Primer sequences used in real-time PCR.

Gene	Primer sequences[1]	Accession No.
SOD	F-5'-CACTGCATCATTGGCCGTACCA-3'	NM_001031215.1
	R-5'-GCTTGCACACGGAAGAGCAAGT-3'	
CAT	F-5'-TGGCGGTAGGAGTCTGGTCT-3'	NM_205064.1
	R-5'-GTCCCGTCCGTCAGCCATTT-3'	
GSH-Px	F-5'-GCTGTTGCCTTCCTGAGAG-3'	NM_001277853.1
	R-5'-GTTCCAGGAGACGTCGTTGC-3'	
HSP70	F-5'-GATCTGGGCACCACGTATTCT-3'	FJ217667.1
	R-5'-GGTTCATTGCCACTTGGTTCTT-3'	
HSP90	F-5'-ACACATGCCAACCGCATTTA-3'	NM_001109785.1
	R-5'-CCTCCTCAGCAGCAGTATCA-3'	
NF-κB	F-5'-GAAGGAATCGTACCGGGAACA-3'	NM_205134
	R-5'-CTCAGAGGGCCTTGTGACAGTAA-3'	
β-actin	F-5'-TTGGTTTGTCAAGCAAGCGG-3'	NM 205518.1
	R-5'-CCCCCACATACTGGCACTTT-3'	

[1]Data form reference Chiang et al. (2009), Ahmadipour and Khajali, (2019), and Madkour et al. (2021)

Abbreviations: SOD, superoxide dismutase; CAT, catalase; GSH-Px, glutathione peroxidase; HSP70, heat shock protein 70; HSP90, heat shock protein 90; NF-κB, nuclear factor-κB.

916 **Table 3**. Effect of dietary antioxidant supplementation in breeder hen diets under heat stress

917 condition on productive performances.

Items	Treatments[1]				Pooled SEM	Contrasts[2]		
	T1	T2	T3	T4		1	2	3
IBW (g)	2795.21	2891.37	2747.18	2785.16	26.644	0.836	0.057	0.614
FBW (g)	3314.48^{a}	3176.57^{ab}	3102.67^{ab}	3048.19^{b}	34.971	0.013	0.215	0.571
FI (g/day/hen)	137.20^{a}	137.22^{a}	135.92^{ab}	135.56^{b}	0.2673	0.103	0.025	0.634
FCR	2.12^{b}	2.15^{ab}	2.16^{a}	2.15^{ab}	0.0044	0.007	0.600	0.431
WI (mL/day/hens)	290.60^{b}	406.57^{a}	370.27^{a}	372.05^{a}	7.7241	0.001	0.015	0.913
EP (g)	88.64^{a}	83.50^{b}	88.62^{a}	89.78^{a}	0.6707	0.372	<0.001	0.524
EW (g)	64.61^{a}	63.83^{b}	62.94^{c}	62.92^{c}	0.0943	<0.001	0<.001	0.925
Fertility (%)	98.86	98.43	99.34	99.14	0.1925	0.798	0.086	0.709
Hatchability (%)	87.63^{b}	87.79^{b}	91.81^{a}	91.71^{a}	0.6394	0.046	0.009	0.957
Fertile period length of sperm (day)	15.04	14.64	14.5	14.33	0.157	0.141	0.556	0.707
Day old chick weight (g)	46.30^{a}	45.55^{a}	44.89^{ab}	43.66^{b}	0.3195	0.009	0.037	0.074
Early dead (%)	2.06^{b}	4.70^{a}	2.76^{ab}	2.67^{ab}	0.3512	0.096	0.022	2.67
Last dead (%)	5.85^{ad}	6.16^{a}	2.83^{c}	3.27^{b}	0.5029	0.123	0.011	0.744
Abnormal chicks (%)	2.55	1.38	1.02	2.21	0.3215	0.172	0.769	0.192
Chick uniformity (%)	75.85^{ab}	76.39^{ab}	82.23^{a}	70.94^{b}	1.6265	0.833	0.953	0.007

918 $^{a-b}$Means within each row with different superscripts are significantly different ($P < 0.05$).

919 [1]T1, thermoneutral zone (23±1°C) + basal diet without supplementation; T2, heat stress

920 (36±1°C, 4 h/day) + basal diet without supplementation; T3, heat stress condition (36±1°C, 4

921 h/day) + basal diet with synthetic antioxidants; T4, heat stress condition (36±1°C, 4 h/day) +

922 basal diets with phytogenic.

923 [2]Orthogonal contrasts: 1) thermoneutral (T1) vs. heat stress conditions (T2, T3, T4); 2) non-

924 supplement (T2) vs. supplement (T3, T4); 3) synthetic antioxidants (T3) vs. phytogenic (T4).

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Table 4. Effect of dietary antioxidant supplementation in breeder hen diets under heat stress condition on blood chemistry.

Items	Treatments[1]				Pooled SEM	Contrasts[2]		
	T1	T2	T3	T4		1	2	3
pH	7.37[b]	7.47[a]	7.46[a]	7.46[a]	0.007	<0.001	0.458	0.859
PCO_2 (mmHg)	50.73[a]	36.90[b]	36.51[b]	36.82[b]	0.9762	<0.001	0.886	0.867
PO_2 (mmHg)	40.09[a]	37.36[b]	36.72[b]	37.18[b]	0.3859	<0.001	0.627	0.641
BE (mmol/L)	4.72[a]	3.17[ab]	2.55[b]	2.41[b]	0.2842	0.002	0.299	0.85
HCO_3^- (mmol/L)	29.94[a]	26.84[b]	26.35[b]	26.20[b]	0.3098	<0.001	0.368	0.842
TCO_2 (mmol/L)	31.33[a]	27.94[b]	27.44[b]	27.35[b]	0.3098	<0.001	0.406	0.902
sO_2 (%)	70.17[b]	77.29[a]	76.11[a]	77.05[a]	0.7313	<0.001	0.662	0.609
iCa (mmol/L)	1.78[a]	1.53[b]	1.51[b]	1.58[b]	0.0263	<0.001	0.860	0.225
Na (mmol/L)	148.35[a]	146.43[ab]	146.37[ab]	146.00[b]	0.3218	0.004	0.744	0.687
K (mmol/L)	5.13[a]	4.92[ab]	5.10[ab]	4.86[b]	0.0348	0.023	0.448	0.011
Glu (mg/dL)	229.77[b]	241.17[a]	246.16[a]	246.47[a]	1.6465	<0.001	0.159	0.947
Hct (%PCV)	28.27[a]	25.64[b]	25.72[b]	25.47[b]	0.3174	<0.001	0.942	0.781
Hb (g/dL)	9.62[a]	8.71[b]	8.75[b]	8.66[b]	0.1076	<0.001	0.966	0.760

[a-b]Means within each row with different superscripts are significantly different (P < 0 .05).

[1]T1, thermoneutral zone (23±1°C) + basal diet without supplementation; T2, heat stress condition (36±1°C, 4 h/day) + basal diet without supplementation; T3, heat stress condition (36±1°C, 4 h/day) + basal diet with synthetic antioxidants; T4, heat stress condition (36±1°C, 4 h/day) + basal diets with phytogenic.

[2]Orthogonal contrasts: 1= thermoneutral (T1) vs. heat stress condition (T2, T3, T4); 2, non-supplement (T2) vs. supplement (T3, T4); 3, synthetic antioxidants (T3) vs. phytogenic (T4).

Abbreviations: PCO_2, Partial pressure of carbon dioxide; PO_2, Partial pressure of oxygen; BE, Base excess; HCO_3^-, concentration of bicarbonate ions; TCO_2, total concentration carbon dioxide; sO_2, saturation of oxygen; iCa, concentration ionized calcium. Na, sodium; K, potassium; Glu, glucose; Hct, hematocrit; Hgb, hemoglobin.

940 **Table 5**. Effect of dietary antioxidant supplementation in breeder hen diets under heat stress
941 condition on antioxidant activity in egg yolk, liver, and breast.

Items	Treatments[1]				Pooled SEM	Contrasts[2]		
	T1	T2	T3	T4		1	2	3
DPPH (%)								
Egg yolk	17.23^{c}	18.20^{c}	47.03^{a}	22.18^{b}	2.2021	<0.001	<0.001	<0.001
Liver	81.82^{b}	86.24^{ab}	87.48^{a}	86.43^{ab}	0.7426	0.003	<0.001	0.567
Breast	19.49^{ab}	18.60^{b}	21.05^{a}	21.35^{a}	0.3478	0.212	0.004	0.733
FRAP (mM TE/g sample)								
Egg yolk	0.42^{b}	0.36^{b}	0.71^{a}	0.40^{b}	0.0299	0.072	<0.001	<0.001
Liver	1.87^{b}	2.14^{b}	2.49^{a}	2.53^{a}	0.0628	<0.001	0.001	0.676
Breast	0.23	0.29	0.29	0.29	0.0111	0.023	0.962	0.934
ABTS (mM TE/g sample)								
Egg yolk	5.46^{a}	5.18^{b}	5.43^{a}	5.42^{a}	0.0336	0.052	0.001	0.921
Liver	35.56^{ab}	34.18^{b}	35.23^{ab}	37.28^{a}	0.4011	1.000	0.027	0.051
Breast	40.28^{ab}	38.29^{b}	45.37^{a}	45.02^{a}	0.8393	0.103	<0.001	0.855
MDA (uM/g sample)								
Egg yolk	28.14^{ab}	32.74^{a}	10.76^{c}	23.94^{b}	1.6502	0.001	<0.001	<0.001
Liver	10.92^{a}	10.90^{a}	8.65^{b}	11.53^{a}	0.3397	0.398	0.274	0.002
Breast	8.26	8.71	7.74	8.68	0.1433	0.716	0.134	0.015

942 [a-c]Means within each row with different superscripts are significantly different (P < 0 .05).

943 [1]T1, thermoneutral zone (23±1°C) + basal diet without supplementation; T2, heat stress
944 condition (36±1°C, 4 h/day) + basal diet without supplementation; T3, heat stress condition
945 (36±1°C, 4 h/day) + basal diet with synthetic antioxidants; T4, heat stress condition (36±1°C,
946 4 h/day) + basal diets with phytogenic.

947 [2]Orthogonal contrasts: 1, thermoneutral (T1) vs. heat stress condition (T2, T3, T4); 2, non-
948 supplement (T2) vs. supplement (T3, T4); 3, synthetic antioxidants (T3) vs. phytogenic (T4).

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Table 6. Effect of dietary antioxidant supplementation in breeder hen diets under heat stress condition on gene related to antioxidant enzyme, pro-inflammatory cytokines, and heat shock proteins expressions.

Items	Treatments[1]				Pooled SEM	Contrasts[2]		
	T1	T2	T3	T4		1	2	3
SOD	1.00^{b}	0.71^{c}	1.56^{a}	1.45^{a}	0.0001	<0.001	<0.001	0.121
CAT	1.00^{ab}	0.35^{b}	1.49^{a}	1.54^{a}	0.0240	0.672	0.003	0.907
GHS-Px	1.00^{c}	0.76^{c}	2.40^{b}	5.42^{a}	0.0001	<0.001	<0.001	<0.001
NF-κB	1.00^{ab}	1.25^{b}	0.64^{ab}	0.42^{a}	0.5257	0.285	0.009	0.381
HSP70	1.00^{b}	1.11^{b}	0.16^{a}	0.15^{a}	0.2252	<0.001	<0.001	0.799
HSP90	1.00^{b}	1.01^{b}	0.20^{a}	0.17^{a}	0.0058	0.011	0.001	0.872

$^{a\text{-}c}$Means within each row with different superscripts are significantly different ($P < 0.05$).

[1]T1, thermoneutral zone (23±1°C) + basal diet without supplementation; T2, heat stress condition (36±1°C, 4 h/day) + basal diet without supplementation; T3, heat stress condition (36±1°C, 4 h/day) + basal diet with synthetic antioxidants; T4, heat stress condition (36±1°C, 4 h/day) + basal diets with phytogenic.

[2]Orthogonal contrasts: 1, thermoneutral (T1) vs. heat stress condition (T2, T3, T4); 2, non-supplement (T2) vs. supplement (T3, T4); 3, synthetic antioxidants (T3) vs. phytogenic (T4).