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Effects of Dietary Antioxidant on Performance and Physiological Responses Following Heat Stress in Laying Hens

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Abstract: Heat stress (HS) causes oxidative damage, increasing mortality and reducing productivity in chickens. The objective of this study was to determine the benefits of antioxidant supplementation in laying hens during HS. Eighty 32-week-old W-36 White Leghorn hens were used in this study. Hens were randomly pair-housed in two adjacent rooms and fed a control diet (CF) or control diet mixed with Agrado Plus Ultra®, an antioxidant, at 160 mg/kg (AF) for two weeks. One room was then subjected to a hot climate (H) (33°C) for 8 days. Physical and physiological data were collected at day 1 and 8 during the treatment. Core body temperature was increased ($p<0.0001$) and BW ($p<0.05$) and liver weight ($p<0.0001$) were reduced in laying hens regardless of treatment. However, compared to its respective controls, the concentrations of heat shock protein 70 (HSP70) were increased in H-AF hens ($p< 0.01$) but not in H-CF hens ($p>0.05$) at 8 days during the process of HS. Similarly, HSP70 mRNA expression tended to increase in H-AF hens only ($p = 0.09$). Heat stress reduced the concentrations of total CO_2 and bicarbonate ($p<0.05$), indicating respiratory alkalosis and decreased vitamin A ($p<0.01$), vitamin E ($p<0.0001$) and glutathione peroxidase ($p<0.05$) concentrations but increased protein carbonyl concentrations ($p<0.05$), indicating protein oxidative damage. A temperature by feed interaction was observed in the concentrations of partial pressure CO_2 (pCO_2, $p<0.05$), superoxide dismutase (SOD, $p = 0.06$) and protein carbonyl ($p = 0.1$). Heat stress-caused decreases in pCO_2 and SOD and increases in protein carbonyl concentrations were found in control hens but not in AF hens. These results suggest antioxidant supplementation attenuates oxidative stress response in laying hens. These data support the hypothesis that supplemental antioxidants improve hen well-being by reducing HS associated physical and physiological damage.

Key words: Heat stress, antioxidant, productivity, physiology, hens

INTRODUCTION

Environmental stressors, such as high ambient temperature, have been known to be deleterious to animal welfare. Heat stress (HS) is a large concern in the poultry industry because hens' narrow thermo neutral zones, especially in high temperature climates in which birds are less able to maintain an optimal body temperature (Deeb and Cahaner, 2001; Kadim *et al*., 2008). Heat stress has been known to cause unstable physiological homeostasis, resulting in a dramatic decrease of egg quality and quantity as well as BW gain and meat quality in chickens (Yahav, 2000; Lin *et al*., 2005; Rozenboim *et al*., 2007).

Chickens, as well as other animals, react to HS through activating the oxidative system which initiates a negative cascade of events to cause further harm to the animals (Mujahid *et al*., 2006; Lin *et al*., 2006; Azad *et al*., 2010a). Oxidative stress results from the production of peroxides and reactive oxygen species (ROS), mainly by the mitochondria (Cadenas and Davies, 2000). It occurs when the respiratory chain reaction is up-regulated, producing additional electrons to enhance ROS formation. The body has protective systems against high levels of ROS to prevent its accumulation, such as antioxidants like vitamin A (VA) and E (VE) (Taniguchi *et al*., 1992) as well as antioxidant enzymes like superoxide dismustase (SOD) and glutathione peroxidase (GPx) (Meister and Anderson, 1983; Thomas, 2000).

A high level of ROS within the body, either through over production of ROS or decreased levels of antioxidants and antioxidant enzyme activity, leads to cell damage, altering functions through interruption of cellular signaling and membranes, resulting from disruption of synthesizing proteins, lipids and DNA bases (Chow, 1991, 2009; Spurlock and Savage, 1993). Oxidative stress involves in the development of many diseases in humans, such as cancer, Parkinson's disease, Alzheimer's disease, heart failure and chronic fatigue syndrome (Singh *et al*., 1995; Cooke *et al*., 2003;

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Kennedy *et al*., 2005; Halliwell, 2007; Valko *et al*., 2007). Similarly to the findings in human oxidative research (Duthie *et al*., 1996; Huang *et al*., 2000; Frei, 2004), amelioration of oxidative stress due to HS has been reported use of dietary supplements, such as ascorbic acid or other vitamins and minerals, which bolsters both the availability of antioxidants as well as the activities of antioxidant enzymes in animals including chickens (Bhattacharya *et al*., 1999; Sahin *et al*., 2003; Sujatha *et al*., 2010).

Ethoxyquin has been commonly used as an antioxidant preservative in animal feed and forage crops (Cabel and Waldroup, 1989). It prevents rancidity by scavenging the harmful products of oxidation to maintain cellular integrity. In rats, it is readily absorbed by the small intestines within 1 h of ingestion; reaching its highest concentration in the liver and adipose tissues (Gupta and Boobis, 2005). In chicks, addition of ethoxyquin in their diet decreased overall antioxidant requirement through the increase of the antioxidant enzyme GPx activity (Combs and Scott, 1974). Ethoxyquin also inhibits the production of peroxide in diet and limits the negative effects of peroxide on broiler performance, such as limiting BW gain and feed efficiency (Shermer and Calabotta, 1985; Cabel *et al*., 1988).

Propyl gallate (PG) and tertiary butylhydroquinone (TBHQ) are additionally synthesized antioxidants used for stabilizing vegetable oils, fats and food against oxidative deterioration (Hawrysh *et al*., 1992; Sasse *et al*., 2008). In additional, PG, as a SOD mimic, protects cells from hydrogen peroxide-induced oxidative stress and DNA damages (Reddan, 2003; Chen, 2007). Through its roles in oxidative stress reduction, PG also enhances immunity through its anti-inflammatory feature (Jung *et al*., 2011), improves glomerular pathological changes in diabetic rats (Tian *et al*., 2011), increases hippocampal neuronal survival following forebrain ischemia (Kawano *et al*., 2012) and extends the normal lifespan (Bains *et al*., 1996). Similarly, Benedetti *et al*. (2008) reported that GP has functions in conferring thermal stress resistance and extends lifespan in tested animals. Butylhydroquinone, similar to the functions of PG, activates endogenous antioxidant defense system in neuronal cells against oxidative insult initiated by free radical-mediated damage (Duffy *et al*., 1998; Kraft *et al*., 2004). Therefore, we hypothesize that laying hen well-being will be improved by adding these compounds into their diet during HS through increasing activity of the antioxidant system to prevent or limit oxidative damage.

Agrado plus® consists of ethoxyquin and TBHQ as main antioxidant contents (Novus International Inc., St Charles, MO). As a dietary antioxidant, it increases feedlot benefits, including decline of morbidity and mortality and increase of daily gains in beef cattle (Kegley *et al*., 2000; Han *et al*., 2002) and improvement of milk yield in dairy cows (Vazquez-Anon and Jenkins, 2007; Wang *et al*., 2010). Agardo Plus Ultra® is recently synthesized antioxidant blend of ethoxyquin and PG (Novus International Inc., St Charles, MO) and has been used to reduce lipid peroxidation in broiler meat (Tavarez *et al*., 2011). The inclusion of this antioxidant supplement increases overall concentrations of antioxidant VA and VE within broiler liver tissue (Tavarez *et al*., 2011), thereby preserving free antioxidant concentrations. We hypothesize that the use of those compounds could reduce harmful effects of oxidative damage in laying hens. Therefore, the objective of this study was to test the effects of an antioxidant mixture, Agrado Plus Ultra®, on laying hens under HS and to determine the responding cellular mechanisms.

MATERIALS AND METHODS

Birds and treatments: Eighty W-36 Hy-line White Leghorn laying hens at 32 weeks of age were randomly transferred to two adjacent climate-controlled rooms at Purdue Animal Research facility. Hens were pair-housed and randomly assigned to a control feed (CF), a regular layer ration, or layer ration mixed with Agrado Plus Ultra® (AF) at 160 mg/kg per manufacture instruction (Novus International Inc., St Charles, MO) and previous study (Tavarez *et al*., 2011). Hens in both rooms were kept for two weeks at a control climate (c) (24°C, 15.6% RH, at 23 heat index). Afterward, one room was subjected to a heat climate (H) (33°C, 23.7% RH, at 31 heat index) for 8 days, resulting in four groups at 10 replications of 20 birds per group: C-CF (control temperature hens fed with control diet), C-AF (control temperature hens fed with Agrado diet), H-CF (heat stressed hens fed with control diet) and H-AF (heat stressed hens fed with Agrado diet). Lighting was at a 16 h light/8 h dark cycle. Hens accessed to water and feed *ad libitum* throughout the experiment.

All hens used in this experiment were housed and cared for under the approval of Purdue Animal Care and Use Committee (PACUC, 00-008-09).

Physical and physiological sampling: Feed intake (FI) was monitored on day 1, 3, 6 and 8. Trough feeders were emptied before the test. During the test, plastic trays were placed individually under feed troughs of each cage to collect wasted feed. A weighed portion of feed was added to the troughs. During each of the FI trials hens were provided with sufficient feed to consume *ad libitum*. At the end of the test, feed remaining in the feed troughs was weighed and plastic trays removed from under the troughs. The waste feed inside the plastic trays was weighed after being separated from manure. Feed intake was calculated using the following formula: FI (g)/hen = (Total grams of feed offered per cage-Grams of feed wasted and leftover/cage)/2 hens/cage.

At 24 h and 8 day during HS treatment (acute and chronic, respectively), physical and physiological

measurements were taken (n = 10/period/treatment). To avoid any errors that might be caused by the time, i.e., the order of sampling, the sampled hens were taken by repeating the cycle of C-CF, H-CF, C-AF and H-AF until the end. Core body temperature (CBT) was obtained with a 4600 Series Precision thermometer (Yellow Springs Instruments, Inc. OH) with a 1-mm pediatric probe that was inserted 5 cm beyond the vent and applying pressure to the dorsal wall of the cloacae (Cheng *et al.*, 2004).

A 10 mL blood sample was randomly collected from one hen per cage by cardiac puncture following sedation with sodium phenobarbitol and before euthanization. Approximately, 0.5 mL volume of whole blood was analyzed for pH, blood gas and ionized calcium concentration within 2 h of being collected. The remaining blood was centrifuged at 700 x g for 20 min at 4°C. The supernatant plasma was then collected and stored at -80°C until analysis.

Following blood sampling, hens were immediately euthanized via cervical dislocation. Hens' BW was assessed. After dissection, the weights of the liver (LW), spleen (SW) and ileum (IW) were collected and the relative weight of each organ was calculated by the formula:

$$\text{Relative organ weight} = \frac{\text{Absolute organ weight}}{\text{BW of the hen (g/kg)}}$$

Tissue samples of approximately 1 cm^3 were taken from the same location of the liver and spleen of each sampled bird and stored at -80°C until analysis.

Western **blot analysis for heat shock protein 70:** Heat shock protein 70 was measured using previously published method (Felver-Gant *et al.*, 2012). Liver tissue of an estimated 1 cm^3 in size was homogenized in 1 x RIPA buffer solution (50mmol/L Trizma-HCL [pH 7.4], 15 mmol/L NaCl, 0.25% deoxycholic acid, 0.1% Triton X, 10mmol/L EDTA, 1 mmol/L Na_2VO_3, with protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO). Following homogenization, the supernatant solution was collected. Protein concentration of each sample was determined and diluted to create an equal protein concentration of 50 µg/µL. 30 µL of diluted sample was then added with 10 µL of a tracking dye and separated by SDS-PAGE on a 10% acrylamide gel through electrophoresis at 130 V for 1.5 h. Proteins were then transferred to a 0.2 µmol/L nitrocellulose membrane by the semi-dry method (Bio-Rad, Hercules, CA). Membranes were blocked in 5% bovine serum albumin in TTBS (50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 0.1% Tween 20). Immunoblotting was performed using mouse monoclonal antibody for HSP70 (Sigma-Aldrich, St Louis, MO) at a dilution of 1:5000. Blots were then incubated with secondary antibody (Cell Signaling Technologies, Danvers, MA) at a dilution of 1:2000 with the addition of a biotinylated protein ladder (Cell Signaling Technologies, Danvers, MA) at a dilution of 1:1000. Signal was detected by autoradiography by chemiluminescence with Super Signal West Pico reagent (Pierce, Rockford, IL). Densitometry analyses of western blots were performed using Kodak EDAS290 imaging system (Kodak, New Haven, CT).

Membranes were stripped and re-probed with mouse monoclonal antibody for Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) at a dilution of 1:20000 (Sigma-Aldrich, St Louis, MO). Similar procedures were the conducted to determine the amount of GAPDH per sample. A ratio of the optic density of HSP70 and GAPDH was then calculated to determine actual amount of HSP70/sample.

Enzyme-linked Immunosorbent Assay (ELISA): Plasma activity of SOD and GPx enzyme were measured using commercially available ELISA kits (Cayman Chemicals, Ann Arbor, MI, USA). Briefly, for GPx activity, plasma samples were initially diluted to 1:10 with included sample buffer (50 mM Tris-HCl, 5mM EDTA, 1 mg/mL BSA at pH 7.6). Sample wells (two well per sample) included 100 µL assay buffer (50 mM Tris-HCl, 5 mM EDTA at pH 7.6), 50 µL reconstituted co-substrate mixture (includes DAPH, glutathione and glutathione reductase; reconstituted using 2 mL HPLC-grade water) and 20 µL of diluted plasma sample. Reactions were initiated by adding 20 µL of Cumene Hydroperoxide. Samples were analyzed in triplicate with absorbance readings taken at 340 nm every minute for 5 min. Curves were standardized through comparison with supplied positive controls. Enzyme activity was reported at nmol/min/mL GPx activity.

For SOD activity, plasma samples were initially diluted to 1:10 with included sample buffer (50 mM Tris-HCl at pH 8.0). Sample wells included 200 µL diluted Radical Detector (tetrazolium salt solution) and 10 µL diluted plasma sample. Reactions were initiated by adding 20 µL Xanthine Oxidase (diluted with sample buffer/instructions) and incubated on a shaker for 20 min. Samples were analyzed in duplicate with absorbance readings of 450 nm. Concentrations were calculated using a standard curve created per instructions with the positive controls. Enzyme activity was reported at units/mL.

Thiobarbituric acid reactive substances, malondialdehyde (MDA) and protein carbonyl, were quantified colorimetrically in plasma samples using commercially available assay kits followed manufactures' instructions (MDA, Cayman Chemicals Ann Arbor, MI and Carbonyl, Cell Biolabs Inc., San Diego, CA). Concentrations were analyzed using absorbance values at 540 nm and 450 nm wavelengths for MDA and Carbonyl, respectively. MDA was calculated using a

malondiadehyde standard curve and results reported as µM. Protein carbonyl was calculated using a protein carbonyl standard curve and results reported as nmol/mg.

Polymerase chain reaction (PCR): Heat shock protein 70 mRNA expression in the liver was detected by quantitative real-time PCR using primers and probes developed elsewhere (Applied Biosystems); forward primer, TCGGCCGCAAGTATGATGA; reverse primer, CGGAAGGGCCAGTGCTT; TaqMan probe, CCACAGTGCAGTCAGA.

Tissue samples were homogenized and RNA was then extracted per instructions using the RNeasy Mini Kit (Qiagen, Valencia, CA). Extracted RNA was quantified using the GeneQuant pro RNA/DNA Calculator (Amersham Biosciences Corp., Piscataway, NJ). Sample RNA was mixed with RNase-free water (Ambion Inc.) to make equal concentrations of RNA. Thirty five µL of diluted samples were then added to 65 µL of master mix for a total of 100 µL. The master mix was made of reagents included in the TaqMan Reverse Transcription Reagent Pack (Applied Biosystems, Foster City, CA) including 50U/µL of Multi-Scribe reverse transcriptase, 25mM $MgCl_2$, 2.5 µM random hexamers, 0.4 U/µL RNase inhibitor, 50 µM dNTPs and TaqMan reverse transcription buffer (TaqMan reverse transcription reagents, Applied Biosystems). Reverse transcription was followed in the Hybaid PCR Express thermo cycler (Midwest Scientific, St. Louis, MO) and amplified using cycling conditions; 50°C for 2 min activation followed by 30 cycles of 95°C for 15 s, 60°C for 1 min and a final stage of 60°C for 5 min with a holding temperature of 4°C. Prior to analysis, stock primers and probes were diluted to 10 µM solutions. The conditions for PCR were a ratio of 3.5 µL of TaqMan probe, 4.5 µL of gene specific TaqMan forward and reverse primer each, 25 µL PCR Mastermix (Applied Biosystems), 7.75 µL RNase-free water (Ambion Inc.) and 5 µL of sample cDNA. Cycling conditions for real-time PCR consisted of an initial step at 50°C for 2 min followed by 40 cycles of 10 min at 95°C, 20 sec at 95°C and 1 min at 60°C. To assure accuracy and consistency, all samples were measured in duplicates and standards in triplicates with a standard deviation of less than 2.0 and a coefficient of variation less than 2.0%.

Blood gas analysis: Samples were kept on ice and analyzed within 2 h based on the recommendation of whole blood gas stability (Clinical Pathological Lab, Purdue Vet School). Samples were analyzed using the Bayer Rapidlab 855 Blood Gas Analyzer (Bayer Healthcare LLC, West Walpole, MA) at the Purdue veterinary clinical pathology lab. Initially, individual blood samples were gently inverted and rolled to ensure proper homogeneity. Samples were then analyzed and calculations performed according to manufactories settings. Bicarbonate (HCO_3^-), total carbon dioxide (TCO_2), base excess (BE) and ionized calcium (IonCa) were reported as mmol/L. Partial pressure CO_2 (pCO_2) and partial pressure O_2 (pO_2) were reported as mmHg, while oxygen saturation (O_2Sat) was reported as a percentage of the volume of oxygen carried to the maximum volume that can be carried by the hemoglobin.

High pressure liquid chromatography (HPLC): Vitamin A and VE concentrations were measured in plasma through HPLC analysis. A C18 column, 4.6 mm by 15 cm, was used at a flow rate of 1.5 mL/min. A 50 µL of plasma sample was injected onto the column with mobile phase (96% methanol, 4% H_2O) and measurements were taken at 325 and 295-325 nm for VA and VE respectively. Analysis of VA and VE concentrations were done following the HPLC procedure previously described (Catignani, 1986).

Statistics analysis: The experimental design was an incomplete randomized block. For the analysis cage (1 bird was sampled per cage) was considered the experimental unit. Feed, treatment and feed x treatment were treated as fixed effects and diet and treatment within cage were considered as a random effect. When measuring organ weight, BW was considered a covariate. Diagnostic tests were run to determine if data had a normal distribution. Data that did not approach a normal distribution were transformed using box-cox transformations as described by Fahey *et al.* (2007). Analysis was done using PROC MIXED model with SAS 9.2 software (SAS Institute Inc., Cary, N.C.). A repeated measures analysis was used for feed intake data. Comparisons were made between diet (with and without Agrado) within treatment (hot or control temperature) and within the diet across the treatments. Data presented in this paper shows the non-transformed values of the data for BW and organ weight, however all P-values were calculated using the corresponding transformed data. Means reported were the least square means (LS Means) with standard error of the mean (SEM). Slice adjustment was used to obtain the simple effects of significant interactions. Significant statistical differences were reported when $p<0.05$ and statistical trends were reported when $0.05<p<0.10$.

RESULTS AND DISCUSSION

Effect of antioxidant supplementation on core body temperature (CBT) in hens following heat stress: High CBT is one of the hallmarks of HS in animals including chickens. In our study, hens exposed to HS had significantly elevated CBT compared to it of controls ($p<0.0001$; Table 1). The peak of increased CBT in both H-AF and H-CF was at day 1 during heat treatment ($p<0.001$), then reduced at day 8 but still significantly

Table 1: effects of antioxidant supplementation on relative organ weights of the hen following heat stress[1]

Treatment[2]	CBT (°C)	BW (kg)	LW	SW	IL
24 h					
C-CF	41.31±0.09	1.46±0.03	35.03±0.68	1.46±0.07	26.39±1.33
H-CF	41.96±0.08**	1.45±0.03	32.82±0.69	1.38±0.08	20.85±1.24*
C-AF	41.21±0.09	1.45±0.03	36.00±0.69	1.42±0.08	24.97±1.29
H-AF	4 42.17±0.08**	1.45±0.03	34.11±0.68	1.41±0.08	22.02±1.26
Day 8					
C-CF	41.33±0.09	1.49±0.03	36.48±0.69	1.36±0.08	28.82±1.31
H-CF	41.69±0.09	1.42±0.03	33.00±0.68*	1.21±0.08	25.62±1.36
C-AF	41.42±0.09	1.45±0.03	36.82±0.68	1.42±0.08	27.34±1.30
H-AF	41.86±0.09*	1.36±0.03	34.10±0.74	1.22±0.08	26.10±1.37
p-value					
Temp	<0.0001	0.0413	<0.0001	0.0599	0.0011
Feed	0.1655	0.1765	0.0634	0.8387	0.7271
Temp*Feed	0.125	0.8043	0.5907	0.9292	0.2266
24 h C-CF vs. H-CF	<0.0001	1.0000	0.3218	0.9971	0.0491
24 h C-AF vs. H-AF	<0.0001	1.0000	0.5638	0.9997	0.8041
Day 8 C-CF vs. H-CF	0.116	0.7918	0.0166	0.8828	0.7511
Day 8 C-AF vs. H-AF	0.0247	0.3395	0.1411	0.6195	0.9989

[1]All means reported are LS Means±standard error created by mixed model analysis (n = 10)
[2]C-AF = control temperature/Agrado diet; C-CF = control temperature/control diet
H-AF = heat temperature/Agrado diet; H-CF = heat temperature/control diet
CBT = core body temperature; IL = relative ileum weight
LW = relative liver weight; SW = relative spleen weight
**, p<0.01 and *, p<0.05 compare with respective controls

Table 2: Effects of antioxidant supplementation on feed intake on day 1, 3, 6 and 8 of the hen following heat stress[1]

Treatment[2]	FI at d 1 (g)	FI at d 3 (g)	FI at d 6 (g)	FI at d 8 (g)
C-CF	77.17±2.29	92.13±3.25	98.50±3.25	92.33±3.25
H-CF	84.66±2.29	63.21±3.25**	74.21±3.25**	70.95±3.39**
C-AF	76.37±2.34	94.96±3.12	97.81±3.12	90.58±3.25
H-AF	87.52±2.29	64.42±3.75**	74.39±3.75**	71.80±3.56**
p-value[3]				
Temp	<0.0001			
Feed	0.7082			
Temp*Feed	0.6605			
C-CF * H-CF	0.6183	<0.0001	<0.0001	0.0013
C-AF * H-AF	0.9172	<0.0001	0.0004	0.0040

[1]All means reported are LS Means±standard error created by mixed model analysis (n = 10)
[2]C-AF = control temperature/Agrado diet; C-CF = control temperature/control diet
H-AF = heat temperature/Agrado diet; H-CF = heat temperature/control diet
FI = feed intake; [3]Repeated measures analysis
**, p<0.01 compare with respective controls

higher in H-AF hens but not in H-CF hens (p<0.05 and p>0.05, respectively). Although HS-associated cellular mechanisms of the different reactions between H-AF and H-CF hens are unclear, it may be related to the differences in their adaptability to HS. Animals including chickens have capability to adapt their environments by changing the activations of their endocrine and central nervous systems. The fever response, for example, is one of adaptive physiological responses in animals to protect themselves, such as enhancing the host immunity against inflammation and infection (Ostberg *et al*., 2000; Lee *et al*., 2012) and reducing psychological stress (Oka *et al*., 2001; Murapa *et al*., 2007). Agrado fed hens, similarly to mammals, may have higher adaptability compared with the H-AF hens by increasing the thermoregulatory set-point, regulating body temperature at a high point. There are several mechanisms associated with the positive protection program in animals: 1) changing the structure of heat-sensitive ion channels at the surface of nerve cells from the mildly warm to hot temperature (Cheng *et al*., 2012) constituting a physiological and psychological stress associated with HS through activated the hypothalamus which is the temperature-regulating center (Nakamura, 2011; Oka *et al*., 2001) and serotonin 1A agonists reduce the psychological stress-induced rise in core temperature in animals (Oka *et al*., 2001) and producing mediators, such as interleukins (ILs), including IL-1 and -6 and HSPs, such as HPS70 (Murapa *et al*., 2007; Felver-gant *et al*., 2012).

Effect of antioxidant supplementation on physical measurements in hens following heat stress: The BW of hens exposed to HS was significantly decreased compared with their respective controls but without feed effects and the interactions between temperature and feed (p<0.05 and p>0.05, respectively; Table 1). Similarly, FI of hens under HS was decreased from day

3 to day 8 during the process of HS ($p<0.0001$; Table 2). These results are in agreement with previous studies in which broilers have decreased growth rate, FI and feed efficiency (Teeter *et al.*, 1985; Geraert *et al.*, 1996). Low energy intake, as a strategy for reduced HS associated damage by reducing metabolic heat production, may benefit by lowering the chickens' body temperature.

This decrease in FI was the greatest at day 3, tended to recover from day 3 to day 6, then kept at a fairly consistent but still significantly reduced from day 6 to day 8 (Table 2). The time sequence of the changes of FI following HS may indicate that the hens had begun to adapt to HS after day 3. Similar to the current findings, Yahav *et al.* (2000) reported 4-7 days to be an adequate amount of time to efficiently control body temperature in high ambient temperature conditions.

Overall, SW, LW and IW were reduced in hens following HS (Table 1). Spleen weight is a commonly used indicator of immunocompetence in birds. Birds with larger SW are better capable of handling a stress load on the immune system (Cheng *et al.*, 2004). In our study, SW tended to decrease under HS regardless of diet ($p = 0.057$; Table 1), which is in agreement with other studies which observed a decrease in SW in broilers following HS (Quinteiro-Filho *et al.*, 2010). Similarly, LW was reduced in HS hens ($p<0.0001$; Table 1). The reduced LW could be related to the reduced FI. However, it is unlikely because, compared with their respective controls; LW was significantly reduced in H-CF hens ($p<0.05$) but not in H-AF hens ($p>0.05$) although the levels of FI were similar between the two group hens during the HS treatment. It is likely due to the nature of the antioxidant present within the feed, which may allow AF hens to have greater stores of energy and higher metabolism than CF hens. Ileum weight in stressed hens was reduced compared with their respective controls (Table 1) but the significantly decrease was found in H-CF hens at day 1 only ($p<0.05$). Similarly to the changes of LW, the changes of IW between H-CF and H-AF hens were unlikely related to the change of FI. These data may indicate that Agrado plus ultra® additively prevents HS associated liver and small intestine damage. Supplemental Agrado plus ultra® prevents oxidative stress from ROS production by mitochondria within liver tissue of the bird, through both up-regulation of antioxidant enzyme activity and limiting the use of antioxidants like VA and VE.

Liver, spleen and small intestine weight, as physical indicators, can be attributed to several main physiological factors. The liver, for example, is the primary site for nutrient absorption, transportation and storage in birds (Manoli *et al.*, 2007) and has an extremely high metabolic activity, with 1000-2000 mitochondria present per hepatocyte (Alberts *et al.*, 2002). The liver is also a primary site for protein metabolism (synthesis and degradation) and biosynthesis and storage of numerous chemicals involved in digestion and regulation of oxidation reactions (Kmiec, 2001). The different changes of those organs' physical characters following HS may associate with hens' physiological changes and adaptability to HS.

Effect of antioxidant supplementation on heat stress protein 70 in hens following heat stress: In general, chickens, as well as all organisms, must be able to adapt to changes in their environment in order to survive. Heat shock proteins, as a kind of extracellular stress protein, are induced by various stressors, including high temperature. Heat shock proteins, such as HPS70, play important roles in organisms' defense mechanism against cell stress responses, attenuating stress-associated damage to maintain organisms' homeostasis to avoid future insults (Javid *et al.*, 2007). Previous studies have shown that, similar to the findings in mammals, HSPs, specifically HSP70, are inducible in chickens when exposed to stress, including crating and HS (Wang and Eden, 1998; Franco-Jimenez and Beck, 2007; Zulkifli *et al.*, 2009). Yahav *et al.* (1996) showed that HSP expression in broilers was positively correlated with increased thermotolerance, i.e., a higher HSP expression was found in hens exposed to severe high temperatures, while lower HSP expression resulted from mild high temperatures. Similar to the previous findings, there were temperature effects on the concentrations of HSP70 in the liver of stressed hens ($p<0.01$, Table 3). Compared to their respective controls, H-AF hens had an increased level of HSP70 in the liver at d 8 of treatment ($p<0.01$) while it was a tendency to increase in H-CF hens ($p = 0.051$). Similarly, a tendency for higher HSP70 mRNA expression existed in H-AF hens ($p = 0.091$) but not in H-CF hens ($p>0.14$) at d 8 compared to their respective controls. This difference evidences that Agrado has functions in regulating HSPs expression in the liver through its antioxidant functions. Supporting the hypothesis, HSPs serve important protective roles in animals and are not appear until temperature reached very high end of the physiological range (Sorger, 1991; Flanagan *et al.*, 1995; Pirkkala, *et al.*, 2001; Gothard *et al.*, 2003). Agrado may prevent HS associated tissue damage by activating thermoregulation system.

Effect of antioxidant supplementation on heat stress associated change in blood gas parameters: A common problem that occurs in some species of animals under HS, exhibiting thermal panting to lower body temperatures, is respiratory alkalosis. This has been observed in birds under HS (El-Hadi and Sykes, 1982; Teeter *et al.*, 1985), resulting from an increased loss of CO_2 from the body through hyperventilation (Borges *et al.*, 2004). Partial pressure CO_2 is a vital measurement of respiratory activity. The measurements

Table 3: Effects of antioxidant supplementation on the physiology of the hen following heat stress[1]

Treatment[3]	HSP-70[2]	HSP-70 mRNA[2]
24 h		
C-CF	136.3±34.4	0.46±0.27
H-CF	156.8±31.2	0.77±0.26
C-AF	167.0±32.7	0.35±0.25
H-AF	194.6±29.1	1.26±0.26
Day 8		
C-CF	154.8±28.8	0.54±0.26
H-CF	248.7±28.8*	1.10±0.26
C-AF	138.5±28.8	0.57±0.26
H-AF	271.1±28.8**	1.65±0.26*
p-value		
Temp	0.0023	0.0005
Feed	0.2296	0.2061
Temp*feed	0.594	0.1385
24 h C-CF * H-CF	0.9922	0.4199
24 h C-AF * H-AF	0.9577	0.2190
Day 8 C-CF * H-CF	0.0506	0.1420
Day 8 C-AF * H-AF	0.0020	0.0910

[1]All means reported are LS Means±standard error created by mixed model analysis (n = 10)
[2]Indicates data have been either logarithmically or square root transformed to meet assumptions of normal distribution
[3]C-AF = control temperature/Agrado diet
C-CF = control temperature/control diet
H-AF = heat temperature/Agrado diet
and H-CF = heat temperature/control diet
HSP-70 = heat shock protein 70 concentration in the liver
HSP-70 mRNA = heat shock protein 70 mRNA expression in the liver
**, p<0.01 and *, 0.05<p<0.1 compare with respective controls

of the total amount of circulating carbon dioxide and HCO_3^-, along with TCO_2, provide information about HS associated disorders such as respiratory alkalosis. Higher blood pH, through low levels of CO_2 in circulation, is common in birds exhibiting respiratory alkalosis (Odom *et al.*, 1986). Although we did not observe any differences in pH among the treatments ($p>0.05$; Table 4), the results indicated that hens regardless diet may have been exhibiting respiratory alkalosis under HS, since under HS hens have decreasing levels of TCO_2, as well as HCO_3^- ($p<0.05$). Interestingly, this change does not correlate with pCO_2, which had no temperature effect ($p>0.05$). A temperature x feed effect was observed in HS hens ($p<0.05$) but in opposite directions, i.e., increasing and decreasing levels of pCO_2 were observed in H-AF and H-CF hens, respectively. While decreased pCO_2 is expected with hens exhibiting respiratory alkalosis due to HS. Circulating concentrations of IonCa tended to have a temperature effect, in which hens under HS had lower levels of IonCa ($p = 0.089$; Table 4). This has been correlated with decreased FI and results in limited calcium available for egg shell formation (Elija and Adedapo, 2006). These results may indicate that HS is a general stressor to all the stressed hens but Agrado may have functions in regulating respiratory reactions in preventing respiratory alkalosis, although no effects on the concentrations of pO_2 (partial pressure of oxygen),O_2Sat (saturation of oxygen) and BE (base excess), which warrants further studies.

Effect of antioxidant supplementation on heat stress associated change in oxidative stress measurements: Oxidative stress occurs when the balance of reactive oxygen species (ROS) and antioxidant (AOX) within the body is altered in favor of ROS, stimulating oxidative damage, such as induction or inhibition of apoptotic pathways (Droge, 2002). This balance can be shifted toward in favor of ROS when circulating concentrations of AOX, like VA and VE, are reduced. Through supplementing hens with VA and VE, the deleterious effects of HS on egg production and the inhibition of immune function can be ameliorated (Puthpongsiriporn *et al.*, 2001; Lin *et al.*, 2002).

Our study showed that, in general, hens under HS had dramatically lower concentrations of circulating VA and VE ($p<0.01$ and $p<0.0001$, respectively; Table 5). There was a temperature x feed interaction effect on the concentration of VE ($p<0.05$) but not on the concentration of VA ($p>0.05$). However, compared to their respective controls, the significantly changes was in the H-AF hens under acute heat stress only ($p<0.05$). Similarly, HS has been reported to decrease antioxidants like VA and VE in birds (Lin *et al.*, 2002). The reasons of reduced plasma concentrations of VE in H-AF hens is not clear but could be related to the distribution of VE within a body. The concentrations of VE cover a wide range in body tissues, such as among blood, liver and fat. Over 90% of total body VE is stored in the adipose tissue (Bouwstra *et al.*, 2008). In the future studies liver tissue or adipose tissue should be used as Bouwstra *et al.* (2008) indicated that using blood value of VE to evaluate an animal's oxidative status should be interpreted with caution. In addition, blood concentrations of antioxidants including VE are not related to dietary intakes but to serum lipids in mammals as that VE is conjugated with and transported by plasma lipoproteins (Herrera and Barbas, 2001).

No treatment effects were observed in thiobarbituric acid reactive substances, indicated as concentrations of malondialdehyde (MDA) in blood ($p>0.05$; Table 5). The change of MDA concentration, an indicator of oxidative damage through tissue oxidation, could be species and tissue associated. Increased MDA concentrations were found in stressed broilers under chronic conditions of HS, (Mujahid *et al.*, 2009; Azad *et al.*, 2010b) but was less evidenced in male laying-type chickens (Azad *et al.*, 2010a). Other studies have shown MDA to be increased under periods of 3 and 6 h of HS (Lin *et al.*, 2006), with the changes being more evident in liver tissue than heart tissue. Another study showed circulating MDA values is not affected by antioxidant supplement in the diet of broilers (Tavarez *et al.*, 2011). Based on current and previous studies, it may suggest that broilers are more susceptible to tissue oxidation than laying hens. However, the changes of MDA levels have been reported in the liver than other organs in birds, this may warrant a further study to determine MDA concentrations in the liver of laying hens under HS.

Table 4: Effect of antioxidant supplementation on blood pH, blood gasses and ionized calcium of the hen following heat stress[1]

Treatment[3]	pH[2]	pCO_2[2]	pO_2[2]	O_2Sat[2]	HCO_3^- (mmol/L)	TCO_2 (mmol/L)	BE (mmol/L)	IonCa (mmol/L)
24 h								
C-CF	1.97±0.00	4.00±0.07	4.72±0.12	9.72±0.10	22.83±0.86	24.56±0.92	-5.51±0.95	1.19±0.03
H-CF	1.97±0.00	3.95±0.07	4.40±0.12	9.57±0.10	21.32±0.80	22.99±0.87	-6.52±0.90	1.14±0.03
C-AF	1.98±0.00	3.89±0.06	4.34±0.12	9.41±0.10	21.97±0.88	23.60±0.94	-6.51±0.92	1.26±0.03
H-AF	1.97±0.00	3.91±0.07	4.50±0.12	9.57±0.10	19.86±0.81	21.30±0.87	-7.69±0.90	1.16±0.03
Day 8								
C-CF	1.96±0.00	4.16±0.07	4.16±0.12	9.43±0.11	22.24±0.86	24.27±0.92	-7.05±0.93	1.36±0.03
H-CF	1.97±0.00	3.94±0.07	4.39±0.13	9.43±0.10	20.51±0.90	22.08±0.96	-7.24±0.95	1.32±0.03
C-AF	1.97±0.00	3.88±0.07	4.36±0.13	9.59±0.11	21.28±0.87	23.57±0.93	-5.75±0.94	1.34±0.03
H-AF	1.97±0.00	4.01±0.07	4.35±0.13	9.42±0.10	21.47±0.90	23.35±0.96	-7.03±0.95	1.34±0.03
p-value								
Temp	0.75	0.56	0.88	0.57	0.039	0.02	0.17	0.089
Feed	0.23	0.08	0.71	0.59	0.34	0.43	0.80	0.54
Temp*Feed	0.12	0.04	0.51	0.64	0.59	0.64	0.64	0.92
24 h C-CF * H-CF	1.0000	0.9995	0.6420	0.9576	0.9048	0.9191	0.9939	0.9861
24 h C-AF * H-AF	0.9975	1.0000	0.9857	0.9597	0.6453	0.6137	0.9837	0.5606
Day 8 C-CF * H-CF	0.8533	0.4290	0.9176	1.0000	0.8601	0.7251	1.0000	0.9949
Day 8 C-AF * H-AF	0.9182	0.9314	1.0000	0.9500	1.0000	1.0000	0.9789	1.0000

[1]All means reported are LS Means±standard error created by mixed model analysis (n = 10)
[2]Indicates data has been transformed either through logarithmic or square root transformation to meet assumptions of normal distribution
[3]C-AF = control temperature/Agrado diet; C-CF = control temperature/control diet
H-AF = heat temperature/Agrado diet; H-CF = heat temperature/control diet
BE = base excess; HCO_3^- = bicarbonate
IonCa = ionized calcium; O_2Sat = saturation of oxygen
pCO_2 = partial pressure carbon dioxide; pO_2 = partial pressure of oxygen
TCO_2 = total carbon dioxide

Table 5: Effects of antioxidant supplementation on measures of oxidative stress of the hen following heat stress[1]

Treatment[3]	MDA uM	Carbonyl[2]	VA ng/mL	VE ng/mL	SOD (u/mL)	GPx
24 h						
C-CF	2.39±0.19	0.57±0.03	520.9±14.6	7.03±0.41	26.36±3.50	35.79±3.29
H-CF	2.61±0.19	0.63±0.03	474.7±14.6	5.43±0.41	14.55±3.50	34.24±3.29
C-AF	2.72±0.18	0.64±0.03	503.0±14.6	8.05±0.41	21.55±3.50	44.77±3.09
H-AF	2.74±0.19	0.62±0.03	481.6±14.6	4.47±0.41*	26.54±3.50	33.34±3.29
Day 8						
C-CF	2.54±0.20	0.60±0.03	506.2±15.3	6.88±0.41	30.03±3.50	26.73±3.29
H-CF	2.73±0.20	0.75±0.03#	463.8±14.6	6.13±0.41	27.15±3.50	21.45±3.29
C-AF	2.73±0.20	0.64±0.03	528.7±15.3	7.21±0.41	21.87±3.50	22.28±3.29
H-AF	2.83±0.19	0.62±0.03	494.4±14.6	6.02±0.41	21.16±3.50	18.12±3.29
p-value						
Temp	0.3379	0.0466	0.0015	<0.0001	0.3004	0.0208
Feed	0.1719	0.7008	0.3211	0.8179	0.4862	0.9734
Temp*Feed	0.5938	0.011	0.4354	0.0461	0.0639	0.3494
24 h C-CF * H-CF	0.9903	0.8429	0.3530	0.1476	0.2806	1.0000
24 h C-AF * H-AF	1.0000	1.0000	0.9645	0.0276	0.9700	0.2172
Day 8 C-CF * H-CF	0.9970	0.0537	0.4933	0.8999	0.9989	0.9442
Day 8 C-AF * H-AF	1.0000	0.9997	0.7351	0.4733	1.0000	0.9845

[1]All means reported are LS Means±standard error created by mixed model analysis (n = 10)
[2]Indicates data has been transformed either through logarithmic or square root transformation to meet assumptions of normal distribution
[3]C-AF = control temperature/Agrado diet; C-CF = control temperature/control diet
H-AF = heat temperature/Agrado diet; H-CF = heat temperature/control diet
Carbonyl = protein carbonyl concentration; GPx = glutathione peroxidase activity
MDA = malondialdehyde concentration; SOD = superoxide dismutase activity
VA = vitamin A concentration; VE = vitamin E concentration
*, 0.05<p>0.01 compare with respective controls

Heat stress had an effect on limiting GPx activity ($p<0.05$) regardless of diets and without a temperature x diet interaction ($p>0.05\%$). This change may be associated with the fasting of hens exhibiting under HS. Fasting has been reported to decrease activities of antioxidant enzymes catalase and CuZn-SOD (copper-zinc superoxide dismutase) in rats (Marczuk-Krynicka *et al.*, 2003). Additionally, mice fasted for 18 h lowered SOD and GPx concentration in the liver (Di Simplicio *et al.*, 1997). However, we did observe a trend for a temperature x feed interaction on SOD activity ($p = 0.064$; Table 5), in which H-AF hens had increasing levels of SOD activity at 24 h post heat treatment while H-CF hens had decreasing levels under HS at both 24 h and 8 days post heat treatment.

Protein carbonyl concentration in plasma increased under HS ($p<0.05$, Table 5), with a tendency for increasing carbonyl concentration in H-CF hens but not

in H-AF hens, especially at 8 days during heat treatment (P = 0.054). The quantification of protein carbonyl is a common biomarker of oxidative stress (Stadtman and Oliver, 1991; Halliwell, 1996). Carbonyl formation occurs through the transformation of amino acid side chains from ROS activity. With increased levels of ROS activity, these oxidized proteins are increased in the circulation (Halliwell, 1996). The current results indicate that hens fed a control diet exhibited oxidative damage, as observed with the increase in carbonyl content. This change did not occur in Agrado hens, suggesting oxidative damage caused by HS was inhibited through the inclusion of the antioxidant supplement. The current results may be indicative of a benefit for feeding the antioxidant supplement, Agrado, to the hen by inducing a positive change for antioxidant activity to prevent oxidative damage. Similar to the hypothesis, Agrado has been used in beef cattle and dairy cows for reducing stress responses (Vazquez-Anon *et al.*, 2008; Wang *et al.*, 2010).

Conclusion: In conclusion, our data suggest that HS causes r espiratory alkalosis and negative effects on production values such as decreased FI and BW of laying hens. These negative changes are likely associated with the onset of oxidative stress, as decreased concentrations of circulating antioxidants VA and VE as well as increased carbonyl concentrations under HS. Agrado fed hens had a great reaction of HSP70 system with less protein oxidative damage following HS. These results provide evidence for further research to examine the strategy for using antioxidant supplementation to prevent heat stress-associated damage in laying hens.

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