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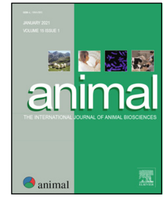

Breast muscle and plasma metabolomics profile of broiler chickens exposed to chronic heat stress conditions

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ABSTRACT

Understanding the variations of muscle and plasma metabolites in response to high environmental temperature can provide important information on the molecular mechanisms related to body energy homeostasis in heat-stressed broiler chickens. In this study, we investigated the effect of chronic heat stress conditions on the breast muscle (*Pectoralis major*) and plasma metabolomics profile of broiler chickens by means of an innovative, high-throughput analytical approach such as the proton nuclear magnetic resonance (**^{1}H NMR**) spectrometry. A total of 300 Ross 308 male chicks were split into two experimental groups and raised in either thermoneutral conditions for the entire rearing cycle (0–41 days) (**TNT** group; six replicates of 25 birds/each) or exposed to chronic heat stress conditions (30 °C for 24 h/day) from 35 to 41 days (**CHS** group; six replicates of 25 birds/each). At processing (41 days), plasma and breast muscle samples were obtained from 12 birds/experimental group and then subjected to ^{1}H NMR analysis. The reduction of BW and feed intake as well as the increase in rectal temperature and heterophil: lymphocyte ratio confirmed that our experimental model was able to stimulate a thermal stress response without significantly affecting mortality. The ^{1}H NMR analysis revealed that a total of 26 and 19 molecules, mostly related to energy and protein metabolism as well as antioxidant response, showed significantly different concentrations respectively in the breast muscle and plasma in response to the thermal challenge. In conclusion, the results obtained in this study indicated that chronic heat stress significantly modulates the breast muscle and plasma metabolome in fast-growing broiler chickens, allowing to delineate potential metabolic changes that can have important implications in terms of body energy homeostasis, growth performance and product quality.

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Implications

In the present study, we have investigated the variations induced by heat stress in muscle and plasma metabolites of broiler chickens through an innovative analytical approach such as the proton nuclear magnetic resonance spectrometry. Our findings indicate that heat stress significantly altered the concentration of a wide number of metabolites involved in crucial biological functions, such as protein and energy metabolism and antioxidant response. Overall, these results contribute in enriching the knowledge regarding the biological mechanisms by which heat stress affects animal productivity and health as well as product quality.

Introduction

According to the Intergovernmental Panel on Climate Change (2018), global warming is likely to reach the 1.5 °C threshold between 2030 and 2052 if the current trend is maintained (+0.2 °C *per* decade). On the other hand, Raftery et al. (2017) suggested that the global temperature increase by 2100 is unlikely to be less than 2 °C. In any case, a substantial increase in magnitude, duration, and frequency of extreme heat waves could be reasonably expected at different latitudes (Russo et al., 2015), thereby augmenting the risk for livestock to be exposed to heat stress condition. Heat stress occurs when the amount of heat produced by the animal exceeds its capacity to dissipate extra-heat to the external environment (Farag and Alagawany, 2018), which is influenced by both environmental factors (e.g. air temperature, humidity and movement, sunlight and thermal irradiation) and animal characteristics (e.g. specie, metabolism rate, and thermoregulatory mechanisms) (Lara and Rostagno, 2013). Homoeothermic organisms are

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able to keep constant body core temperature despite substantial variations in climatic conditions adopting thermoregulatory mechanisms that balance heat production and loss (Renaudeau et al., 2012). Indeed, when exposed to high environmental temperatures, animals employ certain physiological and metabolic adjustments to promote the maintenance of euthermia at the expenses of growth and reproductive aspects, which are not considered metabolic priorities anymore (Baumgard and Rhoads, 2013; Rhoads et al., 2013). This results in serious economic consequences for the livestock industry, which include not only the detriment of productive and reproductive performance but also increased veterinary costs, inconsistent product quality, and greater animal welfare issues (Gonzalez-Rivas et al., 2020). Among livestock, fast-growing broiler chickens are particularly susceptible to environmental challenges such as heat stress, with economic losses related to this condition estimated between 128 and 165 million $/year for the US poultry industry (Lara and Rostagno, 2013). The scarce thermotolerance response observed in modern broiler genotypes can be mainly ascribed to the imbalance between their overwhelming metabolic rate, resulting from the genetic selection for growth performance and feed efficiency, and the limited development of the visceral systems (e.g. cardiovascular and respiratory systems), which play a crucial role in balancing energy expenditure and body water content under adverse environmental conditions (Yahav, 2009).

Although the negative effects of heat stress on growth performance and health status of broiler chickens are widely acknowledged, there is still a paucity of scientific information regarding the impact of such condition on the metabolic and physiological features of the animals, including postabsorptive metabolic dynamics and nutrient repartitioning mechanisms (Baumgard and Rhoads, 2013; Rhoads et al., 2013). To this purpose, a proper application of high-throughput –omics technologies can be useful to shed a light on the complex biological and metabolic aspects of heat-stressed broilers (Zampiga et al., 2018a). For instance, the results reported by Jastrebski et al. (2017), who investigated the hepatic response of broiler chickens to chronic heat stress (35–37 °C, 8 h/day from 21 to 28 days of bird age) through an integrated transcriptomic and metabolomic approach, revealed that heat stress affects multiple metabolic pathways, such as glucose, amino acid, and lipid metabolism along with glutathione production and beta-oxidation. On the other hand, the serum metabolomics profile of chronically heat-stressed broilers (32 °C, 24 h/day from 28 to 42 days of age) allowed to hypothesize that heat stress stimulates protein decomposition likely to provide substrates for energetic purposes as birds are unable to effectively mobilize fats during periods of negative energy balance (Lu et al., 2018).

As regards the skeletal muscle, which is considered a high metabolically active tissue deeply involved in energy homeostasis mechanisms of broiler chickens (Zampiga et al., 2018a), some studies provided evidence that heat stress can modulate the expression level of specific genes involved in important biological pathways. For instance, Zuo et al. (2015) found that the transcriptional profile of breast and thigh muscles of broiler chickens exposed to chronic heat stress (34 °C, 24 h/day from 29 to 49 days of age) was consistent with a reduced protein synthesis and an increased protein degradation, respectively. Similarly, it has been demonstrated that the decreased muscle mass and yield as well as the reduced muscle protein synthesis and amino acid transportation in response to chronic thermal stress (32 °C, 24 h/day from 28 to 42 days of age) were mirrored by the downregulation of genes involved in the insulin-like growth factor-mammalian target of rapamycin pathway (Ma et al., 2018). To the best of our knowledge, no information is currently available to as concerns the molecular response of the chicken skeletal muscle to heat stress at a deeper biological level (e.g. protein and metabolite expression). Indeed, according to previous findings on gene expression, it can be hypothesized that heat stress could profoundly affect the concentration of a wide number of metabolites involved in crucial biological functions related to muscle metabolism and, in turn, to body energy homeostasis. This information is crucial to decipher the molecular mechanisms involved in the reduction of growth performance as well as in the worsening of product quality when broilers are exposed to heat stress. Furthermore, these findings could be useful to identify specific biomarkers related to thermal stress and to delineate targeted mitigation strategies aimed at sustaining quali-quantitative production traits during heat waves. Therefore, the aim of this research was to evaluate, by means of a high-throughput analytical approach such as the proton nuclear magnetic resonance spectrometry (**^{1}H NMR**), the effect of chronic heat stress conditions on the metabolomics profile of the chicken breast muscle (*Pectoralis major*), which represents the most relevant muscle in fast-growing broilers accounting for 20–25% of their live-bodyweight. In addition, also the plasma metabolome was investigated through the same analytical approach to better understand the role of the breast muscle in the systemic metabolism of heat-stressed broilers.

Material and methods

Animals, diet, and environmental conditions

A total of 300 one-day-old Ross 308 male chicks, obtained from the same breeder flock and hatching session, were vaccinated at the hatchery (coccidiosis, infectious bronchitis, Marek's, Newcastle, and Gumboro disease) and then transported to an environmental-controlled poultry facility. Chicks were randomly allocated in 12 concrete floor pens (3.3 m^2 each), equally divided into two rooms (six pens/room) presenting identical features (e.g. pens disposition and characteristics, artificial lighting, ventilation systems, etc.). The only difference between the two rooms was the presence of an electrical heating system, which was used to increase the environmental temperature during the thermal challenge. Each pen presented one circular pan feeder, able to ensure a minimum of 2 cm of front space/bird, and five nipples. Wood shaving was used as litter material (3–4 kg/m^2). Stocking density (maximum 33 kg/m^2) and photoperiod (light: dark = 23:1 during 0–7 and 39–41 days; and 18:6 from 8-38 days) were in compliance with the EU legislation, specifically with the Directive 2007/43/EC for the protection of chickens kept for meat production. As regards the experimental design, birds allocated in the first room were raised in thermoneutral conditions for the entire rearing cycle (0–41 days) (**TNT** group; six replicates of 25 birds/each), with environmental temperature defined according to the age of the birds following the recommendations of the breeding company (i.e. placement: 30 °C; 3 days: 28 °C; 6 days: 27 °C; 9 days: 26 °C; 12 days: 25 °C; 15 days: 24 °C; 18 days: 23 °C; 21 days: 22 °C; 24 days: 21 °C; 27 days-onwards: 20 °C). The birds housed in the second room were raised in analogous environmental conditions to those belonging to the TNT group until 35 days (8:00 AM; 0 day of the heat stress period) and then exposed to chronic heat stress conditions for 6 days (35 days, 8:00 AM – 41 days, 8:00 AM; **CHS** group: 30 °C for 24 h/day; six replicates of 25 birds/each). Environmental temperatures were constantly monitored throughout the challenging period (35–41 days) and they were recorded in both the rooms three times a day (8:00 AM, 4:00 PM and 12:00 PM). To this purpose, three data loggers were placed in representative locations of each room (i.e. beginning, half, and end) approximately at the height of the birds. For each time point, the environmental temperature of the room (TNT or CHS) was obtained by averaging the temperature values recorded by the three data loggers. Relative humidity ranged from 40 to 55% in

both the rooms throughout the trial. The same corn-wheat-soybean meal basal diet (mash form) was provided to both TNT and CHS groups according to a 3-phase feeding programme: starter (0–14 days), grower (15–28 days) and finisher (29–41 days) (Table 1). Birds had free access to feed and water, which were administered *ad-libitum*. Birds were raised, handled and processed according to the Directive 2007/43/EC for the protection of chickens kept for meat production, the Regulation 1099/2009/EC for the protection of animals at the time of killing, and the Directive 2010/63/EU for the protection of animals used for scientific purposes. The experimental protocol was positively evaluated and approved by the Ethical Committee of the University of Bologna (ID: 1031/2019).

Validation of the heat stress model

The efficacy of the heat stress model was evaluated through a multi-criteria approach including different parameters such as rectal temperature, BW, feed intake, and heterophil:lymphocyte (**H:L**) ratio. Rectal temperature was recorded on six birds *per* experimental group (one bird/replication), selected according to the average BW of the corresponding group, at 35, 36, 39 and 41 days of age (i.e. 0, 1, 4, and 6 days of the heat stress period). As regards growth traits, BW was determined pen wise before starting the heat stress procedure and at the end of it (i.e. 35 and 41 days, respectively). Similarly, feed intake during the heat stress period was calculated on a pen basis as the difference between the amount of feed administered at 35 days and the residuals at 41 days. Mortality was recorded daily and used to correct the feed intake data. At 41 days, all birds were slaughtered in a commercial processing plant and carcass and breast yields were obtained on a group basis as previously described (Zampiga et al., 2018b).

Table 1
Composition of the basal diet (in each feeding phase) administered to broiler chickens of both the experimental groups.

Item	Feeding phase		
	Starter (0–14 days)	Grower (15–28 days)	Finisher (29–41 days)
Ingredients (g/100 g)			
Corn	42.2	35.0	27.7
Wheat	10.0	20.0	25.0
Sorghum	0.00	0.00	5.00
Vegetable oil	3.08	4.43	5.48
Soybean meal 48%	23.1	20.6	17.6
Full-fat soybean	10.0	10.0	13.0
Sunflower	3.00	3.00	3.00
Lysine sulphate	0.59	0.55	0.46
DL-Methionine	0.27	0.29	0.30
L-Threonine	0.15	0.14	0.14
Choline chloride	0.10	0.10	0.10
Calcium carbonate	0.91	0.65	0.52
Dicalcium phosphate	1.52	1.20	0.57
Sodium chloride	0.27	0.27	0.25
Sodium bicarbonate	0.15	0.10	0.15
Vitamin-mineral premix[1]	0.50	0.50	0.50
Phytase	0.10	0.10	0.10
Xylanase	0.08	0.08	0.08
Proximate composition[2]			
AME (MJ/kg)	12.9	13.3	13.7
DM (%)	88.6	88.7	88.6
CP (%)	22.7	21.5	19.7
Total lipid (%)	7.06	8.24	9.74
Crude fibre (%)	3.08	3.04	3.07
Ash (%)	5.85	5.17	4.49
Total Ca (%)	0.91	0.80	0.59
Total P (%)	0.63	0.57	0.38

Abbreviations: AME = Apparent Metabolizable Energy.
[1] Provided the following per kg of diet: vitamin A (retinyl acetate), 13 000 IU; vitamin D3 (cholecalciferol), 4 000 IU; vitamin E (DL-α_tocopheryl acetate), 80 IU; vitamin K (menadione sodium bisulfite), 3 mg; riboflavin, 6.0 mg; pantothenic acid, 6.0 mg; niacin, 20 mg; pyridoxine, 2 mg; folic acid, 0.5 mg; biotin, 0.10 mg; thiamine, 2.5 mg; vitamin B12 20 μg; Mn, 100 mg; Zn, 85 mg; Fe, 30 mg; Cu, 10 mg; I, 1.5 mg; Se, 0.2 mg; ethoxyquin, 100 mg.
[2] Calculated values.

Breast muscle and blood sample collection

At slaughter (41 days), 12 birds/group (two birds/pen) were selected according to the average BW of each experimental group (TNT: 2 900 ± 50 g; CHS: 2 450 ± 50 g), clearly identified, and then subjected to breast muscle sampling and blood withdrawal. As described in our previous paper (Zampiga et al., 2018b), *Pectoralis major* muscle samples were consistently obtained from the same portion of the pectoral muscle, placed into a 1.5 mL vial, snap frozen in liquid nitrogen, and then stored at −80 °C until the ^{1}H NMR analysis. Muscle portions presenting visible defects or abnormalities were intentionally excluded during sampling operations. Similarly, blood was obtained from the same 12 birds/group by wing vein withdrawal. Whole blood was used to determine the H:L ratio, whereas plasma was obtained by collecting blood into 4 mL lithium-heparin vials and immediately centrifuging (4 000*g* for 15 min). Plasma was then transferred into 1.5 mL labelled vials and maintained at −80 °C until metabolomic analysis.

Metabolomic analysis

We created an ^{1}H NMR analysis solution with D_2O, containing 3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid sodium salt (**TSP**) 10 mmol/L and NaN_3 2 mmol/L. We set the solution to pH 7.00 ± 0.02 by phosphate buffer 1 M. TSP was selected as a reference for NMR chemical shift, while NaN_3 excluded microbial proliferation. We prepared samples from breast muscle according to our previous works (Zampiga et al., 2018b; Zampiga, 2019) by homogenizing 0.5 g of meat with 3 mL of distilled water. This was done for 2 min, by means of a high-speed disperser (IKA, USA). We centrifuged 1 mL of the obtained mixture for 15 min at 18 630*g* and 4 °C. We added 0.7 mL of supernatant to 0.8 mL of $CHCl_3$, then we vortex mixed for 3 min and centrifuged again at the above conditions to remove fat from samples. We added 0.5 mL of supernatant to 0.2 mL of the ^{1}H NMR analysis solution and we centrifuged one last time at the above conditions. In parallel to meat samples, we prepared plasma samples for ^{1}H NMR analysis by centrifuging 0.65 mL of each sample at 18 630*g* for 15 min and 4 °C. We added 0.5 mL of supernatant to 0.1 mL of the ^{1}H NMR analysis solution. Finally, we centrifuged each sample again at the above conditions. We registered ^{1}H NMR spectra at a frequency of 600.13 MHz and a temperature of 298 K with an AVANCE™ III spectrometer (Bruker, Milan, Italy), equipped with the software Topspin 3.5. We suppressed signals from broad resonances due to large molecules with a CPMG-filter composed by 400 echoes with a τ of 400 μs and a 180° pulse of 24 μs, for a total filter of 330 ms. The pulses' sequence cpmgpr1d allowed us to reduce the water residual signal by presaturation. We acquired each spectrum by summing up 256 transients constituted by 32 000 data points encompassing a window of 7 184 Hz, separated by a relaxation delay of 5 s. We phase adjusted the ^{1}H NMR spectra in Topspin, then exported them to ASCII format by means of the built-in script "convbin2asc". We imported the spectra to R software environment through scripts developed in-house, employed also for any other processing step. We baseline-adjusted the spectra by distinguishing baseline imperfection from ^{1}H NMR signals according to the "rolling ball" principle (Kneen and Annegarn, 1996) implemented in the R package "baseline" (Liland et al., 2010). Signal assignment was performed by comparing their chemical shift and multiplicity with the Human Metabolome Database

(Wishart et al., 2007) and Chenomx software library (Chenomx Inc., Edmonton, Canada, ver. 10). This was done by taking advantage of the "autofit" utility of Chenomx software (Chenomx Inc., ver. 8.3), as visually represented in Supplementary Figs. S1–S10. The molecules of the first sample analysed for each matrix were quantified by means of an external standard. Differences in water content among samples were then taken into consideration by probabilistic quotient normalization (Dieterle et al., 2006). Molecule quantification was performed by means of rectangular integration of one signal per molecule, selected to avoid interferences with other molecules, as visually represented in Fig. S11.

Statistical analysis

Rectal temperatures were statistically evaluated by means of two-way ANOVA, considering the environmental condition (TNT vs. CHS) and the age of the birds (36, 39 and 41 days) as main factors. Rectal temperature values recorded at 35 days were used as baseline and analysed through the Student-T test. Similarly, BW, feed intake and the H:L ratio were analysed applying the Student-T test with the environmental condition as experimental factor. Carcass and breast yield were not subjected to statistical analysis as these parameters were determined on a group basis without replications. As for the metabolomics analysis, the concentrations of each metabolite detected in either breast muscle or plasma samples were normalized by means of Box-Cox transformation (Box and Cox, 2018) and then compared through the Student-T test considering the environmental condition as experimental factor. Principal component analysis models in their robust version (**rPCA**) were generated, centred and scaled to unity variance (Hubert et al., 2005), in order to highlight the trends observed in the breast muscle and plasma samples, respectively. For each rPCA model, a correlation plot, which shows the importance of each molecule over the components of the rPCA model, was obtained with the purpose of revealing the most important metabolites determining the trends observed in the scoreplot. The bird represented the experimental unit for the statistical analysis of rectal temperature, H:L ratio and metabolomics data, whereas the pen was considered as the experimental unit for BW, feed intake and mortality. For all these parameters, statistically significant differences were defined when the corresponding P-value was lower than the 0.05 threshold.

Results

Validation of the heat stress model

The environmental temperatures recorded in both the rooms from 35 to 41 days are depicted in Fig. 1A. Before starting the heat stress procedure (35 days; 8 AM), the environmental temperature was similar between the rooms, with differences lower than 1 °C. During the heat stress period, the environmental temperatures recorded in the CHS room were consistently higher than those detected in the TNT one ($\Delta_{temperature}$: from 5.3 to 11.4 °C). Such differences in environmental temperature were mirrored by changes in bird's rectal temperature (Fig. 1B). Indeed, while no significant difference between the groups was observed before starting the heat stress (35 days; 41.1 vs. 41.0 °C, respectively for TNT and CHS), CHS birds showed significantly higher rectal temperature than TNT ones during the thermal challenge (36 days: 41.0 vs. 42.6 °C; 39 days: 41.3 vs. 42.7 °C; 41 days: 41.1 vs. 42.3 °C, respectively, for TNT and CHS; $P < 0.01$). There was no significant effect of age and interaction between environmental condition and age for rectal temperature. As regards growth traits (Table 2), TNT and CHS broilers showed comparable BW at 35 days before starting the heat stress procedure (2 347 vs. 2 348 g/bird, respectively; $P = 0.98$). As widely expected, six days of chronic heat stress significantly reduced BW at slaughter and overall feed intake during the thermal challenge (2 888 vs. 2 486 and 1 064 vs. 713 g/bird, respectively, for TNT and CHS; $P < 0.001$). Mortality rate was not significantly affected by the environmental condition (1.63 vs. 3.03%, respectively for TNT and CHS; $P = 0.55$), whereas the H:L ratio was significantly higher in CHS birds (0.72 vs. 1.03, respectively for TNT and CHS; $P < 0.05$; Fig. 2). Although not statistically evaluated as recorded on a group basis, carcass and breast yield (calculated as percentage of BW) showed the following values: 72.7 vs. 71.3% and 23.6 vs. 23.1%, respectively for TNT and CHS.

Breast muscle and plasma metabolome

^{1}H NMR spectra were registered on breast muscle and plasma samples and a total of 55 and 52 molecules were quantified, respectively. As for the breast muscle, 26 molecules, showed in Table 3, exhibited significantly different concentrations in response to the environmental condition (TNT vs. CHS). Similarly, the plasma levels of 19 molecules were significantly modified by the thermal challenge, as reported in Table 4. Breast muscle and plasma metabolites were further classified according to their main biochemical characteristics and functions. In the breast muscle (Table 3), the molecules presenting significantly different concentrations were mainly free amino acids (tryptophan, tyrosine, serine, threonine, glycine, glutamine, methionine, proline and alanine), histidine-containing dipeptides (anserine and carnosine) and energy metabolism-related compounds (glucose, guanidinoacetate, creatine, creatinine, hypoxanthine and citrate). As for the plasma, the most relevant changes induced by the thermal challenge concerned the concentration of some free amino acids (alanine, tyrosine, aspartate, β-alanine, glutamine, arginine, lysine, valine and isoleucine) and metabolites that could be generally related to the energy metabolism (glycerol, pyruvate and acetate). Furthermore, two rPCA models (Figs. 3 and 4) were generated according to the concentration of the molecules reported in Tables 3 and 4, respectively. As for the breast muscle, the first two principal components of the rPCA model explained 78.7% of the total variance, with the principal component 1 accounting for 57.5% (Fig. 3). In the second rPCA model (Fig. 4), which was referred to the plasma metabolites, the amount of variance described by the first two extracted components was equal to 86.5% of the total, with the principal component 1 contributing for 62.4%.

Discussion

In the present study, fast-growing broiler chickens were exposed to constant, chronic heat stress conditions approximately in the last week of their rearing cycle (35–41 days of age), as it is generally recognized that broiler susceptibility to heat stress increases with the age of the birds. The effectiveness of the challenging model applied in the present study was validated by the reduction of BW (−14%; $P < 0.001$) and feed intake (−33%; $P < 0.001$), along with the increase in rectal temperature ($P < 0.01$) and H:L ratio ($P < 0.05$), in CHS birds. In particular, the significantly higher rectal temperatures detected in the CHS group clearly show that these birds were not able to properly thermoregulate and dissipate extra-heat (Lu et al., 2018), thereby confirming that the environmental conditions adopted for this group stimulated an effective thermal stress response in the chickens.

Overall, the breast muscle and plasma metabolome showed extensive variations in response to the environmental condition. In this study, glucose concentration was found to be significantly

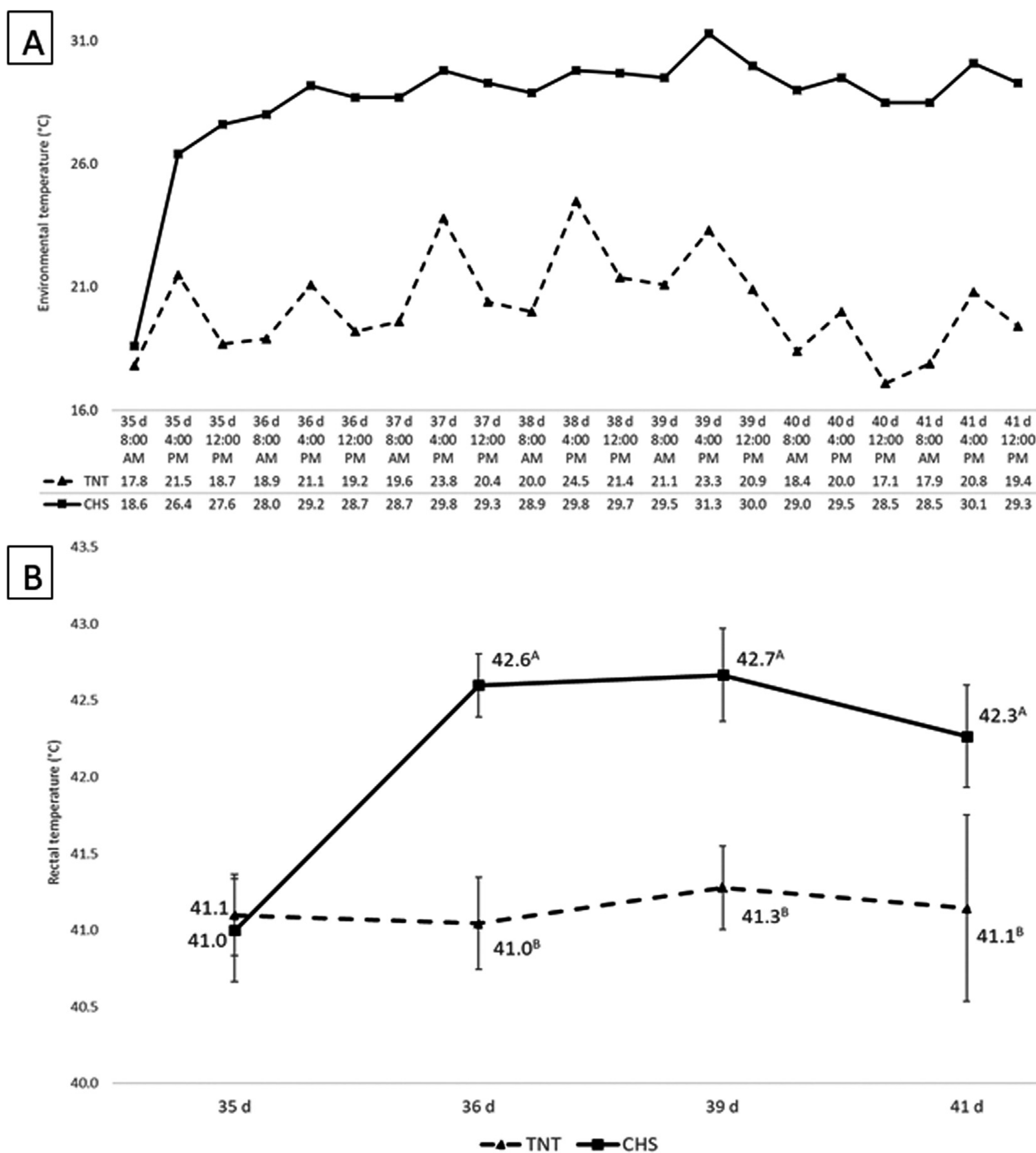

Fig. 1. (A) Environmental temperatures recorded before and during the thermal challenge (35–41 days of bird age; three time points/day: 8:00 AM, 4:00 PM and 12:00 PM) in the TNT and CHS room, and (B) rectal temperatures of broiler chickens raised in either thermoneutral (TNT, N = 6) or chronic heat stress (CHS, N = 6) conditions from 35 to 41 days of age (B). $^{A–B}$Values for the same day with different superscripts differ significantly at P < 0.01.

Table 2
BW, feed intake and mortality of broiler chickens raised in either thermoneutral or chronic heat stress conditions from 35 to 41 days of age.

Variable	TNT (N = 6) Mean	CHS (N = 6) Mean	SEM	P-value
BW 35 days (g/bird)	2 347	2 348	12.2	0.98
BW 41 days (g/bird)	2 888	2 486	76.0	<0.001
Feed intake 35–41 days (g/bird)[1]	1 064	713	0.07	<0.001
Mortality (%)	1.63	3.03	0.05	0.55

Abbreviations: TNT = thermoneutral group; CHS = chronic heat stress group.
[1] Corrected for mortality.

reduced in the breast muscle of CHS broilers, whereas comparable plasma glucose levels were observed among stressed and unstressed birds (3.69 vs. 4.06 mmol/L, respectively for TNT and CHS, P = 0.14). Glucose is one of the most important metabolites involved in body energy homeostasis and serves as an energy source for various tissues, such as brain, erythrocytes, muscles and immune system cells (Scanes, 2015). The *Pectoralis major* muscle of broiler chickens is mainly constituted by glycolytic fast-twitch (type IIb) fibres, which oxidize glycogen and glucose through the glycolysis pathway yielding lactate as final product (Petracci and Cavani, 2012). Therefore, the lower glucose concentration observed in the breast muscle of CHS birds could suggest an increased utilization of this metabolite through the glycolytic pathway. In addition, further meat quality insights revealed a

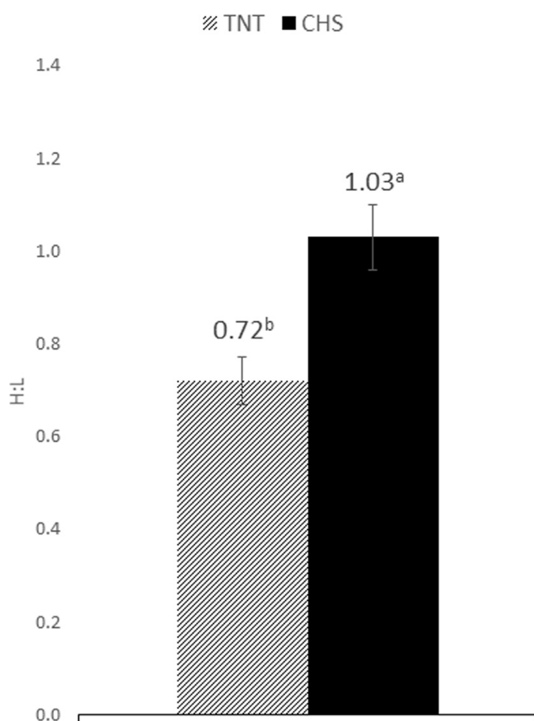

Fig. 2. Heterophil: lymphocyte (H:L) ratio detected in 41-day-old broiler chickens raised in either thermoneutral (TNT, N = 12) or chronic heat stress (CHS, N = 12) conditions from 35 to 41 days of age. [a–b]Values with different superscripts differ significantly at P < 0.05.

limited acidification capacity for the breast muscle of CHS birds (unpublished observations), which could be consistent with an increased glycogenolysis and/or a reduced glycogenosynthesis *in-vivo*. Overall, these results could indicate that chronic heat stress affects carbohydrate metabolism in the breast muscle of fast-growing broilers, with potential negative consequences on body energy homeostasis, growth performance, and meat quality traits.

Furthermore, it is noteworthy to consider that CHS birds were able to maintain similar plasma glucose levels compared to TNT ones despite the significant reduction of feed intake induced by the thermal challenge. During heat stress events, animals are unable to effectively mobilize the adipose tissue, resulting in a condition of "metabolic inflexibility" in which glucose apparently becomes the favoured energy source for the metabolic needs of peripheral tissues (Baumgard and Rhoads, 2013; Rhoads et al., 2013). In this scenario, the liver continues to provide glucose to peripheral tissues likely through an increased gluconeogenesis (Rhoads et al., 2013). This metabolic pathway, which is responsible for the conversion of non-carbohydrate precursors to glucose or glycogen, has the primary function of meeting the body requirements of glucose when carbohydrates are not adequately provided with the diet or from glycogen reserves (Mayes and Bender, 2003), as may occur in heat-stressed animals due to reduced feed intake. In this study, lower concentrations of some gluconeogenic precursors such as certain amino acids (i.e. alanine, tyrosine, aspartate, β-alanine and glutamine), glycerol, pyruvate, and lactate (1.40 vs. 1.13 mmol/L, respectively for TNT and CHS, P = 0.09) were detected in the plasma of CHS birds. Overall, these results could be consistent with an increased uptake of these metabolites by the liver and kidneys to provide substrates for the gluconeogenesis. Consistent with our hypothesis, Jastrebski et al. (2017) recently reported

Table 3
Relative concentration of differentially expressed metabolites in the breast muscle of broiler chickens raised in either thermoneutral or chronic heat stress conditions from 35 to 41 days of age.

Item	TNT (N = 12) Mean	CHS (N = 12) Mean	SEM	Trend[1]	P-value
Mean concentration of histidine-containing dipeptides (mmol/L)					
Anserine	1.30×10^{-2}	1.66×10^{-2}	4.74×10^{-4}	↑	<0.001
Carnosine	3.01×10^{-3}	4.24×10^{-3}	2.93×10^{-4}	↑	0.04
Mean concentration of free amino acids (mmol/L)					
Tryptophan	1.13×10^{-4}	1.01×10^{-4}	2.93×10^{-6}	↓	0.03
Tyrosine	3.68×10^{-4}	2.49×10^{-4}	1.70×10^{-5}	↓	<0.001
Serine	5.48×10^{-4}	4.37×10^{-4}	2.50×10^{-5}	↓	0.02
Threonine	7.14×10^{-4}	4.45×10^{-4}	3.44×10^{-5}	↓	<0.001
Glycine	2.58×10^{-3}	1.55×10^{-3}	1.88×10^{-4}	↓	<0.01
Glutamine	9.21×10^{-4}	6.89×10^{-4}	5.07×10^{-5}	↓	0.02
Methionine	2.96×10^{-4}	2.55×10^{-4}	7.26×10^{-6}	↓	<0.01
Proline	4.56×10^{-4}	2.23×10^{-4}	3.65×10^{-5}	↓	<0.001
Alanine	2.12×10^{-3}	1.55×10^{-3}	9.05×10^{-5}	↓	<0.001
Mean concentration of energetic metabolism-related compounds (mmol/L)					
Glucose	2.54×10^{-3}	2.12×10^{-3}	6.89×10^{-5}	↓	<0.001
Guanidinoacetate	1.82×10^{-2}	2.26×10^{-2}	5.84×10^{-4}	↑	<0.001
Creatine	2.69×10^{-2}	3.26×10^{-2}	7.69×10^{-4}	↑	<0.001
Creatinine	5.71×10^{-4}	7.29×10^{-4}	2.12×10^{-5}	↑	<0.001
Hypoxanthine	4.15×10^{-4}	5.65×10^{-4}	3.74×10^{-5}	↑	0.04
Citrate	8.16×10^{-5}	1.01×10^{-4}	4.85×10^{-6}	↑	0.04
Mean concentration of other metabolites (mmol/L)					
Uridine monophosphate	9.58×10^{-5}	8.56×10^{-5}	1.88×10^{-6}	↓	<0.01
Uridine	5.31×10^{-5}	3.94×10^{-5}	2.64×10^{-6}	↓	<0.01
Mannose	7.49×10^{-5}	5.37×10^{-5}	5.06×10^{-6}	↓	0.03
4-Hydroxyphenylacetate	1.42×10^{-4}	6.84×10^{-5}	1.45×10^{-5}	↓	<0.01
Betaine	1.19×10^{-3}	4.62×10^{-4}	9.38×10^{-5}	↓	<0.001
Dimethyl sulfone	6.84×10^{-5}	7.57×10^{-5}	1.54×10^{-6}	↑	<0.01
Malonate	1.57×10^{-4}	2.13×10^{-4}	1.10×10^{-5}	↑	<0.01
N,N-Dimethylglycine	5.57×10^{-4}	3.24×10^{-4}	3.29×10^{-5}	↓	<0.001
Acetate	2.15×10^{-4}	3.06×10^{-4}	1.69×10^{-5}	↑	<0.01

Abbreviations: TNT = thermoneutral group; CHS = chronic heat stress group.
[1] Variation in response to heat stress: ↑ increase, ↓ decrease.

Table 4
Relative concentration of differentially expressed metabolites in the plasma of broiler chickens raised in either thermoneutral or chronic heat stress conditions from 35 to 41 days of age.

Item	TNT (*N* = 12) Mean	CHS (*N* = 12) Mean	SEM	Trend[1]	*P*-value
Mean concentration of free amino acids (mmol/L)					
Alanine	1.32×10^{-1}	8.16×10^{-2}	7.26×10^{-3}	↓	0.03
Tyrosine	2.57×10^{-2}	1.46×10^{-2}	1.71×10^{-3}	↓	<0.001
Aspartate	9.05×10^{-3}	4.68×10^{-3}	5.89×10^{-4}	↓	<0.001
β-Alanine	6.06×10^{-3}	8.68×10^{-4}	7.07×10^{-4}	↓	<0.001
Glutamine	1.24×10^{-1}	9.74×10^{-2}	6.15×10^{-3}	↓	0.02
Arginine	2.11×10^{-2}	3.47×10^{-2}	1.97×10^{-3}	↑	<0.001
Lysine	3.48×10^{-3}	1.80×10^{-2}	2.45×10^{-3}	↑	<0.001
Valine	2.34×10^{-2}	3.09×10^{-2}	1.69×10^{-3}	↑	0.02
Isoleucine	8.30×10^{-3}	1.07×10^{-2}	5.36×10^{-4}	↑	<0.001
Mean concentration of energetic metabolism-related compounds (mmol/L)					
Glycerol	1.20×10^{-2}	7.83×10^{-3}	6.72×10^{-4}	↓	<0.001
Pyruvate	3.26×10^{-2}	2.91×10^{-2}	1.51×10^{-3}	↓	<0.001
Acetone	2.70×10^{-3}	1.92×10^{-3}	1.45×10^{-4}	↓	<0.01
Mean concentration of other metabolites (mmol/L)					
3-Hydroxyisobutyrate	1.78×10^{-3}	4.28×10^{-3}	3.26×10^{-4}	↑	<0.01
Uridine	2.18×10^{-3}	3.58×10^{-3}	2.38×10^{-4}	↑	<0.001
Mannose	4.78×10^{-3}	6.45×10^{-3}	4.13×10^{-4}	↑	0.02
Myo-inositol	5.97×10^{-2}	4.27×10^{-2}	2.92×10^{-3}	↓	<0.001
Trans-4-Hydroxy-L-proline	1.68×10^{-2}	1.16×10^{-2}	9.36×10^{-4}	↓	<0.01
Acetate	1.21×10^{-2}	8.94×10^{-3}	4.56×10^{-4}	↓	<0.01
Dimethyl sulfone	7.44×10^{-3}	1.27×10^{-2}	1.75×10^{-3}	↑	0.03

Abbreviations: TNT = thermoneutral group; CHS = chronic heat stress group.
[1] Variation in response to heat stress: ↑ increase, ↓ decrease.

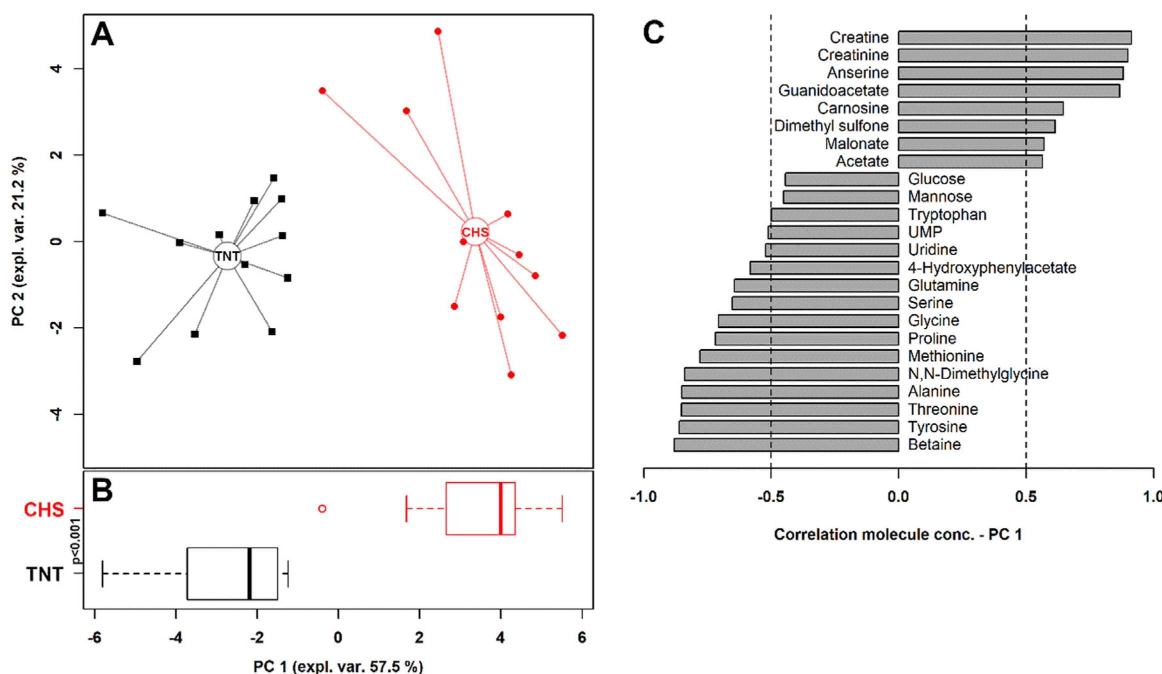

Fig. 3. Robust Principal Component Analysis (rPCA) model built on the space constituted by the concentration of the breast muscle metabolites showing differential expression in 41-day-old broiler chickens raised in either thermoneutral (TNT, *N* = 12) or chronic heat stress (CHS, *N* = 12) conditions from 35 to 41 days of age. (A) In the scoreplot, samples from TNT and CHS chickens are represented with black squares and red circles, respectively. The wide, empty circles represent the means of the samples. (B) Boxplot summarizing the position of the samples along the principal component 1. (C) Loadingplot highlighting the correlation between the concentration of each metabolite and its importance over the principal component 1. For clarity, only significant correlations ($P < 0.05$) are reported. Abbreviations: PC = principal component; expl. var. = explained variance; conc. = concentration, UMP = uridine monophosphate.

that chronic heat stress condition could stimulate hepatic gluconeogenesis in 28-d-old broiler chickens. An increased utilization of amino acids for energy purposes could also explain the reduced concentrations of many free amino acids in the breast muscle of CHS birds (i.e. tryptophan, tyrosine, serine, threonine, glycine, glutamine, methionine, proline and alanine). In this scenario, it could be reasonably hypothesized that the breast muscle could have served as a source of free amino acids that can be used for gluconeogenesis during the period of heat stress. Overall, our results corroborate with the considerations of Rhoads et al. (2013), who revealed that amino acids can be mobilized to provide substrates supporting energy metabolism during periods of inadequate

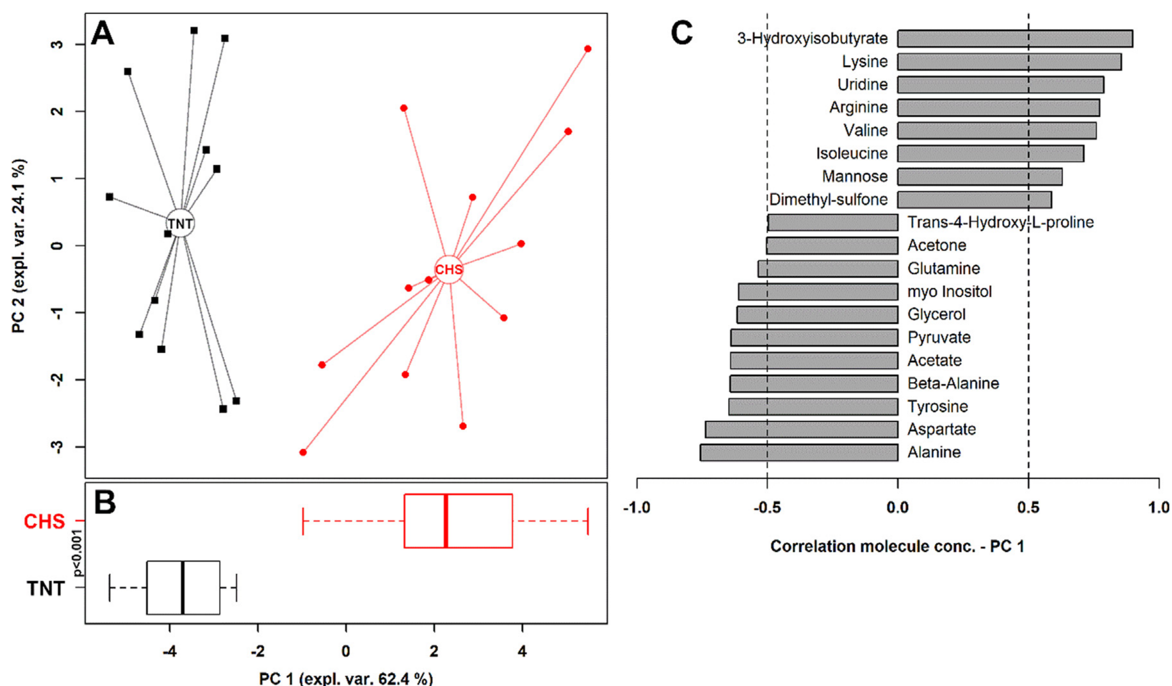

Fig. 4. Robust Principal Component Analysis (rPCA) model built on the space constituted by the concentration of the plasma metabolites showing differential expression in 41-day-old broiler chickens raised in either thermoneutral (TNT, *N* = 12) or chronic heat stress (CHS, *N* = 12) conditions from 35 to 41 days of age. (A) In the scoreplot, samples from TNT and CHS chickens are represented with black squares and red circles, respectively. The wide, empty circles represent the means of the samples. (B) Boxplot summarizing the position of the samples along the principal component 1. (C) Loadingplot highlighting the correlation between the concentration of each metabolite and its importance over the principal component 1. For clarity, only significant correlations ($P < 0.05$) are reported. Abbreviations: PC = principal component; expl. var. = explained variance; conc. = concentration.

nutrient intake or disease. Similarly, it has been supposed that the protein catabolism observed in animals exposed to chronic heat stress is likely to produce glucose through the gluconeogenesis pathway (Belhadj Slimen et al., 2016). Furthermore, Lu et al. (2018) pointed out that protein decomposition could be considered the main factor affecting growth performance and carcass characteristics of chronically heat-stressed broilers, and that the breast muscle could be involved in this catabolic activity. Conversely, some essential amino acids (i.e. isoleucine, valine, arginine and lysine) showed higher concentration in the plasma of CHS birds. Considering the significant reduction of feed intake observed in the CHS group, this outcome is rather counterintuitive although it paves the way to some considerations. Indeed, the higher plasma levels of these amino acids could possibly reflect their limited utilization either for protein synthesis, which was reported to be negatively affected by heat stress (Zuo et al., 2015), or for gluconeogenesis in the case of ketogenic amino acids such as lysine or isoleucine. Taken together, the breast muscle seems to be directly involved in energy homeostatic mechanisms of chronically heat-stressed chickens, likely serving as source of free amino acids that can be used through the gluconeogenesis pathway to maintain adequate blood glucose levels when feed intake is depressed for thermoregulatory reasons.

Furthermore, the ^{1}H NMR approach applied herein revealed other important information about the muscle metabolism of heat-stressed chickens. For instance, metabolites such as creatine, creatinine and guanidoacetate, which were classified among those contributing more on the differences between the two groups (Fig. 3), showed significantly higher concentration in the breast muscle of CHS broilers. Guanidinoacetate is endogenously synthesized from arginine and glycine mainly in the liver and kidneys (Ostojic, 2015). In the muscle, guanidinoacetate is subjected to methylation to form creatine, which, in its phosphorylated form, is a crucial compound for muscle energy metabolism (Ostojic, 2015). Indeed, phosphocreatine could be used to regenerate ATP from ADP with the formation of creatinine (Mayes and Botham, 2003), which was detected to be significantly higher in the breast muscle of CHS birds. Moreover, other metabolites showing lower concentrations in the breast muscle of CHS broilers, such as betaine, N-N-dimethylglycine, glycine and methionine, could be involved either directly or indirectly in the aforementioned biological pathways (Mayes and Botham, 2003). Overall, these results may suggest that creatine and creatine-associated metabolites could play a crucial role in the altered muscle energy metabolism of heat-stressed broiler chickens, likely attempting to restore the energy pool for muscle cells in a period of limited substrate availability.

On the other hand, oxidative stress is thought to be part of the stress response induced by high environmental temperatures (Lin et al., 2006). Oxidative stress stems by an alteration of the steady-state equilibrium between pro- and anti-oxidant compounds, resulting in an excessive production of reactive oxygen species and free radicals. Previous findings on broiler chickens indicated that heat stress determines an overproduction of reactive oxygen species as well as increased lipid and protein peroxidation (Farag and Alagawany, 2018). However, it has also been reported that heat stress upregulates the activity of antioxidant enzymes in liver and serum (Tan et al., 2010) as well as the expression of superoxide dismutase and catalase genes in spleen (Slawinska et al., 2019) likely to maintain the steady-state concentration of free radicals (Belhadj Slimen et al., 2016). Similarly, Jastrebski et al. (2017) observed a remarkable increase of reduced glutathione levels in the liver of heat-stressed broilers. In the present study, higher concentration of two histidine-containing dipeptides, namely carnosine and anserine, was detected in the breast muscle of CHS birds. These metabolites have a remarkable antioxidative capacity acting as free radical scavengers, reducing agents, and copper ion chelators (Fu et al., 2009). Therefore, the increased

concentration of carnosine and anserine in the breast muscle of CHS broilers can be considered as an attempt to counteract the oxidative environment triggered by the thermal challenge, which is consistent with the general observation that chickens seem to adapt to chronic heat stress by upregulating the levels of endogenous antioxidants (Akbarian et al., 2016).

Finally, of particular note from a bioenergetics standpoint is the increased concentration of malonate in the breast muscle of CHS birds. Malonate has been detected in different biological systems including plants and animals (Kim, 2002), and it is recognized as a competitive inhibitor of the enzyme succinate dehydrogenase, which plays a central role both in the Krebs cycle and in the electron transport chain. Therefore, the increased concentration of malonate could negatively affect crucial energy production pathways, with potential implications in terms of body energy homeostasis. However, it should be considered that the chicken breast muscle has a prevalently glycolytic metabolism and thus a scarce presence of mitochondria. Therefore, the potential impact of this molecule could be much more relevant for skeletal muscles presenting an oxidative metabolism, such as those composing the hindquarter, which have not been considered in this study. Further insights are necessary to understand whether such changes in malonate concentration are able to determine physiological consequences and, in general, to clearly define the potential role of this molecule in the muscle metabolism during heat stress.

In conclusion, the results obtained in this study indicated that chronic heat stress significantly modulates the breast muscle and plasma metabolome in fast-growing broiler chickens. Based on these insights, the breast muscle appears to be deeply involved in relevant metabolic functions of heat-stressed broilers, such as energy and protein metabolism as well as antioxidant response, which could have important implications in terms of body energy homeostasis and growth performance. Overall, this information can contribute to add insights regarding the molecular response of meat-type chickens towards thermal stress and can help to shed a light on the factors that underlie the detriment of productivity during warm periods. Further research is warranted to clearly define the role of certain molecules in the metabolic aspects of broilers and to understand whether such changes in muscle metabolome could have consequences on meat quality traits.

Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.animal.2021.100275.

Ethics approval

The research complies with the European legislation and guidelines regarding the protection of chickens kept for meat production (Directive 2007/43), the protection of animals at the time of killing (Regulation 1099/2009) and the protection of animals used for scientific purposes (Directive 2010/63). The experiment was approved by the Ethical Committee of the University of Bologna (ID: 928/2018).

Data and model availability statement

None of the data were deposited in an official repository. The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author contributions

MZ, ACM, SM and **FS** conceived and designed the experiment.

LL and **CZ** performed the ^{1}H NMR spectroscopy experiments and analysed the metabolomics data.

MZ and **FS** interpreted the results and wrote the manuscript.

LL, CZ, ACM and **SM** contributed in revising and reviewing the manuscript.

All authors read and approved the final manuscript.

Declaration of interest

None.

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