ABSTRACT: The aim of the present study was to evaluate the effect of acute heat stress on the production of mitochondrial reactive oxygen species (ROS), the gene expression of the avian uncoupling protein (avUCP) and glutathione peroxidase (GPX 7), and the activity of the enzyme GPX in the liver of meat quail. Two groups of 15 meat quail (Coturnix coturnix japonica) that were 23 d of age were initially housed individually in metallic cages. A period of 7 d was provided for the 2 bird groups to adapt to the cages and to a thermoneutral environment at 25°C with 60% relative humidity. At 30 d of age, 15 quail were exposed to a heat stress (HS) treatment of 34°C for 24 h, humidity 60%, whereas control quail (n = 15) were kept at 25°C. To analyze the production of ROS, 4 quail from each treatment group were slaughtered, and their livers were collected for mitochondrial isolation and to measure the subsequent production of ROS by the mitochondria. Additionally, the livers of 6 animals from each treatment group were collected for total RNA extraction. The cDNA was amplified using primers specific for the target genes, and expression was analyzed using the real-time PCR reaction (qRT-PCR). Five animals from each treatment group were slaughtered to analyze glutathione peroxidase (GPx) activity, which was determined by using of hydrogen peroxide (H$_2$O$_2$), and based on measuring the amount nicotinamide adenine dinucleotide phosphate oxidized. A greater amount of mitochondrial ROS was found in HS animals (0.34 vs. 0.22 nm of ROS produced min$^{-1}$·mg$^{-1}$ of protein, $P < 0.05$) for the reactions that contained only rotenone and in the reactions that were performed with rotenone and antimycin (0.31 vs. 0.23 nm of ROS produced min$^{-1}$·mg$^{-1}$ of protein, $P < 0.05$). Concomitantly, the birds that were subjected to acute heat stress and had a greater amount of ROS production expressed less avUCP mRNA [0.75 arbitrary units (AU) vs. 0.87 AU, $P < 0.05$] and more GPX 7 mRNA (2.37 AU vs. 1.17 AU, $P < 0.01$). The HS quail displayed significantly greater GPx activity in their hepatocytes (47.8 vs. 39.6 nmol of NADPH oxidized per mg of protein per minute, $P < 0.05$). Thus, acute heat stress at 34°C for 24 h affects the production of mitochondrial ROS, the expression of avUCP and GPX 7 mRNA, and the activity of the GPx enzyme in the liver of meat quail.

Key words: antioxidant system, avian uncoupling protein, hydrogen peroxide, oxidative stress

INTRODUCTION

Quail, broiler, layers, and breeders production is affected by high temperatures found in some tropical countries during the summer season. High temperatures can cause damage on their performance including yield, that can be explained by physiological changes in the birds (Geraert et al., 1996; Yunianto et al., 1997).

These physiological changes might partly be caused by the oxidative stress that occurs in animals kept under heat stress (HS) environments. Birds that are subjected to high temperatures may display a reduction in the activity of the mitochondrial respiratory chain, which may induce a greater production of reactive oxygen species (ROS; Yang et al., 2010). Reactive oxygen species are produced by normal cellular processes. However, when produced in large amounts, ROS are frequently associated with disorders, such as apoptosis (Moustafa et al.,...
ROS, gene expression and enzymatic activity

Large amounts of ROS are produced when animals are exposed to oxidative stress, due both to the overproduction of ROS and deficiencies in the antioxidant defense systems (Halliwell and Gutteridge, 2001). The production of ROS has been frequently related to the potential of the mitochondrial membrane and to the expression of the uncoupling protein (UCP; Mujahid et al., 2009).

Glutathione peroxidase (GPx) forms a part of the antioxidant defense system of glutathione and plays a fundamental role in the elimination of ROS by the organism. High temperatures have been demonstrated to affect the activity of GPx (Tan et al., 2010).

In this study, we hypothesized that HS is related to increased ROS production and also shifts the defense capacity of the organism against the ROS by action of glutathione complex and UCP. We performed a trial maintaining quail under comfort and HS environments to evaluate changes in the mitochondrial ROS production, mRNA avian UCP (avUCP), and glutathione peroxidase (GPX 7) expression, and the GPx activity on liver tissue.

MATERIALS AND METHODS

The procedures in this experiment was approved by the Committee on Animal Care of the Brazil.

Experimental Design and Animals

Two groups of 15 meat quail that were 23 d of age were initially housed individually in metal cages. A period of 7 d was provided for the 2 bird groups to adapt to the cages and to a thermoneutral environment of 25 ± 0.9°C with 60 ± 1.2% relative humidity. The thermal comfort zone for quail used in the present study followed recommendation by Pinto et al. (2003).

At 30 d of age (160.1 ± 1.8 g of BW), 15 quail were exposed to 34 ± 0.6°C for 24 h (humidity 60 ± 0.4%; HS treatment), whereas control quail (n = 15) were kept at 25°C. According to Mujahid et al. (2006), the temperature and relative humidity used here for quail can be considered as a HS condition.

The temperature was measured every minute with sensors (developed by the Animal Science Department, Universidade Estadual de Maringa, Maringa, Brazil) that were distributed along the climatic chamber that was attached to a computer.

During the entire experimental period, the animals had free access to water and food. The food was formulated to meet the nutritional requirements of the animals (Rostagno et al., 2005) and consisted of corn and soybean meal that contained 23.9% CP and 2900 Kcal of apparent ME (AME). The animals were weighed at the beginning of the evaluation period (30-d-old quail) and before slaughter. During the entire experiment, a continuous light program was used.

At the end of the experimental period, the birds were slaughtered by cervical dislocation, and the liver was collected for subsequent analysis of mitochondrial ROS production (n = 4), GPX 7 and avUCP mRNA expression (n = 6), and the activity of the GPx enzyme (n = 5).

Reactive Oxygen Species Production

Four quail from each treatment group were slaughtered to analyze ROS production. The livers were collected for mitochondrial isolation and subsequent analysis of the mitochondrial ROS. To isolate the mitochondria, the livers were stored in a chilled beaker containing isolation medium (0.2 M mannitol; 0.075 M sucrose; 2.0 mM Tris-HCl, pH 7.4; 0.2 mM EDTA; 100 μM phenylmethylsulfonyl fluoride (PMSF); and 50 mg% BSA, fatty-acids-free; all the products were obtained from Sigma-Aldrich, St. Louis, MO) and perforated with scissors. The material was transferred to a Dounce homogenizer along with the isolation medium. The liver homogenate was filtered and underwent 2 sequential centrifugations at 536 × g and 7,080 × g for 10 min at −4°C each. Then, the sediment was washed twice by centrifuging at 6,392 × g for 10 min at −4°C. The mitochondria were homogenized in the isolation medium to a final protein concentration of 80 to 100 mg/mL (Bracht et al., 2003).

The protein content of the subcellular fractions was determined as described by Lowry et al. (1951) using BSA as the standard and a spectrophotometer at a wavelength of 700 nm.

The production of mitochondrial ROS, hydrogen peroxide (H₂O₂), was estimated by measuring the linear increase in fluorescence. Hydrogen peroxide induces the oxidation of dichlorofluorescein diacetate (DCFH-DA) and forms the fluorescent compound 2′-7′ dichlorofluorescein (DCF) in the presence of the horseradish peroxidase (HRP) enzyme (Zaccagnino et al., 2009).

Intact mitochondria (10 μL containing ~0.8 mg of protein) were added to 2 mL of buffer containing 250 mM mannitol; 10 mM Heps buffer, pH 7.2; and 1.36 μM DCFH-DA with either 10 mM succinate and 10 μM rotenone or 10 mM succinate, 10 μM rotenone, and 15 μM antimycin. The reaction was initiated by adding 0.4 μM HRP, and the fluorescent signal was measured every minute for 10 min (Rodrigues Siqueira et al., 2005; all the products were obtained from Sigma-Aldrich). The entire assay was performed under agitation in a spectrofluorometer. The results are expressed in nanomoles of ROS produced per minute per milligram of protein.

ROS, gene expression and enzymatic activity

Lipid peroxidation (Shimizu et al., 2006), and various diseases (Hybertson et al., 2011).

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**Gene Expression Analysis**

The livers of 6 animals from each treatment group were collected and stored in Holder RNA (BioAgency Biotecnologia, São Paulo, Brazil) at –20°C until the RNA was extracted. The total RNA was extracted using the reagent Trizol (Invitrogen, Carlsbad, CAA) according to the manufacturer’s guidelines at a concentration of 1 mL for each 100 mg of tissue. All of the materials used were treated with the RNase inhibitor RNase AWAY (Invitrogen). The RNA concentration was measured using a spectrophotometer at a wavelength of 260 nm. The integrity of the RNA was evaluated in a 1% agarose gel in the presence of 0.5 μg/mL ethidium bromide and visualized with ultraviolet light. The RNA samples were treated with DNase I (Invitrogen) to remove residual genomic DNA according to the manufacturer’s recommendations. To produce the cDNA, the SuperScript III First-Strand Synthesis SuperMix (Invitrogen) kit was used according to the manufacturer’s guidelines.

For the real-time PCR reactions, the fluorescent dye SYBR GREEN was used (SYBR GREEN PCR Master Mix, Applied Biosystems, Carlsbad, CA). The real-time PCR analysis was performed using the StepOnePlus v. 2.2 system (Applied Biosystems). The primers used in the reactions to amplify avUCP were designed according to Ojano-Dirain et al. (2007; GenBank accession number AB088685), and the primers used to amplify GPX 7 were designed according to Yarru et al. (2009; GenBank accession number NM_001163245; Table 1). Two endogenous controls were tested, specifically, the β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes. Based on both the expression stability and expression levels, the β-actin gene (GenBank accession number L08165) was determined to be the best reference gene for normalization of quantitative real-time PCR analysis. All analyses were performed in duplicate using a volume of 25 μL.

**Enzymatic Assays**

Five quail from each treatment group were used to analyze the activity of the GPx enzyme. Immediately after slaughter, the liver of each animal was removed, pressed, and stored in liquid nitrogen until analysis. To analyze the enzymatic activity, the samples were weighed and then crushed in liquid nitrogen using a mortar and pestle. The material was then transferred to a chilled test tube, and 10% of the weight of the sample of cold potassium phosphate buffer (K₂HPO₄ and KH₂PO₄ 0.1 M pH 7.4) was added. The samples were homogenized in a Van Potter homogenizer. The liver homogenate was centrifuged at 6,392 × g for 10 min at –4°C, and the enzymatic activity of the supernatant was determined. The concentration of protein was determined according to the method of Lowry et al. (1951) using BSA as the standard.

The activity of the GPx enzyme was determined according to the method of Paglia and Valentine (1967) using H₂O₂. The activity of the enzyme was determined by the amount of nicotinamide adenine dinucleotide phosphate (NADPH) oxidized that was detected using the spectrophotometer at a wavelength of 340 nm to measure the fluorescent signal emitted by the consumed NADPH, the fluorescent signal was measured every 10 s for 50 s. The consumed NADPH was measured based on the decay in absorbance over the evaluated time period.

For the reaction to occur, 350 μL of 171 mM potassium phosphate buffer was added along with 250 μL of 6 mM GSH, 300 μL of 0.9 mM of NADPH, 10 μL of 2 U/mL glutathione reductase (all the products were obtained from Sigma-Aldrich), 40 μL of supernatant, and 520 μL of MilliQ H₂O. The fluorescent signal was recorded every 10 s for 50 s. The activity is expressed as the nanomoles of NADPH oxidized per mg of protein per minute.

**Statistical Analysis**

The results are shown as the mean and the SD. The UNIVARIATE procedure was applied to verify the normality of all the data. The statistical analysis was performed using Student’s t test (P < 0.05) to compare the 2 treatment groups (SAS Inst. Inc., Cary, NC).

**RESULTS**

The quail BW at the beginning of the evaluation period (30 d old) for both birds groups was 160.1 ± 1.8 g. On the thermal comfort, the birds showed average and SEM of feed intake, final BW, and daily weight gain and feed conversion ratio of 26.3 ± 0.4 g, 167.5 ± 2.7 g, 7.1 ± 0.2 g, 3.7 ± 0.1 g/g, respectively, during the 24-h period of evaluation. Otherwise, HS quail showed 4.7 ± 0.2 g, 140.3 ± 3.2 g of feed intake and final BW, respectively. Quail under HS lost BW (~19.9 ± 0.6 g) during this 24-h period.

The production of H₂O₂ was greater in the mitochondria of the HS birds. When the production of ROS was measured with rotenone alone, it was 0.34 vs. 0.22 nm of ROS produced min⁻¹ · mg⁻¹ of protein (P < 0.05), and

<table>
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<th>Table 1. Real-time PCR (qRT-PCR) primers</th>
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<td>Gene</td>
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<td>GPX 7</td>
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<td>avUCP¹</td>
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<td>β-actin</td>
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¹avUCP, mRNA avian uncoupling protein.
when the production of ROS was measured in the presence of rotenone and antimycin, it was 0.31 vs. 0.23 nm of ROS produced min\(^{-1}\) \cdot mg\(^{-1}\) of protein \((P < 0.05; \text{Fig. 1})\).

The HS led to less expression of mRNA \(avUCP\) in the liver of the quail \((P < 0.05; \text{Fig. 2A})\). Unlike the expression of mRNA \(avUCP\), the HS birds expressed more mRNA \(GPX 7\) in their livers \((P < 0.01; \text{Fig. 2B})\).

The activity of the GPx enzyme was verified using a spectrophotometer at a wavelength of 340 nm to measure the fluorescent signal emitted by the consumed NADPH, which was measured based on the decay in absorbance over the evaluated time period. The absorbance values observed during the initial measurements for the different animals groups were evaluated and are shown in Fig. 3. The rate of decay in absorbance \((\Delta A)\) was different between the treatment groups, which resulted in a significant difference in the activity of the enzyme in the liver of quail in the 2 treatment groups (Fig. 3).

Along with the greater production of ROS and the greater expression of mRNA \(GPX 7\), the HS animals also displayed greater activity in the liver than the animals in the thermoneutral group (47.8 vs. 39.6 nmol of NADPH oxidized per mg of protein per minute, \(P < 0.05; \text{Fig. 4})\).

**DISCUSSION**

We observed a huge loss of BW in quail under HS environment, even with the period of evaluation being only 24 h. We cannot explain completely the loss in BW, but the literature indicates that broilers under high temperatures increase plasma corticosterone concentrations, which stimulates a large increase in body protein breakdown (Yunianto et al., 1997); a reduction in the relative weights of many organs such as intestine, liver, gizzard, lungs, proventriculus, and heart (Oliveira Neto et al., 2000); a reduction in the absorptive area (villus) of the intestine (Mitchell and Carlisle, 1992); a reduction in yield (Geraert et al., 1996) and increased water consumption (Oliveira Neto et al., 2000). All these physiological changes may indicate that quail probably lost body protein and also had more liquid feces, which can lead to a greater discharge of the intestine content.

Heat stress caused by high temperatures is also related to the production of mitochondrial ROS (Tan et al., 2010; Yang et al., 2010). To verify the production of ROS by the mitochondria isolated from the hepatocytes of the quail and to ensure that all of the free radicals produced were measured, assays were performed with rotenone alone, which blocks the I complex of the respiratory chain, and with rotenone and antimycin, blocks the III complex of the chain. Based on these assays, the birds exposed to 34°C for 24 h produced more mitochondrial ROS than did control birds. These results were found independent of how the reaction was performed. The broiler chickens that were subjected to acute HS also displayed greater levels of mitochondrial ROS when either glutamate or succinate was used as the substrate in the electron transport chain (Mujahid et al., 2006), which indicates that the production of ROS is increased when the animals are exposed to HS independent of the substrate that is used.
The mechanisms involved in the increased production of ROS when animals are exposed to HS may be associated with a leakage of electrons from the mitochondrial chain, oxidation of the proteins from the chain complexes, a reduction in the activity of the chain complexes, or other forms of damage that occur in the mitochondria. This damage may be related to body temperature because an increase in the production of ROS is observed followed by a reduction in BW gain by the birds with a greater body temperature (Mujahid et al., 2005).

The increased production of ROS appears to be involved in a cycle in which escaped electrons, due to disturbances in the respiratory chain, increase the production of free radicals, which causes more harm to the mtDNA and causes additional production of ROS (Tengan et al., 1998). Thus, perfect activity of the respiratory chain complexes is required to maintain system equilibrium. However, exposure to acute HS inhibits certain complexes of the chain (Tan et al., 2010).

The production of ROS in animals exposed to high temperatures has also been correlated with the potential of the mitochondrial membrane and to the expression of the UCP gene (Fink et al., 2005). A greater membrane potential is related to greater ROS production, and greater UCP mRNA production is associated with the production of fewer free radicals and to less cellular damage as a result of ROS. A greater membrane potential and less avUCP mRNA expression is observed in animals subjected to HS (Mujahid et al., 2009).

The UCP can reduce ROS production by affecting decoupling during the production of ATP (Abe et al., 2006). The expression of UCP is influenced by environmental factors, such as HS (Mujahid et al., 2006) and nutritional status (Evock-Clover et al., 2002).

Along with a greater production of H₂O₂, the HS quail displayed significantly less avUCP mRNA expression in the liver. Thus, as in broiler chickens (Mujahid et al., 2006), acute stress in quail may lead to a greater production of mitochondrial ROS, which may be caused by reduced avUCP mRNA expression. Therefore, maintaining the appropriate levels of avUCP mRNA transcripts could help to combat the overproduction of ROS and help combat the oxidative stress that is caused by acute HS (Mujahid et al., 2009). Conversely, the effect of greater ROS production can also be analyzed by comparing it to the expression of UCP. Greater ROS production and greater protein oxidation are consistently observed in birds with reduced food intake, which suggests that this factor may alter the expression of mitochondrial genes involved in the production of ATP and thus in the efficiency of the ability of the animals to convert food into body mass (Ojano-Dirain et al., 2007).

The glutathione (GSH) antioxidant defense system and other systems protect the cell from damage caused by mitochondrial ROS. In addition to GSH, the glutathione system is composed of the enzymes glutathione oxidase, GPx, and glutathione reductase. Therefore, the function of this system depends on the activity of the complete compound. To reduce oxidative species, glutathione is oxidized to glutathione disulfide (GSSG) by the GO and GPx enzymes. The glutathione reductase enzyme is responsible for regenerating GSH from GSSG, which allows the same molecule to be used more than once to eliminate ROS (Huber et al., 2008).

Exposure of the quail to HS also affects the expression of GPX7 mRNA in the liver. The mRNA abundance appears to affect the activity of GPx (Tiedge et al., 1997). In the present study, we observed that acute HS caused increased expression of GPX7 mRNA and the GPx activity. Similar results were also found by other authors who suggested that this increase in activity may be an attempt by the organism to combat ROS, which also experiences increased production under these conditions (Pamok et al., 2009; Tan et al., 2010; Yang et al., 2010).

In general, our results suggest that exposure to HS at 34°C for 24 h leads to greater H₂O₂ production, partly by inducing a reduction in avUCP mRNA expression. In addition, HS also leads to greater expression and activity of
the GPx enzyme, which is involved in protecting the cell from ROS.

Thus, complementary studies that use biomarkers are necessary to measure oxidative stress and confirm whether the applied stress is sufficient to establish a state of oxidative stress in the cell that causes greater ROS production and impairs the antioxidant defense system.

In conclusion, acute HS at 34°C for 24 h affects the production of mitochondrial ROS, the expression of avUCP and GPX 7 mRNA, and the activity of the GPx enzyme in the livers of meat quail.

LITERATURE CITED


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