Heat stress modifies the lactational performances and the urinary metabolomic profile related to gastrointestinal microbiota of dairy goats

Alexandra Contreras-Jodar[1¶], Nazri Nayan[2¶], Soufiane Hamzaoui[1,#b], Gerardo Caja[1*], Ahmed A.K. Salama[1,#c¶*]

[1] Group of Research in Ruminants (G2R), Department of Animal and Food Sciences, Universitat Autonoma de Barcelona, Bellaterra, Barcelona, Spain

[2] Department of Animal Science, Faculty of Agriculture, University Putra Malaysia, Serdang, Selangor, Malaysia

[#b] Current address: Department of Agronomic Science, University of Bouira, Bouira, Algeria

[#c] Current address: Dairy and Food Science Department, South Dakota State University, Brookings, South Dakota, United States of America

* Corresponding authors:

gerardo.caja@uab.es (GC), ahmed.salama@sdstate.edu (AAKS)

¶These authors contributed equally to this work

Abstract

The aim of the study was to identify the candidate biomarkers of heat stress (HS) in the urine of lactating dairy goats through the application of proton Nuclear Magnetic Resonance (^{1}H NMR)-based metabolomic analysis. Dairy does (n = 16) in mid-lactation were submitted to thermal neutral (TN; indoors; 15 to 20°C; 40 to 45% humidity) or HS (climatic chamber; 37°C day, 30°C night; 40% humidity) conditions according to a crossover design (2 periods of 21 days). Thermophysiological traits and lactational performances were recorded and milk composition analyzed during each period. Urine samples were collected at day 15 of each period for ^{1}H NMR spectroscopy analysis. Principal component analysis (PCA) and partial least square–discriminant analysis (PLS-DA) assessment with cross validation were used to identify the goat urinary metabolome from Human Metabolome Data Base. HS increased rectal temperature (1.2°C), respiratory rate (3.5-fold) and water intake (74%), but decreased feed intake (35%) and body weight (5%) of the lactating does. No differences were detected in milk yield, but HS decreased the milk contents of fat (9%), protein (16%) and lactose (5%). Metabolomics allowed separating TN and HS urinary clusters by PLS-DA. Most discriminating metabolites were hippurate and other phenylalanine (Phe) derivative compounds, which increased in HS vs. TN does. The greater excretion of these gut-derived toxic compounds indicated that HS induced a harmful gastrointestinal microbiota overgrowth, which should have sequestrated aromatic amino acids for their metabolism and decreased the synthesis of neurotransmitters and thyroid hormones, with negative impact on milk yield and composition. In conclusion, HS markedly changed the thermophysiological traits and lactational performances of dairy goats, which were translated into their urinary metabolomic profile through the presence of gut-derived toxic compounds. Hippurate and other Phe-derivative compounds are suggested as urinary biomarkers to detect heat stressed dairy animals in practice.

Introduction

Exposure to high ambient temperature induces several physiological responses in order to maintain body homeostasis. Animals suffer from heat stress (HS) when physiological mechanisms fail to counterbalance an excessive heat load [1]. Exposure of dairy animals to HS results in a decline in their productive [2] and reproductive [3] performances due to a strong metabolic disruption. Dairy animals under HS typically show decreased feed intake, increased water consumption and thermophysiological traits, such as respiratory rate and rectal temperature, when compared to thermoneutral (TN). Usually, HS reduces milk yield and impairs milk composition in dairy goats [4]. Although these negative effects on milk

63 production are traditionally attributed to a decline in feed intake, pair-fed TN experiments
64 have shown that intake only accounts for 35-50% of milk yield reduction in dairy cows [5,
65 6]. Therefore, there is a specific effect of HS that disrupts body metabolism and milk
66 secretion which remains unknown.

67 Biofluid assessment by Nuclear Magnetic Resonance (NMR) spectroscopy can shed
68 some light on the physiological mechanisms occurred in animals when exposed to HS.
69 Proton (^{1}H) NMR, together with multivariate statistical analysis, has been successfully used
70 as metabolite profiling method to study the metabolic changes in HS rats [7]. This robust
71 and reliable technique provides vast information on metabolome dynamics and metabolic
72 pathways [8]. The ^{1}H NMR spectra derives from thousands of metabolite signals that
73 usually overlap, adding complexity to data processing. Computer-based data reduction and
74 multivariate statistical pattern recognition methods, such as principal component analysis
75 (PCA) and partial least square–discriminant analysis (PLS-DA), have shown to be beneficial
76 techniques to get the most from the information obtained in the ^{1}H NMR spectra for
77 classification purposes [9, 10].

78 To our knowledge, no studies have been carried out to evaluate urine metabolomics
79 of dairy goats. The aim of this study was to identify the candidate biomarkers of HS
80 through the application of ^{1}H NMR-based metabolomic urinalysis of dairy goats.
81

82 Material and methods

83 Animals and treatments

84 Animal care conditions and management practices agreed with the procedures stated by
85 the Ethical Committee of Animal and Human Experimentation (reference CEEAH#09/771)

of the Universitat Autonoma of Barcelona (UAB) and the codes of recommendations for livestock wellbeing of the Ministry of Agriculture, Food and Environment of Spain.

Sixteen multiparous Murciano-Granadina dairy does (43.5 ± 1.6 kg body weight), lactating and open, from the herd of the SGCE (Servei de Granges i Camps Experimentals) of the UAB in Bellaterra (Barcelona, Spain), were blocked in 2 balanced groups at mid-lactation (81 ± 3 days-in-milk; 2.00 ± 0.04 L/day). Does were adapted to metabolic cages for 2 weeks before the start of the experiment and the groups randomly allocated to 2 ambient conditions treatments according to a 2 × 2 (treatment × period) crossover design. Treatments were TN (indoors shelter; 15 to 20°C and 45 ± 5% relative humidity) and HS (climatic chamber 4 × 6 × 2.3 m with temperature-humidity control system, Carel Controls Ibérica, Barcelona, Spain; 37 ± 0.5°C during the day, and 30 ± 0.5°C during the night; 40 ± 5% humidity and 90 m^3/h continuous air turnover). Day-night length was set to 12-12 hours and the experimental periods lasted 21 days (14-days adaptation, 5-days measurements, 2-days washout). Temperature-humidity index (THI) calculated according to NRC (1971) resulted THI_{TN} = 59 to 65 and THI_{HS} = 75 to 83. Experimental conditions were similar to those detailed by Hamzaoui et al. [11].

Does were milked once-a-day (0800) with a portable machine (Westfalia-Separator Ibérica, Granollers, Spain) set at 42 kPa, 90 pulses/min and 66% pulsation ratio and provided with volumetric recording jars (3 L ± 5%). The milking routine included cluster attachment, machine milking, machine stripping before cluster removal, and teat dipping in an iodine solution (P3-ioshield, Ecolab Hispano-Portuguesa, Barcelona, Spain). Feed was offered ad libitum at 0930 (130% feed intake of the previous day) and consisted of a total mixed ration (dry matter, 89.9%; net energy for lactation, 1.40 Mcal/kg; crude protein, 17.5%; organic matter, 87.3%; neutral detergent fiber, 34.4%; acid detergent fiber, 21.8%;

on dry matter basis). Ration ingredients were (as fed): alfalfa hay, 64.2%; ground barley, 9.6%; beet pulp, 9.6%; ground corn, 8%; soybean meal, 3.3%; sunflower meal, 3.2%; molasses, 1%; salt, 0.6%; sodium bicarbonate, 0.3%; mineral and vitamin complex, 0.2% (Vitafac premix, DSM Nutritional Products, Madrid, Spain). Water was permanently available and offered at room temperature in water bowls connected to individual tanks of 20 L. A sawdust drip tray under each water bowl was used to collect spilled water.

Sampling and measurements

Thermophysiological traits and lactational performances of the goats

Does were weighed at the start and the end of each period using an electronic scale (True-Test SR2000, Pakuranga, New Zealand; accuracy, 0.2 kg). Rectal temperature (digital clinical thermometer, ICO Technology, Barcelona, Spain; accuracy, 0.1°C) and respiratory rate (flank movements during 60 s) were recorded daily at 0800, 1200, and 1700 throughout the experiment. Milk yield (volume) was recorded daily throughout the experiment and milk samples collected weekly for composition (NIRSystems 5000, Foss, Hillerød, Denmark). Feed and water intakes were calculated by weight from the daily refusals and feed samples were collected daily and composited by period for analyses. Feed composition was determined according to analytical standard methods [12].

Urine sampling and preparation

Urine samples from each doe were collected at micturition on the morning of day 15 of each period (n = 32) and stored at −20°C until ^{1}H NMR analysis.

Preparation of samples for ^{1}H NMR spectroscopy was done according to Beckonert et al. [8]. Briefly, phosphate buffer solution (pH 7.4) was prepared with sodium phosphate

dibasic (Na_2HPO_4; 99.95% trace metals basis, anhydrous, Sigma-Aldrich, Germany), sodium phosphate monobasic (NaH_2PO_4; 99.95% trace metals basis, anhydrous, Sigma-Aldrich) and sodium azide (NaN_3; Sigma-Aldrich). Deuterium oxide (D_2O; 99.9 atom % D, Sigma-Aldrich), containing 0.75% 3-(trimethylsilyl) propionic-2,2,3,3-d4 acid (TSP) sodium salt as NMR reference compound, was added before the flask was filled up to 25 mL with milli-Q water (EMD Millipore, Darmstadt, Germany). The flask was shaken thoroughly and left in a Clifton sonicator (Nickel Electro, Weston-super-Mare, United Kingdom) at 40°C until the salts were dissolved. The prepared phosphate buffer solution was stored at 4°C. Urine samples were thawed in a water bath, thoroughly shaken and spun for 5 min at 12,000 × *g* in a swing-bucket rotor (Hettich, Tuttlingen, Germany) at 4°C. Then, 400 µL of the urine sample were transferred into Eppendorf tubes and mixed thoroughly with 200 µL of cold phosphate buffer solution. All the tubes were then centrifuged for 5 min at 12,000 × *g* at 4°C and 550 µL of the final mixture transferred into 5-mm NMR tubes (VWR International Eurolab, Barcelona, Spain). The prepared NMR tubes were immediately put on ice and sent to the NMR Service of the UAB for ^{1}H High Resolution NMR Spectroscopy.

NMR spectroscopy

^{1}H NMR spectra were acquired on a Bruker Avance-III spectrometer (Bruker BioSpin, Rheinstetten, Germany) operating at a ^{1}H NMR frequency of 600 MHz and a temperature of 298°K, controlled by a Burner Control Unit-extreme regulator. A 5 mm Triple Resonance Broadband Inverse probe with *z*-gradients and inverse detection was used and controlled by TopSpin2.1 software (Bruker, Germany). One-dimensional ^{1}H NMR spectra were obtained using an one-dimensional Nuclear Overhauser Enhancement Spectroscopy (NOESY) pulse sequence. The solvent signal was suppressed by pre-saturation during

158 relaxation and mixing time. A total of 32 scans and 2 dummy scans were performed to
159 produce 32,768 data points for each spectrum using a relaxation delay of 2.0 s with a pulse
160 power level of 54 dB and an acquisition time of 2.6 s. Spectral width (δ) used for all data
161 collected was 12.0 ppm, and 0.3 Hz exponential line broadening was applied for the
162 Fourier-transform of the raw data. ^{1}H NMR spectra were phased, baseline corrected, and
163 corrected for chemical shift registration relative to the TSP reference compound previously
164 indicated (δ = 0.0 ppm) in TopSpin 2.1.(data in S1 File).
165

166 ## Statistical analyses

167 **Thermophysiological and performance analysis**

168 Data were analyzed by the PROC MIXED for repeated measurements of SAS v. 9.1.3 (SAS
169 Inst. Inc., Cary, North Carolina, USA). The statistical mixed model contained the fixed
170 effects of environmental treatment (TN vs. HS), the period (1 and 2) and measuring day (1
171 to 19), and the random effects of the animal (1 to 16), the interactions (treatment × day
172 and treatment × period), and the residual error. Differences between least squares means
173 were determined with the PDIFF option of SAS. Significance was declared as $P<0.05$.
174

175 **NMR data pre-processing and analysis**

176 Pre-treatment of raw spectral data is critical for generating reliable and interpretable
177 models using multivariate analysis techniques. Nevertheless, metabolic fingerprinting
178 datasets acquired from ^{1}H NMR spectrometers suffer from imprecisions in chemical shifts
179 due to temperature, pH, ionic strength and other factors. Therefore, models generated
180 using multivariate analysis may fail to identify separations between classes, and their
181 loadings can be difficult to interpret due to over-abundance of variables. To mitigate these

182 complications each spectrum was uniformly divided into 'bins' and the signal intensities
183 within each bin were integrated to produce a smaller set of variables according to Worley
184 and Powers [13]. In this way, each dataset was divided in bins of 100 (i.e., from 0.0003 to
185 0.03 ppm) using R software v. 3.2.3 [14]. After binning, alignment and normalization of
186 spectra were accomplished to ensure that all observations were directly comparable. In
187 this sense, urine spectra were normalized to creatinine methyl resonance intensity at δ =
188 3.05 ppm and then $\log_2$ transformed. Regarding variable selection, raw ^{1}H NMR spectral
189 data were edited by excluding the regions outside the chemical shift range of δ = 9.0-0.5
190 ppm, and also the residual peak of the imperfect water suppression (δ = 5.5-4.6 ppm).
191 Following the recommendations of Pechlivanis et al. [15], the spectral regions of histidine,
192 1-metylhistidine, and 3-methylhistidine (δ = 8.17-7.87, δ = 7.15-7.01, and δ = 3.77-3.71
193 ppm, respectively) were also removed because of the sensitivity to small pH differences
194 among urine samples.
195 Once ^{1}H NMR pre-processing data was completed, data were subjected to
196 multivariate statistical analysis. Initially, PCA was performed without considering the class
197 information for samples examination and search for outliers. Then, PLS-DA with leave-one-
198 out cross validation was also performed on the datasets using the pls package of R
199 software [16]. PLS-DA allowed individual samples to be classified according to the
200 respective class prior to analysis (TN or HS). Model strength was assessed using both R^2
201 and Q^2 statistical parameters. While R^2 values reported the total amount of variance
202 explained by the model, the Q^2 reported model accuracy as a result of cross-validation.
203 Aside from its theoretical maximum value of 1, for biological models, an empirically
204 inferred acceptable value is ≥ 0.4 [9]. The resulting Q^2 statistic was compared to a null
205 distribution to test model significance ($P<0.05$).

206 Interpretation of multivariate analysis was performed through scores and loadings
207 plots according to its contribution to the separation between groups. For biomarker
208 search, PLS-DA loadings were sorted by absolute values, being the first ones the
209 metabolites responsible of the separation between experimental groups. Because both
210 PCA and PLS-DA analysis may be influenced by variable correlations, the intra- and the
211 inter-class variance of metabolites may have no significant differences in the one-
212 dimensional statistical analysis [17]. For this reason, spectral bins were also selected using
213 a Volcano plot with paired student *t* test analysis between HS over TN cohorts to get a
214 general overview of the data (log_2 fold change thresholds, ≤1.5 and ≥1.5; P<0.01). All ^1H
215 NMR data pre-processing, statistical analysis and the generated plots were performed
216 using R software v. 3.2.3 [14].
217
218 **Metabolite assignment**
219 Chemical shifts linked to the highest loading values found in PLS-DA were annotated for
220 metabolite assignment as HS biomarker candidates. The candidate chemical shifts and
221 corresponding metabolites were assigned using the Human Metabolome Database [18]
222 and queried in KEGG (Kyoto Encyclopedia of Genes and Genomes) database to know in
223 which metabolic pathways they were involved.
224
225

226 Results and discussion

227 Effects of heat stress on thermophysiological and lactational performances
228 of the goats

The effects of the experimental HS conditions on thermophysiological and lactational performances of the dairy goats are summarized in Table 1. Rectal temperature and respiratory rate increased during the day in both groups of does, following the expected circadian rhythm and the daily THI pattern in both TN and HS conditions. The greatest values were observed in the HS does at 1700, the increases being 1.2°C and 3.5-fold ($P<0.001$) when compared to TN does. On average, feed intake decreased 35% in HS ($P<0.001$) when compared to TN does but, in contrast, water consumption increased 74% ($P<0.001$). Furthermore, HS does lost 115 g/d of body weight, whereas TN goats won 162 g/d, on average ($P<0.001$). Obtained results agreed with those reported for the same breed of dairy goats in late-lactation and under similar HS conditions [11].

Reducing feed intake is a way to decrease heat production in warm environments because heat increment of feeding, especially in ruminants, is an important source of heat production [4]. Moreover, increased water consumption under HS conditions is mainly used for boosting latent heat losses by evaporation (e.g., sweating and panting). Despite this, no differences in milk yield were observed, although milk composition markedly worsened. Milk fat, protein and lactose contents varied by −9%, −16% and −5%, respectively (Table 1; $P<0.01$), which would severely compromise the milk transformation to dairy products [19]. Consequently with the decrease in milk composition, fat corrected milk yield also varied by −14% ($P<0.05$).

Table 1. Thermophysiological and lactational performances of dairy goats under thermal neutral (TN) and heat stress (HS) conditions. Values are least square means and standard error of the means (SEM).

Item	Treatment		SEM	P value
	TN	HS		
Rectal temperature, ºC				
0800	38.5	39.1	0.08	<0.001
1200	38.7	39.7	0.07	<0.001
1700	38.7	39.9	0.09	<0.001
Respiratory rate, breaths/min				
0800	27	69	4	<0.001
1200	39	131	6	<0.001
1700	37	130	6	<0.001
Performances				
Dry matter intake, kg/d	2.26	1.47	0.09	<0.001
Water intake, L/d	6.1	10.6	1.0	<0.001
Final body weight, kg	48.6	39.8	1.8	<0.001
Body weight variation, kg	3.5	−2.1	1.0	<0.001
Milk yield, L/d	1.88	1.79	0.11	0.413
FCM[1] yield, L/d	2.17	1.86	0.13	0.017
Milk composition, %				
Fat	3.98	3.64	0.13	0.009
Protein	3.40	2.85	0.10	<0.001
Lactose	4.51	4.30	0.07	0.003

[1]Fat corrected milk at 3.5%; FCM = L × [0.432 + 0.162 × (fat, %)], being L liters of milk.

Although our does were less sensitive to HS than dairy cows with regard to feed intake and milk yield, the effects of HS on milk fat content and fat corrected milk were contradictory when compared to cows. So, despite the typical fat depression seen in commercial dairy cow farms during the summer, Rhoads et al. [5] and Shwartz et al. [20] reported 9% increase or no change in milk fat content, at the short- or mid-term, respectively, in HS vs. TN dairy cows. On the other hand, the above indicated negative effect of HS on the milk protein content of our goats (i.e., −16%), was greater than

reported by Rhoads et al. ([5], −5%) and Shwartz et al. ([20], −9%) in dairy cows, and herewith in the same breed of dairy goats in late lactation ([11], −13%). The negative effects of HS on the lactational performances of dairy ruminants are usually attributed to the decline in feed intake, but pair-fed experiments under TN conditions have shown that feed intake only explains approximately a half of the fall in milk yield and body weight in dairy cows [5, 6]. Therefore, similar responses were expected in our dairy goats.

As an intermediate conclusion, the thermophysiological and lactational performance responses observed, clearly evidenced that our HS does (kept at THI = 75 to 83) were under severe stress on the days at which the urine samples for ^{1}H NMR-metabolomics assessment were collected (day 15).

NMR urinary spectroscopy of the goats

Comparison of ^{1}H NMR urinary mean spectra for the TN and HS lactating does is shown in Fig 1. Resonance assignments reported in the figure were made from the known chemical shifts and coupling patterns of urine spectra previously described in humans [14, 21].

Fig 1. One-dimensional ^{1}H NMR spectra at 600 MHz of urine from representative thermoneutral (TN) and heat stressed (HS) dairy does. Dominant metabolites were: 1, creatinine; 2, creatine; 3, trimethyl-N-oxide; 4, urea; 5, branched-chain amino acids and organic acids; 6, glycine; 7, allantoin; 8, alanine; 9, N-acetyl glycoprotein; 10, glutamate; 11, succinic acid; 12, citric acid; 13, aromatic signals; 14, hippuric acid.

S1 Table. ^{1}H NMR data matrix of normalized and binned spectral data. HS, heat-stressed lactating dairy does; TN, thermal neutral lactating dairy does.

At first sight, visible differences in urine metabolites were found between HS and TN groups. Spectral region from δ = 8.0-6.5 ppm showed higher excretion compounds in the

283 HS doe group. On the contrary, all excreted compounds that lay on the δ = 4.5-0.5 ppm
284 spectral region appeared to be at lower concentrations in the HS group. More detailed
285 analysis of metabolic differences between these two thermal conditions were obtained
286 from the multivariate PCA and PLS-DA data analyses and the Volcano plot.

287 First, the Volcano plot (Fig 2) showed that TN does excreted a greater number of
288 urinary metabolites (i.e., higher number of left-sided spots) than HS does. Most probably,
289 this was a consequence of the metabolic sparing of nutrients of the HS does, which loosed
290 weight as a result of their negative energy balance, to cope with the HS conditions.

291 **Fig 2. Volcano plot based on fold change (log_2) and P value ($-log_{10}$) of all spectral bins of**
292 **^{1}H NMR urinary spectroscopy of thermoneutral (TN) and heat stressed (HS) lactating**
293 **dairy does. Red circles indicate the spectral bins that showed significant changes and**
294 **absolute fold changes greater than 1.5.**

295 Regarding the multivariate analysis, PCA was initially applied to the ^{1}H NMR spectra.
296 Based on the principle of minimum differentiation, no samples were identified as outliers
297 according to Hotelling's T^2 (95% interval of confidence). Therefore, all samples remain for
298 subsequent PLS-DA in order to identify the metabolic differences between HS and TN dairy
299 does. The PLS-DA scores plot showed a slight distinguishable separation between HS and
300 TN datasets (Fig 3).

301 **Fig 3. PLS-DA scores plot of the first two principal components of ^{1}H NMR urinary spectra**
302 **of thermoneutral (TN) and heat stressed (HS) lactating dairy does.**

303 The separation along the x-axis (PLS-DA component 1) represents differences related
304 to the environmental treatment. All other variations in the NMR data are visualized as
305 separation in the y-axis direction (second component). The cross-validation of plasma

metabolomics PLS-DA models (first 2 components) gave $R_x^2 = 0.54$, $R_y^2 = 0.17$, and $Q^2 = 0.47$. The R^2 and Q^2 values in the model were higher than in the random model ($P<0.01$). The top-ranking urinary metabolites responsible for discriminating HS does were hippurate (δ = 7.54 ppm, 7.83 ppm and 7.63 ppm) and the phenylalanine (Phe) derivative compounds OH-phenylacetate (δ = 7.27 ppm), OH-phenylacetylglycine (δ = 7.20 ppm) and phenylglyoxylate (δ = 7.62 ppm). All of them were excreted in greater concentrations in the urine of HS when compared to TN does. Table 2 shows the excreted metabolites with the greatest quantitatively change under HS conditions, according to the results of the univariate and multivariate analyses.

As it can be observed, of them come from certain gastrointestinal bacteria that generate uremic toxins that are absorbed into the blood and mainly cleared by the kidney [22, 23]. The greater excretion of these metabolites, also known as gut-derived uremic toxins and mammalian-microbial cometabolites [24], may be related to an abnormal overgrowth of gastrointestinal microbiota that is commonly accompanied with intestinal hyper-permeability [23, 25, 26].

Table 2. Selected metabolites contributing to the classification of the urine metabolome of thermoneutral and heat stressed lactating dairy does.

Pathway	Chemical shift (δ, ppm)	Metabolite	Fold change*	P value
Phenylalanine (Phe) metabolism	7.83, 7.63, 7.54	Hippurate	2.74	<0.001
Microbial metabolism in diverse environments	7.62	Phenylglyoxylate	1.59	<0.001
	7.27	OH-phenylacetate	1.51	<0.001

Tyrosine (Tyr) metabolism	7.20	OH-phenylacetylglycine	2.16	<0.001

* Metabolites with positive fold change values means that they are excreted in greater concentrations under heat stress conditions

The increase of gut-derived uremic toxins reflected alterations in the gastrointestinal environment due to the metabolic impact of HS. In fact, it is well known that under HS conditions, mammals redistribute blood to the periphery for heat dissipation purposes, while vasoconstriction occurs in the gastrointestinal tract [27] that leads to tissue hypoxia and oxidative stress [28]. Moreover, lower rumen pH has been reported as a side effect in HS goats [29] and it may compromise the integrity of the gastrointestinal tract barrier [2].

Hippurate and other Phe-derivative compounds are produced by the aerobic and anaerobic degradations of aromatic amino acids (e.g., Phe and Tyr) and dietary polyphenols by the gastrointestinal microbiota [22, 30, 31]. Although they are usually found in plasma and excreted in urine by crossing the cellular and tissue barriers (gastrointestinal epithelium, lymphatic barrier and liver), high levels of Phe-derivatives in urine are related to gastrointestinal leaky stages [32]. Moreover, high levels of gut-derived uremic toxins seem to affect both the cellular protein expression and the activity of the cyclooxygenase-2 (COX-2) enzyme, which plays a major role in the regulation of inflammation trough the production of prostaglandins; so, when COX-2 activity is speed up, inflammation increased [33]. Some Phe-derivatives also produce cytotoxic effects by the inhibition of cell pores opening and the production of reactive oxygen species [34].

Among Phe-derivatives, hippurate has a strong association with diet and gastrointestinal microbiota and its production requires of both microbial and mammalian metabolisms [24]. Gastrointestinal bacteria produce benzoic acid from dietary aromatic

348 compounds, which is absorbed into the blood. Because of the toxicity of benzoic acid, it is
349 conjugated with glycine in the mitochondrial matrix of the liver and renal cortex to form
350 hippuric acid [24, 25], which is later filtered in the kidneys and finally excreted in urine as
351 hippurate [24, 35]. The main elimination route for hippurate is the active renal tubular
352 secretion and its disruption results in its accumulation in the blood [24]. Hippurate is a
353 uremic toxin that participates in the correction of metabolic acidosis by stimulating
354 ammoniagenesis, a dominant and adaptive mechanism of proton excretion. Moreover, it
355 interferes several metabolic processes, such as: inhibition of glucose utilization by the
356 kidney and muscle, modulation of fatty acid metabolism and regulation of acid-base
357 balance by stimulating kidney's ammoniagenesis, among others, as reviewed by Dzúrik et
358 al. [35].

359 It might also be noted that, additionally to the production of gut-derived uremic toxins
360 from dietary aromatic amino acids by the gastrointestinal microbiota, Phe is known to be
361 an essential amino acid for most animals, including ruminants [36]. It is also the precursor
362 of Tyr, that is essential for the synthesis of thyroid hormones and levodopa
363 neurotransmitter. Previous studies have evidenced a strong decrease in plasma thyroid
364 hormones (i.e., TSH, T4 and T3) of steers under HS conditions [37], which means that the
365 basal heat production may in fact decrease when Phe and Tyr are scarce. Moreover, the
366 rate of milk production is markedly affected by thyroid hormones, which modulate the
367 nutrient partitioning towards milk production [38]. On the other hand, a decrease in the
368 dopaminergic neurons activity was also observed in HS calves [39]. The drop of levodopa
369 synthesis may be the result of the hypersecretion of its antagonist prolactin, as observed
370 in response to HS in goats [40], ewes [41] and cows [42]. Prolactin is not only a hormone
371 related to milk production, but has a broad variety of biological functions related to

372 thermoregulation and water balance. The increase in plasma prolactin is not reflected in
373 an increase in milk yield, as seen in dairy ruminants under HS conditions [11, 43]. Alamer
374 [44] concluded that the mammary gland experiences a down-regulation of prolactin
375 signaling pathways that could partially explain the depressed milk production of dairy
376 cows during HS.

377 Conclusions

378 Heat stress caused marked changes in thermophysiological traits and lactational
379 performances of dairy goats, which were translated into their ^{1}H NMR metabolomic
380 urinary profile. These changes were related to the over-excretion of gut-derived toxic
381 compounds generated by the gastrointestinal microbiota with expected decreases in the
382 bioavailability of aromatic amino acids and impairment of the synthesis of thyroid
383 hormones and neurotransmitters (i.e., levodopa, serotonin), which compromised the milk
384 production of dairy goats. In practice, the use of hippurate and other phenylalanine
385 derivatives are suggested as urinary biomarkers to identify heat stressed animals.

386 Acknowledgements

387 The authors are grateful to the technical team of SGCE (Servei de Granges i Camps
388 Experimentals) of the UAB for the care of the animals.

389

390 References

391 **1.** Bernabucci U, Lacetera N, Baumgard LH, Rhoads RP, Ronchi B, Nardone A.
392 Metabolic and hormonal acclimation to heat stress in domesticated
393 ruminants. Anim. 2010;4:1167–83. pmid: 22444615.
394 **2.** Baumgard LH, Rhoads RP. Effects of heat stress on postabsorptive metabolism
395 and energetics. Annu Rev Anim Biosci. 2013;1:311–37. pmid: 25387022.

3. Hansen PJ. Effects of heat stress on mammalian reproduction. Philos Trans R Soc Lond B Biol Sci. 2009; 364:3341–50. pmid: 19833646.
4. Salama AAK, Caja G, Hamzaoui S, Badaoui B, Castro-Costa A, Façanha DE et al. Different levels of response to heat stress in dairy goats. Small Rumin. Res. 2014;121:73–9.
5. Rhoads ML, Rhoads RP, VanBaale MJ, Collier RJ, Sanders SR, Weber WJ et al. Effects of heat stress and plane of nutrition on lactating Holstein cows: I. Production, metabolism, and aspects of circulating somatotropin. J Dairy Sci. 2009;92:1986–97. pmid: 19389956.
6. Wheelock JB, Rhoads RP, VanBaale MJ, Sanders SR, Baumgard LH. Effects of heat stress on energetic metabolism in lactating Holstein cows. J Dairy Sci. 2010;93:644–55. pmid: 20105536.
7. Ippolito DL, Lewis JA, Yu C, Leon LR, Stallings JD. Alteration in circulating metabolites during and after heat stress in the conscious rat: potential biomarkers of exposure and organ-specific injury. BMC Physiol. 2014;14:14. pmid: 25623799.
8. Beckonert O, Keun HC, Ebbels TMD, Bundy J, Holmes E, Lindon JC et al. Metabolic profiling, metabolomic and metabonomic procedures for NMR spectroscopy of urine, plasma, serum and tissue extracts. Nature Protocols. 2007;2:2692–703. pmid: 18007604.
9. Westerhuis JA, Hoefsloot HCJ, Smit S, Vis DJ, Smilde AK, van Velzen EJJ et al. Assessment of PLSDA cross-validation. Metabolomics. 2008;4:81–9.
10. Yamamoto H, Yamaji H, Abe Y, Harada K, Waluyo D, Fukusaki E et al. Dimensionality reduction for metabolome data using PCA, PLS, OPLS, and RFDA with differential penalties to latent variables. Chemom Intell Lab Syst. 2009;98:136–42.
11. Hamzaoui S, Salama AAK, Albanell E, Such X, Caja G. Physiological responses and lactational performances of late lactating dairy goats under heat stress conditions. J Dairy Sci. 2013;96:6355–65. pmid: 23958010.
12. AOAC (2003) Official Methods of Analysis of the Association of Official's Analytical Chemists. 17th Edition, Association of Official Analytical Chemists (AOAC), Arlington, Virginia.
13. Worley B, Powers R. Multivariate analysis in metabolomics. Curr Metabolomics. 2013;1:92–107. pmid: 26078916.
14. R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. 2015. URL https://www.R-project.org/.
15. Pechlivanis A, Kostidis S, Saraslanidis P, Petridou A, Tsalis G, Mougios V et al. H-1 NMR-Based Metabonomic Investigation of the Effect of Two Different Exercise Sessions on the Metabolic Fingerprint of Human Urine. J Proteome Res. 2010;9:6405–16. pmid: 20932058.
16. Mevik B, Wehrens R., Hovde Liland K. Pls: Partial Least Squares and Principal Component Regression. R package version 2.5-0. 2015. Available from: http://CRAN.R-project.org/package=pls.
17. Ji P, Wei Y, Hua Y, Zhang X, Yao W, Ma Q et al. A novel approach using metabolomics coupled with hematological and biochemical parameters to explain the enriching-blood effect and mechanism of unprocessed Angelica

sinensis and its 4 kinds of processed products. J Ethnopharmacol . 2017;211:101–16. pmid: 28958590.

18. Wishart DS, Knox C, Guo AC. 2009. HMDB: a knowledgebase for the human metabolome. Nucleic Acids Res. 2009;37 (Database Issue):D603–610. pmid: 18953024.

19. Salama AAK, Caja G, Hamzaoui S, Such X, Albanell E, Badaoui B et al. 2016. Chap. 2. Thermal stress in ruminants: Responses and strategies for alleviation. In: Animal Welfare in Extensive Systems. J. Villalba, X. Manteca (eds.). The animal Welfare Series, 5M Publishing, Sheffield, UK, pp. 11–34.

20. Shwartz G, Rhoads ML, VanBaale MJ, Rhoads RP, Baumgard LH. Effects of a supplemental yeast culture on heat-stressed lactating Holstein cows. J. Dairy Sci. 2009;92:935-42. pmid: 19233786.

21. Bouatra S, Aziat F, Mandal R, Guo AC, Wilson MR, Knox C et al. The human urine metabolome. PLoS One 2013;8:e73076. 10.1371/journal.pone.0073076. pmid: 24023812.

22. Martin AK. The origin of urinary aromatic compounds excreted by ruminants. 1. The metabolism of quinic, cyclohexanecarboxylic and non-phenolic aromatic acids to benzoic acid. British J Nutr. 1982;47:139–54. pmid: 7059566.

23. Ramezani A, Raj DS. The gut microbiome, kidney disease, and targeted interventions. J Am Soc Nephr. 2014;25: 657–70. pmid: 24231662.

24. Lees HJ, Swann JR, Wilson ID, Nicholson JK, Holmes E. Hippurate: the natural history of a mammalian-microbial cometabolite. J Proteome Res. 2013;12:1527-46. pmid: 23342949.

25. Nicholson JK, Holmes E, Wilson ID. Gut microorganisms, mammalian metabolism and personalized health care. Nat Rev Microbiol. 2005;3:431–8. pmid: 15821725.

26. Pearce SC, Mani V, Weber TE, Rhoads RP, Patience JF, Baumgard LH, Gabler NK. Heat stress and reduced plane of nutrition decreases intestinal integrity and function in pigs. J Anim Sci. 2013;91:5183–93. pmid: 23989867.

27. Kregel KC, Wall PT, Gisolfi CV. Peripheral vascular responses to hyperthermia in the rat. J Appl Physiol. 1988;64:2582–8. pmid: 340344.

28. Lambert GP. Stress-induced gastrointestinal barrier dysfunction and its inflammatory effects. J Anim Sci. 2009;87(14 Suppl):E101–8. pmid: 18791134.

29. Castro-Costa A, Salama AAK, Moll X, Aguiló J, Caja G. Using wireless rumen sensors for evaluating the effects of diet and ambient temperature in nonlactating dairy goats. J Dairy Sci. 2015;98:4646–58. pmid: 25958285.

30. Macfarlene GT, Macfarlene S. Human colonic microbiota: ecology, physiology and metabolic potential of intestinal bacteria. Scand J Gastroenterol. 1997;32 (Suppl 222):3–9. pmid: 9145437.

31. Pallister T, Jackson MA, Martin TC, Zierer J, Jennings A, Mohney RP, MacGregor A, Steves CJ, Cassidy A, Spector TD, Menni C. Hippurate as a metabolomic marker of gut microbiome diversity: Modulation by diet and relationship to metabolic syndrome. Sci Rep. 2017;7:13670. pmid: 29057986.

32. Fedotcheva NI, Kazakov RE, Kondrashova MN, Beloborodova NV. Toxic effects of microbial phenolic acids on the functions of mitochondria. Toxicol Lett. 2008;180:182–8. pmid: 18634861.

33. Karlsson PC, Huss U, Jenner A, Halliwell B, Bohlin L, Rafter JJ. Human fecal water inhibits COX-2 in colonic HT-29 cells: role of phenolic compounds. J Nutr. 2005;135:2343–9. pmid: 16177193.

34. Galati G, Lin A, Sultan AM, O'Brian PJ. Cellular and in vivo hepatotoxicity caused by green tea phenolic acids and catechins. Free Radic Biol Med. 2006;40:570–80. pmid: 16458187.

35. Dzúrik R, Spustová V, Krivosíková Z, Gazdíková K. Hippurate participates in the correction of metabolic acidosis. Kidney Int. 2001;78:S278–81. pmid: 11169026.

36. Patton RA, Hristov AN, Parys C, Lapierre H. Relationships between circulating plasma concentrations and duodenal flows of essential amino acids in lactating dairy cows. J Dairy Sci. 2015;98: 4707–34. pmid: 25912872.

37. Kahl S, Elsasser TH, Rhoads RP, Collier RJ, Baumgard LH. Environmental heat stress modulates thyroid status and its response to repeated endotoxin challenge in steers. Domest Anim Endocrinol. 2015;52:43–50. pmid: 25804834.

38. Hurley WL. Adrenal and thyroid hormones; 2010 [cited 25 July 2018]. In: Lactation biology website. University of Illinois, Urbana-Champaign, IL. Available from: http://ansci.illinois.edu/static/ansc438/Lactation/adrenalthyroid.html

39. Tucker HA, Chapin LT, Lookingland KJ, Moore KE, Dahl GE, Evers JM. Temperature effects on serum prolactin concentrations and activity of dopaminergic neurons in the infundibulum/pituitary stalk of calves. Proc Soc Exp Biol Med. 1991;197:74–6. pmid: 1902294.

40. Sergent D, Berbigier P, Kann G, Ravault JP. Effect of prolactin inhibition on thermo-physiological parameters and on plasma prolactin and cortisol concentrations in male Creole goats. Reprod Nutr Dev. 1985;25:629–40.

41. Colthorpe KL, Anderson ST, Martin GB, Curlewis JD. Hypothalamic dopamine D1 receptors are involved in the stimulation of prolactin secretion by high environmental temperature in female sheep. J Neuroendocrinol. 1998;10:503–9. pmid: 9700677.

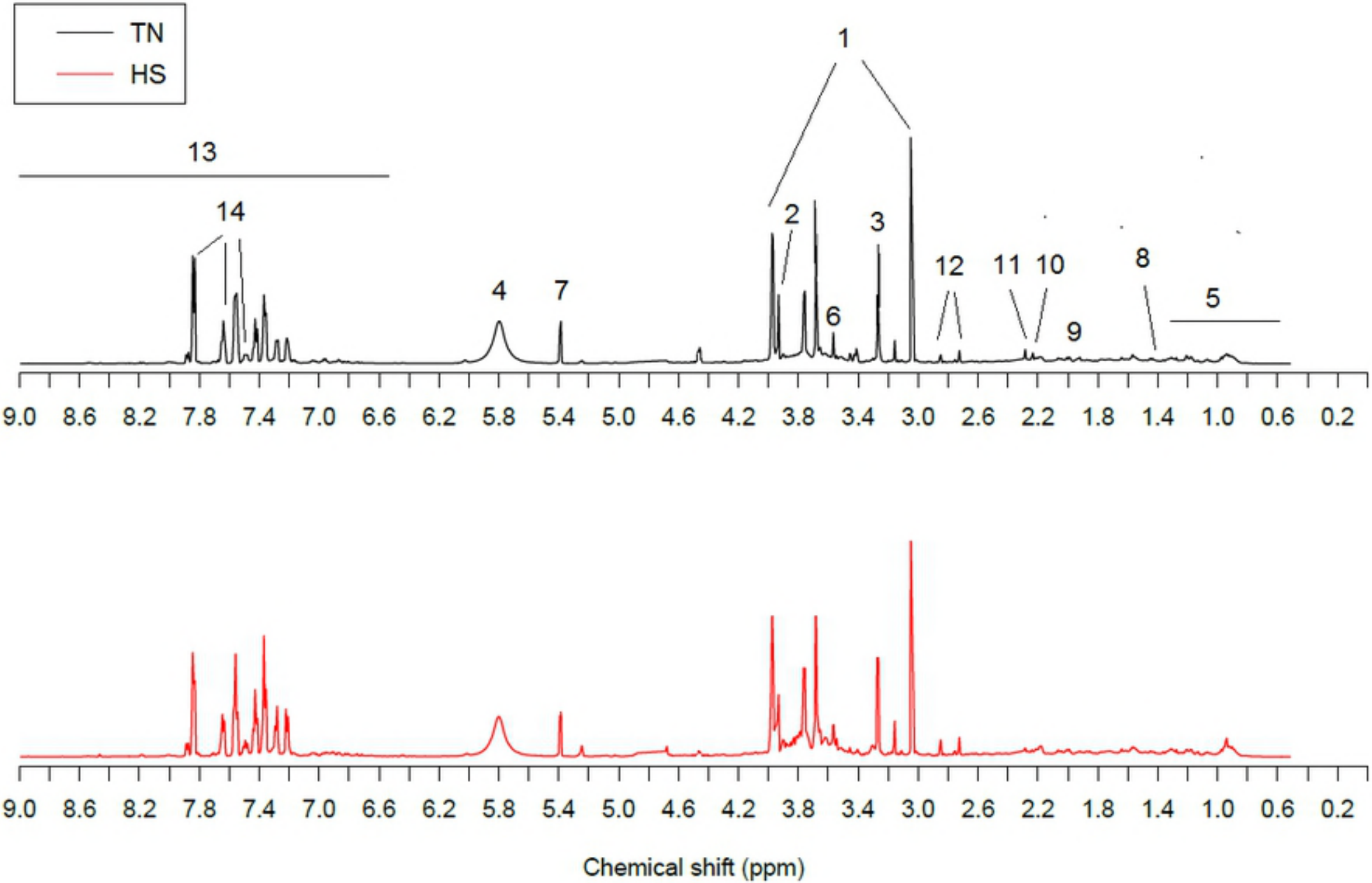
42. Ronchi B, Stradaioli G, Supplizi AV, Bernabucci U, Lacetera N. Influence of heat stress or feed restriction on plasma progesterone, oestradiol-17b, LH, FSH, prolactin and cortisol in Holstein heifers. Livest. Prod Sci. 2001;68:231–41.

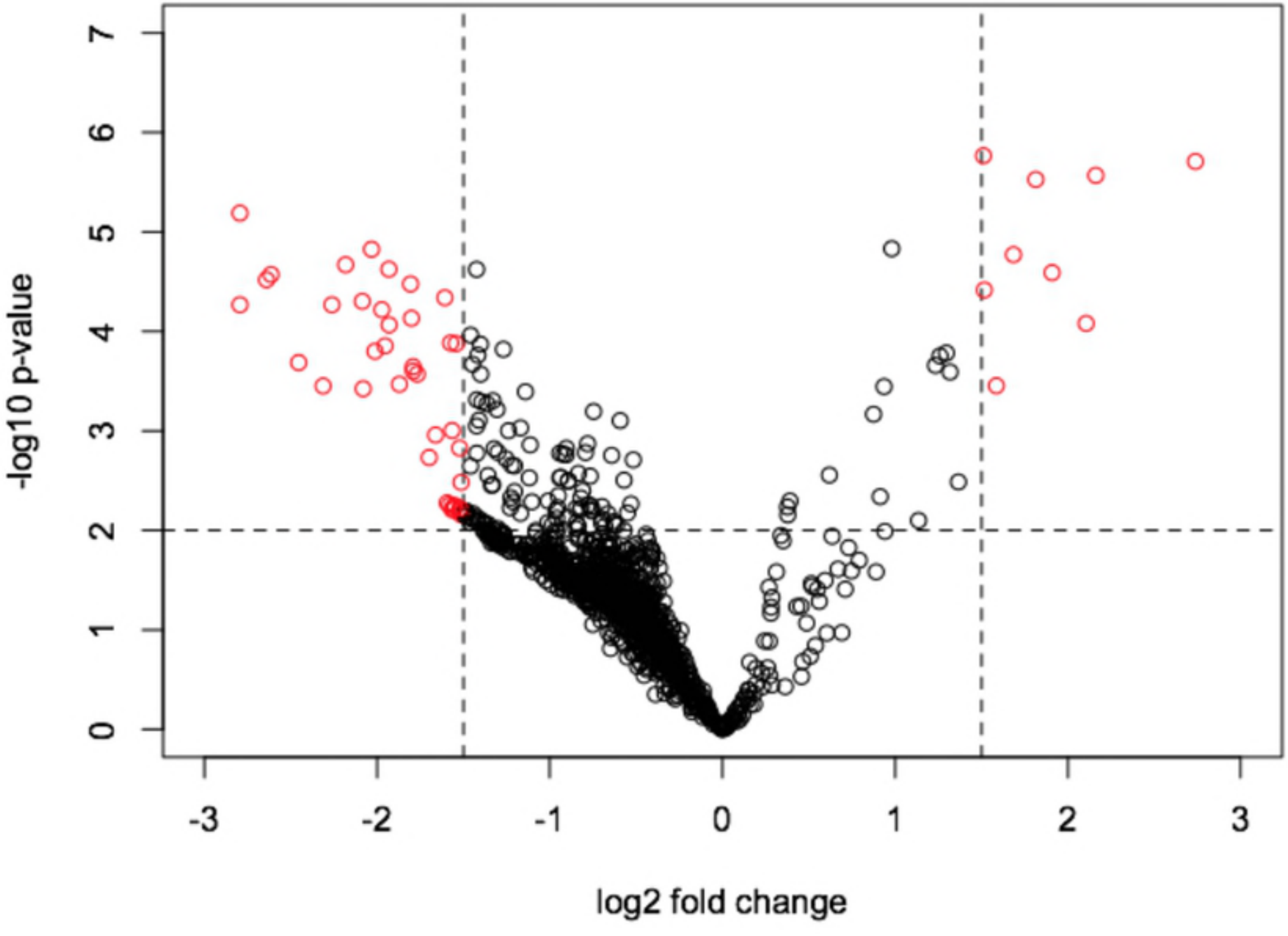
43. Gao ST, Guo J, Quan SY, Nan XM, Fernández MVS, Baumgard LH, Bu DP. The effects of heat stress on protein metabolism in lactating Holstein cows. J Dairy Sci. 2017;100:5040–9. pmid: 28390717.

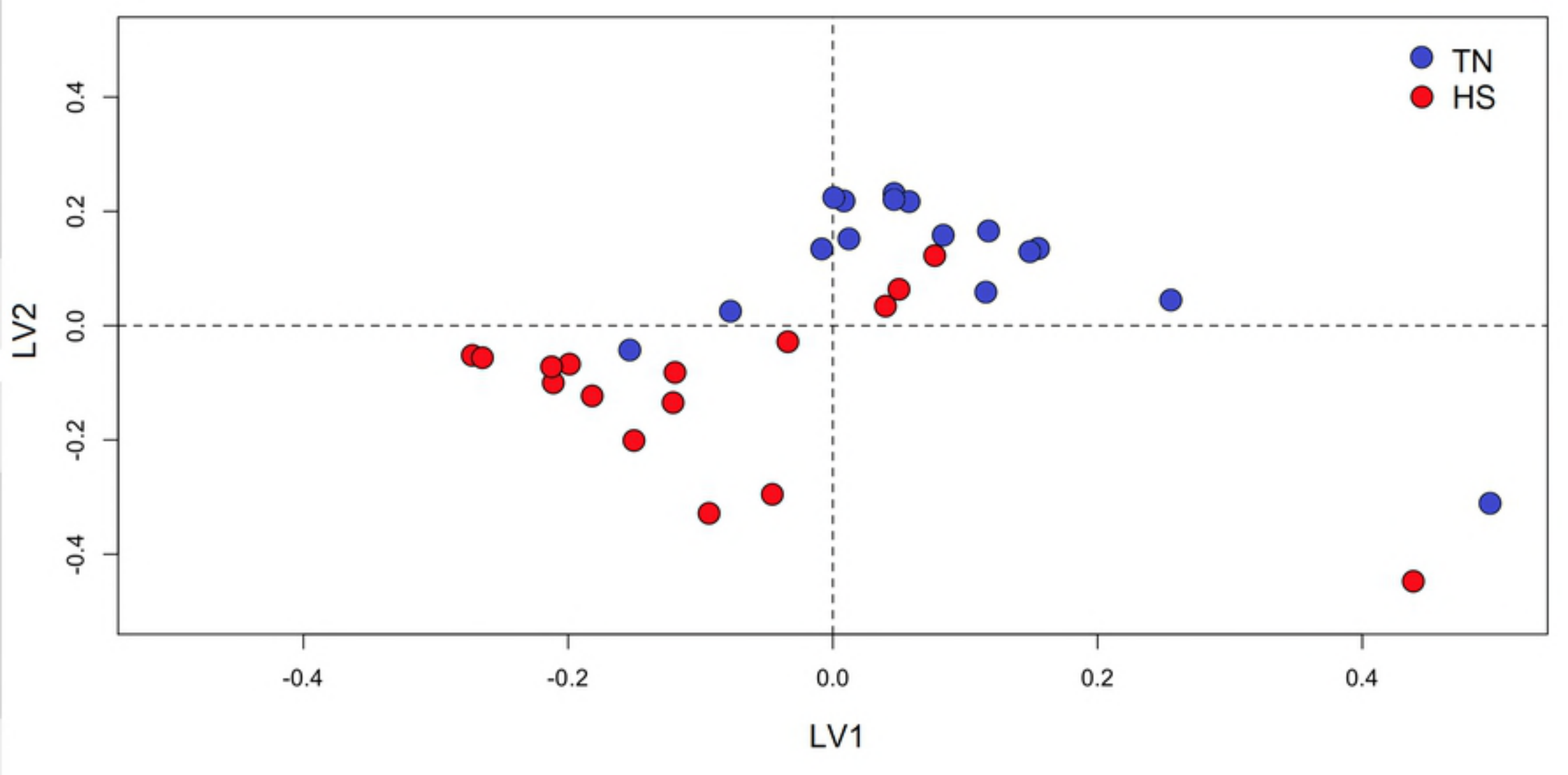
44. Alamer M. The role of prolactin in thermoregulation and water balance during heat stress in domestic ruminants. Asian J Anim Vet Adv. 2011;6:1153–69.

Supporting information

S1 Dataset. ^{1}H NMR data matrix of normalized and binned spectral data. HS, heat-stressed lactating dairy does; TN, thermal neutral lactating dairy does.


TN
HS
1
2
3
4
5
6
7
8
9
10
11
12
13
14
9.0 8.6 8.2 7.8 7.4 7.0 6.6 6.2 5.8 5.4 5.0 4.6 4.2 3.8 3.4 3.0 2.6 2.2 1.8 1.4 1.0 0.6 0.2
9.0 8.6 8.2 7.8 7.4 7.0 6.6 6.2 5.8 5.4 5.0 4.6 4.2 3.8 3.4 3.0 2.6 2.2 1.8 1.4 1.0 0.6 0.2
Chemical shift (ppm)


-log10 p-value
0
1
2
3
4
5
6
7
-3
-2
-1
0
1
2
3
log2 fold change


TN
HS
LV2
LV1
0.4
0.2
0.0
-0.2
-0.4
-0.4
-0.2
0.0
0.2
0.4