Research, Society and Development, v. 9, n. 8, e63985059, 2020
(CC BY 4.0) | ISSN 2525-3409 | DOI: http://dx.doi.org/10.33448/rsd-v9i8.5059

Estresse térmico agudo promove alterações morfológicas e moleculares no coração de frangos de corte

Acute thermal stress promotes morphological and molecular changes in the heart of broiler chickens

El estrés térmico agudo promueve cambios morfológicos y moleculares en el corazón de los pollos de engorde

Recebido: 28/05/2020 | Revisado: 30/05/2020 | Aceito: 10/06/2020 | Publicado: 24/06/2020

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Resumo

Este estudo teve como objetivo compreender os possíveis efeitos do estresse térmico agudo (32ºC, 12 horas) na temperatura corporal por meio de duas vias de medida (via sonda e retal), nos aspectos morfométricos do coração e da artéria aórtica, na expressão gênica (superóxido dismutase, glutationa peroxidase-3, óxido nítrico sintase induzida, enzima conversora de angiotensina e proteína de transferência de colesterol esterificado), nos parâmetros inflamatórios (mieloperoxidase e N-acetilglicosaminidase), parâmetros de estresse oxidativo e níveis de nitrito em frangos de corte (Cobb 500) aos 42 dias de idade. Foram utilizados 36 frangos de corte com 42 dias de idade distribuídos em esquema fatorial 2x2: dois ambientes térmicos (conforto a 18°C e estresse a 32°C) e dois métodos de aferição da temperatura corporal (via probe e via retal). O estresse térmico desencadeou aumento na temperatura corporal independentemente da via de aferição. Houve efeito significativo na espessura da parede da artéria aorta e nos diâmetros látero lateral e ântero posterior (P<0,05). Da mesma forma, houve diferença nas dosagens de hidroperóxidos lipídicos, na quantificação das espécies reativas de oxigênio e na dosagem de nitrito (P<0,05). A quantificação do mRNA dos genes óxido nítrico sintase induzida, enzima conversora de angiotensina e proteína de transferência de colesterol esterificado foram significativamente maior nos animais submetidos ao estresse térmico. Assim, pode-se concluir que o estresse térmico agudo foi capaz de promover diversas alterações morfológicas e moleculares no coração e na artéria aorta de frangos de corte.

Palavras-chave: Avicultura; Células cardíacas; Parâmetros bioquímicos.

Abstract

This study aimed to understand the possible effects of acute thermal stress (32ºC, 12 hours) on body temperature using two measurement methods (via probe and rectal), on the

morphometric aspects of the heart and aortic artery, on gene expression (superoxide dismutase, glutathione peroxidase-3, nitric oxide synthase, angiotensin-converting enzyme and esterified cholesterol transfer protein), inflammatory parameters (myeloperoxidase and N-acetylglycosaminidase), oxidative stress parameters and nitrite levels in broilers (Cobb 500) at 42 days of age. 36 broilers with 42 days of age were used, distributed in a 2x2 factorial scheme: two thermal environments (comfort at 18ºC and stress at 32ºC) and two methods of measuring body temperature (via probe and rectal). Thermal stress triggered an increase in body temperature regardless of the measurement method. There was a significant effect on the thickness of the aortic artery wall and on the lateral lateral and posterior antero diameters (P <0.05). Likewise, there was a difference in the dosages of lipid hydroperoxides, in the quantification of reactive oxygen species and in the dosage of nitrite (P <0.05). The quantification of the mRNA of the induced nitric oxide synthase, angiotensin-converting enzyme and esterified cholesterol transfer protein genes were significantly higher in animals subjected to heat stress. Thus, it can be concluded that acute thermal stress was able to promote several morphological and molecular changes in the heart and aorta artery of broilers.

Keywords: Poultry; Cardiac cells; Biochemical parameters.

Resumen

Este estudio tuvo como objetivo comprender los posibles efectos del estrés térmico agudo (32ºC, 12 horas) en la temperatura corporal utilizando dos métodos de medición (a través de sonda y rectal), en los aspectos morfométricos del corazón y la arteria aórtica, en la expresión génica (superóxido dismutasa, glutatión peroxidasa-3, óxido nítrico sintasa, enzima convertidora de angiotensina y proteína de transferencia de colesterol esterificada), parámetros inflamatorios (mieloperoxidasa y N-acetilglucosaminidasa), parámetros de estrés oxidativo y niveles de nitrito en pollos de engorde (Cobb 500) a los 42 días de edad. Se utilizaron 36 pollos de engorde con 42 días de edad, distribuidos en un esquema factorial 2x2: dos ambientes térmicos (comodidad a 18ºC y estrés a 32ºC) y dos métodos para medir la temperatura corporal (a través de sonda y rectal). El estrés térmico provocó un aumento de la temperatura corporal, independientemente del método de medición. Hubo un efecto significativo en el grosor de la pared de la arteria aórtica y en los diámetros antero lateral y posterior (P <0.05). Asimismo, hubo una diferencia en las dosis de hidroperóxidos lipídicos, en la cuantificación de especies reactivas de oxígeno y en la dosis de nitrito (P <0.05). La cuantificación del ARNm de la sintasa de óxido nítrico inducida, la enzima convertidora de

angiotensina y los genes de proteína de transferencia de colesterol esterificada fueron significativamente mayores en animales sometidos a estrés por calor. Por lo tanto, se puede concluir que el estrés térmico agudo fue capaz de promover varios cambios morfológicos y moleculares en el corazón y la arteria aorta de los pollos de engorde.

Palabras clave: avicultura; células cardíacas; parámetros bioquímicos.

1. Introduction

The genetic improvement provided a great advance in the growth and development of broiler chickens, allowing fast weight gain, low feed conversion, high carcass yield and increased muscle tissue deposition, especially in the pectoral region (Sahraei, 2014). However, the accelerated increase in muscle mass may make the animals susceptible to the development of metabolic disorders due to the mismatch between accelerated weight gain and the inherent lack of adaptation of the cardiorespiratory apparatus (Tickle et al., 2014).

The inability of the cardiorespiratory apparatus to adjust to the body mass demand is more evident in situations of high ambient temperature, since broilers are extremely sensitive to abrupt temperature variation (Crandall & Wilson, 2015). Thus, temperature increase is considered one of the most important environmental stressors in poultry farming in tropical countries, negatively impacting food consumption, weight gain, morbidity rate and mortality due to multiple pathophysiological changes related to this condition (El-Tarabany, 2016; Moretti et al., 2020).

Among the major pathophysiological changes, oxidative stress and metabolic disorders such as sudden death syndrome, acute hyperthermia, respiratory alkalosis and electrolyte imbalance stand out (Rani et al., 2016). Oxidative stress can increase lipoperoxidation causing several functional changes in the cells. The compounds generated by oxidative stress (superoxide radicals ($O_2^{\bullet}$), hydroxyl ($OH^{\bullet}$) and hydrogen peroxide (H_2O_2)) are highly reactive and modify several biologically active macromolecules such as proteins, lipids and nucleic acids, on the other hand, metabolic disturbances are associated with economic and productive losses due to the high mortality rate in these animals (Akbarian et al., 2016; Roushdy et al., 2018).

In this context, the understanding of how thermal stress can interfere not only in the morphometry of the heart and the aorta, but also in specific metabolic processes at the cellular and molecular level, becomes increasingly important. Therefore, this study aimed to understand the possible effects of acute thermal stress (32ºC, 12 hours) on body temperature

by means of two pathways of measurement (via probe and rectal), on morphometric aspects of the heart and aortic artery, gene expression (superoxide dismutase (*SOD*), glutathione peroxidase 3 (*GPX3*), induced nitric oxide synthase (*iNOS*), angiotensin converting enzyme (*ACE*), and cholesteryl ester transfer protein (*CETP*)), inflammatory parameters (myeloperoxidase (MPO) and N-acetylglicosaminidase (NAG)), oxidative stress parameters (superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), lipid hydroperoxides (LOOH) and reactive oxygen species (ROS)) and nitrite levels in broiler chickens at 42 days old.

2. Methodology

The research is explanatory and experimental, and the field experiment was carried out in the bioclimatology shed at Fazenda Experimental de Iguatemi (FEI) belonging to the State University of Maringá (UEM). Subsequently, the analyzes, being of a quantitative nature, were carried out in the Histology Laboratory of the Department of Morphological Sciences (DCM) -UEM and in the Gene Z Molecular Genetics Laboratory of the Zootechnics department- UEM. This study was conducted according to the specifications of the Committee of Ethics in the Use of Animals of the State University of Maringá (nº.4000170615).

2.1. Animals

Thirty-six male broilers (Cobb 500) raised according to the Cobb lineage guide up to 42 days old were used. These birds were distributed in a 2 x 2 factorial scheme with two environments (thermal comfort at 18ºC and thermal stress at 32 ºC for 12 hours) and two temperature measurement (via probe and rectal). The diets were formulated to attend nutritional requirements during the periods from 1 to 21 days and from 22 to 42 days, water and feed were supplied *ad libitum* throughout the breeding period. (Table 1).

Table 1 - Experimental Diets.

Nutrients	1-21 days	22-42 days
Crude Protein (%)	22	20
Energy (Kcal)	3053	3169
Methionine+Cistine Digestive	0.856	0.810
Lys Digestive	1.199	1.080
Tryp Digestive	0.244	0.218
Threonine Digestive	0.779	0.700
Isoleucine Digestive	0.855	0.772
Valine Digestive	0.923	0.842
Arginine Digestive	1.384	1.243
Sodium	0.200	0.19
Calcium	0.876	0.68
Phosphor	0.450	0.35

Source: Autors

Presentation of the experimental diet provided throughout the period (1 to 21 days of age and 22 to 42 days of age) according to the necessary requirements of crude protein, energy and amino acids in each growth phase, as seen in Table 1.

2.2. Body temperature monitoring

For monitoring body temperature, the birds within each experimental group (comfort and stress) were divided into two groups: via probe and rectal (n = 7).

2.2.1. Via Probe

The monitoring of the temperature via probe was carried out continuously in the 12 hours of experimentation that preceded the slaughter, and the readings were recorded every 10 minutes. Therefore, a data logging system (*iButton® data loggers, Embedded Systems*, KY, USA) was surgically implanted in the coelomic cavity of the animals, 7 days before the data collection period (animal recovery periods). The *data logger* placement (1.5 cm diameter x 0.6 cm height) was performed after general anesthesia with isoflurane, feather removal and

local asepsis with 2% chlorhexidine. Subsequently, muscle layers and skin were sutured, and the animals were treated with two doses of enrofloxacin (10 mg/kg, i.m) and flunixinameglumine (2.5 mg / kg, i.m), one at the end of surgery and the other after 6 hours. For data collection, the *data loggers* were removed and analyzed at the time of slaughter.

2.2.2. Via Rectal

The rectal body temperature measurement was performed in both groups, comfort and stress environment, hourly, during the experimental period (12 hours prior to slaughter) by means of a digital thermometer with a prominent and rounded bulb that facilitates the introduction and decrease the discomfort of animals. The birds were placed in lateral decubitus position and the thermometer was inserted 2.5 cm above the cloaca of the animals.

2.3. Morphometric analyzes and biochemical tests

2.3.1. Heart collection

After slaughter, the birds were submitted to thoracotomy, to the opening of the fibrous pericardium and to the surgical removal of the organ by means of an incision on the large vessels of the base. The heart was immediately weighed on an analytical balance (Shimadzu®) and perfused with Saline Phosphate Buffer (PBS; 0.1 M; pH 7.4) solution through the myocardial tissue for complete removal of the blood housed in its chambers.

In sequence, the hearts were cut immediately below the atrioventricular septum and the cardiac tissue above this septum was used for the morphometric analyzes of the heart. Thus, the atria were readily fixed in 4% paraformaldehyde for 72 hours and thereafter stored in 70% alcohol. The atrioventricular septum tissue was subdivided, preserved in liquid nitrogen and subsequently stored in a freezer at -80°C until the accomplishment of the biochemical tests that included the evaluations of the inflammatory parameters, the nitrite dosage and the analyzes of the parameters of oxidative stress.

2.3.2. Morphometric analyzes of the atrium and aortic artery

The previously stored atria were used to capture images that were used to measure the cardiac chambers (atrial and latero-lateral and anteroposterior diameters, thickness of the right

and left atrial walls, and interatrial septal thickness) and aortic artery (wall thickness, laterolateral and anteroposterior diameters).

To capture the images, a magnifying glass (Motic® TIM-2B) coupled to a camera (OPTON®) was used, and all images were analyzed using the ImageJ® program (IMAGE PROPLUS® 5.2 from Media Cybertechnics, São Paulo, Brazil). The calibration for standardization of the capture was done using a calibration strip of 7500 μm. To avoid interference of live weight of birds, measurements were corrected according to the mean relative body weight using the formula: [(heart weight/live weight) * 100], which resulted in the correction factor of 0.61% for the animals of the thermal comfort group and 0.58% for the animals of the thermal stress group.

2.3.3. Biochemical tests

To obtain the homogenate used in the biochemical assays, the cardiac tissue was weighed and homogenized in 200 mM potassium phosphate buffer, pH 6.5. Part of this homogenate was used for quantification of glutathione levels (GSH) and part was centrifuged for 20 minutes at 9,000 g. The supernatant resulting from this centrifugation was used for the analysis of the enzymes catalase (*CAT*), superoxide dismutase (*SOD*) and glutathione s-transferase (*GST*), and for the measurement of lipid hydroperoxide levels (*LOOH*). The precipitate was used for the analysis of myeloperoxidase (*MPO*) and N-acetylglucosamidase (*NAG)* enzymes.

Quantitation of *GSH* levels was determined by adding 12% trichloroacetic acid to the homogenate. The solution was homogenized, centrifuged for 15 minutes at 9,700 g and Tris buffer (0.4 M, pH 8.9) was added to the 96-well microplates. The reaction was started with the addition of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB; 1 mM) and the reading was performed within 5 minutes in a spectrophotometer at 415 nm. The values obtained were interpolated on a standard *GSH* curve and expressed in μg GSH/g tissue (Sedlak & Lindsay, 1968).

Measurement of *CAT* activity was performed by diluting the supernatant in potassium phosphate buffer (0.2 M, pH 6.5) in the ratio of 1:10. In a 96-well plate, the sample was homogenized in a solution containing tris-HCl-EDTA buffer (0.1M; pH 8.5), distilled water and hydrogen peroxide (H_2O_2). The readings were made at 240 nm. The results were expressed as μmol/min/mg protein (Aebi, 1984).

The dosage of *SOD* activity considered its ability to inhibit auto-oxidation of

pyrogallol. The supernatant was homogenized in tris-HCl buffer (200 mM) and EDTA (2 mM; pH 6.5) and 1 mM pyrogallol was added. The solution was incubated at room temperature for 20 minutes and the reaction was quenched with 1N HCl. The solution was centrifuged for 4 minutes at 14,000 g and the supernatant was pipetted into microplates for spectrophotometer reading at 405 nm. The amount of protein that inhibits the reaction by 50% (IC_{50}) equals 1 (U) unit of *SOD*. The results were expressed as U of *SOD*/mg protein (Marklund & Marklund, 1974).

For the analysis of the enzymatic activity of GST, the sample was diluted in potassium phosphate buffer (0.1M; pH 6.5) and then pipetted into a 96-well plate. The reaction was started by the addition of a solution containing potassium phosphate buffer (0.1 M, pH 6.5), 1-chloro-2,4-dinitrobenzene (CDNB) and GSH. The spectrophotometer was read at 340 nm using the extinction coefficient of 9.6 mmolar/cm. The results were expressed as µmol/min/mg protein (Warholm et al., 1985).

Total lipid hydroperoxides (*LOOH*) were determined by the oxidation test of iron II in the presence of orange xylenol. The samples were homogenized in 90% methanol at the ratio of 1:10 and centrifuged for 30 minutes at 10000 g at 4 °C. The resulting supernatant and the reaction medium (90% methanol, xylenol orange, 25 mM sulfuric acid, 4 mM butylated hydroxytoluene and 250 mM ammonium ferrous sulfate) were pipetted into a 96-well plate which was incubated for 30 minutes at room temperature and out of the light. The reading was performed in a spectrophotometer with a wavelength of 560 nm. The concentration of *LOOH* was determined from the extinction coefficient of 4.3 mmol/cm and the results were expressed as mmol/mg tissue (Jiang et al., 1991).

MPO activity was assessed by resuspending the precipitate in 80 mM potassium phosphate buffer containing 0.5% hexadecyltrimethylammonium. Samples were homogenized and centrifuged at 4 °C for 20 minutes at 11,000 g. In a 96 well-plate, the supernatant and a solution containing sodium phosphate buffer (0.1 M, pH 7.4) and 0.017% hydrogen peroxide were added. The reaction was started with tetramethylbenzidine and after 3 minutes of incubation at 37 °C, the reaction was quenched with sodium acetate (1.46 M, pH 3.0). The reading was carried out in a spectrophotometer at 620 nm and the results were expressed as unit of optical density (D.O)/min/mg protein (Bradley et al., 1982; De Young et al., 1989).

For the assay of *NAG* activity, samples containing the supernatant were incubated with citrate buffer (5 mM, pH 4.5) in the presence of p-nitrophenyl-N-acetyl-β-D-glucosaminide (2.24 mM). The microplates were incubated for 60 minutes at 37°C and the reaction was

quenched with glycine buffer (200 mM, pH 10.4). The reading was performed in a spectrophotometer at 405 nm and the results were expressed as unit of optical density (O.D.)/mg protein/hour (Bailey, 1988).

Calculations of the biochemical assays considered the protein concentration determined by the BCA™ protein assay kit (PIERCE, BCA protein), which adopts bovine albumin as standard. The test was performed following the manufacturer's instructions on a 96 well-plate.

For the determination of the reactive oxygen species (*ROS*), more specifically the superoxide radical anion, samples stored in a freezer at -80 ºC were weighed and homogenized in tris-EDTA buffer (5 mM, pH 8.5). After incubation with dichlorofluorescein diacetate (DFD, 1 mM) for 40 minutes in the dark, the fluorescence was measured in a spectrofluorimeter. All measurements were performed using wavelength with excitation of 488 nm and emission of 520 nm and the results were expressed in nmol of DFD/mg protein (Brandt & Keston, 1965).

2.3.4. Dosing of nitrite levels

The nitrite levels were obtained by means of the Greiss Reaction. The cardiac tissue was homogenized in sodium phosphate buffer solution (0.1M PBS, pH 7.4) and centrifuged for 10 minutes at 3000g. Subsequently, the supernatant was added to the Greiss reagent (solution containing phosphoric acid, sulfanilamide and N-1-naphthalylethylenediamide) and nitrite levels were measured by spectrophotometer reading at 570 nm in 96-well plates. The nitrite concentration was calculated using a standard curve constructed in the range of 100 µM to 1.56 µM sodium nitrite and nitrite levels were expressed as µM/µL (Tiwari et al., 2011).

2.4. Gene expression

For the analysis of gene expression, cardiac tissue samples from eight animals/group were collected, immediately stored in liquid nitrogen and stored in a freezer at -80ºC until *RNA* extraction. Total *RNA* was extracted using TRIzol® reagent (Invitrogen, Carlsbad CA, USA) according to the manufacturer's standards.

The *RNA* concentration was measured via a spectrophotometer (Nanodrop 2000c, Thermo Fisher Scientific ™) at wavelength 260 nm and the integrity of the *RNA* was evaluated on 1% agarose gel stained with SYBR® Safe DNA Gel Stain and visualized in

ultraviolet light. Total *RNA* samples were treated with *DNAase* I, Amplification Grade (Invitrogen, Carlsbad CA, USA) according to the manufacturer's instructions.

For the synthesis of complementary *DNA* (*cDNA*), the SuperScript ™ III First-Strand Synthesis SuperMix kit (Invitrogen Corporation, Brazil) was used and reactions were performed according to the manufacturer's guidelines. Shortly after cDNA synthesis, the samples were stored at -20°C until the time of use. Real-time PCR reactions (polymerase chain reaction - RT-qPCR) were performed using the SYBRTM Green PCR Master Mix fluorescence compound (Applied BiosystemsTM, USA) according to the manufacturer's instructions.

The primers used in the reactions were designed according to the sequences of the genes deposited at www.ncbi.nlm.nih.gov (Table 2): superoxide dismutase (*SOD*), glutathione peroxidase 3 (*GPX3*), induced nitric oxide synthase (*iNOS*), angiotensin converting enzyme (*ACE*), and cholesteryl ester transfer protein (*CETP*).

Table 2 - Sequence of primers used in the polymerase chain reaction (real-time PCR qRT-PCR).

Genes	Sequence of primer (5'-3')	Access number	Amplicon (pb)
SOD	F-AGATGGCAGTGGGAAATGAG	NM_205064	110
	R-ACTCAAGACAGCAGAGTAGT		
GPX3	F-GGAGAGGGAGAAGGTGAAAT	NM_001163232.2	137
	R-TGGCCACGTTGACAAAGA		
iNOS	F-TCCTGAGTTCTGTGCCTTTG	U46504.1	92
	R-GTTCATCTCTTCACCCACTG		
ACE	F- TCACCCGCATCCTCAATAAG	NM_001167732.1	128
	R-GTTGTACTCCTTCAGCTCATCC		
CETP	F-TTTAAAGGAGATCCCAAGGAG	NM_001034814.2	135
	R- ACTCACATCTTCAGCCATACA		
β-actin	F-GCCAACAGAGAGAAGATGAC	L08165	113
	R-CACCAGAGTCCATCACAATA		

Source: Authors.

Primer sequence of all analyzed genes with β-actin as endogenous control, in addition to the access number for eventual research as well as the size of each primer, as seen in Table 2.

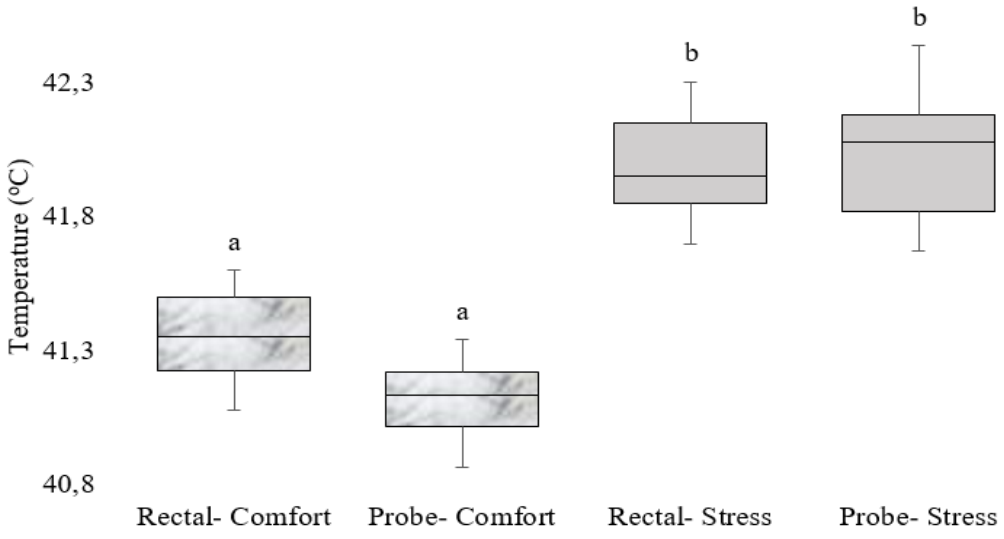
2.5. Statistical analysis

The analyzes of the studied variables were obtained through the analysis of the statistical premises of normality of residues through the Shapiro-Wilk test and homogeneity of the variances through the Bartley test. The data of all the variables, except for the rectal temperature, were analyzed considering two treatments (comfort and thermal stress) using the SAS GLM procedure, and the means were compared by the F test (P <0.05) (SAS INSTITUTE, 2004). Results were expressed as mean and standard deviation. The $2^{-\Delta ct}$ method was used for the relative expression analyzes, and its results were expressed as arbitrary unit (AU). The experiment was conducted in a 2 x 2 factorial scheme, being two environments (comfort and stress) and two measurement systems (probe and rectal).

3. Results and Discussion

The analysis of the body temperature according to the measurement ways and the environments to which the animals were kept showed interaction between the factors. Animals maintained in a thermal stress environment had their body temperatures elevated (P <0.0001) regardless of the measurement used (via probe: 42.01°C; via rectal: 41.97°C) (Figure 1). In the animals at comfort temperature, the measurement via probe was more sensitive to temperature variation (via probe: 41.24°C; via rectal: 41.38°C) (Figure. 2).

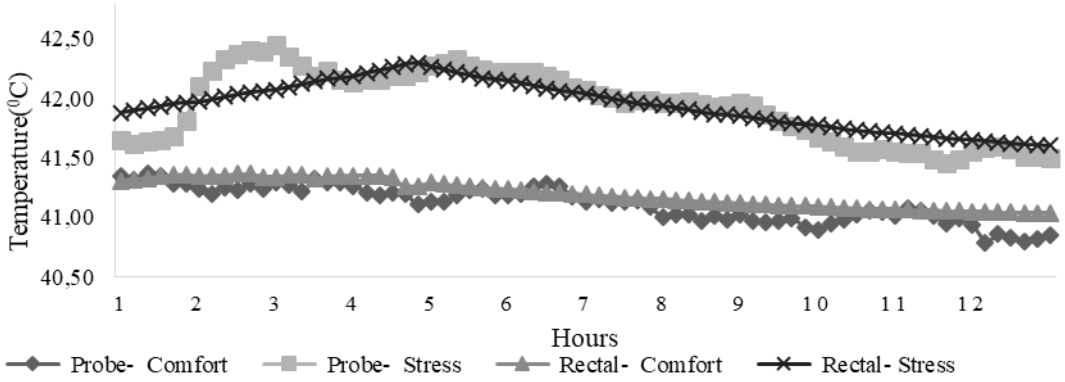
Figure 1 - Body temperature measured via probe and rectal in conditions of comfort and thermal stress.



[a,b,*]Mean values significantly different (P <0.05). Source: Authors.

Comparison of body temperature of 42-day-old chickens, measured in two ways - by probe and rectal in two environments - thermal comfort and thermal stress, regardless of the measurement method, thermal stress raised the animals' body temperature, as seen in Figure 1.

Figure 2 - Body temperature of the broilers at 42 days submitted to the comfort and thermal stress environment considering the probe or rectal measurement pathways for an uninterrupted period of 12 hours.



Source: Authors.

Body temperature behavior of broilers in the 12-hour period, measured in two ways - by probe and rectal in two environments - thermal comfort and thermal stress. The thermal stress raised the animals' body temperature in the first hour of heat elevation that was maintained in the next 12 hours of heat, as seen in Figure 2.

In adult birds, body temperature ranges from 40 to 41ºC, this temperature being maintained by homeostatic reactions that balance the heat production generated by the metabolic processes of the main organs of the body and the dissipation of the body to the environment (Sellier et al., 2014). Birds subject to high environmental temperatures present decreased feed intake, increased water intake and behavioral changes becoming prostrate and panting, keeping their wings high, ventral region exposed, feathers erect, and the beak half open (signs observed in birds of the present study), such modifications favor peripheral vasodilation that intensifies blood flow to the body surface (Mascarenhas et al., 2018).

In this study, the body temperature was measured by two pathways, one surgically introduced into the coelomic cavity in direct contact with internal organs such as the intestine (via probe) and the other by the introduction of a thermometer in the cloaca (rectal). The use of the probes allowed the body temperature to be measured more accurately, revealing that in

the first hours of heat stress there was a greater change in the internal temperature, and later, although the temperature remained above the homeostasis temperature, the body tended to maintain the most constant internal temperature. This behavior is probably related to biochemical and molecular mechanisms that prevent organic collapse.

There was no significant effect of the environment (comfort and stress) on measures related to the latero-lateral and anteroposterior diameters of the heart (Table 3). Similar results were found in relation to the thickness of the right and left atrial walls and the interatrial septum. However, thermal stress altered measurements of the aortic artery, such as wall thickness (3,399 mm, P = 0.0023), latero-lateral diameter (7,887 mm, P = 0,0106) and anteroposterior diameter (6.068 mm; P = 0.0155). Thus, although the wall thickness of this vessel increased, its diameters decreased significantly (Figure 3).

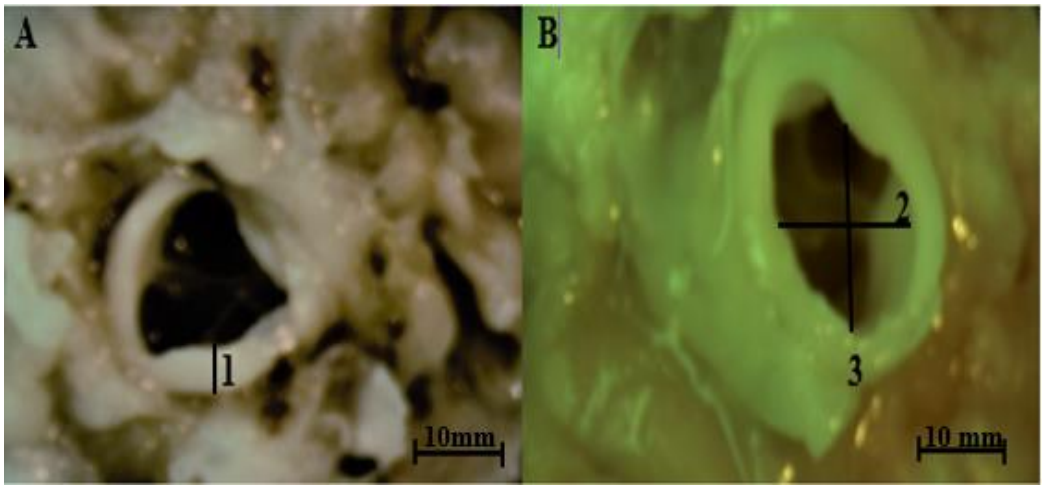
Table 3- Heart morphometry of broiler chickens at 42 days old, presented as mean (standard deviation).

	LLD (mm)	APD (mm)	RAW (mm)	LAW (mm)	ISA (mm)	AAT (mm)	LDA (mm)	APDA (mm)
Comfort	36.359 (1.676)	30.422 (2.155)	4.92 (0.73)	9.84 (0.624)	8.529 (1.870)	2.870 [b] (0.266)	11.573 [a] (2.859)	7.562 [a] (0.843)
Stress	37.21 (12.261)	36.123 (2.857)	4.024 (0.95)	8.977 (1.113)	8.86 (2.175)	3.399 [a] (0.247)	7.887 [b] (1.498)	6,068 [b] (1.119)
Probability	0.4383	0.3302	0.0715	0.0988	0.7651	0.0023	0.0106	0.0155

Latero-lateral diameter (LLD), anteroposterior diameter (APD), right atrial wall (RAW), left atrial wall (LAW), interatrial septum (IAS), aortic artery thickness (AAT), lateral diameter of the aorta (LDA), anteroposterior diameter of the aortic artery (APDA). [a,b]Mean values significantly different (P <0.05). Source: Authors.

Heat stress caused changes in the morphometric measurements of the heart aortic artery thickness (AAT), lateral diameter of the aorta (LDA), anteroposterior diameter of the aortic artery (APDA), as seen in Table 3

Figure 3 - Photomicrographs of aorta arteries. Images used for morphometric analysis



Source: Authors.

A: chicken aorta artery maintained in a thermal comfort environment; B: chicken aorta artery maintained in a thermal stress environment. 1: arterial wall thickness; 2: latero-lateral diameter; 3: anteroposterior diameter.

Morphometric variation in chicken aorta artery promoted by heat stress, as seen in Figure 3

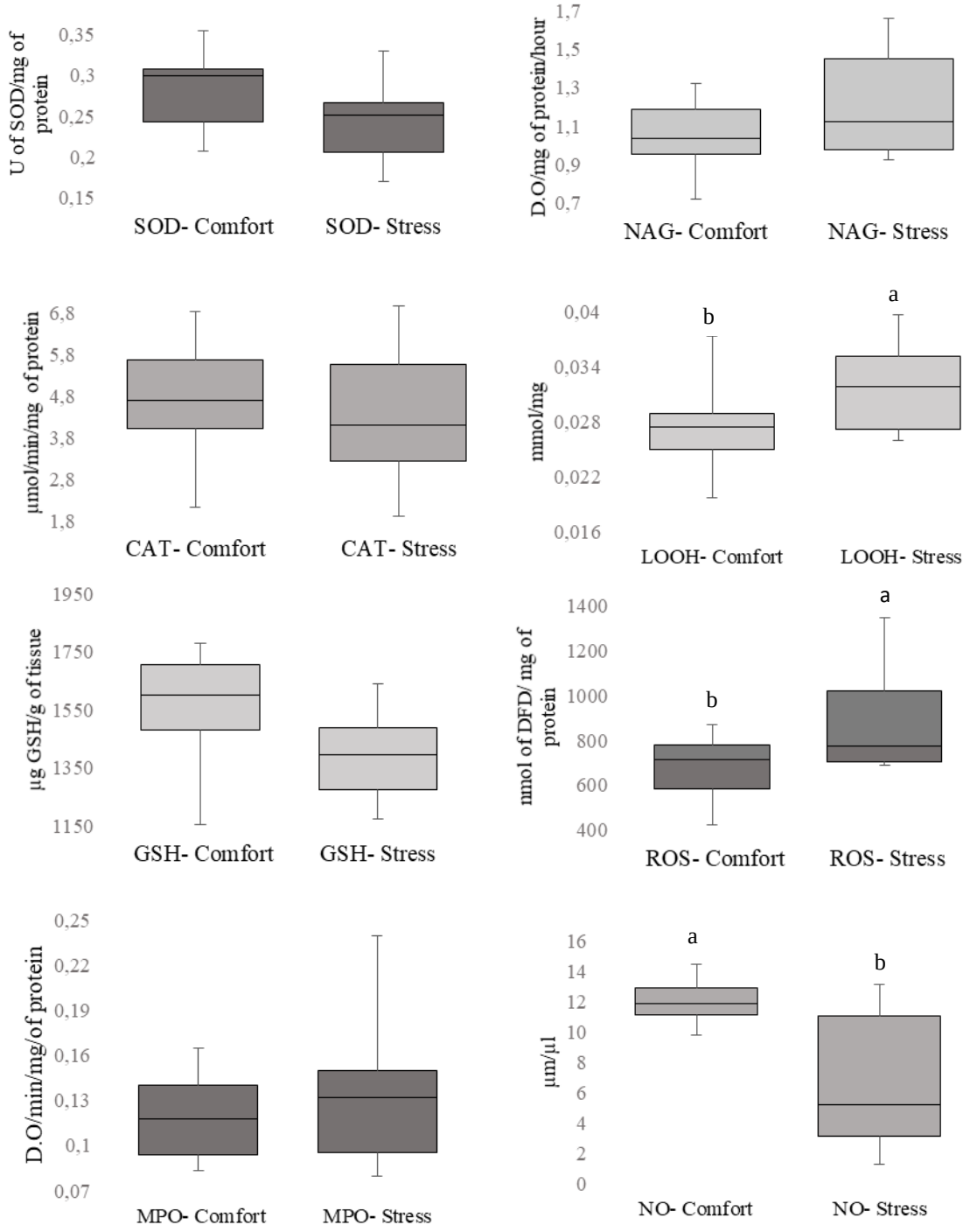
Studies have shown that environments whose temperature exceeds 30 ºC predispose the appearance of several metabolic disturbances in broilers (Roushdy et al., 2018). Thus, small oscillations in body temperature caused by environmental factors are strictly controlled by the biological rhythm which regulates the metabolism to maintain body homeostasis (Crandall & Wilson, 2015). When the primary mechanisms of heat dissipation (radiation, conduction, and convection) fail, latent mechanism activation causes cardiorespiratory rate elevation, increase in cardiac output, blood flow, and cutaneous vasodilation (McCafferty et al., 2017).

Considering the occurrence of increased cardiorespiratory frequency, cardiac output, blood flow and vascular motricity, our study evaluated the influence of the increase in ambient temperature on some morphometric parameters of the heart and aorta. In fact, increased systemic blood pressure has been pointed out as one of the most important physiological adaptations associated with hyperthermia (Tuleta et al., 2011). In this sense, studies have shown that the maintenance of blood pressure and vascular integrity is obtained by structural and functional adaptations in the organs of the circulatory system and that non-adaptation may predispose the organism to cardiovascular disorders (Liu et al., 2015). Thus, it can be inferred that the morphometric changes related to the wall thickness and the latero-

lateral and anteroposterior diameters of the aorta artery identified in the present study are understood as adaptations induced by heat stress in the period to which the birds were submitted.

The results of the biochemical tests are presented in Figure 4. Was verified the significant effect of the ambient temperature on the quantification of lipid hydroperoxides (P = 0.0230), *ROS* (P = 0.0283) and on the nitric oxide (P = 0.0363). The temperature was able to increase the amount of lipid hydroperoxides (0.032 mmol/mg tissue), *ROS* (883.61 nmol DFD/mg protein) and decrease the nitric oxide dosage (7.734) in cardiac tissue.

Figure 4 - Biochemical assays: superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), myeloperoxidase (MPO), N-acetylglicosaminidase (NAG), lipid hydroperoxides (LOOH), reactive oxygen species (ROS) and nitric oxide (NO).



[a,b] Mean values significantly different (P <0.05). Source: Authors.

Comparison of inflammatory parameters MPO and NAG, parameters of oxidative stress (SOD, CAT, GSH, LOOH and ROS) and nitric oxide (NO) in the heart of broilers at 42 days in an environment of thermal stress at 32ºC, as seen in Figure 4.

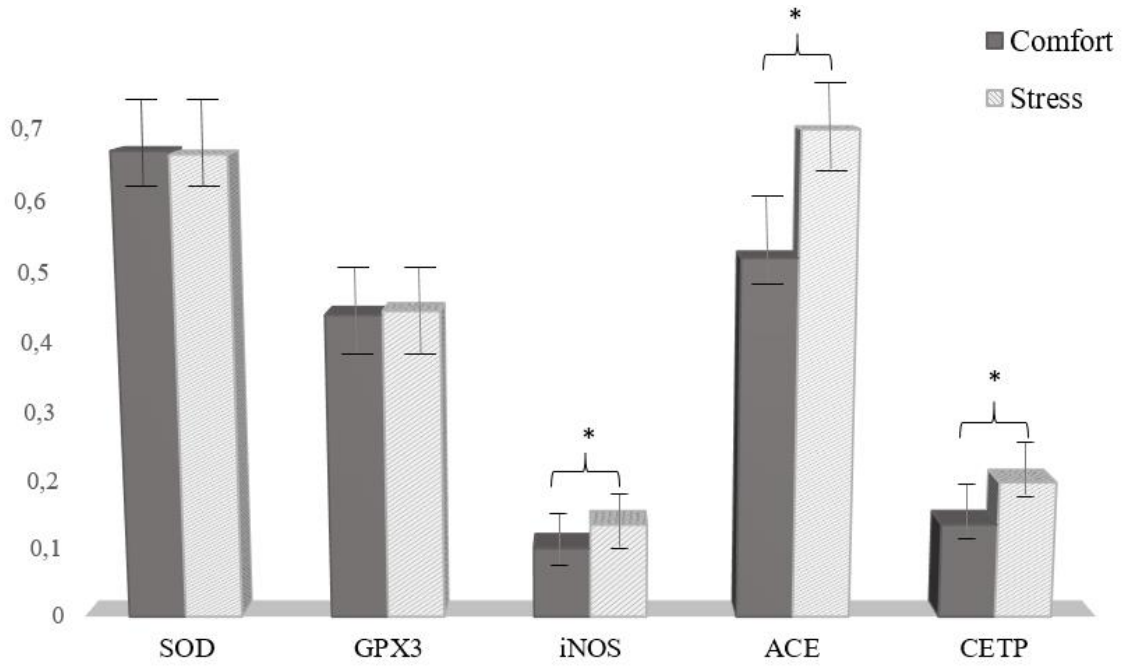
In addition to morphological changes, alterations in the visceral body temperature may modify biochemical and physiological mechanisms, compromising the metabolic and enzymatic functioning of the organism (Zaboli et al., 2016). In this way, incidences of metabolic cellular disorders of cardiovascular origin may be related to the imbalance between the antioxidant systems and the overproduction of reactive oxygen species. However, the activation of such antioxidant systems depends, among other factors, on the time and intensity of exposure to the stressor (Akbarian et al., 2016; Rani et al., 2016). Perhaps because of this, our results showed no significant difference in the activities of the *SOD*, *CAT*, *GSH*, *MPO* and *NAG* enzymes measured in the heart of broilers at the end of the 12-hour heat stress period. However, this doesn't mean that these defense systems are not responding to the heat challenge, since changes may have occurred prior to the time being analyzed.

Additionally, the general imbalance between antioxidant systems and the overproduction of reactive oxygen species (*ROS*) is related to lipid peroxidation whose occurrence generates a wide variety of products resulting from cellular oxidation, among which lipid hydroperoxides (*LOOH*), these compounds predispose to the destruction of the cellular lipid membrane and this disorder has been associated with several cardiovascular problems (Rani et al., 2016). In fact, our results showed a direct correlation between the maintenance of broiler chickens in the acute heat stress environment and the higher production of *ROS* and *LOOH* by cardiac cells. Several mechanisms act to ameliorate the occurrence of metabolic disorders of cardiovascular origin which include, in addition to antioxidant systems, the bioactivity of nitric oxide (*NO*) from the vascular endothelium (Hayashi et al., 2018). In the cardiovascular system, for example, *NO* has beneficial effects such as vasodilation, anti-inflammatory action, anticoagulant, pro-fibrinolytic, inhibition of aggregation and adhesion of platelets and leukocytes. In addition, it reduces the expression of proinflammatory genes and is related to thermoregulatory pathways (Alberghina et al., 2015).

In the present study, we verified that the birds submitted to thermal stress had lower *NO* levels in the cardiac tissue when compared to those maintained in thermal comfort. There are three isoforms of nitric oxide synthase (*NOS*) which play important and independent roles: neuronal (*nNOS*) - expressed in central nervous system specific neurons, endothelial (*eNOS*) - a physiological vasodilator, and inducible (*iNOS*) - related to the immune system of endothelial cells, vascular smooth muscle and cardiac myocytes. The increase in the expression of *iNOS* induced by thermal stress is related to the inflammatory response induced by lipopolysaccharide (*LPS*) and cytokines such as interleukin 1 (*IL-1*), interferon-γ (*IFN-γ*) and tumor necrosis factor-α (*TNF-α*) (Förstermann & Sessa, 2011). Thus, thermal stress

causes the enzyme involved to be responsible for *NO* synthesis, causing the cardioprotective function attributed to *NO* to be severely compromised (Alberghina et al., 2015).

Figure 5- Expression of superoxide dismutase (*SOD*), glutathione peroxidase 3 (*GPX3*), induced nitric oxide synthase (*iNOS*), angiotensin converting enzyme (*ACE*), and cholesteryl ester transfer protein (*CETP)* in the heart of broilers at 42 days old undergoing thermal stress for 12 hours.



*Significantly different mean values (P <0.05). Source: Authors.

Heat stress promotes increased expression *iNOS, ACE* e *CETP* of chicken heart genes, as seen in Figure 5.

NO is related to the thermoregulatory pathways and therefore exerts an indirect control over blood pressure (Alberghina et al., 2015). The most specific control of blood pressure is performed, among other factors, by the renin-angiotensin system which additionally relates to electrolytic homeostasis through the angiotensin-converting enzyme (*ACE*), therefore, several organs (including heart, kidneys, brain and blood vessels) have a very active renin-angiotensin system in order to increase the availability of angiotensin II, this is because this hormone plays an important role in cardiovascular homeostasis, contributing to cardiac and vascular remodeling in pathological conditions (Farag et al., 2015).

Our results showed an increase in *mRNA* levels of the *ACE* gene due to acute thermal stress. Although this seems beneficial at first, it is necessary to consider the fact that thermal stress severely alters the hydroelectrolytic balance (Rodrigues et al., 2019). Thus, inadequate

ACE increase followed by increased production of angiotensin II may favor the onset of hypertension and increased cardiovascular morbidity and mortality. This fact occurs as angiotensin II activates signaling molecules in several important cell pathways, including transcription of kinases, activation of cytokines, integrins and adhesion molecules, resulting in inflammatory processes (Farag et al., 2015).

Considering that inflammatory processes may be associated with the onset of cardiovascular disorders, some enzymes with high pro-inflammatory power should be carefully monitored (Ruparelia et al., 2017). An example is the cholesteryl ester transfer protein (*CETP*) whose action is considered pro-atherogenic. This enzyme acts by transferring the esterified cholesterol of high-density lipoproteins (*HDL*) while receiving triglycerides from low density lipoproteins (*LDL*), intermediate density (*LDL*) and very low density (*VLDL*) (Martinelli et al., 2018).

Our results showed higher expression of the *CETP* gene in birds maintained under a thermal stress environment. Thus, it can be assumed that in these animals there was a decrease in *HDL* whose functions include an antiatherogenic and stimulatory activity of the enzyme *eNOS*. On the other hand, this condition progressively increases *LDL*, which is related to multiple atherogenic, proinflammatory, immunogenic, apoptotic and cytotoxic properties. Such factors in association may raise rates of cardiovascular disease and animal mortality (Li et al., 2017).

4. Final Considerations

- The body temperature of broilers varied according to the ambient temperature when measured via probe and rectal.
- There was an increase in the thickness of the aortic artery wall and a decrease in its latero-lateral and anteroposterior diameters, increase in *LOOH* dosage, *ROS* quantification and decrease in *NO* dosage, increase in the gene expression of *iNOS*, *ACE*, and *CETP*.
- Therefore the acute thermal stress (32°C; 12 hours) was able to promote several morphological, biochemical and molecular changes in the heart and the aorta artery of broilers.

Acknowledgements

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível

Superior - Brasil (CAPES) - Finance Code 001.

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