Universitat Autònoma de Barcelona

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DOCTORAL THESIS

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Tesi doctoral presentada per Alexandra Contreras Jodar, dirigida pel Dr. Gerardo Caja López i Dr. Ahmed Salama del Departament de Ciència Animal i del Aliments de la Universitat Autònoma de Barcelona.

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Vist i plau

Dr. Gerardo Caja López

Dr. Ahmed A.K. Salama

A la meva família per recolzar-me en totes i cada una de les meves decisions. Per la seva entrega incansable i infinita que tant he agraït en la recta final de la meva tesi.

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SHORT BIOGRAPHY

Alexandra Contreras Jodar was born in Barcelona in 1985, she graduated in Agricultural Engineering in 2009 at the Polytechnic University of Catalonia, with an award for her degree project. Then she performed a Master's degree in Research in Animal and Food Science at Autonomous University of Barcelona where she was subsequently hired at the Animal Nutrition and Welfare Service as researcher assitant. In 2012, she obtained a FPI fellowship from the Spanish Ministry of Economy and Business to perform a Master's degree in Bioinformatics for Genomics and Drug Design and a PhD at the Animal and Food Science Department of the Autonomous University of Barcelona. During the last four years, she also stayed abroad for a short-term period at the Department of Health, Animal Science and Food Safety of the University of Milan and the Mammalian NutriPhysioGenomics Laboratory at the University of Illinois at Urbana-Champaign.

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LIST OF ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
ADH	Aldosterone hormone
ATP	Adenosine triphosphate
BHBA	β-hydroxybutyrate
BW	Body weight
CRH	Corticotropin-releasing Hormone
DM	Dry matter
FC	Fold change
GH	Growth hormone
GWAS	Genome-wide association studies
HPA	Hypothalamic-pituitary-adrenal
HPT	Hypothalamic-pituitary-thyroid
HS	Heat stress
HSE	Heat Shock Element
HSF	Heat Shock transcription Factor
HSP	Heat Shock Protein
IPCC	Intergovernmental Panel on Climate Change
LPS	Lipopolysaccharide
LV	Latent variable
MCFA	Medium-chain fatty acids
MDA	Malondialdehyde
mtDNA	Mitochondrial DNA
NADPH	Nicotinamide adenine dinucleotide phosphate
NEFA	Non-sterified fatty acids
NMR	Nuclear Magnetic Resonance
NO	Nitric oxide
NOS	Nitric oxide synthase
NRC	National Research Council
PFTN	Pair-fed thermoneutral
PLS-DA	Partial Least Squares-Discriminant Analysis
PRL	Prolactin
PVN	Paraventricular nucleus

ROS	Reactive oxygen species
RR	Respiratory rate
RT	Rectal temperature
SAM	Sympathetic-adrenal-medullary
SCC	Somatic cell count
SCFA	Short chain fatty acids
SNP	Single nucleotide polymorphism
T3	Triiodothyronine
T4	Thyroxine
TCA	Tricarboxylic acid
THI	Temperature Humidity Index
TN	Thermal neutral
TRH	Thyrotropin-releasing hormone
TSH	Thyrotropin hormone

The use of omic-methodologies in the assessment of heat-stressed lactating dairy goats

Heat stress (HS) causes significant losses in the dairy goat industry when temperature-humidity index (THI) is >75. 'Omic' technologies offer a holistic approach to figure out how goats cope with HS and to find biomarkers. With this aim, 3 experiments (Exp.) were carried out using Murciano-Granadina dairy goats and a climatic chamber with metabolic boxes. Lactating does (n = 32) were fed a total mixed ration, freely watered and milked ×1 daily under different climatic conditions. They were: TN (thermal neutral, THI = 59-65) and HS (day, THI = 86; night, THI = 77). Photoperiod (light-dark) was constant (12-12 h). Physiological and performance traits were recorded daily, milk composition sampled weekly and BW at the start and the end of each Exp. period.
In Exp.1, changes in blood transcriptome of 2 groups of 4 does (n = 8), under TN or HS were studied for 35 d. In addition to performance impairment, microarrays of blood samples at d 35, revealed that HS up-regulated 55 genes and down-regulated 88. Dynamic Impact Approach analysis showed 31 biological pathways affected by HS. Effects were negative in these related with leukocyte transendothelial migration, cell adhesion, hematopoietic cell lineage, Ca and PPAR signaling, whereas were positive on those activating nucleotide metabolism. In conclusion, HS worsened milk performances and altered the functionality of immune cells, which may result in a less competent immune system for fending-off diseases. In Exp.2, HS candidate biomarkers in urine were assessed by ^{1}H NMR (proton Nuclear Magnetic Resonance)-based metabolomics. Does (n = 16) were submitted to the TN and HS conditions in a crossover design lasting 35 d. Partial least square-discriminant analysis with cross validation were used to separate between TN and HS clusters. Discriminating metabolites were Phenilalanine (Phe) derivative toxic compounds (OH-phenylacetate, OH-phenylacetylglycine, phenylglyoxylate and hippurate), which increased in HS vs. TN does. Increased urinary excretion of these compounds indicated a harmful gastrointestinal microbiota overgrowth by HS, which sequestrated dietary aromatic amino acids. Consequently, HS does should have decreased the synthesis of neurotransmitters and thyroid hormones, impairing milk yield and composition. In conclusion, lactational impairment of HS does was reflected in their metabolome by the presence of gut-derived toxic compounds in urine. Phe derivatives and hippurate were identified as key urinary biomarkers of HS dairy goats. In Exp.3, lactating dairy goats (n = 8) were submitted to the TN and HS conditions for 15 d and milk candidate biomarkers assessed by ^{1}H NMR. On d 12, does were challenged with *E. coli* lipopolysaccharide (LPS) or saline (CON) by udder-half and milk samples collected post-challenge (h 0, 4, 6, 12 and 24). Treatments were: TN (CON and LPS) and HS (CON and LPS). Milk citrate increased in HS revealing a shift in macrophages' function (i.e., transporting mitochondrial citrate to cytosol to produce inflammatory mediators). Differences between TN and HS in response to LPS over time where observed by PLS-DA. Milk metabolome in TN-LPS udder halves was less affected and restored earlier than in HS-LPS halves. Most discriminating metabolites were choline, N-acetylcarbohydrates, L-lactate, β-hydroxybutyrate (BHBA) and phosphocholine. Overall, milk metabolomic profiles were markedly affected by ambient and udder health conditions. Citrate and choline indicated the occurrence of oxidative and inflammatory stages in HS and LPS stressed mammary glands, respectively, and were proposed as key biomarkers in milk.

Ús de metodologies 'òmiques' en l'avaluació de l'estrés per calor en cabres en lactació

L'estrés per calor (EC) genera pèrdues significatives en l'industria lletera caprina quan l'índex de temperatura-humitat (THI) és > 75. Les tecnologies '*òmiques'* ofereixen una perspectiva holística de com les cabres combaten l'EC i poden permetre trobar biomarcadors. Amb aquest objectiu, es van portar a terme 3 experiments (Exp.) utilitzant cabres lleteres Murciano-Granadines i una càmbra climàtica amb caixes metabòliques. Les cabres lleteres (n = 32) es van alimentar amb una ració total mesclada i es van munyir ×1 diàriament sota dos condicions climàtiques. Aquestes foren: TN (termo-neutralitat, THI = 59-65) i EC (dia, THI = 86; nit, THI = 77) amb fotoperíode (llum-foscor) constant (12-12 h). Es van controlar a diari diferents variables fisiològiques i productives, setmanalment la composició de la llet i a l'inici i al final de tots els períodes Exp, el pes corporal. En l'Exp.1, es van estudiar els canvis transcriptòmics en sang de 2 grups de 4 cabres (n = 8) sota TN o EC durant 35 dies. Els microarrays en mostres de sang van revelar que l'EC va augmentar l'expressió de 55 gens i en va reduir la de 88 gens. L'anàlisi *Dynamic Impact Approach* va indicar 31 rutes biològiques afectades per EC. Els efectes van ésser negatius per a la migració transendotelial dels leucòcits, l'adhesió cel·lular, el llinatge de cèl·lules hematopoiètiques, i la senyalització de Ca i PPAR, mentre que es va activar el metabolisme dels nucleòtids. En conclusió, l'EC va afectar negativament a la producció de llet i va alterar la funcionalitat de les cèl·lules immunitàries, fet que podria resultar en un sistema immunitari amb menor capacitat per combatre malalties. En l'Exp.2, es van avaluar candidats de biomarcadors d'EC en orina mitjançant ^{1}H RMN (Resonància Magnètica Nuclear de protons). Les cabres (n = 16) es van sotmetre a les condicions de TN i EC segons un disseny *crossover* durant 35 d. Es va emprar un anàlisi de mínims quadrats discriminatoris parcials (PLS-DA) amb validació creuada que va permetre separar els grups TN i EC. Els metabòlits discriminants entre grups van ser derivats tòxics de la fenilalanina (Phe) (OH-fenilacetat, OH-fenilacetilglicina, fenilglioxilat i hipurat) que van augmentar en EC, respecte a TN. Una major excreció d'aquests compostos en orina va indicar que l'EC indueix un sobrecreixement de la microbiota gastrointestinal nociva segrestant els aminoàcids aromàtics de la ració. En consequència, l'EC hauria de disminuir la síntesi de neurotransmissors i hormones tiroidees, abaixant el rendiment i la composició de la llet. En conclusió, la minvada lletera de les cabres EC es va reflexar en el seu metaboloma per la presència de compostos tòxics derivats del tracte gastrointestinal en l'orina. Es van identificar els derivats de Phe i l'hipurat com biomarcadores d'EC en orina de cabres lleteres. En l'Exp.3, les cabres lleteres (n = 8) es van sotmetre a les condicions de TN i EC durant 15 dies i es van avaluar biomarcadors candidats en llet mitjançant ^{1}H RMN. El dia 12, se les va exposar a lipopolisacàrid d'E. *coli* (LPS) o salí (CON) en una de les mamelles i es varen prendre mostres de llet a les 0, 4, 6, 12 y 24 h. El citrat en llet va augmentar en EC indicant un canvi en la funció mitocondrial dels macròfags (i.e. transport de citrat de la mitocòndria al citosol para produir mediadors inflamatoris). El metaboloma en llet de les mamelles TN-LPS va quedar menys afectat i va recuperar el seu nivell basal en un període més curt que en les mamelles EC-LPS. Els metabòlits discriminants van ésser la colina, els N-acetil-carbohidrats, L-lactat, β-hidroxibutirat i la fosfocolina. Per tant, el perfil metabolòmic de la llet es va veure molt afectat per les condicions ambientals i de salut de la mamella. El citrat i la colina van indicar l'aparició d'estadis oxidatius e inflamatoris en la glàndula mamària sota EC o LPS, respectivament, pel que es proposen com a biomarcadors en llet.

Uso de metodologías 'ómicas' en la evaluación de estrés por calor en cabras en lactación

El estrés por calor (EC) causa pérdidas significativas en la industria lechera caprina cuando el índice de temperatura-humedad (THI) es >75. Las tecnologías '*ómicas'* ofrecen un enfoque holístico de cómo las cabras se enfrentan al EC y pueden permitir encontrar biomarcadores. Con este objetivo, se llevaron a cabo 3 experimentos (Exp.) usando cabras lecheras Murciano-Granadinas y una cámara climática con cajas metabólicas. Las cabras lecheras (n = 32) se alimentaron con una ración total mezclada y se ordeñaron ×1 diariamente bajo dos condiciones climáticas. Éstas fueron: TN (termo-neutralidad, THI = 59-65) y EC (día, THI = 86; noche, THI = 77) con fotoperiodo (luz-oscuridad) constante (12-12 h). Se controlaron a diario distintas variables fisiológicas y productivas, semanalmente la composición de leche y al inicio y al final de todos los periodos Exp., el peso corporal. En el Exp.1 se estudiaron los cambios transcriptómicos en sangre de 2 grupos de 4 cabras (n = 8) bajo TN o EC durante 35 d. Los microarrays revelaron que el EC aumentó la expresión de 55 genes y la redujo en 88. El análisis *Dynamic Impact Approach* indicó 31 rutas biológicas afectadas por EC. Los efectos fueron negativos para la migración transendotelial de los leucocitos, la adhesión celular, el linaje de células hematopoyéticas, y la señalización de Ca y PPAR, mientras que se activó el metabolismo de nucleótidos. En conclusión, el EC afectó negativamente a la producción de leche y alteró la funcionalidad de las células inmunitarias, lo que podría resultar en un sistema inmunitario con menor capacidad para combatir enfermedades. En el Exp.2, se evaluaron candidatos de biomarcadores para EC en orina mediante ^{1}H RMN (Resonancia Magnética Nuclear de protones). Las cabras (n = 16) se sometieron a las condiciones de TN y EC según un diseño *crossover* durante 35 d. Se utilizó un análisis de mínimos cuadrados discriminatorios parciales (PLS-DA), con validación cruzada, que permitió separar los grupos TN y EC. Los metabolitos discriminantes fueron derivados tóxicos de la fenilalanina (Phe) (OH-fenilacetato, OH-fenilacetilglicina, fenilglioxilato e hipurato), que aumentaron en EC, respecto a TN. Una mayor excreción de estos compuestos en orina indicó que el EC indujo un sobrecrecimiento de microbiota gastrointestinal dañina, secuestrando los aminoácidos aromáticos de la ración. En consecuencia, el EC debió disminuir la síntesis de neurotransmisores y hormonas tiroideas, disminuyendo la producción y composición de leche. En conclusión, el descenso de producción lechera de las cabras EC se reflejó en su metaboloma por la presencia de compuestos tóxicos urinarios procedentes del tracto gastrointestinal. Se identificaron varios derivados de Phe y al hipurato como biomarcadores de EC en la orina de cabras lecheras. En el Exp.3, las cabras lecheras (n = 8) se sometieron a condiciones TN y EC durante 15 d y se evaluaron biomarcadores candidatos en leche mediante ^{1}H RMN. En el día 12, se realizó un reto con lipopolisacárido de *E. coli* (LPS) o salino (CON) en una mitad de la ubre y se tomaron muestras de leche a 0, 4, 6, 12 y 24 h. El citrato en leche aumentó en EC, indicando un cambio en la función mitocondrial de los macrófagos (i.e., transporte de citrato de la mitocondria al citosol para producir mediadores inflamatorios). El metaboloma en leche de las medias ubres TN-LPS quedó menos afectado y recuperó su nivel basal en menos tiempo que en las EC-LPS. Los metabolitos discriminantes fueron la colina, N-acetil-carbohidratos, L-lactato, β-hidroxibutirato y fosfocolina. Por tanto, el perfil metabolómico de la leche se vió muy afectado por las condiciones ambientales y de salud de la ubre. El citrato y la colina indicaron la aparición de estados oxidativos e inflamatorios en glándulas mamarias bajo EC o LPS, respectivamente, por lo que se proponen como biomarcadores en leche.

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CHAPTER 1

Literature review

CHAPTER 1
LITERATURE REVIEW

1.1. Introduction

Livestock might experience heat stress (**HS**) during hot weather, particularly if the heat episodes are prolonged or temperatures are extremely high for short periods. As a result, HS may reduce feed intake, growth, production, reproduction, welfare, and health of the animals, which in turns affects the production potential and the quality of the products, causing relevant economic losses in livestock (St-Pierre *et al.* 2003).

Nowadays, global mean surface temperature is expected to increase 0.3 to 4.8ºC by the end of the 21st century (IPCC, 2014), with greater warming in the Northern hemisphere. Farmers are concerned on the practical consequences and research in this field is needed to understand the HS limits, its signs and effects, as well as the management practices that can mitigate its consequences.

The impact of HS depends on the physiological stage of life cycle and the adaptations of given species and breeds. Thus, ruminants are less susceptible to HS than monogastrics based on their ability to dissipate heat through sweating (West, 2003), their efficient heat exchange in the long nasal cavities and turbinates (Baker and Hayward, 1968) and by the special vascular carotidal-ophthalmic rete (Taylor, 1966) used as an alternative "brain-cooling system" (Elkhawad, 1992). However, among ruminants, those specialized in dairy production are more sensitive. Dairy animals have an antagonistic relationship between milk production traits and resistance to HS because they have to deal with higher levels of heat increment associated to their high level of nutrition which increases their efforts for the regulation of body temperature (Hansen, 2007).

Among domestic ruminant species, goats are thought to be less susceptible to HS than cattle and sheep. They are widely distributed in regions with arid and warm climatic conditions and own unique characteristics such as water conservation capability, higher sweating rate, lower metabolic weight and higher skin temperature (Lu, 1989; Silanikove, 2000). Because of these characteristics, goats are supposed to cope heat stress better than sheep and cattle. However, reduced feed intake, production impairment and lower quality of cheese occur in goats under HS (Hamzaoui *et al.*, 2013; Abdel-Gawad, 2012).

Despite goat milk represented less than 2.5% of the world's total milk production in 2017 (828 Mt) (FAOSTAT, 2019), worldwide goat milk production has more than doubled during the last 50 yr and, if this trend is maintained, it is expected to increase by approximately 9.7 Mt (53%) by 2030 (Pulina *et al.*, 2018). Dairy goat farms spread on subtropical-temperate areas of Asia, Europe, and Africa. Although dairy goat's farms are mainly located in low-income, food-deficit countries, where their products are a primary food source, they are also present in high-income and technologically developed countries where goat's milk is sold as high quality and gourmet food (Silanikove *et al.*, 2010; Pulina *et al.*, 2018).

Consequently, in addition to the expectation of global warming, the problem of HS have grown due to the genetic improvement of animals with higher milk yield during the last decades and the greater demand of dairy products due to increase in world's population (Godfray and Garnett, 2014). Moreover, this demand also increased as the income in many countries increased (Haq and Ishaq, 2011), particularly in China (Qian *et al.*, 2011).

Despite the large number of studies on dairy cows, little is known on the effects of HS in dairy goats and other small ruminants, where a rapid diagnosis of HS symptoms and strategies for mitigation are needed.

1.2. Measuring heat stress level

1.2.1. Measurements on animals

Monitoring animal thermophysiological traits (e.g., body temperature, respiratory, heart or sweating rate) is a key point to detect thermal stress. Increased body temperature is the critical reaction when animals are exposed to environmental temperatures above the thermoneutral zone (Maurya *et al.*, 2007) and it is an excellent indicator of the animal's susceptibility to thermal stress (Lefcourt *et al.*, 1986, Salama *et al.*, 2016). Body temperature is widely used to evaluate the thermal balance and usually monitored as rectal (**RT**), vaginal, tympanic or rumen temperatures (Lees *et al.*, 2018). However, each body temperature measurement location has either physical, logistical or physiological limitations. Moreover, many physiological and environmental factors affect body temperature making detection of outliers challenging (Halachmi *et al.*, 2019).

In intensive conditions, several approaches to measure body temperature have been

used in cattle, such as temperature-sensing ear tags, intravaginal and intrarectal thermosensor devices, reticulum rumen boluses, and thermal imaging cameras (Caja *et al.*, 2016; Koltes *et al.*, 2018). However, technical difficulties increase when body temperature needs to be measured under extensive conditions (Salama *et al.*, 2016).

When body temperature increases due to thermal load, a common animal strategy to dissipate body heat is through evaporation by increasing respiration rate (**RR**) and sweating. In practice, measuring RT or RR for each individual animal can be difficult and time-consuming. For that reason, measuring 10% of animals in the herd, or even a few animals, would be enough to detect HS (Salama *et al.*, 2016). However, it should be taken into consideration that dairy animals undergo periods of their lifespan that are more sensitive to HS than other periods. These include the neonatal and early growth, reproduction and lactation periods (Collier *et al.*, 1982) as well as the production level (i.e., high productive animals are considered more sensitive) and there is the possibility of existence of microclimates at different locations in the same farm. Moreover, an increase in RT is not always accompanied by an increase of RR. Thus, Caja *et al.* (2019) pointed out that there is no relationship between these two variables under acute HS (i.e., 2 h length) in dairy ewes (**Fig. 1**).

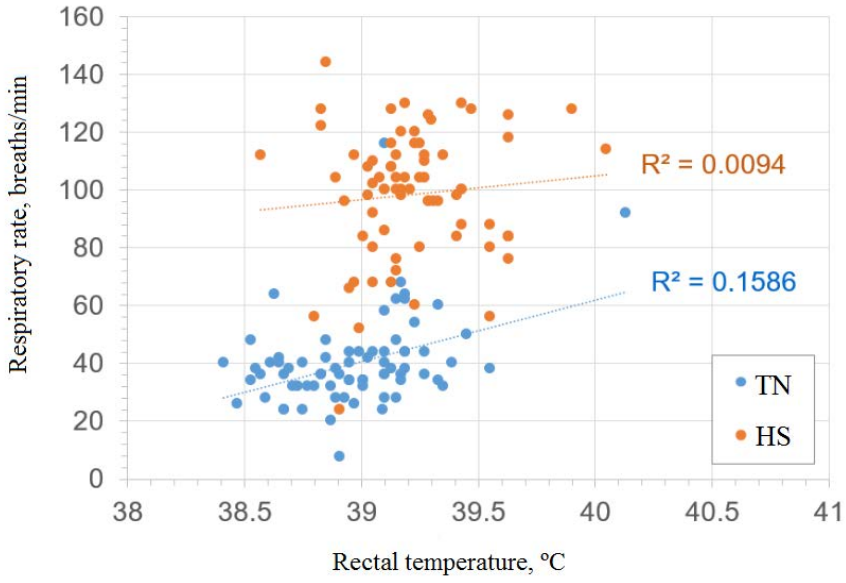


Figure 1. Relationship between temperature and respiration in thermal neutral (TN) and acute heat stress (HS) conditions in lactating dairy ewes (Caja *et al*., 2019).

On the contrary, significant correlations were observed in lactating dairy goats between RT and RR under different environmental conditions and HS lengths (i.e.,

thermal neutral, acute and chronic stresses; **Fig. 2**). However, the correlation becomes weaker along time, as an RT×week interaction was observed ($P < 0.05$), resulting in lower RR values and slope during the second week ($r^2 = 0.09$) compared to the first ($r^2 = 0.34$).

As ambient temperature increases, pulse rate, as well as blood flow, increases to transfer heat from the animal core to the periphery (Marai *et al.*, 2007). Nevertheless, differences in pulse rate were observed in cattle depending on the severity of HS. Thus, Kibler and Brody (1951) reported a decrease in pulse rate under chronic and moderate HS, whereas Whittow (1971) reported increased pulse rate when subjected to acute and severe HS. Therefore, pulse rate is not considered to be a fully reliable indicator for HS, as RT and RR are (Lemerle and Goddard, 1986), although there is some recent controversy.

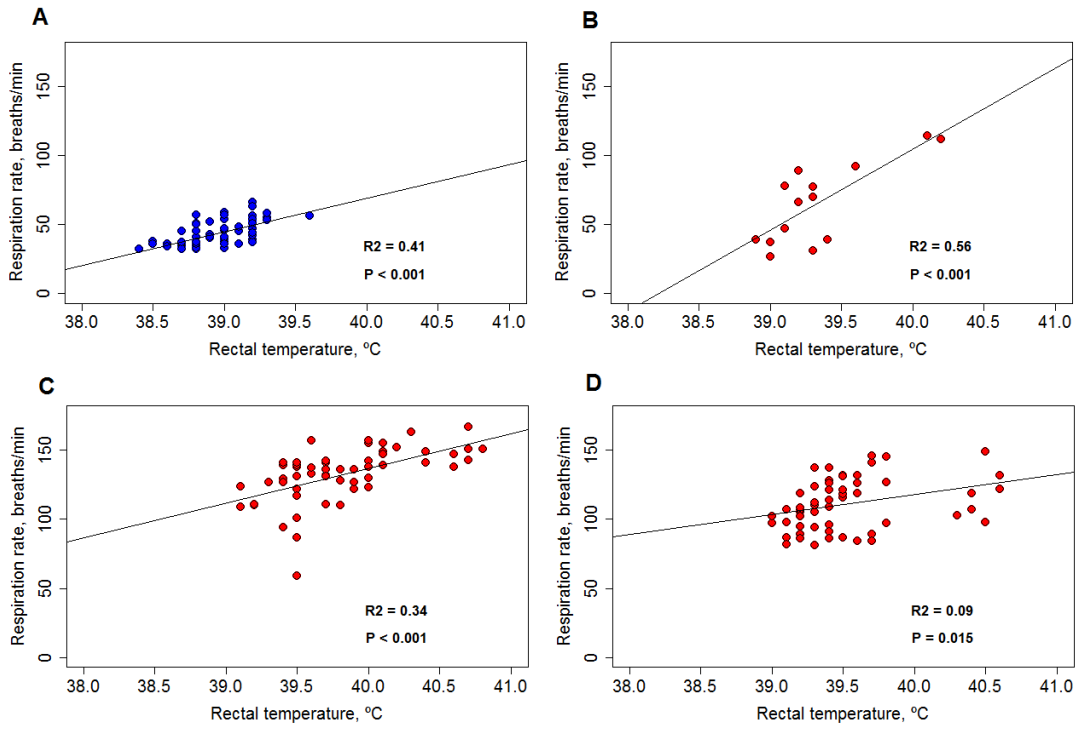


Figure 2. Relationship between temperature and respiration in lactating dairy goats, under: A) thermal neutral, B) acute heat stress, C) chronic heat stress at wk 1, and D) chronic heat stress at wk 2.

Body gain depends on the interaction between genetic background and the environment by well-balanced available nutrients, hormones and enzymes (Marai *et al.,* 2007). Thus, reduction in feed intake is commonly observed under HS in order to

reduce the post-prandial heat production for HS alleviation purposes and consequently, goat body weight (**BW**) is also affected under HS conditions (Hamzaoui *et al.*, 2013; Castro-Costa *et al*., 2015; Pragna *et al*., 2018). Moreover, differences in the impact of HS on feed intake and BW were observed among goat breeds by Pragna *et al*. (2018). Therefore, the magnitude of the changes in both feed intake and BW may explain the adaptive capability of goats under HS conditions.

1.2.2. Temperature humidity indices

As mentioned above, monitoring animals are key points but difficult for a large number of animals and dependent on management conditions. Therefore, a temperature humidity index (**THI**) has been proposed as a general alternative to determine the extent to which the animals are under HS. In this sense, THI is a single value index developed to assess the discomfort related to ambient T combined with relative humidity (**RH**), and indicates the thermal stress intensity. When the ambient T reach levels that causes animal discomfort, RH becomes a key factor in maintaining the homeostasis of the animal as it have impact on the rate of evaporative loss through panting and sweating. Although THI was originally developed by Thom (1959) to detect HS in humans, later on THI was adapted to cattle (Bianca, 1962; Berry *et al.*, 1964; NRC, 1971; Yousef *et al.*, 1985; Mader *et al.*, 2006; Berman *et al.*, 2016) and sheep (Marai *et al.*, 2001).

Different equations have been developed using the dry bulb temperature ($\mathbf{T_{db}}$) along with either wet bulb temperature ($\mathbf{T_{wb}}$), dew point temperature ($\mathbf{T_{dp}}$) and HR to measure water vapor content, as summarized in Table 1.

Using a range of environmental parameters in the different THI equations for cattle shown in Table 1, the calculated values resulted in a strong variability of THI (Table 2). Thus, although the mean coefficient of variation was 14.4%, the greatest coefficient was found when lower T and higher HR occurs, indicating that THI may not be adequate to monitor animals under cold conditions. The equation proposed by Marai *et al.* (2007) for sheep was not included in this discussion because the values are in a completely different scale with regard to those for cattle.

Most of the indices shown in Table 1 were evaluated by Bohmanova *et al.* (2007) as potential predictors of HS and milk yield losses of dairy cattle in two different climates (i.e., hot and semiarid vs. hot and humid) in the USA. They demonstrated that THI indices differ in their ability to detect HS depending on the climate type. Thus, THI indices with larger weights on HR seem to be more suitable for humid climates,

whereas in climates where HR levels that do not compromise evaporative cooling (i.e., panting and sweating), THI indices with most emphasis on T_{db} are desirable (like in most Spanish conditions).

Table 1. List of most known temperature-humidity indices (THI) to monitor thermal discomfort.

Author (year)	Target especies	Equation[1]
Thom (1959)	Humans	$THI = [0.4 \times (T_{db} + T_{wb})] \times 1.8 + 32 + 15$
Bianca (1962)	Humans	$THI = (0.15 \times T_{db} + 0.85 \times T_{wb}) \times 1.8 + 32$
Bianca (1962)	Cattle in chamber	$THI = (0.35 \times T_{db} + 0.65 \times T_{wb}) \times 1.8 + 32$
NRC (1971)	Cattle	$THI = (0.55 \times T_{db} + 0.2 \times T_{dp}) \times 1.8 + 32 + 17.5$
NRC (1971)	Outdoor cattle	$THI = (0.81 \times T_{db} + 0.143 \times RH + 0.0099 \times T_{db} \times RH) + 46.3$
Yousef (1985)	Cattle in chamber	$THI = T_{db} + 0.36 \times T_{dp} + 41.2$
Marai *et al.* (2007)	Sheep	$THI = T_{db} - (0.31 - 0.31 \times RH) \times (T_{db} - 14.4)$
Mader *et al.* (2006)	Cattle	$THI = (0.8 \times T_{db}) + (RH / 100) \times (T_{db} - 14.4) + 46.4$
Berman *et al.* (2016)	Cattle warm-humid	$THI = 3.43 + (1.058 \times T_{db} - 0.293 \times RH + 0.0164 \times T_{db} \times RH) + 35.7$

[1] T_{db} = dry bulb temperature (ºC), T_{wb} = wet bulb temperature (ºC), T_{dp} = dew point temperature (ºC) and RH = relative humidity (%).

In an attempt to improve these equations, some authors tried to develop new thermal indices using RR and RT, as dependent response variables, by applying multiple regressions in formulas containing ambient temperature and radiation (Yamamato *et al.*, 1994). These authors identified solar radiation as a crucial environmental parameter with regard to heat load of animals. In addition, other scientists developed thermal indices including solar radiation, wind speed and rainfall, as well as duration of exposure (Gaughan *et al.*, 2008; Zimbelman *et al.*, 2009; Mader *et al.*, 2010). However, the proposed formulas include too many variables not easy to collect on the field and with no advantages over other THI equations, as concluded by Zimbelman *et al.* (2009).

Table 2. Values of temperature-humidity indices (THI) for cattle according to the source and ambient conditions.

Ambient conditions				THI equations						Descriptive statistics		
T_{db}, ºC	HR, %	T_{wb}, ºC	T_{dp}, ºC	Bianca (1962)	NRC (1971)	NRC (1971)	Yousef (1985)	Berman *et al.* (2016)	Mader *et al.* (2006)	Mean	SD	CV, %
0	20	-4.5	-20	26.8	42.3	49.2	34	43.5	33.3	38.2	8.2	22
	50	-2.7	-9	28.8	46.3	53.5	38	39.2	24.5	38.4	10.7	28
	80	-1.1	-3	30.8	48.4	57.7	40.1	34.9	15.7	37.9	14.6	38
10	20	2.6	-12	41.4	55.1	59.2	46.9	53.5	47.1	50.5	6.5	13
	50	5.6	0	44.9	59.4	66.5	51.2	52.2	43.3	52.9	8.8	17
	80	8.3	7	48	61.9	73.8	53.7	50.9	39.4	54.6	11.9	22
20	20	9.4	-3	55.6	68.2	69.3	60.1	63.5	61	63	5.2	8
	50	13.8	10	60.8	72.9	79.6	64.8	65.2	62	67.6	7.2	11
	80	17.7	17	65.3	75.4	89.8	67.3	66.9	63.1	71.3	10	14
30	20	15.8	5	69.4	81	79.4	73	73.5	74.9	75.2	4.3	6
	50	22.1	18	76.7	85.7	92.6	77.7	78.2	80.8	82	6.1	78
	80	27.1	26	82.6	88.6	105.8	80.6	82.9	86.8	87.9	9.3	11
40	20	22.2	13	83.1	93.8	89.5	85.9	83.5	88.7	87.4	4.1	5
	50	30.4	27	92.7	98.8	105.7	90.9	91.2	99.6	96.5	5.9	6
	80	36.6	36	100	102.1	121.8	94.2	98.9	110.5	104.6	10	10

[1] T_{db} = dry bulb temperature (ºC), T_{wb} = wet bulb temperature (ºC), T_{dp} = dew point temperature (ºC) and RH = relative humidity (%).

The large differences of THI values among authors shown in Table 2 evidence the limitations of using THI as the unique indicator of HS. The THI weaknesses is attributed to its assumption that all animals react similarly to environmental stressors, and because it does not take into account the breed, the genotype, age or level of production (Hammami *et al.*, 2013; Serradilla *et al.*, 2017). Moreover, the THI do not differentiate climates or production systems nor the statistical models and methods used to detect the critical limits in each study (Carabaño *et al.*, 2016) but also it does not take into account that animals are capable to acclimate or adapt to HS conditions. Thus, THI that causes HS in early spring may not cause HS at the end of summer (L.H. Baumgard, personal communication, June 12, 2019).

In any case, THI from the NRC (1971) is still the most used equation in cattle worldwide and several authors made efforts to create thresholds depending on the effect of THI on milk yield and milk composition in both dairy cows and goats. There is no consensus on the THI upper critical limit value for milk yield. In this sense, some authors set the upper critical limit around a THI = 60-62 in Central Europe (Hammami *et al.*, 2013; Gorniak *et al.*, 2014; Lambertz *et al.*, 2014), although a higher upper critical limit (i.e., THI = 73-76) was found for Holstein dairy cows in the Mediterranean region (Bernabucci *et al*., 2014; Carabaño *et al.*, 2014).

In dairy goats, studies have been carried using the THI developed for dairy cattle because of the lack of THI designed specifically for goats. Moreover, fewer studies are carried out to describe at which THI threshold the milk production decays. Vitaliano *et al.* (2012) proposed THI > 79 as a dangerous condition for Saanen goats, whereas Silanikove and Koluman (2015) set THI > 80 as modest effect on milk yield, and a danger effect at THI > 85. However, exposure of dairy goats to THI = 79 decreased milk yield in Alpine but not in Nubian goats (Brown *et al.*, 1988); so differences between authors and in the genetic background for adaptive traits and milk production should be taken into account and invalidate the generalization of the previously proposed thresholds.

Salama *et al.* (2016) pointed out that the criteria for identifying the upper critical THI may be not well defined and proposed to use the THI value that causes rectal temperatures above 39.0ºC. However, in a recent study on Murciano-Granadina dairy goats, values of THI = 74 caused a RT = 39.4ºC with greater water intake and respiratory rate, but without impact on feed intake or milk production (Caja *et al.*, 2019).

1.3. Responses of ruminants to heat stress

Most animals exhibit several behavioral responses to adapt to HS conditions (i.e., shade seeking, amount of water consumption and drinking frequency, standing, lying and feeding time, defecation and urination frequency) as indicated by Ratnakaran *et al.* (2017). Seeking for shade is a common behavioral response which helps ruminants to avoid direct solar radiation. When heat load increases, ruminants tend to reduce lying time and to increase standing time (Schutz *et al.*, 2010; Hamzaoui, 2014). These positional changes increase their body surface which promote evaporative heat loss and avoid radiative and convective heat from the ground (Kamal *et al.*, 2016).

Feeding ruminants during the hottest hours of the day generally increases the metabolic heat increment and their body temperature. Consequently, under HS they tend to reduce their feed intake (Kadzere *et al.*, 2002; Brown-Brandl *et al.*, 2006) and rumination (Collier *et al.*, 1982), which contribute to decline rumen pH by altering the ratio acetate/propionate and decreasing the total volatile fatty acid production (Collier *et al.*, 1982) and the productive performance of cattle (Tapki and Sahin, 2006).

Water consumption dramatically increases during severe hot environmental conditions. Water is vital under HS and it seems to be preferred with a moderate temperature, neither too cold nor too hot (Machado *et al.*, 2004). Despite this, many experts recommend offering cold water during HS or water below the body temperature, with the aim of reducing the total heat load, but it looks to be a bad recommendation.

While animals modify their behavior to cope with the environmental conditions, their physiology is also modified. So, the molecular, cellular and metabolic responses reported under HS conditions are thereafter described.

1.3.1. Molecular responses to heat stress

1.3.1.1. Oxidative stress

Heat stress is known to cause a disturbance in the cell steady-state concentrations of pro-oxidants and antioxidants in the cells whose lead to an over-production of free radicals and by-products of the oxygen (i.e., reactive oxygen species or **ROS**) and nitrogen metabolism (i.e., reactive nitrogen species or **RNS**) inducing oxidative and nitrosative stress. A summary of the main ROS and RNS formed under hyperthermia and their oxidant properties are shown in Table 3.

Table 3. Main reactive oxygen (ROS) and nitrogen (RNS) species (adapted from Di Meo *et al.*, 2016).

Species	Symbol	Name	Properties
ROS	O^{-}_{2}	Superoxide anion	Powerful oxidant
	OH^{-}	Hydroxyl	Extremely reactive
	H_2O_2	Hydrogen peroxide	Oxidant, diffuses across membranes
RNS	$NO^{\cdot}$	Nitric oxide	Relatively unreactive
	$NO_2^{\cdot}$	Nitrogen dioxide	Strong oxidant
	$ONOO^{-}$	Peroxynitrite	Powerful oxidant

ROS are generated in several cellular systems localized on the plasma membrane, in the cytosol, in the peroxisomes, and on membranes of mitochondria and endoplasmic reticulum. This occurs because HS leads to the production of transition metal ions through increasing the rate of iron release from blood ferritin. These ions make electron donations to oxygen (O_2), forming H_2O_2 and O^{-}_{2}. While H_2O_2 is reduced to OH^{-} radicals (Liochev and Fridovich, 1999), the O^{-}_{2} is precursor of ROS and mediator in oxidative chain reactions (Turrens, 2003). On the other hand, ROS are also generated by the activation of nicotinamide adenine dinucleotide phosphate (**NADPH**) oxidase caused by HS. The NADPH oxidase converts NADPH to NADP+, increasing the mitochondrial NADP+/NADPH ratio (Moon *et al.*, 2010). Furthermore, thiols, hydroquinones, catecholamines and flavins are able to undergo redox reactions, producing ROS in the cytosol (Freeman and Capo, 1982).

RNS are formed in mitochondrias and peroxisomes (reviewed by Di Meo *et al.*, 2016) because over-production of ROS stimulates the mitochondrial and peroxisomal nitric oxide synthase (NOS) which stimulates the breakdown of arginine to citrulline and releases NO (Tatoyan and Giulivi, 1998). The formation of NO· binds to heme groups from cytochromes oxidase inhibits mitochondrial respiratory chain that, in turn, enhances O^{-}_{2} formation (Poderoso *et al.*, 1996). Moreover, the reaction of NO· with O^{-}_{2} formed in the mitochondrial respiratory chain drives to the pathological inhibition of mitochondrial function by $ONOO^{-}$ (Radi *et al.*, 2002).

Uncontrolled production of both ROS and RNS leads to free-radical-mediated chain reactions that indiscriminately target DNA (Richter *et al.*, 1988), proteins (Stadtman and Levine, 2000) and lipids (Rubbo *et al.*, 1994) that triggers the regulation of the pathways of apoptosis (Belhadj Slimen *et al.*, 2014) and autophagy (Filomeni *et al.*, 2015). However, there are organisms adapted to a coexistence with ROS and RNS by

utilizing them as signaling molecules that regulate a wide variety of physiological functions that enable the cells to survive when normally it should be lethal. Thus, ROS and RNS play a crucial role in gene activation (mainly the Heat Shock Response), cellular growth, and modulation of chemical reactions in the cell. Moreover, ROS and RNS are upstream regulators of the antioxidant capacity of the cells by increasing the mitochondrial expression of antioxidant enzymes (i.e., SOD or superoxide dismutase; CAT or catalase; and GPX or glutathione peroxidase) and increasing the use of endogenous antioxidants reserves, like glutathione and vitamins E, C and A (Lallawmkimi, 2009; Kumar *et al.*, 2011; Pandey *et al.*, 2012). A general picture of the mechanisms involved in the molecular response under hyperthermia are shown in **Fig 3**.

Accordingly, higher antioxidant enzyme levels improve the thermal resistance (Omar *et al.*, 1987) as well as some nutritional strategies to alleviate HS effects recommend the supplementation of antioxidants in different animal models (Sahin *et al.*, 2003; Megahed *et al.*, 2008; Ajakaiye *et al.*, 2011; Alhidary *et al.*, 2012; McKee and Harrison, 2013).

1.3.1.2. Mitochondrial damage

Mitochondria are subcellular organelles that sustain life through energy transformation and intracellular signaling, and are a key component of the stress response; moreover, they are the main cellular component of oxygen metabolism and account for 85 to 90% of the oxygen consumed by the cell (Chance *et al.*, 1979).

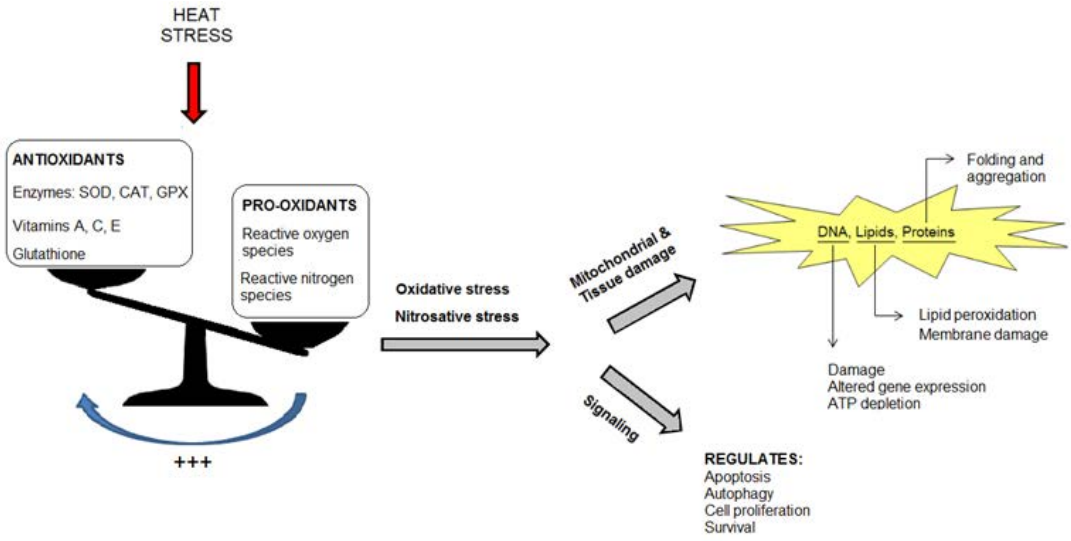


Figure 3. Molecular response to oxidative and nitrosative stresses caused by hyperthermia.

Because of their bacterial origin, mitochondria are the only organelle who contain their own genome, with lack of introns, being more susceptible to damage in comparison to nuclear genome (Yakes and Van Houten, 1997). The mitochondrial genome (**mtDNA**) encodes essential genes for energy production of the respiratory chain.

The respiratory chain is located in the inner mitochondrial membrane called *cristae* (crests) and contains 5 enzyme complexes - I, II, III, IV and V - that transport energy in the form of electrons derived from nutrients, initially catabolized by TCA cycle and β-oxidation. The energy used from electron transport stored across the cristae is used for several functions, being these the adenosine triphosphate (**ATP**) production, which fuels most cellular reactions involved in gene expression, chromatin remodeling, ion homeostasis, protein and hormone synthesis, secretion, neurotransmitter release and reuptake, and muscle contraction, among others. Thus, mitochondria influence gene expression within the cell nucleus and the physiological regulation across the organism (Picard and McEwen, 2018).

When the steady state concentration of ROS is disturbed, mitochondria are the first cellular organelles to be damaged and their functions are strongly impaired. Mitochondrial membrane constituents are particularly sensitive to oxidative damage by ROS. The major phospholipid components of the mitochondrial membranes are rich in polyunsaturated fatty acids (**PUFA**), which ROS undergo peroxidation in the double bonds through a chain of oxidative reactions (Belhadj Slimen *et al.*, 2014). Malondialdehyde (**MDA**) is the main product of PUFA peroxidation. The MDA reacts with DNA bases causing gene mutations (Trachootham *et al.*, 2008).

HS causes mitochondrial protein oxidation and denaturation, which is triggered by ROS (Belhadj Slimen *et al.*, 2014; Tomanek, 2015; Klumpen *et al*., 2017). Uncontrolled levels of ROS induce the inactivation of the respiratory chain, via the oxidation of complexes I, II, IV and V (England *et al.*, 2004). Moreover, the over-production of ROS caused by hyperthermia not only oxidize the mitochondrial proteins and lipids causing mitochondrial damage, but also induces intra-mitochondrial Ca^{2+} overload causing cytotoxicity and triggering apoptotic cell death (Orrenius *et al.*, 2003) through the opening of permeability transition pores and the activation of initiators and effectors of apoptotic proteins, such as the Bcl-2 family members and caspases (Crompton *et al.*, 2002).

Hsu *et al.* (1995) and Lewandowska *et al.* (2006), among other authors, reported

histological abnormalities and altered morphology of mitochondria under HS conditions. Mainly, changes in location (Hsu *et al.*, 1995) and structure (Song *et al.* 2000) of mitochondrias. Thus, under HS, mitochondria aggregates are observed within the subsarcolemmal space, swollen with their inner membrane (i.e., cristae) broken, along with lower matrix density. The mitochondrial matrix contains enzymes of the Kreb cycle and β-oxidation pathway that metabolize energy food substrates. Consequently, mitochondrial damage may be responsible of the cell inability to meet the increased energy requirements of HS animals (Belhadj Slimen, 2016). Mitochondrial damage caused by HS is summarized in **Fig. 4**.

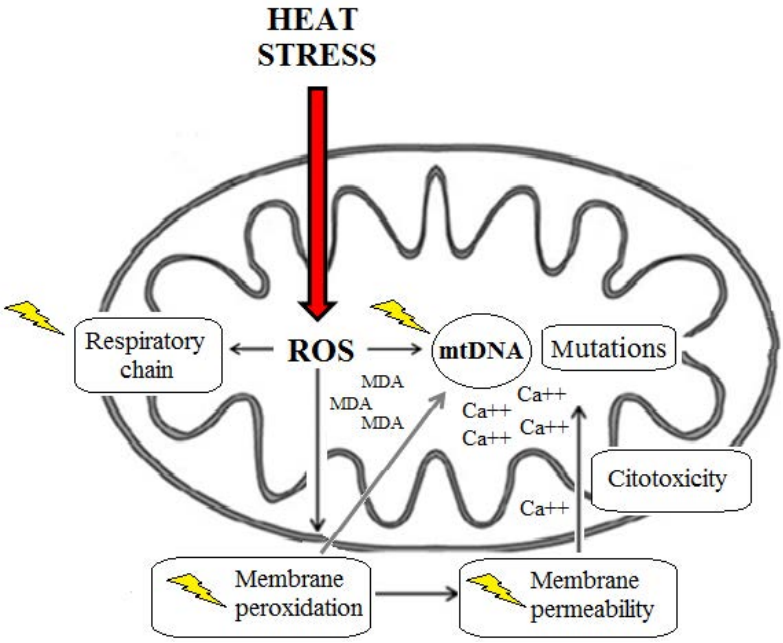


Figure 4. Reactive oxygen species (ROS) and mitochondrial damage produced by heat stress.

HS can lead to mitochondrial damage through overproduction of ROS. It includes mutations in mtDNA, shutdown of mitochondrial energy production through damaged mitochondrial respiratory chain, mitochondrial membrane permeability, and elevations of Ca^{2+} which disturbs Ca^{2+} homeostasis.

1.3.1.3. Proteostasis

Cellular exposure to hyperthermia induces oxidative stress that drives to anomalies in cellular function (Sonna *et al.*, 2002), which include defects in protein structure,

morphological changes due to cytoskeleton rearrangements, shifts in metabolism, alterations in cell membrane dynamics and fluidity. The excessive ROS production activates an activation of transcription factors by a signaling cascade (Katschinski *et al.*, 2000).

These alterations invoke large changes in gene transcription and protein synthesis known as the Heat Shock Response (**HSR**); timing and success of these alterations determine cell survival and adaptation or cell death (reviewed by Collier *et al.*, 2008). In this way, the HSR involves a general inhibition of protein synthesis, except for the activation of the heat shock transcription factors (**HSFs**). Thus, HSFs binds to the heat shock elements (**HSEs**) located in the promoter region of the heat shock genes inducing the expression of the heat shock protein (**HSP**) genes.

Several isoforms of HSFs are present in different livestock species. The isoforms HSF-1 to HSF-4 have been reported till date in large eukaryotes. Isoform HSF-2 is activated by other cellular stress than thermal stress, HSF-3 has been reported to be present only in poultry and HSF4 acts as a repressor of certain genes during HS (Nakai *et al.*, 1997; Tanabe *et al.*, 1997). However, HSF-1 has been mainly studied and reported to have a direct correlation with thermotolerance in livestock (Archana *et al.*, 2017).

HSPs are a large group of chaperone proteins that are classified into several families according to their molecular size and amino acid similarity (e.g., Hsp27, Hsp60, Hsp70, Hsp90, Hsp100). The HSPs functions are related to the correct folding, unfolding and refolding of denatured proteins caused by ROS and NOS stresses, thus preventing their improper folding or further damage or degradation (Tytell and Hooper, 2001).

Understanding the regulation of HSP induction may be important for HS alleviation strategies. Thus, HS exposure and recovery effect on HSP expression contribute to HS tolerance via prevention of protein misfolding and removal of protein aggregations (Collier *et al.*, 2008). Among the cited HSP families, at least Hsp27 and Hsp70 are associated with heat exposure (Belhadj Slimen *et al.*, 2014). In farm animals, HS increase both Hsp70 and Hsp90 in sheep (Romero *et al.*, 2013), buffalo (Kapila *et al.*, 2013), cattle (Deb *et al.*, 2014), and goats (Dangi *et al.*, 2014). On the other hand, it has been reported that lower heat tolerance is associated to a higher expression of Hsp72 (Lacetera *et al.*, 2006).

When the heat shock response fails to repair damaged proteins and stop the oxidative damage propagation, the synthesis of Hsp27 and Hsp70 stops, leading proteolysis by the

proteasome and allowing cells to undergo apoptosis.

The proteasome are protein complexes located both in the nucleus and in the cytoplasm that breaks peptide bonds (Peters *et al.*, 1994). Its main function is the removal of damaged proteins to avoid protein accumulation and aggregation. Thus, the damaged proteins enter the ubiquitin-proteasome system (**UPS**) where they are tagged for degradation with a small protein that attaches to substrates, called ubiquitin, and is catalyzed by a cascade of enzymes called ubiquitin ligases (Hershko and Ciechanover, 1998). Once a protein is tagged with a single ubiquitin molecule, this is a signal to other ligases to attach additional ubiquitin molecules. The result is a polyubiquitin chain that is bound by the proteasome, allowing it to degrade the tagged protein into short peptides and to amino acids through the aminopeptidases (reviewed by Rousseau and Bertolotti).

The TOR complex 1 (TORC1) integrates protein and amino acid homeostasis. Thus, under nonlimiting nutrient conditions, TORC1 is activated to promote anabolic processes to synthesize nutrients. However, when a negative energy balance occurs, like in HS animals, autophagy is induced (Nakatogawa *et al.*, 2009) by the inhibition of the TORC1, allowing cellular anabolism and up-regulating the UPS. Elevated protein catabolism enables the degradation of unwanted proteins into free amino acids, which will support the synthesis of new stress proteins. This metabolic reprogramming of the cells is essential to maintain cell viability. A general picture of the HSR is summarized in **Fig. 5**. Therefore, HSP expression acts as a potential indicator of animal adaptation to environmental stress and the level of stress tolerance (Hansen, 2004). Additionally, elevation or prolongation of the HSP response would also improve thermo-tolerance in bovines via manipulation of the genes controlling the expression of HSF1 (Archana *et al.*, 2017).

1.3.2. Cellular response to heat stress

HS affects both the fluidity and the stability of cellular and subcellular organelle membranes and inhibits receptors as well as transmembrane transport proteins function as a cellular adaptation to HS. Thus, membrane lipids decrease in molecular order to hyperfluidity forming transient non-bilayer lipid structures through the unsaturation of their fatty acids (Park *et al.*, 2005). This alteration in the cellular membrane conformation increases ion fluxes (i.e. Ca^{2+}, Na^{+}, K^{+}, H^{+}) and induces changes in gene expression although a loss of membrane integrity (Wrabl *et al.*, 2002; Park *et al.*, 2005).

The influx of Ca^{2+} stimulates the activity of several signaling cascades (i.e., calmodulin-dependent protein kinases, inositol triphosphate production, etc.), while alterations in Na^+/H^+ exchanges and Na^+, K^+, ATPase result in hyperpolarized membrane potential (Park *et al.*, 2005).

Moreover, HS has a strong impact on transmembrane proteins function (i.e., occludins, claudins, and junctional adhesion molecules) affecting the tight junction barrier among cells (Fanning *et al.*, 1998). It is known, through both human and animal studies, that disruption of the intestinal tight junction barrier is an important pathogenic factor leading to an increase of intestinal epithelial permeability to luminal antigens including endotoxins (Pearce *et al.*, 2013). HSF-1 was observed to be upstream regulator of occludin gene activity and protein expression (Dokladny *et al.*, 2008).

On the other hand, HS influences on the cell's cytoskeleton organization and shape, the mitotic apparatus and the intracytoplasmic membranes, such as the mitochondrial, endoplasmic reticulum and lysosomes. Moreover, HS is not only responsible of protein denaturation, but also induces their aggregation into the nuclear matrix (Park *et al.*, 2005). This aggregation increases the nuclear protein concentration (Streffer, 1988), impairing many molecular functions such as DNA synthesis, replication and repair, cell division and nuclear enzymes and DNA polymerases functions (Higashikubo *et al.*, 1993).

1.3.3. Metabolic response

1.3.3.1. Neuroendocrine system

The hypothalamus is the organ that regulates the maintenance of homeothermy in animals through complex mechanisms. The centre of body temperature control is located in the preoptic area of the anterior hypothalamus and it is regulated by afferent information related to temperature. The sum of both hypothalamus and temperature receptors in the nerve pathways, determine the level of the temperature control mechanisms (Morrison and Nakamura, 2011).

When the behavioral response is not enough to maintain the body temperature in a steady-state, neuro-endocrine readjustments through the hypothalamic-pituitary-adrenal (**HPA**), sympathetic-adrenal-medullary (**SAM**) and hypothalamic-pituitary-thyroid (**HPT**) axes are activated to compensate the thermal imbalance (Afsal *et al.*, 2018). The neuroendocrine response to HS in dairy goats is summarized in **Fig. 5**.

The HPA axis is the primary response of adaptive mechanisms to stress in order to help the animal to cope with extreme environmental conditions (Afsal *et al.*, 2018). When the HPA axis is activated, the hypothalamus release corticotrophin-releasing hormone (**CRH**) and vasopressin peptides. These peptides act synergically on the anterior pituitary gland to release adrenocorticotropic hormone (**ACTH**). When ACTH binds to the adrenal cortex receptors, it stimulates the release of glucocorticoids and, through the sympathetic-adrenal-medullary (**SAM**) axis, the secretion of dopamine, epinephrine and norepinephrine (i.e., catecholamines). These hormones participate in the response from specific target receptors, eventually returning homeostasis to its normal stage (Nejad *et al.*, 2014).

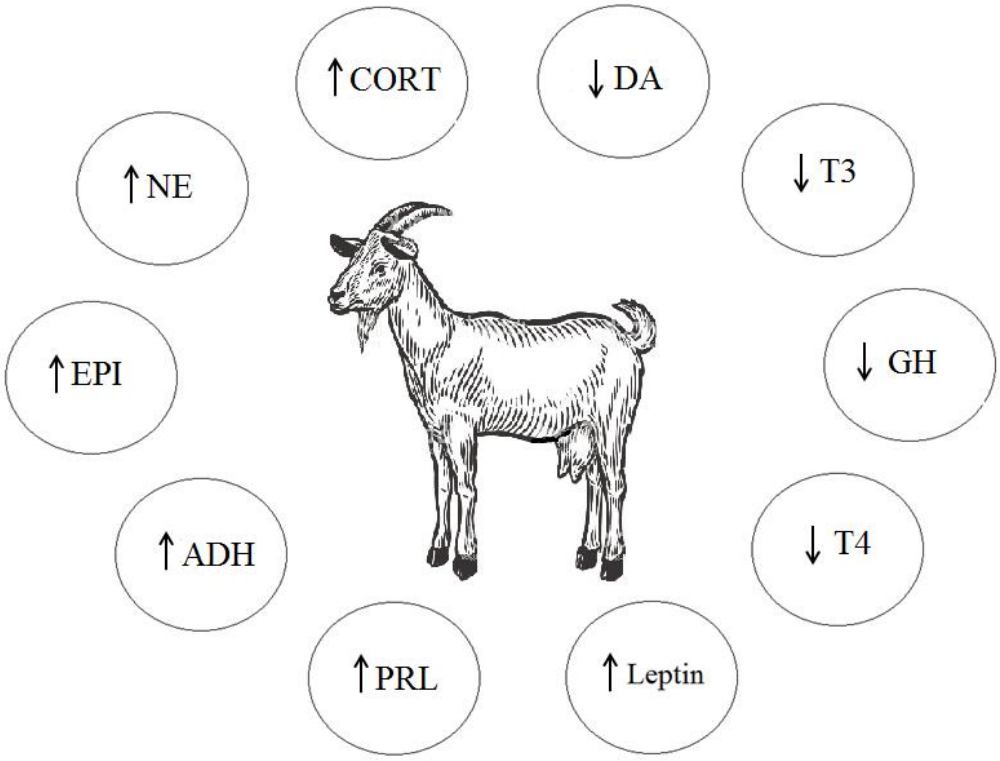


Figure 5. Main neuroendocrine responses to heat stress in dairy goats (CORT, cortisol; DA, dopamine; T3, triiodothyronine; GH, growth hormone; T4, thyroxine; PRL, prolactine; ADH, aldosterone; EPI, epinephrine; NE, norepinephrine)

The energy stored in animals for production purposes is deviated from the neuroendocrine response to help the animal to cope with stressful conditions through the alteration of metabolic rate, peripheral circulation, respiration, and energy availability (Niyas *et al.*, 2015; McMorris, 2016). However, it is known that prolonged stimulation of the HPA axis results in suppression of immune function and reproductive

performance as it is commonly observed in animals under HS (De Castro *et al.*, 2014).

On the other hand, aldosterone (**ADH**) is an antidiuretic steroid hormone released from the cortex of the adrenal glands that helps to maintain the Na^+/K^+ balance within the body. The ADH regulates blood osmolarity by increasing the water absorption in the kidneys, which also assists the excretion of concentrated urine in HS goats (Kaliber *et al.*, 2016). Thus, the ADH level in plasma was reported to be higher in dehydrated HS goats as well as in HS goats with freely available water (reviewed by Gupta and Mondal, 2019).

Additionally, the HPT axis plays a critical role for the regulation of both thermogenesis (Silva, 2006) and energy consumption. Therefore, HPT is an axis to be taken into consideration in the adaptation to HS through the action of thyroid hormones (Bernabucci *et al.*, 2010). The HPT axis is under the control of neurons located in the medial region of the paraventricular nucleus of the hypothalamus that synthesizes and releases thyrotropin-releasing hormone (**TRH**) into the pituitary gland (Fekete and Lechan, 2013). The TRH stimulates the release of **TSH** (thyroid stimulant hormone or thyrotropin) from the anterior pituitary, which in turn stimulate the synthesis and release of thyroid hormones (triiodothyronine, **T3;** thyroxine, **T4**) in the target thyroid gland. The integrated functioning and signaling of the thyroid hormones allow for the adjustment of metabolic rates in favor of decreased energy utilization and heat production during exposure to elevated humidity and temperature that demand for normalization of body core temperatures (Baumgard and Rhoads, 2012). Thyroid hormones are transitorily activated under both acute and chronic stresses as a result of the stimulatory effect of glucocorticoids on the pituitary thyrotrope, leading to an increase of TSH concentration (Shagi *et al.*, 2016). However, under HS conditions, a decreased HPT activity was observed in farm animals (Sejian *et al.*, 2010), with reduced circulating concentrations of T4 (Collier *et al.*, 1982; Kahl *et al.*, 2015) and T3 (Magdub *et al.*, 1982; Kahl *et al.*, 2015), apparently in an effort to reduce the metabolic heat production (Afsal *et al.*, 2018). In fact, Pereira et al (2008) concluded that the degree of reduction in circulating T3 is related to the ability of different cattle breeds to adapt to HS. This link between thyroid hormones and thermophysiological mechanisms was also observed by Silva (2006), when hypothyroid rodents under cold conditions showed to develop greater degree of hypothermia than the normothyroid ones.

Moreover, dopamine also falls in HS animals (Tucker *et al.*, 1991) which is also associated with greater levels of prolactin (**PRL**) when the cause of stress is only the

thermal load (Morehead and Gala, 1987). A significant negative correlation between RT and peripheral PRL concentration was observed in several species, including goats (Sergent *et al.*, 1985). When PRL was pharmacologically suppressed, an increase in RT was detected (Faichney and Barry, 1986). Therefore, this rise in PRL values enables animals to tolerate HS (Mills and Robertshaw, 1981). The PRL, despite being a lactogenic hormone, is involved in several functions as a cytokine, stimulates the Hsp60 in rats (Stocco *et al.*, 2001) and protects the cell from hyperthermia (Collier *et al.*, 2008). Surprisingly, despite the higher plasma PRL levels, a greater milk production is not observed in HS dairy animals. Thus, a down-regulation of PRL signaling pathways is observed in the mammary gland, which could partially explain for the depressed milk production in HS dairy animals **(see chapter 3.5)**.

On the other hand, although leptin is a hormone that regulates feed intake, body weight, metabolism, reproduction and adaptation in animals (Bagath *et al.*, 2016), it also participates in the HPT axis regulation. Leptin oversees the energy homeostasis of dairy cows by regulating glucose metabolism and insulin sensitivity (Min *et al.*, 2016). Several studies reported how leptin increases under HS conditions in dairy cows (Min *et al.*, 2015), dairy goats (Agarwal *et al.*, 2009) and rats (Morera *et al.*, 2012). Therefore, the primary target site for leptin that mediates its effect on the HPT axis, abolishes both the nutritional stress and the leptin-induced regulation of HPT axis. However, further research on the leptin-HPT link is needed because it stills remains not fully understood.

1.3.3.2. Post-absorptive metabolism and tissue mobilization

The decrease in feed intake is a highly-conserved response among species when environmental temperatures increase (Baumgard and Rhoads, 2012), presumably in an attempt to reduce the animal metabolic heat production (West, 2003; Gao *et al.*, 2017). Despite of feed intake, HS also modifies the utilization of substrate, the post-absorptive metabolism and the metabolic hormonal responses affecting the energy partitioning for promoting body heat loss.

In dairy animals, it was assumed that the decrease in feed intake is the main responsible of the negative effects in body weight and milk production and composition. However, pair-fed experiments that included thermoneutral controls restricted at the same level as their HS counterparts, demonstrated that the decrease in feed intake only explains 35 to 50% milk yield reduction in lactating dairy cows (Rhoads *et al.*, 2009; Wheelock *et al.*, 2010). However, no pair-fed experiments have

been performed in dairy goats, but similar effects are expected although they are less sensitive to HS than dairy cows.

Fat reserves. Surprisingly, despite the marked reduction in nutrient intake, no adipose tissue mobilization but a decrease in muscle mass has been extensively observed in HS ruminants (Baumgard *et al.*, 2013). Therefore, dramatic metabolic and hormonal changes should occur when they are exposed to a severe HS.

Regarding the adipose tissue mobilization, the metabolic profile of HS lactating dairy ruminants differs from that expected under negative energetic balance in TN conditions (Shwartz *et al.*, 2009). Thus, HS lactating ruminants are primarily characterized by low NEFA blood levels (Rhoads *et al.*, 2009), despite the 2-fold increase of NEFA levels observed in those pair-fed under thermoneutral conditions (Wheelock *et al.*, 2010). In dairy goats, Hamzaoui *et al.* (2013) reported that HS does only mobilize fat during the first week of HS (i.e., greater values of plasma NEFA) but then, despite of being under strong negative energy balance, no differences in NEFA levels were observed until the end of the experiment. These results agree with those observed in dairy cows, where HS did not cause significant changes in blood NEFA (Itoh *et al.*, 1998; Baumgard and Rhoads 2007; Wheelock *et al.*, 2010). Others authors even observed a decrease from 30 to 60% of NEFA levels in HS dairy cows (Cowley *et al.*, 2015; Lamp *et al.*, 2015; Gao *et al.*, 2017).

The failure to increase NEFA as a result of fat mobilization, contradicts what it should be expected by HS because the marked increase in the levels of circulating catabolic hormones, such as cortisol, norepinephrine and epinephrine (Collier *et al.*, 1982) that are known for stimulating lipolysis and adipose mobilization. On the contrary, HS in cows seems to increase the basal insulin levels and to stimulate its response by increasing the sensitivity of insulin and leading to a potent antilipolytic effect that reduces body fat mobilization (Vernon, 1992; Baumgard *et al.*, 2012). Surprisingly, small ruminants under HS are able to maintain similar blood glucose levels compared with TN animals, with no change in blood insulin concentration as observed in lactating dairy goats (Sano *et al*., 1985; Hamzaoui, 2014) and non-lactating ewes (Achmadi *et al*., 1993; Sano *et al*., 1983). Therefore, making adipose tissue insensitive to insulin seems to be a key to improve the HS adaptation of dairy cows but not in small ruminants.

In this sense, other mechanisms could explain the ability of HS goats to maintain blood glucose. First, kidneys may play an important role as they can produce glucose

from ketone bodies (Krebs *et al.*, 1965; Gerich *et al*., 2001). The greater levels of BHB found in the urine of HS goats (Salama *et al*., 2014) might be the source that arrived to kidneys and accelerated the gluconeogenesis, as proposed by Kaufman and Bergman (1971) in sheep. Second, the decrease in milk lactose secretion by 5% in HS goats (Hamzaoui *et al*., 2012) may spare some glucose in blood (Sano *et al*., 1985).

Muscle degradation. As ruminants obtain little glucose directly from diet digestion, gluconeogenesis is vital for supplying extra-hepatic tissues with glucose (Rhoads *et al.*, 2011). Consequently, under HS, energy deficit it is not provided from adipose tissue but skeletal muscle mobilization (Shwartz *et al.*, 2009). Thus, amino acids (**AA**) are used for gluconeogenesis to meet glucose requirements, as reflected by the strong decrease in gluconeogenic precursors, such as some essential (i.e., Ile, Lys) and non-essential AA (i.e., Ser, Arg, Gly, Cys) observed in HS dairy cows (Gao *et al.*, 2017). Moreover, the AA are also used for the synthesis of acute-phase proteins in response to inflammation (LH Baumgard, personal communication, June 12, 2019) agreeing with the increase of haptoglobin also reported in HS dairy goats (Hamzaoui *et al.*, 2013).

Postabsorptive protein metabolism is also affected by HS, as illustrated by changes in the quantity of carcass lean tissue in different species (Close *et al.*, 1971; Lu *et al.*, 2007) and reflected in the reduced RNA/DNA synthesis capacity (Velichko *et al.*, 2012). Moreover, skeletal muscle catabolism is clearly increased during HS, as reflected by indicators of muscle breakdown found in HS animals. So, increased levels of urinary and plasma 3-methyl-histidine, in cows (Schneider *et al.*, 1988; Kamiya *et al.*, 2006) and pigs (Cruzen *et al.*, 2015), as well as, of creatine in sheep (Bell *et al.*, 1989) and cows (Schneider *et al.*, 1988; Abeni *et al.*, 2007) were reported. Cruzen *et al.* (2015) supported these findings although in pigs, after 12 h of HS, the skeletal muscle proteome differed from those in TN.

Regarding skeletal muscle metabolism, an augmented intramuscular carbohydrate use was observed when animals were submitted to HS conditions (Febbraio, 2001). However, the increased glycolysis reported in hot environments may not be due to increased glucose uptake, but to an increased intramuscular glycogen breakdown because of the anaerobic metabolism (Febbraio, 2001). Thus, this phenomenon may explain why energy efficiency is srongly compromised under HS conditions (Rhoads *et al*., 2016).

Moreover, as aforementioned, HS causes protein damage. When re-folding of the damaged proteins cannot be achieved, these proteins are also degraded. In the process,

the α-amino groups of AA are removed, and the resulting carbon skeleton converted into a major metabolic intermediate. Most AA are converted into urea through the urea cycle, whereas their carbon skeletons are transformed into acetyl CoA, acetoacetyl CoA, pyruvate, or other intermediates of the citric acid cycle. Hence, FA, ketone bodies, and glucose are formed from AA. Thus, increased levels of urea in plasma, milk or urine, as a product of nucleotide catabolism, are expected in HS animals, such as reported in cows (Kamiya *et al.*, 2006; Abeni *et al.*, 2007; Shwartz *et al.*, 2009; Wheelock *et al.*, 2010; Gao *et al.*, 2017), heifers (Nardone *et al.*, 1997; Ronchi *et al.*, 1999) and steers (O'Brien *et al.*, 2010). However, once again, no differences were reported neither in sheep (Bell *et al.*, 1989) nor goats (Hamzaoui *et al.*, 2013).

1.3.4. Immune system

Environmental temperatures out of comfort zone are a potent stressor that not only reduce the productive and reproductive performances, but also depresses the immune system activity. Moreover, it is closely related to the neuroendocrine changes that occur when animals are exposed to stress. Acute stress induces an immunological profile in which low energy consuming components are enhanced and high energy consuming ones are suppressed (Segerstrom and Miller, 2004). Therefore, cells and antibodies are mobilized but blood cells production is inhibited. On the contrary, chronic stress induces adaptive weighing of costs and benefits of immunity against other potential uses of energy (Segerstrom, 2007). Thus, immune functions suppressed include protein and cell production and cell function.

Heat stress is known to threat the resources and as an adaptive mechanism; thus, energy is directed away from the immune system and toward protecting or restoring the damaged cells caused by HS. Part of this adaptation is mediated by the increase of cortisol released by the activation of the HPA axis, as observed in HS sheep (McManus *et al.*, 2009; Sejian *et al.*, 2014). Cortisol inhibits several signaling pathways that repress multiple inflammatory genes (Busillo and Cidlowski, 2013). Although cortisol generally suppresses immunity, if high levels of cortisol are present for long time, the cortisol receptors of immune cells become desensitized (i.e., chronic stress) and, as a consequence, inflammation escapes from cortisol control (Miller and Cohen *et al.*, 2002).

On the other hand, previous studies reported that HS induces apoptosis of T and B

cells and increases the number of NK cells (Hammami *et al.*, 1998). Moreover, the production of interleukin 2 (IL-2) by T cells, and the production of immunoglobulins by B cells, is also suppressed during HS. In addition, HS impairs macrophage function by the inhibition of its phagocytotic and chemotaxic activities. Moreover, Chen *et al.* (2018) reported increased levels of cytokines IL-1ß, IL-6, IFN-γ, and TNF-α in HS dairy cattle. Increased levels of norepinephrine, because the SAM axis and adrenal medulla activation, also suppresses T-cell activities as Nagai and Iriki (2001) pointed out.

HS impairs innate and acquired immune status in dairy cows during the transition period, as indicated by the defects in neutrophil function and immunoglobulin secretion (do Amaral *et al.* 2011). Furthermore, HS during pregnancy compromises the passive immunity of offspring from birth to weaning, suggesting that the immune function of calves is also compromised (Tao *et al.* 2012). Moreover, HS decreases the abundance of components of the plasma complementary system, suggesting that HS results in impaired immune function in lactating dairy cows (Tian *et al.*, 2015). However, the precise mechanisms underlying impaired immune function in lactating dairy cattle during HS remain undefined. All these alterations make the cells less efficient and therefore they may increase the likelihood of infection, as supported by the increased cases of lameness in HS dairy cows during lactation (Cook *et al.*, 2007) and of intra-mammary infections during summer (Waage *et al.*, 1998).

On the other hand, is well known that HS impairs the gut integrity causing endotoxemia, as a result of the release of their lipopolysaccharides (LPS) from the gut (reviewed by Baumgard *et al.*, 2015). During HS, blood flow is diverted from the viscera towards the skin, in an attempt to dissipate heat (Lambert *et al.*, 2002). The reduced blood flow to the gut results in an inadequate supply of nutrient and oxygen, causing both hypoxia and cell atrophy (Kregel *et al.*, 1988; Hall *et al.*, 1999), resulting in ATP depletion and increased ROS and NOS (Hall *et al.*, 2001). This situation contributes to tight junction dysfunction and to an increased passage of luminal content (i.e., LPS) into the portal blood (Hall *et al.*, 2001; Pearce *et al.*, 2013b). When pathogens invade, the innate immune cells of the mucosa layer recruits and regulate the function of innate and adaptive immune system, which is essential to fight against infection (Blikslager *et al.*, 2007). Nevertheless, the immune system becomes less efficient under HS, as above discussed, and glucose requirements for an activated immune cell function markedly increase (Baumgard *et al.*, 2012).

1.3.5. Lactation performance

Heat-stressed animals reduce feed intake in an attempt to reduce animal's metabolic heat production as digesting and processing nutrients generates heat (Collin *et al.*, 2001), especially in ruminants because rumen fermentation (West, 2003). It is well known that HS reduces milk yield and impairs milk composition in dairy animals (see reviews of Sevi and Caroprese, 2012; Salama *et al.*, 2014; Das *et al.*, 2016). However, greater milk losses have been reported in HS dairy goats in early-lactation (Sano *et al.*, 1985) than in mid- or late-lactation (Hamzaoui *et al.*, 2012, 2013). Among livestock dairy species, goats are supposed to be the most tolerant to elevated ambient temperatures (Silanikove, 2000) and milk production changes are less marked than in sheep (Peana *et al.*, 2007) or cows (Shwartz *et al.*, 2009; Wheelock *et al.*, 2010; Gao *et al.*, 2017). Nevertheless, milk composition worsened in dairy goats in all lactation stages (Hamzaoui *et al.*, 2012, 2013).

Although the negative effects of HS observed in milk yield and composition were traditionally attributed to a decline in feed intake, this statement was refuted by pair-feeding experiments. Rhoads *et al.*(2009) and Wheelock *et al.* (2010) reported that the decrease in feed intake under TN conditions only accounts for 35 to 50% of milk yield reduction in dairy cows. No information is available in dairy goats.

In this sense, decreased milk protein content was observed in HS dairy cows, sheep and goats. Regarding protein profile, total casein concentration decreased in HS dairy cows (Bernabucci *et al.*, 2015; Cowley *et al.*, 2015) and the casein variants (α-, β- and κ-casein), with the exception of the γ-casein produced by degradation of true casein fractions, showed the lowest values in summer whereas the highest were observed in winter (Bernabucci *et al.*, 2015). In dairy goats, depressed casein and whey protein contents were also observed under HS, although the decrease in milk protein content reported (i.e., −6 to −13%; Brasil *et al.*, 2000; Hamzaoui *et al.*, 2013) was greater than in HS dairy cows (i.e., −5 to −9%; Rhoads *et al.*, 2009; Shwartz *et al.*, 2009). Thus, the specific mechanisms regulating milk protein synthesis during HS are incomplete known and likely involve changes in several biological systems.

Decreased milk protein during HS might be the result of increased protein turnover, AA competition between casein and structural proteins, as well as increased casein degradation (Bequette and Backwell, 1997). Moreover, HS induces cell apoptosis and disturbs the normal biological activity of the cells (Hu *et al.*, 2016), the specific down-

regulation of mammary protein synthesis (Cowley *et al.*, 2015) and the post-absorptive metabolism and energy partitioning changes. As a result, the delivery of protein precursors to the mammary gland is reduced. Gao *et al.* (2017) observed 78% decreased plasma free AA in HS dairy cows, concluding that the contribution of AA to the circulating glucose pool increases during HS, but also the AA were used to synthesize **HSP** to fight against denaturation of proteins.

In relation to other milk components, decreased milk fat content was observed by Rhoads *et al.* (2009), although not significant changes were observed by other authors in dairy cows (Shwartz *et al.*, 2009; Gao *et al.*, 2017) nor goats (Hamzaoui *et al.*, 2013). However, milk FA profile of HS cows showed a decrease in the content of short-chain and medium-chain FA, and an increase in that of long-chain FA from fat mobilization (Hammami *et al.*, 2015; Liu *et al.*, 2017). HS also reduced the level of some polar lipid classes, especially lysophosphatidylcholine, which was proposed to be a lipid marker for HS in dairy cows (Liu *et al.*, 2017).

Lactose in milk decreased by 1 to 5% under HS conditions in dairy cows (Rhoads *et al.*, 2009; Shwartz *et al.*, 2009; Wheelock *et al.*, 2010) and dairy goats (Brasil *et al.*, 2000; Hamzaoui *et al.*, 2012). This reduction in lactose may spare some glucose in blood as 80 to 85% of glucose in blood is used by the mammary gland for lactose synthesis in goats (Sano *et al.*, 1985) and cows (Bickerstaffe *et al.*, 1974).

1.4. Heat stress in the 'omics' era

'Omics' is a term that refers to a broad field of study in biology of the recently developed high-throughput technologies that include genomics, transcriptomics, proteomics, metabolomics and metagenomics. These technologies offer a holistic, instead of a reductionist, view of the biological phenomena by the evaluation of the metabolism in a whole organism, tissue, or cell at a molecular level. Application of omics approaches, combined with the use of appropriate bioinformatics tools, have been of great help for understanding the physiological changes of organisms under different phenomena (Wheeler *et al.*, 2010).

In recent years, many researchers used the omics techniques to identify the biological markers of HS in dairy cattle. Their results expanded the current knowledge that serve as reference point in breeding for the improvement of genetic potential in many livestock species (Sejian *et al.*, 2019), offering a new perspective for future research.

Selection for thermotolerance has been employed to choose individuals that are genetically resistant to HS, with promising results by using the introgression of specific genes from cattle breeds that are tolerant into dairy breeds that are sensitive to HS. One gene, found in the Senepol cattle of the Caribbean island of St. Croix (Olson *et al.*, 2003) and associated to tropical breeds derived from Spanish Criollo (Huson *et al*., 2014) was identified as the *SLICK* locus. Other breeds from South America carrying the gen are the Carora, Criollo-Limonero and Romosinuano (Huson *et al*., 2014). The gene is inherited as a single dominant which haplotype produces a short and sleek hair coat (Olson *et al.*, 2003). Holstein cows with the introgression of the SLICK haplotype had superior thermoregulatory ability compared with non-slick animals and experience a lower depression in milk yield during the summer (Dikmen *et al.*, 2014). On the other hand, although rectal temperature during HS is heritable with an estimate of 0.17 in Holstein cows (Dikmen *et al.*, 2012), it seems to be a negative genetic correlation between HS tolerance and milk yield in dairy cattle. So, identification of genetic markers for heat tolerance that are not related to milk yield should be distinguished for heat tolerance selection without impairing milk yield (Dikmen *et al.*, 2012).

The new **genomic** technologies permit the identification of specific genes that are associated with sensitivity or resistance to stress and offer the possibility of improving the resistance of domestic animals to hyperthermia, without interfering with their productive ability (Collier *et al.*, 2008). Using the whole genome sequencing of 2 goat breeds opposite for heat tolerance may allow the identification of regions with selective sweeps and candidate mutations that may have an important role in their phenotypic differentiation (e.g., Alpine vs. Nubian goats), revealing genomic regions, genes or polymorphisms of interest in the selection for heat tolerant animals.

Transcriptomics is the study of the complete set of RNA transcripts that are produced by the genome, under specific circumstances or in a cell. Comparison of transcriptomes allows the identification of genes that are differentially expressed in distinct cell populations, or in response to different treatments. The transcriptomic response of bovines to HS was reviewed by Collier *et al.* (2008) and the transcriptomic profile of dairy animals compared, between HS and TN conditions, in blood (Kolli *et al.*, 2014; Salama *et al.*, 2014), milk cells (Salama *et al.*, 2014), mammary epithelial cells (Kapila *et al.*, 2016; Salama *et al.*, 2018), liver (Shahzad *et al.*, 2015), endometrium (Sakumoto *et al.*, 2015) and granulosa cells (Vanselow *et al.*, 2016), among others.

Several enrichments of pathways were observed to be common in different tissues analyzed under HS conditions. Thus, apoptosis, chaperon activity, oxidative stress and pro-inflammatory transcripts were up-regulated, whereas signal transduction and cell cycle related genes were down-regulated, independently of the transcriptomic profile of the analyzed tissue. Considering these studies, it can be concluded that HS compromises the immune system and the lactational and reproductive performances of dairy animals. Moreover, at liver level, energy metabolism from lipids, carbohydrates and AA is affected by HS in dairy cows as observed by Shahzad *et al.* (2015). In this sense, the decreased level of FA synthesis, oxidation, re-esterification, and synthesis of lipoproteins produced by HS in dairy cows leads to hepatic lipidosis, along with a down-regulation of glycan synthesis to counteract the lipidosis progression. Furthermore, an up-regulation of genes related to gluconeogenesis and use of glucose as an energy source, was also observed.

On the other hand, changes in the transcriptome of goat's milk cells under HS conditions were reported by Salama *et al.* (2014). Obtained results showed that HS down-regulated the gene expression of casein, fat and lactose synthesis and up-regulated genes related to milk cathepsins, which is in accordance to the decrease observed in milk protein and milk fat composition. Methionine or methionine and arginine supplementation were used by Salama *et al.* (2018) as an alleviation strategy against hyperthermia and impaired lactational performance in bovine mammary cells. The authors concluded that AA supplementation might increase cell survival and decrease the apoptosis of HS-bovine mammary epithelial cells, with positive effects on mammary metabolism.

Besides transcriptomic studies, some genome-wide association studies (GWAS) have been performed with the aim of identifying the genomic regions associated with heat tolerance in cattle, based on production traits (Hayes *et al.*, 2009; Biffani *et al.*, 2015) or thermophysiological responses (Dikmen *et al.*, 2015). Performing GWAS analysis for thermotolerance in dairy ruminants requires the use of principal component analysis (Carabaño *et al.*, 2014) due to the difficulty of linking milk recording data with phenotypes by measuring the HS response independently from lactation number or lactation stage as both are highly correlated (Carabaño *et al.*, 2017).

Proteomics is the large-scale study of a set of proteins produced in an organism, system, or biological context. To some degree, the proteome reflects the underlying transcriptome. However, protein activity is also modulated by many factors, in addition

to the expression level of the relevant gene. This technique was employed to comprehensively investigate the proteomic profile in plasma of HS vs. TN dairy cows (Min *et al.*, 2016). Results showed that many factors in the complement system are down-regulated in blood by HS, as well as some apolipoproteins and the enzyme phosphatidylcholine-sterol acyltransferase. This study suggested that lipid transport is hindered in HS dairy cows. Taking into consideration that HS cows showed a reduced adipose tissue mobilization (Wheelock *et al.*, 2010), it strongly suggests that the increase in fatty liver without increased adipose mobilization and the reduced rate of lipolysis, would happen in HS cows. Aminoacylase-1 was up-regulated in the plasma of HS dairy cows (Min *et al.*, 2016). This enzyme catalyzes the hydrolysis of N-acetylated peptides and is involved in the final release of free AA (Perrier *et al.*, 2005). The up-regulation of aminoacylase-1 during HS might catalyze more free AA into the plasma, which agrees with the altered AA metabolic profiles identified in the metabolomic studies (Tian *et al.*, 2015). The results presented here further suggest that HS may cause nitrogen repartitioning in dairy cows. Thus, through integrative analyses of plasma proteomics and metabolomics data and the metabolic response, there is a strong evidence of reduced lipolysis, increased glycolysis, and catabolism of AA in dairy cows exposed to HS.

Metabolomics represents a powerful platform for the identification of metabolites as biomarkers associated with physiological alterations resulting from environmental influences. These techniques were employed to comprehensively investigate the discrimination of plasma, milk and liver metabolic profiles between HS and TN dairy cows (Tian *et al.*, 2015, 2016; Fan *et al.*, 2018), as well as plasma metabolomic profile in rats (Ippolito *et al.*, 2014) and chicks (Tomonaga *et al.*, 2018). Some metabolites were identified as metabolic differences during HS, and these potentially biomarkers were involved in the superpathways of carbohydrate, AA, TCA cycle, lipid, gut microbiome-derived metabolism and cofactors and vitamins. Some of them, unique and common potential biomarkers that are summarized in **Table 3**.

A general pattern can be observed in ketone bodies and products of the nucleotide metabolism, where a lower level of these metabolites is found and is linked to the negative energy balance and the cell apoptosis caused by HS, respectively. Higher vitamin and cofactors products of metabolism were found in line with the observed increased vitamin metabolism and requirements under HS (Clarkson, 1993). Comparing the difference of milk metabolites with previously identified biomarkers in plasma

during HS, significant correlations between the levels of lactate, pyruvate, creatine, acetone, β-hydroxybutyrate, trimethylamine, oleic acid, linoleic acid, lysophosphatidylcholine 16:0, and phosphatidylcholine 42:2 were found, indicating that the blood-milk barrier may become more permeable under HS. These 10 biomarkers in milk may represent the metabolomic alterations in blood during HS (Tian *et al.*, 2016). It is noteworthy that HS results in the increase in lactate and pyruvate in both plasma and milk, further reflecting enhanced anaerobic glycolysis in HS dairy cows. Increased metabolite concentrations were found in the liver of HS cows related to nucleotide metabolites (i.e., orotic acid, uridine 5'-monophosphate, uric acid, adenosine monophosphate, uridine and uracil) suggesting an increased purine-pyrimidine metabolism (Fan *et al.*, 2018) that can be potential biomarkers in genetic selection for HS tolerance to in dairy ruminants. Others that deserve consideration are the lower level AA found (i.e., glycine, proline, isoleucine), the higher level of the ketone β-hydroxybutyrate in all liver, milk and plasma (Tian *et al.*, 2015, 2016; Fan *et al.*, 2018) and of trimethylamine (i.e., gut microbiome-derived metabolism) in both plasma and milk fluids (Tian *et al.*, 2015, 2016).

The **microbiome** has emerged as a new player on behavior, physiology and stress because of its significant effects on the brain-gut axis, having an important role in the maintenance of host homeostasis. Nowadays, it is thought that there is a crosstalk communication between microbiome and brain-gut axis through the enteric nervous system (i.e., vagus, sympathetic and spinal nerves) and humoral pathways, including cytokines, hormones, and neuropeptides as signaling molecules (Mayer *et al.*, 2014). For this reason, microbiomics are gaining attention in research in the last years and some trials were conducted to link the activity of the HPA axis and stress on microbial composition. Some of them were done in social-stressed mice (Bailey *et al.*, 2011), weaning stressed dairy cows (Davis *et al.*, 2016), HS-laying hens (Song *et al.*, 2014) and HS-dairy cows (Chen *et al.*, 2018).

HS-dairy cows decreased the diversity of the fecal microbial population, and resulted in a higher expression of pathways related to diseases, environmental adaptation and related to the immune system, whereas it lowered the expression of metabolic related pathways. Moreover, Chen *et al.* (2018) concluded that thermal load affects fecal microbiome composition, being poorer in high heat sensitive cows. In HS-laying hens, lower viable counts of Lactobacillus and Bifidobacterium, and increased viable counts of Coliforms and Clostridium were observed (Song *et al.*, 2014). Regarding microbial

composition in HS-dairy cows, a correlation between lower milk oxytocin levels and T3 levels was found, and with higher cortisol levels and with inflammatory mediators, IL-1ß and TNF-α (Chen *et al.*, 2018). These correlations suggest that HS induces changes in microbial composition and provides further evidence for the possible role of the brain-gut axis in the physiological characteristics and immune activities in dairy cows.

Overall, heat tolerance is a complex phenomenon that requires integration of fine phenotypes and different omics information to provide accurate tools for animal breeding without damaging productivity. Technological developments to make on-farm implementation feasible and with greater insight into the key metabolic biomarkers and genes involved in heat tolerance are needed (Carabaño *et al.*, 2017).

Tables 4.1 to 4.3 summarize the main effects of HS on metabolic indicators.

Table 4.1. Changes induced by heat stress in the metabolomic biomarkers according to the source.

		Dairy cows						Rat
		Milk[1]		Plasma[2]		Liver[3]		Plasma[4]
Metabolic pathway	Metabolite or Technique	^{1}H NMR	LC-MS/MS	^{1}H NMR	LC-MS/MS	LC-MS/MS	GC/MS	LC-MS/MS
Carbohydrate	Glucose (Glu)			–	–	+	–	
	Fructose						–	
	Galactose-1P	+						
	Fructose 1,6 2-P					–		
	Glyceraldehyde 3-P					–		
	Glu 6-P/ Fructose 6-P					–		
Energy	Fumaric acid	–				–		
	Ketoglutaric acid					–		
	Lactic acid	+		+	+	+		
	Malic acid					–	+	
	Oxalacetic acid					+		
	Pyruvic acid	+		+		+		
	Succinic acid					–	+	
Ketone	Acetoacetic acid					+		+
	β-hydroxybutyrate	+				+		
Nucleotide metabolism	Allantoin						+	
	Orotate					+	+	
	Uridine 5'- 1-P					+		
	Adenosine 1-P					+		
	Urea		+		+	+	+	
	Uridine					+	+	
	Uracil					+		
Gut microbiome-derived metabolism	Isobutyrate			–				
	Hippurate							+
	Trimethylamine	–		–				
	Trimethylamine N-oxide			–				

[1] Tian *et al.* (2016), [2] Tian *et al.* (2015), [3] Fan *et al.* (2018), [4] Ippolito *et al.* (2014).

Table 4.2. Changes induced by heat stress in the metabolomic biomarkers according to the source: Lipids

		Dairy cows						Rat
		Milk[1]		Plasma[2]		Liver[3]		Plasma[4]
Metabolic pathway	Metabolite or Technique	^{1}H NMR	LC-MS/MS	^{1}H NMR	LC-MS/MS	LC-MS/MS	GC/MS	LC-MS/MS
Lipid	Acetoacetate			+				
	Acetone	+		+				
	Butyrate	+						
	Choline			+	+	+		
	Fatty acids	+						
	HDL			–				
	VLDL/LDL			–				
	N-acetylsugar	–						
	Total cholesterol	–						
	PUFA	+						
	Phosphorylcholine	–						
	Phosphatidylcholine		–		–			
	Monoacylglycerol		–					
	Diacylglycerol		–					
	Tryacylglycerol		–					
	Sphyngomyelin		–					
	Arachidonic acid				+			
	Linoleic acid		+		+			
	Oleic acid		+		+			
	UFA	+						

[1] Tian *et al.* (2016), [2] Tian *et al.* (2015), [3] Fan *et al.* (2018), [4] Ippolito *et al.* (2014).

Table 4.3. Changes induced by heat stress in the metabolomic biomarkers according to the source: Amino acids.

		Dairy cows						Rat
		Milk[1]		Plasma[2]		Liver[3]		Plasma[4]
Metabolic pathway	Metabolite or Technique	^{1}H NMR	LC-MS/MS	^{1}H NMR	LC-MS/MS	LC-MS/MS	GC/MS	LC-MS/MS
Amino acid	Ala						–	
	Arg				+			–
	Asp					–	–	
	Betaine			–				–
	Citrulline				+	–		+
	Creatine	+			+			
	Creatinine			+		+		+
	Cys						+	
	Glu					–	+	
	Gln							
	Gly			+	–	–		–
	His					–		
	Ile	–		+		–		+
	Leu			+		+		+
	Lys			+		+		
	Met					–		+
	Ornithine				+			
	Orotate	–				+		
	Phosphocreatine	–						
	Phe							
	Pro	–		+	+	–		–
	Ser						–	
	Thr			+		–		–
	Tyr							+
	Trp							+
	3-metyl His							+
	Val					–		+

[1] Tian *et al.* (2016), [2] Tian *et al.* (2015), [3] Fan *et al.* (2018), [4] Ippolito *et al.* (2014).

CHAPTER 2

Objectives

CHAPTER 2

OBJECTIVES

The broad goal of the current thesis was the evaluation of the chronic heat stress response, as well as the use of *'omic'* methodologies in the assessment of heat-stressed lactating dairy goats that could cover the existent gaps in our current knowledge.

More specifically, the objectives were:

1. To evaluate the thermophysiological traits (i.e., rectal temperature, respiratory rate, feed intake, water consumption, body weight) and milk production (i.e., milk yield and composition) of thermoneutral vs. chronic heat-stressed lactating dairy goats.
2. To evaluate the mechanisms behind the immune-dysfunction observed in the white blood cells of lactating dairy goats under chronic heat stress conditions, when compared to thermoneutral, through blood transcriptomics.
3. To elucidate the differences in the mammary gland response to an intramammary LPS challenge in dairy goats under thermoneutral or heat stress conditions.
4. To identify the candidate biomarkers of heat stress through the application of ^{1}H NMR-based metabolomics in urine and in milk of lactating dairy goats.

CHAPTER 3

Transcriptomics in blood cells of heat stressed dairy goats

fCHAPTER 3

Effects of chronic heat stress on lactational performance and the transcriptomic profile of blood cells in lactating dairy goats[1]

3.1. ABSTRACT

High temperature is a major stress that negatively affects welfare, health, and productivity of dairy animals. Heat-stressed animals are more prone to disease, suggesting that their immunity is hindered. Although productive and physiologic responses of dairy animals to heat stress are well known, there is still limited information on the response at the transcriptome level. Our objective was to evaluate the changes in performance and blood transcriptomics of dairy goats under heat stress. Eight multiparous Murciano-Granadina dairy goats in mid-lactation were assigned to 1 of 2 climatic treatments for 35 d. Treatments and temperature-humidity index (THI) were: (1) thermal neutral (TN: n=4;15–20 °C, 40–45%, THI = 59–65), and (2) heat stress (HS: n= 4; 12 h at 37 °C–40%, THI = 86; 12 h at 30 °C–40%, THI = 77). Rectal temperature, respiratory rate, feed intake and milk yield were recorded daily. Additionally, milk composition was evaluated weekly. Blood samples were collected at d 35 and RNA was extracted for microarray analyses (Affymetrix GeneChip Bovine Genome Array). Differences in rectal temperature and respiratory rate between HS and TN goats were maximal during the first 3 d of the experiment, reduced thereafter, but remained significant throughout the 35-d experimental period. Heat stress reduced feed intake, milk yield, milk protein and milk fat contents by 29, 8, 12, and 13%, respectively. Microarray analysis of blood revealed that 55 genes were up-regulated, whereas 88 were down-regulated by HS. Bioinformatics analysis using the Dynamic Impact Approach revealed that 31 biological pathways were impacted by HS. Pathways associated with leukocyte transendothelial migration, cell adhesion, hematopoietic cell lineage, calcium signaling, and PPAR signaling were negatively impacted by HS, whereas nucleotide metabolism was activated. In conclusion, heat stress not only negatively affected milk production in dairy goats, but also resulted in alterations in the functionality of immune cells, which would make the immune system of heat-stressed goats less capable of fending-off diseases.

[1]This article was published in: Contreras-Jodar A, Salama AAK, Hamzaoui S, Vailati-Riboni M, Caja G and Loor JJ. 2018. Effects of chronic heat stress on lactational performance and the transcriptomic profile of blood cells in lactating dairy goats. *J Dairy Res*. 85(4):423-430.

3.2. INTRODUCTION

The increase of global mean surface temperature by the end of the 21st century relative to around the millennium is likely to be 0·3 to 4·8 °C according to the last assessment report of the Intergovernmental Panel on Climate Change (IPCC, 2014), with greater warming in the Northern Hemisphere. Under high environmental temperature and humidity, the ability of animals to dissipate heat is reduced, causing increases in their body temperature and heat stress (HS). It is expected that farm animals will face heat waves at a higher frequency, intensity and duration, which will negatively affect their health, welfare and performance (Salama *et al.* 2016). Furthermore, warmer and wetter weather (i.e., warmer winters) encourages the survival of disease vectors, which would make animals more exposed to diseases. The correlation between HS and susceptibility of dairy animals to both noninfectious and infectious diseases further complicates the issue.

Cases of lameness associated with an increase of total standing time per day due to HS in lactating dairy cows were reported in summer (Cook *et al.* 2007). There is also an increment of intra-mammaryinfections in dairy cattle (Waage *et al.* 1998) and sheep (Sevi and Caroprese, 2012) during summer, leading to drastic economic losses. Thompson *et al.* (2014) showed that HS in the dry period negatively affected the immune response later in lactation when cows received a Staphylococcus aureus challenge; compared with cooled cows, the non-cooled cows had lower neutrophil counts and milk somatic cell count (SCC). They also found that non-cooled cows during the dry period had higher incidence of mastitis after parturition. In dairy goats, Love (2015) also observed that the increase in SCC in milk after a LPS challenge was delayed until 4 h compared to only 2 h in thermal neutral (TN), and SCC in TN recovered faster.

Understanding the molecular mechanisms of the immune-dysfunction observed under HS conditions could uncover targets for development of new strategies to improve animal immunity under HS conditions. Our hypothesis was that heat stress would negatively affect milk production and the functionality of immune cells, which could explain deteriorated performance and the greater incidence of diseases in dairy animals under high ambient temperatures. Therefore, the aim of the present study was to evaluate the effect of heat stress for 5 weeks on milk yield, milk composition, and blood transcriptomics in dairy goats.

3.3. MATERIALS AND METHODS

Animal care conditions and management practices agreed with the procedures stated by the Ethical Committee of Animal and Human Experimentation of the Universitat Autonoma de Barcelona (UAB) and the codes of recommendations for the welfare of livestock of the Ministry of Agriculture, Food and Environment of Spain.

3.3.1. Animals, treatments, and management conditions

Eight multiparous Murciano-Granadina lactating dairy goats (43.3 ± 1.6 kg BW; 81 ± 3 d of lactation; 2.00 ± 0.04 L/d milk), with healthy and symmetrical udders from the herd of the SGCE (Servei de Granges i Camps Experimentals) of the UAB were used. Goats were kept in individual metabolic cages (1·5 m × 0·50 m) throughout the experiment. Goats were divided into 2 balanced groups (n= 4 each) according to body weight, milk yield, and milk composition recorded before the experiment. Goat groups were submitted to 2 different environmental conditions for 35 d. Treatments and temperature–humidity index (THI) were thermal neutral (TN; 15 to 20 °C and 45 ± 5% relative humidity; THI = 59 to 65) and heat stress (HS; 37 ± 0·5 °C during the day, and 30 ± 0·5 °C during the night and 40 ± 5% relative humidity; THI = 75 to 83). The THI values were calculated according to NRC (1971) as follows:

$$THI = (1.8 \times Tdb + 32) - [(0.55 - 0.0055 \times RH) \times (1.8 \times Tdb - 26 \times 8)],$$

where Tdb is the dry bulb temperature (°C) and RH is the relative humidity (%). Values of THI greater than 80 are considered to cause moderate HS in dairy goats (Silanikove and Koluman, 2015).

Throughout the experiment (mid-December to mid-February), the TN goats were kept indoors and the temperature was maintained at 15 to 20 °C with the help of electric heater equipped with a thermostat (3.5 kW; General Electric, Barcelona, Spain). Temperature and relative humidity averaged 16.7 ± 0.3 °C and 45 ± 5% (THI = 61) for the TN goats. The HS goats were in a 4 × 6 × 2.3 m isolated chamber (Euroshield, ETS Lindgren-Euroshield Oy, Eura, Finland) provided with a temperature and humidity

controlling system (Carel Controls Ibérica, S.L., Barcelona, Spain). A continuous 90 m^3/h air turnover was maintained throughout the experiment.

Goats had a 2-wk pre-experimental period under TN conditions for the adaptation to the diet and to metabolic cages. Photoperiod was maintained constant at 12–12 h light–dark (0900 to 2100) and data of environmental temperature and humidity were recorded every 10 min using 2 data loggers (Opus 10, Lufft, Fellbach, Germany).

Feed was offered ad libitum at 0930 h (120% intake of the previous day) and consisted of a total mixed ration (alfalfa hay, 70%; ground barley grain, 14.4%; corn flour, 8.4%; soybean meal, 2.5%; soybean hulls, 4.3%; molasses, 0.3%; salt, 0.01%; sodium bicarbonate, 0.03%; carbonate, 0.02%; dicalcium phosphate, 0.01%; calcium carbonate, 0.01%; and CVM for goats, 0.02%). The ration contained (on DM basis) 17.5% CP, 43.8% NDF, 27.0% ADF, and 1.41 Mcal NEL. Additionally, mineral and vitamin blocks were freely available (Na, 16%; Ca, 12%; bicarbonate and seaweed, 12%; P, 5.5%; Mg, 2.2%; Zinc oxide, 2000 mg/kg; manganese sulfate, 1000 mg/kg; potassium iodide, 60 mg/kg; cobalt, 40 mg/kg; ironsulfate, 40 mg/kg; sodium selenite, 15 mg/kg; yeasts and S. cerevisiae, 10 mg/kg; vitamin A, 120 000 IU/kg; vitamin D3, 32 000 IU/kg; vitamin E, 120 mg/kg).

Goats were milked once daily (0800 h) with a portable milking machine (Westfalia-separator Ibérica, Granollers, Spain). Milking was conducted at a vacuum pressure of 42 kPa, a pulsation rate of 90 pulses/min, and a pulsation ratio of 66%. The milking routine included cluster attachment without udder preparation or teat cleaning, machine milking, machine stripping before cluster removal, and teat dipping in an iodine solution (P3-ioshield, Ecolab Hispano-Portuguesa, Barcelona, Spain).

3.3.2. Measurements and analyses

Rectal temperatures (RT) and respiratory rates (RR) were recorded daily at 0800, 1200, and 1700 h. The RT was measured by a digital clinical thermometer (ICO Technology, Barcelona, Spain; range, 32 to 43·9 °C; accuracy, ±0·1 °C). The RR was calculated as the number of flank movements during 60 s.

Feed intake was recorded daily by an electronic scale (model Fv-60 K; A&D Mercury PTY, Thebarthon, Australia; accuracy, ±20 g) and water consumption was

daily measured by an electronic scale (model JC30; JC Compact, Cobos Precision, Barcelona, Spain; accuracy, ±10 g). Trays with saw dust were put below the drinking troughs and weighed once daily to take into account water wastes.

Milk yield (kg/d) of individual goats was recorded daily throughout the experiment by the electronic scale used for water consumption measurement. Milk composition was evaluated weekly. A milk sample of approximately 100 ml was collected and preserved with an antimicrobial tablet (Bronopol, Broad Spectrum Microtabs II, D&F Control Systems, San Ramon, CA) at 4 °C until analysis. Refrigerated milk samples were sent to the Laboratori Interprofessional Lleter de Catalunya (Allic, Cabrils, Barcelona, Spain) for the analyses of total solids (TS), fat, protein (N× 6.38), lactose, and SCC using Milkoscan (MilkoScan FT2– infrared milk analyzer, Foss 260, DK-3400 Hillerød, Denmark) and an automatic cell counter (Fossomatic 5000, Foss Electric, Hillerød, Denmark) previously calibrated for goat milk.

3.3.3. Blood sampling and microarrays

At d 35, blood samples were collected in 10-ml vacutainers containing EDTA (BD Diagnostics, Franklin Lakes, NJ, USA) and kept on ice. The RNA was extracted from the whole blood immediately using the RiboPure-Blood Kit (Thermo Fisher Scientific, Madrid, Spain). The integrity of the total RNA was assessed by Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA), and the RIN averaged 8.51 ± 0.23. The extracted RNA was frozen at −80 °C until analysis.

For microarrays, the 24,000 transcript AffymetrixGeneChip Bovine Genome Array and the 3' IVT Express Kit (Affymetrix, Santa Clara, CA) were used. Five μg of total RNA from each sample were first reverse-transcribed to the single-stranded cDNAs using a T7 promoter-oligo(dT) primer. The double-stranded cDNA was then synthesized using DNA polymerase and RNase H, and used as templates for the in vitro transcription to generate multiple copies of biotin-modified aRNA. The full-length biotinylated aRNA was fragmented into 35- to 200-base fragments and then hybridized to GeneChip Bovine Genome Arrays for 16 h at 45 °C in a rotating Affymetrix GeneChip Hybridization Oven 320. After hybridization, arrays were washed and stained in an automated Affymetrix GeneChip Fluidic Station F450. The arrays were scanned with an Affymetrix GeneChip Scanner 3000 and the images quantified using Affymetrix GeneChip Operating Software.

3.3.4. Statistical analyses

3.3.4.1. Physiological and performance data

Data were analyzed by the PROC MIXED for repeated measurements of SAS v. 9.1.3 (SAS Inst. Inc., Cary, NC, USA). The statistical mixed model contained the fixed effect of environmental treatment (TN vs. HS), measuring day (1 to 35), and the random effect of the animal (1 to 8), the interaction (treatment × day), and the residual error. Differences between least squares means were determined with the PDIFF option of SAS.

3.3.4.2. Microarray gene expression data analysis

Computational and statistical analyses were carried out using Bioconductor (http://www.bioconductor.org/) packages of R software (version 3.0.3). The gene expression profiles (CEL format) of the 8 chips were converted into expression values using the Microarray Suite 5.0 (MAS5) function of the Affy package of R. The raw data were background corrected and normalized using log2-transformation. To filter out uninformative data, the Affy Absent/Present algorithm was run and non-expressed transcripts were excluded. The selection of differentially expressed transcripts was based on Student's t-test for comparison of means for each transcript between HS and TN groups. The false discovery rate for differentially expressed transcripts was controlled according to the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995) with an adjusted $P < 0.05$.

3.3.4.3. Functional bioinformatics analysis using the Dynamic Impact Approach

The Affy probeset IDs were transformed to Entrez Gene IDs using the bovine.db package of Bioconductor and DAVID program (https://david.ncifcrf.gov). The obtained Entrez Gene IDs were submitted for the functional analysis of DEG by the Dynamic Impact Approach (DIA; Bionaz *et al.* 2012), which relies on the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database. The DIA calculates the 'impact' and the 'flux' (i.e. direction of the impact, increase or decrease) using the entire list of DEG mapped to the corresponding biological pathways in the KEGG database. Thus, the change in flux of a metabolic or signaling pathway is determined by the significant change (i.e. P value) and the magnitude of the change (i.e. by including the fold change)

for proteins involved in the pathway. For the current analysis, the standard settings were used: at least 4 genes or 20% of the genes (whichever bigger) of each pathway were represented on the array.

3.4. RESULTS AND DISCUSSION

3.4.1. Effects of heat stress on performance

The effects of HS on RT and RR during the experimental period are shown in Fig. 1. Goats exposed to HS had the maximum values of RT and RR during the first 3 d and then decreased ($P < 0.05$), but remained greater ($P < 0.001$) than the TN throughout the experiment. The greatest values of RT and RR were observed in the HS goats at 1700, the increases being 1 °C and 3.3-fold ($P < 0.001$) on average when compared to TN at 1700 throughout the experiment. Pugh and Baird (2012) reported that reference RR for adult goat ranges between 15 and 30 breaths/min. The RR values in our HS goats were greater that these reference values at all day time points (0800, 1200, and 1700) throughout the 35 experimental days.

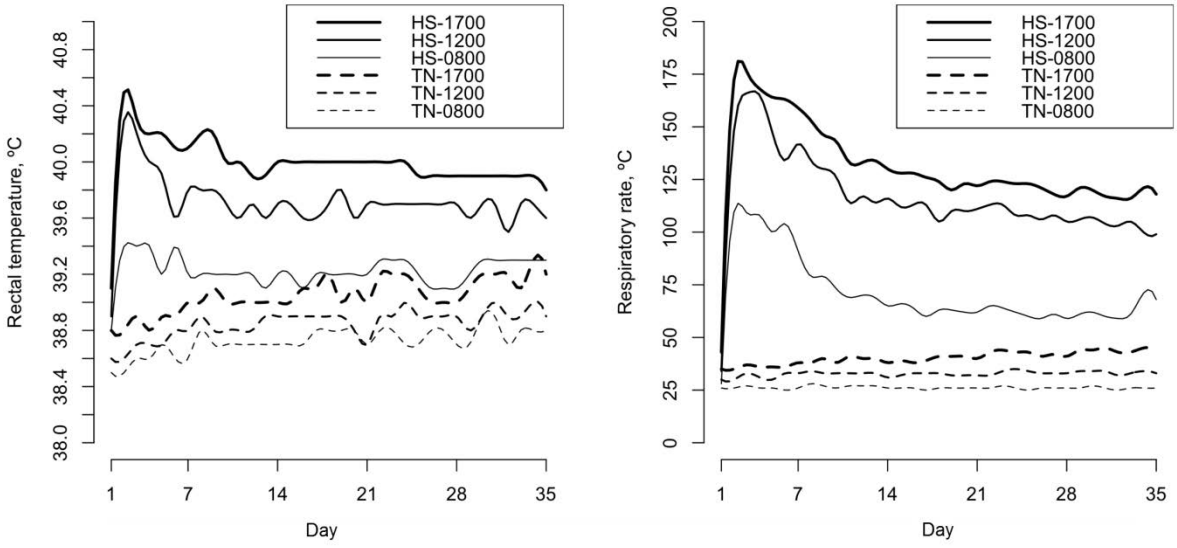


Figure 1. Rectal temperature and respiration rate throughout the day (08.00, 12.00, and 17.00) in Murciano-Granadina dairy goats under thermal neutral (TN; n=4) and heat stress (HS; n=4) conditions in mid lactation. 13.5% FCM = kg of milk yield × [0.432 + 0.162 × (fat %)].

On average, DM intake decreased by 29% in HS ($P < 0.001$) when compared to TN goats (Table 1). In contrast, water consumption increased 69% under HS conditions. Obtained results agree with those reported for the same breed of dairy goats in late-lactation under similar HS conditions (Hamzaoui *et al.* 2013) and heat-stressed dairy cows (Wheelock *et al.* 2010; Gao *et al.* 2017).

Compared to TN, milk yield, fat-corrected milk, milk fat, and milk protein of HS goats decreased ($P < 0.001$) by 8, 15, 12, and 13%, respectively (Table 1). Goats in the current experiment were in mid lactation and milk production losses were greater than those observed by Hamzaoui *et al.* (2013) in late-lactating goats. The negative effects of HS on the lactational performance of dairy cows, goats, and sheep are well known and are usually attributed to the decline in feed intake and direct effect on mammary gland (Baumgard and Rhoads, 2013; Salama *et al.* 2016).

By d 35, HS goats were still having greater ($P < 0.001$) rectal temperature and respiration rate (+0.69 °C and 1.5 fold, respectively) and were producing less amounts of milk. This clearly indicates that at d 35, goats were under significant HS when blood samples were collected for microarray analyses.

Table 1. Lactational performance of Murciano-Granadina dairy goats under thermal neutral (TN; n = 4) and heat stress (HS; n = 4) conditions in mid lactation.

	Treatment			Effect
Item	TN	HS	SED	*P*- Value
Feed intake, kg DM/d	2.49	1.77	0.04	0.001
Water consumption, l/d	5.9	10.0	0.34	0.001
Milk yield, kg/d	1.70	1.56	0.15	0.001
3.5% FCM[1], kg/d	1.90	1.62	0.02	0.001
Milk composition, %				
Total solids	12.9	11.8	0.11	0.001
Fat	4.26	3.76	0.10	0.001
Protein	3.74	3.26	0.09	0.001
Casein	3.20	2.83	0.08	0.001

3.4.2. Identification of differentially expressed genes in response to HS

Gene expression was evaluated in the total blood cells, but the signal intensity of globin genes was low and did not affect the detection of gene expression. Among the 24,128 probes contained in the GeneChip, 14,316 probes were filtered out due to absent or very low expression levels. The remaining 9,812 transcripts were subjected to a Student's t-test for comparison of expression means. Benjamini-Hochberg corrected transcripts ($P < 0.05$) were used for further analysis. After conversion of transcripts to their related corresponding genes, the statistical analysis revealed that HS resulted in 143 differentially expressed genes (DEG; 55 upregulated and 88 downregulated). Furthermore, the fold change (FC) among DEG was modest (only 42 genes had FC ≥ 1, Tables 2 and 3).

Table 2. Top upregulated genes of blood cells in heat-stressed Murciano-Granadina dairy goats for 35 d compared with thermal neutral counterparts.

Gene symbol	Entrez Gene	log2 FC
CENPT	513195	2.52
ENTPD5	957	2.37
GAS2	614840	2.32
CLEC14A	509367	2.25
GPBP1	613751	2.22
APOL3	512905	1.82
SCYL2	782059	1.79
RCN3	522073	1.65
RGS22	617133	1.61
ANGPT2	282141	1.48
FMO5	788719	1.39
PLDN	614408	1.37
WDR73	783802	1.34
SAAL1	113174	1.33
RNF149	284996	1.28

Table 3. Top downregulated genes of blood cells in heat-stressed Murciano-Granadina dairy goats for 35 d compared with thermal neutral counterparts.

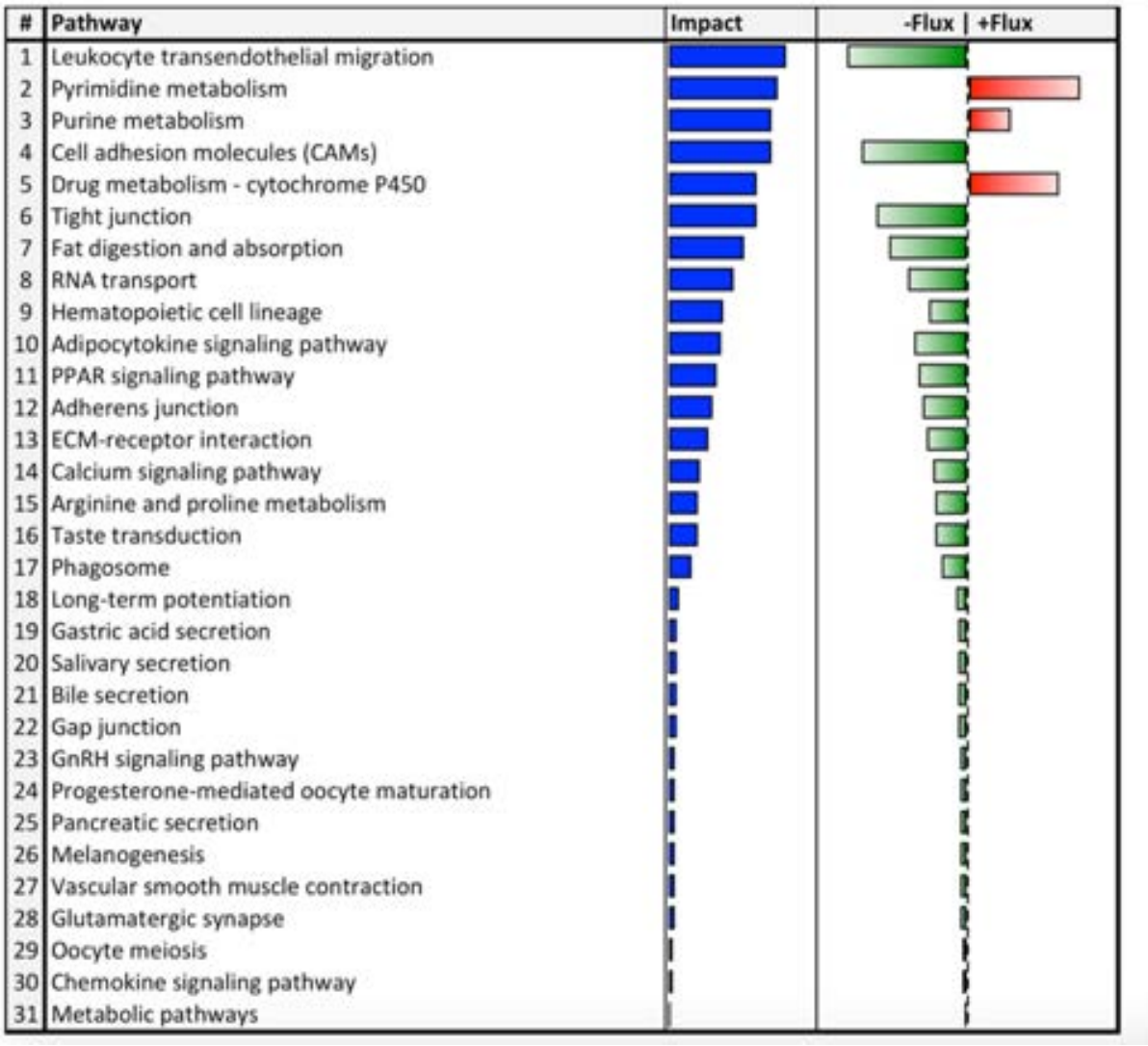
Gene symbol	Entrez Gene	FC
HGD	3081	-3.66
RAVER2	522059	-3.06
WNT5A	7474	-2.69
C1H21ORF7	540879	-2.68
CLDN11	508268	-2.56
SMN1	281492	-2.02
GCFC	514918	-2.00
CBLB	868	-1.95
ARHGEF17	9828	-1.95
OPN1LW	282293	-1.82
ANKRD1	510376	-1.73
MARCH9	540871	-1.63
APBB2	323	-1.41
PDE1A	281969	-1.38
PTGFRN	538209	-1.36
CD36	281052	-1.26
PTPRB	505696	-1.23
MTCH1	781257	-1.16
TARP	100335800	-1.15
PYCR2	504987	-1.14
GPSM2	513654	-1.12
EXTL1	511060	-1.09
ADCY8	535017	-1.04
TXNDC15	79770	-1.00
MCTP1	79772	-1.00

This could be due to the fact that blood samples were collected at d 35 of HS, and it could be possible that more changes in gene expression would have been detected if samples were collected at the first 3–4 d of HS. As shown in Fig. 1, HS goats experienced the highest RR and RT values during the first few days of HS and partially recovered thereafter, but differences remained significant until day 35. So, transcriptomics evaluation at day 35 reflects the chronic effect of heat stress.

3.4.3. Functional bioinformatics analysis of differentially expressed genes

The functional analyses using the 143 DEG revealed that 31 biological pathways (3 upregulated and 28 down-regulated) were impacted by HS (Fig. 2).

Figure 2. Biological pathways impacted by heat stress in blood cells of Murciano-Granadina dairy goats in mid lactation. Blue bars denote the impact, whereas red and green bars denote the direction of the impact (red = increase; green = decrease)



The upregulated pathways were involved in apoptosis and cell death (i.e. pyrimidine metabolism, purine metabolism, drug metabolism–cytochrome P450 and RNA transport pathways). Some of the 28 downregulated pathways were mainly related to immune cell proliferation and migration (i.e. leukocyte transendothelial migration, cell adhesion molecules, RNA transport, hematopoietic cell lineage and ECM-receptor interaction),

lipid metabolism (i.e. adipocyte signaling pathway and PPAR signaling pathways), and tissue repair (i.e. PPAR signaling pathway, arginine and proline metabolism and phagosome).

3.4.4. Transcriptional activity and cell death

A strong activation of pyrimidine and purine pathways (pathways 2 and 3) was observed jointly with xenobiotic degradation via cytochrome p450 (pathway 5) and a downregulation of RNA transport (pathway8) in HS goats (Fig. 2). The activation of pyrimidine and purine pathways in the current study is consistent with the up-regulation of these pathways by HS in other studies involving plasma metabolomics (Ippolito *et al.* 2014), transcriptomics of different tissues (liver, heart, kidney and lung) in rats (Stallings *et al.* 2014), and transcriptomics of blood in zebu cattle (Kolli *et al.* 2014). The final end-product of the up-regulation of pyrimidine and purine pathways is urea, which has been reported to increase in both blood and urine of heat-stressed dairy cows (Wheelock *et al.* 2010; Gao *et al.* 2017).

Heat stress has been shown to increase cell oxidative stress and the accumulation of free radicals and peroxides, causing protein unfolding and cellular damage in different animal models, including sheep (Chauhan *et al.* 2014) and goats (Di Trana *et al.* 2006). The activation of cytochrome p450 pathway by HS in the current study could be related to the fact that cytochrome p450 enzymes are necessary for the cell to get rid of the free radicals and peroxides.

Although there was no change in the expression of heat-shock protein genes at d 35 of HS, we observed an upregulation in the *NUP88* (Nucleoporin 88) protein coding gene, which is related to the cellular response to heat stress pathway and regulates the HSF-1-mediated heat shock response. Additionally, several pro-apoptotic protein-coding genes such as *GAS2*, *SCYL2*, *RNF149* and caspases were upregulated (Table 1).

As HS inhibits DNA, RNA and protein synthesis, the down-regulation of the RNA transport pathway (Fig. 2) could be related to the lower synthetic capacity observed under HS conditions (reviewed by Collier et al. 2008). This could be due to protein aggregation, which is considered a mitigating effect of protein misfolding. In this sense, this response could explain the greater number of down-regulated genes in various HS experiments in different tissues and organisms (current study; Kolli *et al.* 2014; Stallings *et al.* 2014). In accordance with this notion, mammary gland cells have lower

protein synthetic capacity in heat-stressed goats (Hamzaoui *et al*. 2013) and the *EIF4EBP1* gene (inhibitor of protein synthesis) is upregulated in bovine mammary cells under heat stress (Salama *et al*. 2015).

3.4.5. Proliferation and migration of immune cells

White blood cells or leukocytes are a diverse group of cell types that mediate the body's immune response. Leukocytes have a common origin in hematopoietic stem cells that differentiate into diverse functional cell types (Seita and Weissman, 2010). They circulate through the blood and lymphatic system and are recruited to sites of tissue damage and infection (Geissmann *et al.* 2008). Immune cells have different lifespans and are continuously replaced. As the hematopoietic cell lineage (pathway 9) was downregulated by HS (Fig. 2), a reduction in the generation and differentiation of new leukocytes is expected, hence, negatively impacting the immune response. Results agree with those of Lacetera *et al.* (2005) who observed a reduced proliferation of peripheral blood mononuclear cells collected from HS dairy cows in response to mitogenic stimulation. Additionally, heat stress reduced the proliferation of lymphocytes in sheep (Sevi and Caroprese, 2012).

The leukocyte transendothelial migration pathway (pathway 1), related to the movement from the blood to tissues, was also downregulated by HS (Fig. 2). In fact, both innate and adaptive immune responses are not acquired as long as leukocytes do not cross blood vessels (Muller, 2011). This process occurs through diapedesis, in which the leukocytes have to go through 4 steps: rolling, activation, adhesion and finally, locomotion through the tight junctions or through the endothelial cell itself. This leukocyte movement is controlled by cell adhesion molecules (pathway 4), their ligands, and the interaction with extracellular matrix receptors (pathway 13) in endothelial cells (Etzioni, 1996). As shown in Fig. 2, both pathways were downregulated by HS. In addition, for an efficient transmigration, the Ca^{2+} signaling transducer is needed for the loss of cellular junctions (Huang *et al.* 1993). The down-regulation in cell adhesion molecules (pathway 4) and Ca^{2+} signaling (pathway 14; Fig. 2) together with the leukocyte transendothelial migration capacity (pathway 1; Fig. 2), clearly indicate a lower ability of leukocyte migration in HS animals that likely would increase the susceptibility to infectious diseases. These results could explain the slower somatic cell

migration from blood to LPS-challenged mammary glands in HS compared with TN lactating dairy goats (Love, 2015).

3.4.6. Lipid metabolism of blood cells

A strong relationship exists between lipid composition of immune cells and their functions (Calder,2008). In the present study, we observed an altered lipid metabolism as HS downregulated the signaling pathways of peroxisome proliferator-activated receptor gamma (PPARγ) and adipocytokine signaling (pathways 10 and 11, respectively; Fig. 2).

PPARγ belongs to the nuclear receptor superfamily and acts as a lipid sensor in various tissues and cell types to modulate gene expression by binding DNA. PPARγ controls many lipid metabolism related genes (Chawla *et al.* 2001). Therefore, its downregulation by HS in the present study could be associated with a dysregulation in lipid formation and metabolism, leading to significant defects in immune cells function (Calder, 2008).

Adipocytokines (leptin and adiponectin) derive from the adipose tissue or the immune cells that infiltrate fat depots. In the current study, the downregulation of the adipocytokine signaling pathway (presumably leptin signaling) by HS could have a negative effect on immune cell function. Leptin has a diversity of physiologic roles associated with metabolism and energy homeostasis, and transmits information on energy availability and immune capability (Matarese *et al.* 2005). Leptin regulates adaptive and innate responses both in normal and pathological conditions (Fernández-Riejos *et al.* 2010). Furthermore, altered levels of leptin are related to diverse inflammatory conditions (Fantuzzi, 2005), affecting cell–cell signaling, thymic homeostasis, hematopoietic cell lineage, and cytokine production (Matarese *et al.* 2005).

3.4.7. Inflammatory response and tissue repairing

Heat stress induces an inflammatory state as observed by the increase of TNF-α and IL-6 in long-term heat-stressed dairy cows (Min *et al.* 2016). The mononuclear phagocytic system is part of innate immunity and, in the current study, the phagosome pathway was downregulated by HS (pathway 17; Fig. 2). According to Murray and Wynn (2011), macrophages mediate defense of the host from a variety of pathogens,

have anti-inflammatory function, regulate wound healing by engulfing pathogens and apoptotic cells, and produce immune effector molecules. Through their ability to clear pathogens and instruct other immune cells, macrophages are essential for protecting the organism, but also contribute to the origin and development of inflammatory diseases.

Because both are essential for platelet activation and aggregation, the downregulation of both PPARγ and calcium signaling compromises the repair of damaged tissues (Razzell *et al.* 2013). Furthermore, it is well known that PPARγ inhibits the expression of proinflammatory genes and also activates arginases (Munder, 2009) that have been shown to decrease the magnitude of inflammatory responses and promote adequate wound healing (Murray and Wynn, 2011). Accordingly, we observed a down-regulation of arginine and proline metabolism pathway (pathway 15; Fig. 2) by HS.

3.5. CONCLUSIONS

Heat stress negatively affected milk yield and milk components in dairy goats, and resulted in a dramatic increase in rectal temperature and respiratory rate, especially during the first few days of heat stress. The impaired lactational performance was accompanied by immune-dysfunction. The decrease in the hematopoiesis and leukocyte diapedesis might compromise the innate and the adaptive immune response. In addition, the disruption of lipid metabolism would significantly affect immune cell functionality due to altered PPARγ activation and, thus, an inadequate modulation of gene expression. Overall, a lower capacity of phagocytosis not only would compromise the defense of the organism against an eventual infection, but could also lead to a pathological inflammatory state with a decrease in the capacity of platelet activation and aggregation for tissue repair.

CHAPTER 4

Urine metabolome in heat stressed goats

CHAPTER 4

Heat stress modifies the lactational performances and the urinary metabolomic profile related to gastrointestinal microbiota of dairy goats[1]

4.1. ABSTRACT

The aim of the study is 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 the 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 sequestered aromatic amino acids for their metabolism and decreased the synthesis of neurotransmitters and thyroid hormones, with a 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.

4.2. 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 (Bernabucci *et al.*, 2010). Exposure of dairy animals to HS results in a decline in their productive

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(Baumgard and Rhoads, 2013a) and reproductive (Hansen, 2009) performances due to a strong metabolic disruption. Dairy animals under HS typically show decreased feed intake, increased water consumption and altered thermophysiological traits, such as respiratory rate and rectal temperature, when compared to thermoneutral (TN) ones. Usually, HS reduces milk yield and impairs milk composition in dairy goats (Salama *et al.*, 2014). Although these negative effects on milk production are traditionally attributed to a decline in feed intake, pair-fed TN experiments have shown that intake only accounts for 35 to 50% of milk yield reduction in dairy cows (Rhoads *et al.*, 2009a; Wheelock *et al.*, 2010). Therefore, there is a specific effect of HS that disrupts body metabolism and milk secretion which remains unknown.

Bio-fluid assessment by Nuclear Magnetic Resonance (NMR) spectroscopy can shed some light on the physiological mechanisms that occur in animals when exposed to HS. Proton (^{1}H) NMR, together with multivariate statistical analysis, has been successfully used as a metabolite profiling method to study the metabolic changes in blood (Tian *et al.*, 2015), milk (Tian *et al.*, 2016) and liver (Fan *et al.*, 2018) of HS dairy cows, as well as in plasma of HS growing pigs (Dou *et al.*, 2017) and rats (Ippolito *et al.*, 2014). This robust and reliable technique provides vast information on metabolome dynamics and metabolic pathways (Beckonert *et al.*, 2007). The ^{1}H NMR spectra are derived from thousands of metabolite signals that usually overlap, adding complexity to data processing. Computer-based data reduction and multivariate statistical pattern recognition methods, such as principal component analysis (PCA) and partial least square–discriminant analysis (PLS-DA), have been shown to be beneficial techniques to get the most from the information obtained in the ^{1}H NMR spectra for classification purposes (Westerhuis *et al.*, 2008; Yamamoto *et al.*, 2009).

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

4.3. MATERIAL AND METHODS

4.3.1. Animals and treatments

Animal care conditions and management practices of the study were approved by the Ethical Committee of Animal and Human Experimentation (CEEAH Approval No. 09/771) of the Universitat Autonoma of Barcelona (UAB) and agreed 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 into 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. There were two 21-day experimental periods (14 days for adaptation, 5 days for measurements, and 2 days for washout) during which both treatments were sequentially applied to each doe. As a result, a total of 16 observations per variable were obtained for each treatment. Treatments were TN (indoor shelter; 15 to 20°C and 45 ± 5% relative humidity) and HS (climatic chamber 4 m × 6 m × 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. Temperature-humidity index (THI), calculated according to NRC in 1971, resulted in THI_{TN} = 59 to 65 and THI_{HS} = 75 to 83. Experimental conditions were similar to those detailed by Hamzaoui *et al.* (2013).

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 hours (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.

4.3.2. Sampling and measurements

4.3.2.1. 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 hours throughout the experiment. Milk yield (volume) was recorded daily throughout the experiment, and milk samples were 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 (AOAC, 2003).

4.3.2.2. 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.* (2007). Briefly, a phosphate buffer solution (pH 7.4) was prepared with sodium phosphate dibasic (Na_2HPO_4; 99.95% trace metals basis, anhydrous, Sigma-Aldrich Merck; Darmstadt Germany), sodium phosphate monobasic (NaH_2PO_4; 99.95% trace metals basis, anhydrous, Sigma-Aldrich Merck) and sodium azide (NaN_3; Sigma-Aldrich Merck). Deuterium oxide (D_2O; 99.9 atom % D, Sigma-Aldrich Merck), containing 0.75% 3-(trimethylsilyl) propionic-2,2,3,3-d4 acid (TSP) sodium salt as the 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.

4.3.2.3. 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 a one-dimensional Nuclear Overhauser Enhancement Spectroscopy (NOESY) pulse sequence. The solvent signal was suppressed by pre-saturation during relaxation and mixing time. A total of 32 scans and 2 dummy scans were performed to produce 32,768 data points for each spectrum using a relaxation delay of 2.0 s with a pulse power level of 54 dB and an acquisition time of 2.6 s. Spectral width (δ) used for all data collected was 12.0 ppm, and 0.3 Hz exponential line-broadening was applied for the Fourier Transform of the raw data. ^{1}H NMR spectra were phased, baseline corrected, and corrected for chemical shift registration relative to the TSP reference compound previously indicated (δ = 0.0 ppm) in TopSpin 2.1.

4.3.3. Statistical analyses

4.3.3.1. Thermophysiological and performance analysis

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

4.3.3.2. NMR data pre-processing and analysis

Pre-treatment of raw spectral data is critical for generating reliable and interpretable models using multivariate analysis techniques. Nevertheless, metabolic fingerprinting datasets acquired from ^{1}H NMR spectrometers suffer from imprecisions in chemical

shifts due to temperature, pH, ionic strength and other factors (Worley and Powers, 2013). Therefore, models generated using multivariate analysis may fail to identify separations between classes, and their loadings can be difficult to interpret due to an over-abundance of variables. To mitigate these complications, each spectrum was uniformly divided into 'bins' of 20 signals, and the signal intensities within each bin were integrated to produce a smaller set of variables (i.e., from 0.0003 to 0.007 ppm) using R software v. 3.2.3 (R Core Team, 2015). After binning, alignment and normalization of spectra were performed to ensure that all observations were directly comparable. In this sense, urine spectra were normalized to creatinine methyl resonance intensity at δ = 3.05 ppm and then $\log_2$ transformed. Regarding variable selection, raw ^{1}H NMR spectral data were edited by excluding both the regions outside the chemical shift range of δ = 9.0-0.5 ppm and the residual peak of the imperfect water suppression (δ = 5.5-4.6 ppm). Following the recommendations of Pechlivanis *et al.* (2010), the spectral regions of histidine, 1-metylhistidine, and 3-methylhistidine (δ = 8.17-7.87, δ = 7.15-7.01, and δ = 3.77-3.71 ppm, respectively) were also removed because of the sensitivity to small pH differences among urine samples.

Once ^{1}H NMR pre-processing data were completed, data were subjected to multivariate statistical analysis. Initially, PCA was performed without considering the class information for samples examination and search for outliers. Then, PLS-DA with leave-one-out cross-validation was also performed on the datasets using the pls package of R software (Mevik *et al.*, 2015). PLS-DA allowed individual samples to be classified according to the respective class prior to analysis (TN or HS). Model strength was assessed using both R^2 and Q^2 statistical parameters. While R^2 values reported the total amount of variance explained by the model, the Q^2 reported model accuracy as a result of cross-validation. Aside from its theoretical maximum value of 1, for biological models, an empirically inferred acceptable value is $\geq$ 0.4 (Westerhuis *et al.*, 2008). The resulting Q^2 statistic was compared to a null distribution to test model significance ($P <$ 0.05).

Interpretation of multivariate analysis was performed through scores and loadings plots according to their contribution to the separation between groups. For biomarker searches, PLS-DA loadings greater than |0.0005| were selected according to their absolute magnitude values. Consequently, metabolites responsible for the separation between experimental groups were those with the highest values. Moreover, a Volcano plot with paired Student t test analysis between HS over TN cohorts was performed to

get a general overview of the data (log_2 fold change thresholds, ≤ 1.5 and ≥ 1.5; P < 0.01) and to identify metabolites with a significant effect. The false discovery rate (FDR) for differentially excreted metabolites was controlled according to the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995) with an adjusted P < 0.05. Volcano plots are suitable as complementary analysis because both PCA and PLS-DA analysis may be influenced by variable correlations and the intra- and inter-class variance of metabolites may have no significant differences in the univariate analysis (Ji *et al.*, 2017). All ^{1}H NMR data pre-processing, statistical analysis and the generated plots were performed using R.

4.3.3.3. Metabolite assignment

Chemical shifts linked to the highest loading values found in PLS-DA were annotated for metabolite assignment as HS biomarker candidates. The candidate chemical shifts and corresponding metabolites were assigned using the Human Metabolome Database (Wishart *et al.*, 2009) and queried in KEGG (Kyoto Encyclopedia of Genes and Genomes) database to know in which metabolic pathways they were involved.

4.4. RESULTS AND DISCUSSION

4.4.1. Effects of heat stress on thermophysiological and lactational performances 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 hours, 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 gained 162 g/d, on average (P < 0.001). Results obtained agreed with those reported for the same breed of dairy goats in late-lactation and under similar HS conditions (Hamzaoui *et al*., 2013).

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).

	Treatment			
Item	**TN**	**HS**	**SEM**	***P* value**
Rectal temperature, ºC				
0800 hours	38.5	39.1	0.08	<0.001
1200 hours	38.7	39.7	0.07	<0.001
1700 hours	38.7	39.9	0.09	<0.001
Respiratory rate, breaths/min				
0800 hours	27	69	4	<0.001
1200 hours	39	131	6	<0.001
1700 hours	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.

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 (Das *et al*., 2016). 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 into dairy products (Salama *et al.*, 2014). Consequently with the decrease in the content of milk components, fat-corrected milk yield also varied by −14% ($P < 0.05$).

Although our does were less sensitive to HS than were 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.* (2009a) and Shwartz *et al.* (Shwartz *et al.*, 2009) reported a 9% increase or no change in milk fat content, in 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 that reported by Rhoads *et al.* (2009a, −5%) and Shwartz *et al.* (2009, −9%) in dairy cows, and also in the same breed of dairy goats in late lactation (Hamzaoui *et al.*, 2013, −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 half of the fall in milk yield and body weight in dairy cows (Rhoads *et al.*, 2009a; Wheelock *et al.*, 2010). Consequently, the other half should be explained by unknown mechanisms induced by HS. Therefore, similar responses were expected in our dairy goats.

As an intermediate conclusion, the thermophysiological and lactational performance responses observed clearly demonstrated 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).

4.4.2. NMR urinary spectroscopy of the goats

A 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 (Worley and Powers, 2013; Bouatra *et al*., 2013).

At first glance, visible differences in urine metabolites were found between HS and TN groups. The spectral region from δ = 8.0-6.5 ppm showed higher excretion

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

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

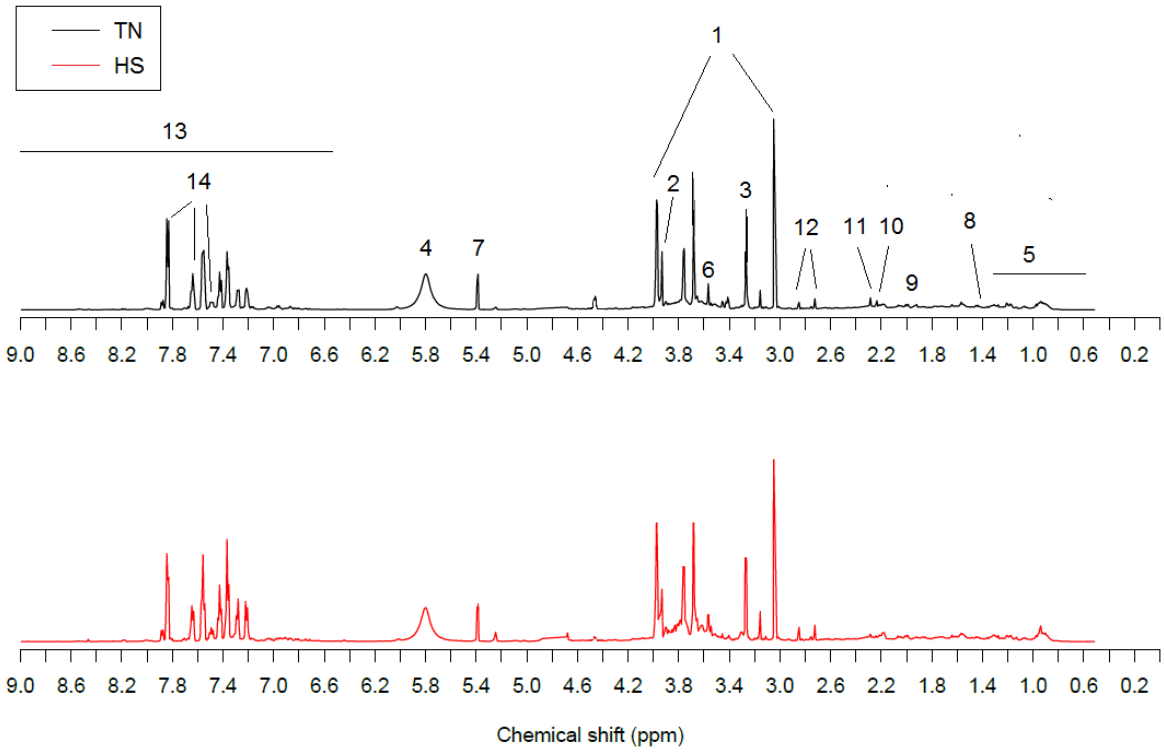

Fig 1. One-dimensional 1H NMR spectra at 600 MHz of urine from representative thermoneutral (TN) and heat-stressed (HS) dairy does. Dominant metabolites were: 1, creatinine; 2, creatine; 3, trimethyl-N-oxide; 4, urea; 5, branched-chain amino acids and organic acids; 6, glycine; 7, allantoin; 8, alanine;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

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

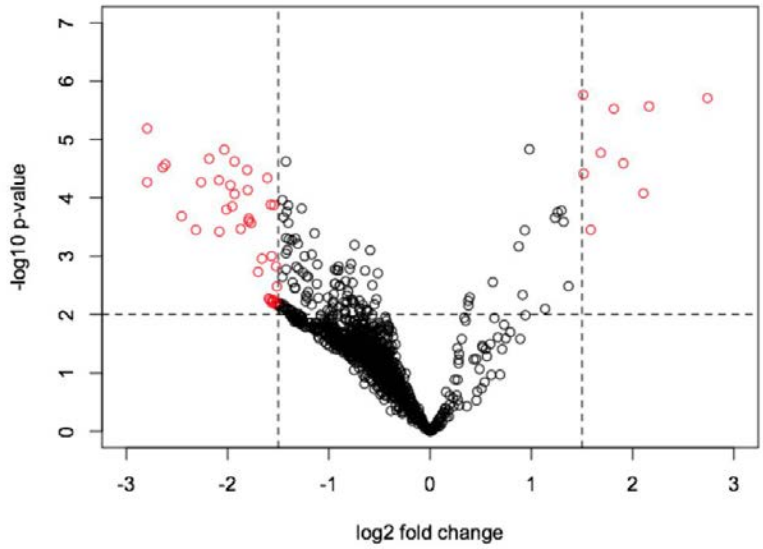

Fig 2. Volcano plot based on fold change ($\log_2$) and *P* value ($-\log_{10}$) of all spectral bins of ^{1}H NMR urinary spectroscopy of heat-stressed (HS) vs. thermoneutral (TN) lactating dairy does. Red circles indicate the spectral bins that showed significant changes and absolute fold changes greater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

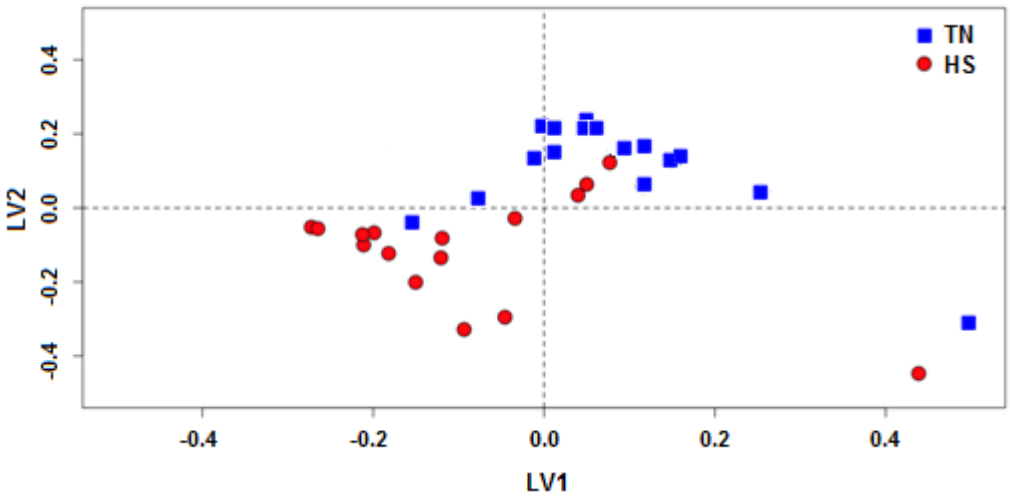

Fig 3. PLS-DA scores plot of the first two principal components of 1H NMR urinary spectra of thermoneutral (TN) and heat-stressed (HS) lactating dairy does.

The separation along the x-axis (PLS-DA component 1) represents differences related to environmental treatment. All other variations in the NMR data are visualized as separation in the y-axis direction (second component). The cross-validation of urine 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 those in the random model ($P < 0.01$). Although the top-ranking metabolites responsible for discriminating HS does were related to gut-derived uremic toxins or mammalian-microbial cometabolites (i.e. hippurate, OH-phenylacetate , OH-phenylacetylglycine, phenylglyoxylate and trimethylamine N-oxide), the thresholds applied for Volcano Plot, allowed to identify a total of 15 metabolites as candidates for urine biomarkers in HS does (Table 2). Thus, by-products of autophagy (i.e. 3-methyladenine) and energy reservoirs for muscle contraction (i.e. phosphocreatine) were also overexcreted under HS conditions. On the other hand, 8 metabolites where detected as underexcreted, some of them related to vitamin metabolism (i.e. cholecalciferol, pyridoxal, β-alanine) and carbohydrate metabolism (i.e. glycogen, galactitol) among others.

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 (Kregel *et al*., 1988) that leads to tissue hypoxia and oxidative stress (Lambert, 2009). Moreover, lower rumen pH has been reported as a side-effect in HS goats (Castro-Costa *et al.*, 2015) that leads to an abnormal overgrowth of gastrointestinal microbiota, a compromised integrity (Baumgard and Rhoads, 2013) and hyper-permeability of the gastrointestinal tract barrier (Nicholson *et al.*, 2005; Pearce *et al.*, 2013c; Ramezani and Raj, 2014). Therefore, these toxins found in plasma and excreted in urine, cross the cellular and tissue barriers (gastrointestinal epithelium, lymphatic barrier and liver) are absorbed into the blood and mainly cleared by the kidney (Ramezani *et al.*, 2014; Martin, 1982).

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 [34–36]. 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 through the production of prostaglandins; so, when COX-2 activity is sped up, inflammation increases (Karlsson *et al.*, 2005). Some Phe-derivatives also

produce cytotoxic effects by the inhibition of cell pores opening and the production of reactive oxygen species (Galati *et al.*, 2006).

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

Metabolic pathway	Chemical shift (δ, ppm)	Metabolite	Fold change*	*P* value
Phenylalanine (Phe)	7.83, 7.63, 7.54	Hippurate	2.74	< 0.001
Tyrosine (Tyr)	7.20	OH-Phenylacetylglycine	2.16	< 0.001
Microbial metabolism	3.27	Trimethylamine N-oxide	2.11	< 0.001
Nucleotide	3.97	3-Methyladenine	1.82	< 0.001
Arginine (Arg) and Proline (Pro)	3.93	Phosphocreatine	1.69	< 0.001
Microbial metabolism	7.62	Phenylglyoxylate	1.59	< 0.001
Microbial metabolism	7.27	OH-Phenylacetate	1.51	< 0.001
Microbial and purine metabolism	5.30	Allantoic acid	−1.52	< 0.001
Vitamin D	0.56, 0.53	Cholecalciferol	−1.57	< 0.001
Glucagon signaling	5.40	Glycogen	−1.66	< 0.001
Galactose	3.69	Galactitol	−1.81	< 0.001
Neurodegeneration	2.86	6OH-Dopamine	−1.87	< 0.001
Vitamin B6	5.29, 5.28, 5.27	Pyridoxal	−2.03	< 0.001
Pantotenate and CoA biosynthesis and pyrimidine metabolism	3.17	β-Alanine (Ala)	−2.09	< 0.001
Histamine	3.28	Histamine	−2.64	< 0.001

* Metabolites with positive fold change values mean that they are excreted in greater concentrations under heat-stressed conditions. Those metabolites with negative fold change values are excreted in higher concentrations under thermoneutral conditions.

Among Phe-derivatives, hippurate has a strong association with diet and gastrointestinal microbiota, and its production requires of both microbial and mammalian metabolisms (Lees *et al.*, 2013). Gastrointestinal bacteria produce benzoic acid from dietary aromatic compounds, which is absorbed into the blood. Because of the toxicity of benzoic acid, it is conjugated with glycine in the mitochondrial matrix of the

liver and renal cortex to form hippuric acid (Nicholson *et al.*, 2005; Lees *et al.*, 2013), which is later filtered in the kidneys and finally excreted in urine as hippurate (Lees *et al.*, 2013; Dzúrik *et al.*, 2001). The main elimination route for hippurate is the active renal tubular secretion and its disruption results in its accumulation in the blood (Lees *et al.*, 2013). Hippurate is a uremic toxin that participates in the correction of metabolic acidosis by stimulating ammoniagenesis, a dominant and adaptive mechanism of proton excretion. Moreover, it interferes with several metabolic processes, such as: inhibition of glucose utilization by the kidney and muscle, modulation of fatty acid metabolism and regulation of the acid-base balance by stimulating the kidneys' ammoniagenesis, among others, as reviewed by Dzúrik *et al.* (2001).

Among these gut-derived metabolic compounds, changing levels of trimethylamine N-oxide in plasma and milk were also observed in HS dairy cows (Tian *et al.*, 2015, 2016). Contradictory, these authors pointed out a lower level of this metabolite found in milk and plasma, while we observed an overexcretion of this compound through the urine.

It might also be noted that, in addition to the production of gut-derived uremic toxins from dietary aromatic amino acids by the gastrointestinal microbiota, Phe is known to be an essential amino acid for most animals, including ruminants (Patton *et al.*, 2015). It is also the precursor of Tyr, which is essential for the synthesis of thyroid hormones and the levodopa neurotransmitter. Previous studies have shown a strong decrease in plasma thyroid hormones (i.e., TSH, T4 and T3) in different ruminant models (Collier *et al.*, 1982; Kahl *et al.*, 2015; Hooper *et al.*, 2018), which means that the basal heat production may, in fact, decrease when Phe and Tyr are scarce. Moreover, the rate of milk production is markedly affected by thyroid hormones, which modulate the nutrient partitioning towards milk production (Hurley, 2010). On the other hand, a decrease in the dopaminergic neurons activity was also observed in HS calves (Tucker *et al.*, 1991). The drop of levodopa synthesis may be the result of the hypersecretion of its antagonist prolactin, as observed in response to HS in goats (Sergent *et al.*, 1985), ewes (Colthorpe *et al.*, 1998) and cows (Ronchi *et al.*, 2001). Prolactin is not only a hormone related to milk production, but also has a broad variety of biological functions related to thermoregulation and water balance. The increase in plasma prolactin is not reflected in an increase in milk yield, as seen in dairy ruminants under HS conditions (Hamzaoui *et al.*, 2013; Gao *et al.*, 2017). Alamer (2011) concluded that the mammary gland

experiences a down-regulation of prolactin-signaling pathways that could partially explain the depressed milk production of dairy cows during HS.

Increased concentration of 3-methyladenine in urine is associated with increased autophagy (Wu *et al.*, 2010). Autophagy controls the proteostasis in organisms (reviewed by Dokladny *et al.*, 2015) and HS is an extracellular stressor that alters the folding capacity of a cell leading to the accumulation of misfolded or unfolded proteins (Tytell and Hooper, 2001). Under stress conditions, eukaryotic cells increase the employ of autophagy to remove misfolded proteins, large protein aggregates, and whole damaged organelles inaccessible to smaller proteolytic systems (Klionsky and Codogno, 2013). Moreover, under negative energy balance, as commonly observed in HS animals, autophagy is an adaptive mechanism that provides biofuel from degraded macromolecules to maintain sufficient ATP production for adaptive macromolecular synthesis to survive stressful conditions (Choi *et al.*, 2013). One of the end products of protein catabolism is urea. An increased concentration of urea in blood, milk and urine is commonly observed in HS dairy cows (Shwartz *et al.*, 2009; Wheelock *et al.*, 2010; Gao *et al.*, 2017; Fan *et al.*, 2018) as a result of the strongly up-regulated pathway of nucleotides metabolism during HS (Contreras-Jodar *et al.*, 2018). Urea excretion peaks were compared between HS and TN does, but no differences were found in our study (P = 0.48) in agreement with that previously reported in the uremia of HS dairy does (Hamzaoui *et al.*, 2013). Thus, because cows do not have very many active sweat glands, we speculate that a greater portion of urea may be lost in the sweat of goats when compared to cows.

On the other hand, the lower urinary excretion of metabolites related to vitamin metabolism (i.e. cholecalciferol, pyridoxal, β-alanine) may be a reflection of the commonly reported increased vitamin requirements of animals under thermal load (Clarkson, 1993).

4.5. CONCLUSIONS

Heat stress caused marked changes in thermophysiological traits and lactational performances of dairy goats, which were translated into their ^{1}H NMR metabolomic urinary profile. These changes were mainly related to the over-excretion of gut-derived toxic compounds generated by the gastrointestinal microbiota with expected decreases in the bioavailability of aromatic amino acids and impairment of the synthesis of thyroid

hormones and neurotransmitters (i.e., levodopa, serotonin), which compromised the milk production of dairy goats. In practice, the use of hippurate and other phenylalanine derivatives are suggested as urinary biomarkers to identify heat-stressed animals.

CHAPTER 5

Milk metabolome of heat stressed and mammary LPS challenged dairy goats

CHAPTER 5

Milk yield, milk composition, and milk metabolomics of dairy goats intramammary-challenged with lipopolysaccharide under heat stress conditions[1]

5.1. ABSTRACT

Heat stress and mastitis are major economic issues in dairy production. The objective was to test whether goat's mammary gland immune response to *E. coli* lipopolysaccharide (LPS) could be conditioned by heat stress (HS). Changes in milk composition and milk metabolomics were evaluated after the administration of LPS in mammary glands of dairy goats under thermal-neutral (TN) or HS conditions. Heat stress reduced feed intake and milk yield by 28 and 21%, respectively. Mammary treatment with LPS resulted in febrile response that was detectable in TN goats, but was masked by elevated body temperature due to heat load in HS goats. Additionally, LPS increased milk protein and decreased milk lactose, with more marked changes in HS goats. The recruitment of somatic cells in milk after LPS treatment was delayed by HS. Milk metabolomics revealed that citrate was a significant marker for HS, whereas choline, phosphocholine, N-acetylcarbohydrates, lactate, and ß-hydroxybutyrate were markers of inflammation with different pattern according to the ambient temperature (i.e. TN vs. HS). In conclusion, changes in milk somatic cells and milk metabolomics indicated that heat stress affected the mammary immune response to simulated infection, which could make dairy animals more vulnerable to mastitis.

5.2. INTRODUCTION

The negative effects of heat stress (HS) on the productivity of dairy animals in terms of milk yield, milk composition and milk quality are well documented (Salama *et al*., 2014; Das *et al*., 2016). Despite advances in cooling systems and environmental management, HS constitutes to be a significant cost for the dairy industry (St-Pierre *et al*., 2003). Goats, originated from hot and arid zones, are considered less sensitive to HS compared to cows. However, milk production losses have been reported in heat-stressed dairy goats, especially at early stages of lactation (Hamzaoui *et al*., 2013; Contreras-Jodar *et al*., 2018).

The effect of HS on performance (e.g. milk yield, milk composition, feed intake, body temperature, respiratory rate) has been intensively evaluated in dairy animals (Salama *et al*., 2014; Das *et al*., 2016). However, only a few studies evaluated the omics

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of biofluids and tissues in animals exposed to HS such as cow's blood plasma (Tian *et al*., 2015), cow's milk (Tian *et al*., 2016), cow's liver (Fan *et al*., 2018) and goat's urine (Contreras-Jodar *et al*., 2019).

Besides the negative impact of HS on milk production, HS has been found to disrupt the immune function (Contreras-Jodar *et al*., 2018). With regard to mammary immunity during HS, available data indicate that mammary immunity might be compromised by HS. Thompson *et al*. (2014) reported that cows without cooling during the dry period have higher incidence of mastitis in the ensuing lactation. At the systemic level, Contreras-Jodar *et al.* (2018) evaluated the transcriptomics of blood immune cells in heat-stressed goats and detected a decrease in the hematopoiesis and leukocyte diapedesis, which might compromise the innate and the adaptive immune response. They also reported a disruption in lipid metabolism of immune cells, which would significantly affect their functionality. Limited information is available on the metabolomics response of the mammary gland to infection under controlled HS conditions. The evaluation of milk metabolomics produced from goats under thermal-neutral (TN) or HS conditions with and without intramammary infection would lead to the detection of biomarkers for infection in both TN and HS conditions. As far as we know, milk metabolomics of TN and HS with and without mammary inflammation have not been evaluated in dairy animals. Obtained results would ultimately help in the development of new strategies to improve animal welfare and productivity under adverse conditions. Therefore, the aim of the present study was to evaluate the effect of both heat stress and simulated intramammary infection on milk yield, milk composition, and milk metabolomics in dairy goats.

5.3. MATERIAL AND METHODS

5.3.1. Animals, treatments and management conditions.

Animal care conditions and management practices were approved by the Ethical Committee of Animal and Human Experimentation of the Universitat Autonoma de Barcelona (UAB), following procedures described in the Spanish and EU legislations (R.D. 53/2013, and Council Directive 2010/63/EU). Eight multiparous lactating Murciano-Granadina dairy goats (42 ± 2 kg of body weight; 101 ± 5 days in milk; 2.2 ± 0.1 L milk/d) from the herd of the Servei de Granges i Camps Experimentals of the

UAB were used. Udders were confirmed to be healthy by culturing separate udder half milk onto Columbia 5% blood agar and assessing over 48 h.

Goats were divided into 2 balanced groups regarding milk yield and milk composition. The 2 groups were randomly assigned to 2 environment treatments for 15 d. Treatments were: 1) thermal-neutral (TN; 15 to 20°C and 45% relative humidity), and 2) heat stress (HS; 12-h day at 35°C and 40% relative humidity, and 12-h night at 28°C and 40% relative humidity). In each environment treatment, one udder half was injected with *E. coli* lipopolysaccharide (LPS) or with 0.9% saline (CON) at d 12 after the a.m. milking. Consequently, there were 4 treatment combinations: TN-CON, TN-LPS, HS-CON, and HS-LPS. The LPS udder halves aseptically received 10 μg *E. coli* LPS (O55:B5; Sigma-Aldrich, St Louis, MO) dissolved in 2 mL sterile 0.9% saline (0.9% NaCl; Braun; Barcelona, Spain), whereas the CON halves were administered with 2 mL saline. Before injection teat openings were disinfected with 70% alcohol.

For the TN goats, the temperature was maintained at 15 to 20ºC with the help of electric heater equipped with a thermostat (3.5 kW; General Electric, Barcelona, Spain) when necessary. The HS goats were kept in a 4×6×2.3 m climatic chamber (Euroshield, ETS Lindgren-Euroshield Oy, Eura, Finland) provided with a temperature and humidity controlling system (CAREL Controls Ibérica, S.L., Barcelona, Spain). A continuous 90 m^3/h air turnover was maintained throughout the experiment. Temperature-humidity index (THI) was calculated according to NRC (1971) and was THI_{TN} = 59 to 65 and THI_{HS} = 83-day, 74-night.

Goats had a 2-wk pre-experimental period under TN conditions for the adaptation to the diet and experimental conditions. Photoperiod was maintained constant at 12-12 h light-dark (09.00 to 21.00) and data of environmental temperature and humidity were recorded every 10 min using 2 data loggers (Opus 10, Lufft, Fellbach, Germany).

The total mixed ration was distributed individually to each goat once daily after the a.m. milking and adjusted at 30% orts based on the previous day intake. The ration was formulated to cover requirements using INRAtion 4.07 software and consisted of (as fed) alfalfa hay 60.4%, ground barley grain 15%, beet pulp 9.1%, ground corn grain 7.5%, soybean meal 3%, sunflower meal 3%, molasses 1%, salt 0.6%, sodium bicarbonate 0.2%, and vitamin-mineral corrector for goats 0.2%. The ration contained (on DM basis) 15.1% CP, 39.3% NDF, 28.6% ADF, and 1.54 Mcal NE_L. Mineral and vitamin blocks as well as water were freely available for each goat.

Goats were milked twice daily (0800 and 1600 h) using a portable milking machine (Westfalia Seperator Iberica SA, Granollers, Spain) set at 42 kPa, 90 pulses/min, 66% pulsation ratio. No udder preparation was done before milking. After milking teats were dipped in iodine solution (P3-io shield; Ecolab Hispano-Portuguesa S.L., Barcelona, Spain).

5.3.2. Measurements, sampling, and analyses

Rectal temperatures (RT) and respiratory rates (RR) were recorded daily at 0800, 1200, and 1700 h. The RT was measured by a digital clinical thermometer (ICO Technology, Barcelona, Spain; range, 32 to 43.9ºC; accuracy, ± 0.1ºC). The RR was calculated as the number of flank movements during 60 s. From d 12 to 15, RT and RR were measured at 0, 4, 8, 12, 24, 48 and 72 h after the intramammary LPS challenge.

Milk yield (kg/d) was daily recorded at each milking. Feed intake was calculated daily by the difference between the weight of the feed offered and the weight from the refusals using an electronic scale (model Fv-60K; A&D Mercury PTY, Thebarthon, Australia; accuracy, ± 20 g). Water consumption was daily measured by an electronic scale (model JC30; JC Compact, Cobos Precision, Barcelona, Spain; accuracy, ± 10g). Trays with saw dust were put below the drinking troughs and weighed to take into account water wastes. Feed samples were collected daily, composited, and analysed (AOAC, 2003).

For the LPS challenge test, milk yield for each udder half was weighed at the regular milking schedule from d 12 to 15. Milk samples from each udder half were collected at 0, 2, 4, 6, 8, 10, 12, 24, 36, 48 and 72 h after LPS challenge for composition. Milk samples (100 mL) were collected and preserved with an antimicrobial tablet (Bronopol, Broad Spectrum Microtabs II, D&F Control Systems, San Ramon, CA) at 4°C until analysis. Milk contents of total solids, fat, protein, lactose, and SCC were determined using Milkoscan (MilkoScan FT2 - infrared milk analyzer, Foss 260, DK-3400 Hillerød, Denmark) and an automatic cell counter (Fossomatic 5000, Foss Electric, Hillerød, Denmark) previously calibrated for goat milk. For milk metabolomics, additional milk samples (10 mL) without preservatives were collected at 0, 4, 6, 12, and 24 h after the LPS challenge and frozen at −80ºC until metabolomics analysis.

5.3.3. Sample preparation and NMR spectroscopy procedures

Milk samples (n = 80) were prepared for metabolomics analysis according to Beckonert *et al*. (2007). Briefly, a phosphate buffer (pH 7.4) solution was prepared with Na_2HPO_4, NaH_2PO_4, NaN_3, and D_2O (Sigma-Aldrich Merck; Darmstadt, Germany). The solution was thoroughly shaken and left in a Clifton sonicator (Nickel Electro, Weston-super-Mare, United Kingdom) at 40 ºC until the salts were dissolved.

After thawing at room temperature, 4 mL of milk were transferred to a filtration tube (Amicon Ultra-4, membrane PLGC Ultracel-PL, 10 kDa) and centrifuged at 22 ºC for 20 min at 5,000 × *g* in a swing-bucket rotor (Hettich, Tuttlingen, Germany). Then, 400 µL of the ultra-filtrated milk were transferred into Eppendorf tubes and mixed thoroughly with 200 µL of cold phosphate buffer solution. Then, 550 µL of the final mixture were transferred into 5 mm-NMR tubes (VWR International, Eurolab, Barcelona, Spain). The prepared NMR tubes were immediately placed in ice and sent for NMR analysis.

^{1}H NMR Spectra were acquired on a Bruker Avance-III spectrometer (Bruker BioSpin, Rheinstetten, Germany) operating at a proton NMR frequency of 600 MHz ^{1}H and a temperature of 298ºK controlled by BCU-extreme regulator. A 5 mm Triple Resonance Broadband Inverse (TBI) probe with *z*-gradients and inverse detection was controlled by TopSpin 3.1 software (Bruker, Germany). One-dimensional ^{1}H NMR spectra were obtained using a one-dimensional nuclear overhauser enhancement spectroscopy pulse sequence. All data were collected with a spectral width (δ) of 12.0 ppm, and 0.3 Hz of exponential line broadening was applied for the Fourier Transform of the raw data. All NMR spectra were phased and baseline corrected using TopSpin 3.1 software.

5.3.4. Statistical analyses

5.3.4.1. Thermophysiological and lactational performance data

The PROC MIXED for repeated measurements of SAS (version 9.1.3; SAS Institute Inc., Cary, NC) was used. The statistical model contained the fixed effects of environmental treatment (TN and HS), day, measuring hour (for RT and RR), the random effect of the animal and interactions of environmental treatment × day; environmental treatment × measuring hour; day × measuring hour, environmental treatment × day × measuring hour, and the residual error. For the LPS challenge data,

the fixed effects of mammary challenge (CON and LPS) and time after challenge (0, 2, 4, 6, 8, 10, 12, 24, 36, 48 and 72 h), and the random effect of udder half nested within the animal were added to the model. Logarithmic transformations (log_{10}) of SCC values were used in the statistical analysis. Differences between least squares means were determined with the PDIFF option of SAS.

5.3.4.2. NMR data processing and analysis

Imprecisions in chemical shifts could occur due to differences in temperature, pH, ionic strength among others (Worley and Powers, 2013). Therefore, each dataset was uniformly divided into bins of 100 by increasing the interval width from 0.0003 to 0.0300 ppm using the R software v. 3.2.3 (R Core Team, 2015). Then, the raw ^{1}H NMR spectral data were edited by excluding the regions outside the chemical shift (δ) range of 8.0 to 0.5 ppm, and also the residual peak of the imperfect water suppression (δ = 5.0 to 4.6 ppm).

A principal component analysis (PCA) was performed without considering the class information to check for possible outliers. Then, Partial Least Squares-Discriminant Analysis (PLS-DA) multivariate analysis with leave-one-out cross validation was performed on the datasets using pls package of R software (Mevik *et al*., 2015). The optimum number of latent variables for each PLS-DA model was chosen by plotting the root mean square error of prediction of the cross validation against the number of latent variables. The minimum indicated the number of factors that minimize the error of prediction and consequently, the number of latent variables chosen to build the model. Model strength was assessed using both R^2 and Q^2 statistical parameters. While R^2 values report the total amount of variance explained by the model, the Q^2 reports model accuracy as a result of cross-validation. Aside from its theoretical maximum of 1, an empirically inferred value of $Q^2 \geq 0.4$ is considered acceptable for a biological model (Westerhuis *et al*., 2008). The resulting Q^2 statistic was compared to a null distribution to test model significance ($P < 0.05$).

Interpretation of multivariate analysis was performed through scores and loadings plots according to its contribution to the separation between groups. For biomarker identification, PLS-DA loadings were sorted by its absolute value, with the greater ones being the metabolites responsible of the separation between experimental groups.

Chemical shifts linked with the highest loading values found in PLS-DA were annotated for metabolite assignment. Furthermore, the important metabolites were also statistically analysed using a mixed model for repeated measurements containing the fixed effects of environmental treatment (TN vs. HS), mammary challenge (CON vs. LPS), sampling hour (0, 4, 6, 12 and 24 h) and their interactions, and the random effect of udder half nested within the animal. The chemical shifts were assigned to their corresponding metabolites according to Sundekilde *et al.* (2013) and the Human Metabolome Database (Wishart *et al*., 2009).

5.4. RESULTS AND DISCUSSION

5.4.1. Effects of heat stress on physiological and productive performance

The performance data of TN and HS goats from d 1 to 11 are shown in Table 1. A significant increase in RT and RR was observed in both TN and HS goats throughout the day in accordance with the expected circadian rhythm and the daily THI pattern in both TN and HS conditions. Maximum RT difference (+1.65°C; $P < 0.001$) and RT (+141 breaths/min; $P < 0.001$) between HS and TN goats occurred at 1700 h. Increased respiration rate under HS conditions is a known mechanism for dissipating the thermal load by evaporation.

The DM intake decreased by 27% in HS ($P < 0.001$) when compared to TN goats (Table 1). In contrast, water consumption increased by 68% ($P < 0.001$) under HS conditions. Obtained results agree with those reported for the same breed of dairy goats under similar HS conditions4, 5. The decrease in feed intake is a highly-conserved response among species when environmental temperature increases and occurs in an attempt to reduce animal's metabolic heat production (Baumgard and Rhoads, 2013). Increased water intake under HS conditions is mainly used for boosting latent heat loss by evaporation (e.g., sweating and panting; Kadzere *et al*., 2002).

Heat-stressed goats tended to decrease ($P < 0.10$) milk yield by 21% compared to the TN. Among livestock species, goats were reported to have the most tolerance to elevated ambient temperatures (Silanikove, 2000), and milk production changes are less pronounced compared to sheep (Peana *et al*., 2007) and cows (Wheelock *et al*., 2010).

These thermophysiological and lactational performances clearly indicates that at d 11, goats were under significant HS, at which time the intramammary LPS challenge was carried out.

Table 1. Physiological and productive responses of dairy goats to thermal neutral (TN) and heat stress (HS) conditions. SED = standard error of the difference.

	Treatment			Effect
Item	TN	HS	SED	*P*- Value
Rectal temperature, ºC				
0800 h	38.54	39.25	0.19	0.001
1200 h	38.57	40.03	0.18	0.001
1700 h	38.80	40.45	0.19	0.001
Respiratory rate, breaths/min				
0800 h	34	81	10	0.001
1200 h	39	171	10	0.001
1700 h	46	187	10	0.001
Feed intake, kg DM/d	2.59	1.87	0.24	0.026
Water consumption, kg/d	6.0	10.1	1.1	0.001
Milk yield, kg/d	2.75	2.18	0.34	0.099

5.4.2. Responses to intramammary LPS challenge under thermo-neutral and heat stress conditions

The intramammary infusion of E. coli LPS is a known method to evaluate mammary immunity (Schmitz *et al.*, 2004) as it mimics responses to natural mastitis but without causing a true infection (Shuster *et al.*, 1991). One of the main questions addressed by the current study was whether heat stress alters responsiveness of lactating dairy goats to intramammary endotoxin challenge. To answer this question, both TN and HS goats

received LPS in one udder half, whereas the other udder half was injected with saline from d 12 to 15.

As shown in Figure 1, rectal temperatures in HS goats were greater ($P < 0.05$) than in TN at all-time points in agreement with results in Table 1. The local treatment with LPS in one udder-half resulted in systemic increment ($P < 0.01$) in rectal temperature (Figure 1). In both TN and HS goats, rectal temperatures at 4, 8 and 12 h after challenge were greater ($P < 0.01$) than at 0 h. From 24 h onwards, rectal temperatures returned to basal values. Similarly, intramammary injection of endotoxin in one udder quarter results in significant increment in rectal temperatures at 5 to 8 h post challenge (40.5 to 41.1 ºC) in dairy cows (Perkins *et al.*, 2002; Vernay *et al.*, 2012; Gross *et al.*, 2018). Body temperature has immunomodulatory functions (Zhang *et al*., 2008) as hyperthermia increases cytokine and nitric oxide production after the challenge by endotoxins (Lee *et al*. 2012).

Rectal temperature in TN goats at 8 h (39.9 ºC) was greater ($P < 0.05$) than values at 4 (39.0 ºC) or 12 h (39.2 ºC), with no differences between 4 and 12 h values. The RT temperatures recorded at 4, 8 and 12 h post challenge in TN goats were greater than normal daily values recorded in the days before the LPS challenge (38.5 to 38.8 ºC; Table 1). In HS animals, RT did not vary between 4 (40.4 ºC), 8 (40.7 ºC), or 12 h (40.5 ºC) post challenge, but were greater ($P < 0.001$) than the remaining time points. Additionally, these RT values in HS goats at 4, 8 and 12 h after LPS injection were similar to the normal RT values recorded during the days before the LPS challenge (39.3 to 40.5 ºC; Table 1). The high ambient temperatures in case of HS goats precluded the detection of the increment in RT at 4 to 12 h after LPS injection. Consequently, the ambient temperature should be considered for the interpretation of data when dealing with detection of infections. Thus, under TN conditions an increase in body temperature would indicate a febrile response to an infection, whereas under HS conditions the febrile response could be masked by a greater heat load.

Respiratory rate at all time points was greater ($P < 0.05$) in HS than in TN goats (Figure 1). From 0 to 8 h rectal temperature increased by similar magnitude in TN (+1.57 ºC) and HS (+1.40 ºC) goats, However, respiratory rate did not change in TN, but increased ($P < 0.001$) by 115 breaths/min in HS goats. Similar to our results in TN goats, intramammary infusion with endotoxin resulted in no change in respiratory rate or even a slight decrease (Perkins *et al.*, 2002). However, other researchers reported increases in respiratory rate after intramammary challenge by LPS (Gross *et al.*, 2018).

In HS goats, the increment in respiratory rate at 4 to 12 h is a consequence of the increment in the ambient temperature (from 28 to 35 ºC) rather than the febrile response to LPS.

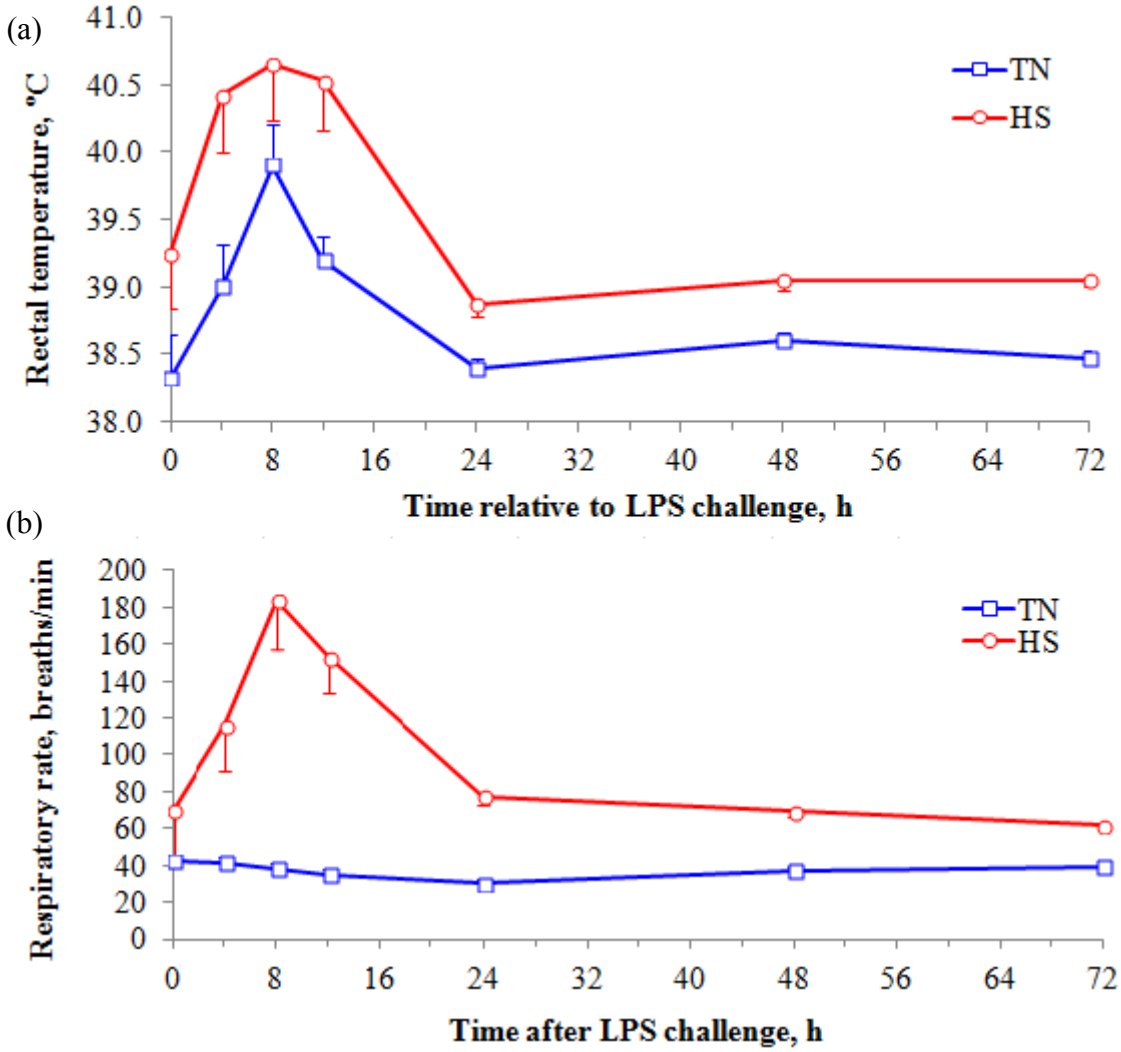

Figure 1. Hourly means and SEM for rectal temperature (a) and respiratory rate (b) following the intramammary injection of 10 µg E. coli endotoxin in thermo-neutral (TN; □) and heat stress (HS; ○) goats.

Milk yield was numerically lower (P = 0.219) by 13% in LPS udder-halves (1077 mL/d) compared to CON udder-halves (1240 mL/d) with no significant interaction between LPS treatment and temperature effects (P = 0.697). However, an interaction between time after challenge and LPS treatment was detected (P = 0.044) due to the fact that milk yield in CON half-udders was greater (P < 0.05) at 24 h (1248 mL/d) than at 0

h (1080 mL/d) and steadied thereafter, while milk yield in LPS halves did not vary throughout the challenge period (1073 mL/d on average). Kobayashi *et al.* (2013) indicated a decrease in milk secretion during LPS induced mastitis.

Milk fat was not affected by ambient temperature or endotoxin infusion (Figure 2a). The only time point at which LPS reduced milk fat was at 4 h when LPS tended to reduce ($P < 0.10$) milk fat by 20% in both TN and HS goats. The initial peak of milk fat at 2 h is most likely due to the fact that milk collected a very short time after milking is mainly alveolar milk which is rich in fat content (McKusick *et al.*, 2002). After 6 h the gland synthesized more milk stores in both cisternal and alveolar compartments, which reduced the fat concentration.

With regard to milk protein (Figure 2b), LPS challenge resulted in greater milk protein (3.72 vs. 3.08% for LPS and CON udder-halves, respectively; $P < 0.01$), whereas HS reduced milk protein numerically (3.25 vs. 3.55% for HS and TN, respectively; $P = 0.188$). Greater milk protein contents in LPS challenged mammary glands were also observed in dairy ewes (Castro-Costa *et al*., 2014) and cows (Auldist *et al.*, 1995), which has been attributed to the influx of blood-borne proteins (Auldist *et al.*, 1995). From 2 to 24 h the increment in milk protein caused by LPS injection was more marked in HS (+1.08 points) than in TN goats (+0.63 points). Additionally, the difference between CON and LPS halves in milk protein started to be significant ($P < 0.01$) at h 4 in HS goats, whereas in TN goats the difference started to be significant ($P < 0.05$) later at h 8. This finding could indicate different timing in the inflammation process under HS conditions. Significant difference in milk protein between CON and LPS halves in both TN and HS disappeared from h 36 onwards.

Lactose content (Figure 2c) was not affected by HS ($P > 0.05$), but LPS challenge induced a dramatic decrease ($P < 0.001$) in lactose concentration with the lowest levels observed at 6 h post-challenge (-1.1 points in TN-LPS and -2.0 points in HS-LPS). Lactose levels in TN-LPS and HS-LPS were recovered at 24 and 36 h post challenge, respectively when compared to their correspondent CON halves. This could indicate that mammary tight junctions in HS goats could have remained opened for longer time than in TN animals. Compared to TN-LPS, HS-LPS had lower milk lactose at 4 to 10 h after LPS infusion ($P = 0.009$ to 0.056). The decrease in lactose content in LPS challenged mammary glands was also observed in dairy ewes (Castro-Costa *et al*., 2014). The drop in lactose in milk is an indicator of tight-junction leakiness (Stelwagen *et al.*, 2000) since lactose partially moves to blood. LPS is the specific ligand of TLR4

(toll-like receptor-4) stimulating the translocation of NFkB transcription factor from the cytoplasm to the nucleus (Ibeagha-Awemu *et al.*, 2008). The activation of NFkB pathway increases tight junction permeability through expression changes of claudins in the mammary gland (Kobayashi *et al.*, 2013). The higher losses of milk lactose detected in HS-LPS can be explained by greater tight junction leakiness, since HS has been shown to compromise tight junction integrity in other tissues such as the gastrointestinal barrier (Gupta *et al.*, 2017; Contreras-Jodar *et al.*, 2019).

Heat stress had no effect on SCC ($P > 0.10$), but LPS challenge induced acute inflammation that was reflected by an increase in log10 SCC from 6.04 to 6.78 ($P < 0.001$) on average (Figure 2d). The SCC is accepted worldwide as an indicator of immune response of the mammary gland to invading microorganisms. The increment in SCC following intramammary LPS challenge is caused by the infiltration of blood immune cells into the mammary gland (Vernay *et al.*, 2013; Gross *et al.*, 2018). By 72 h, SCC values in LPS halves were still greater than their own initial values at 0 h in TN ($P < 0.08$) and HS ($P < 0.001$) goats. The half-life of LPS in the mammary gland is not known. However, previous studies in dairy cows indicated that immune-stimulating effects of intramammary LPS last longer than 12 h even if the cows are milked 12 h after LPS administration40. In the current study, the effect of LPS on SCC lasted for at least 72 h even though the goats were milked 6 times after the administration of LPS. It seems that LPS can stand for a prolonged period in the udder, and some LPS traces may stay in contact with mammary tissues, causing the flux of SCC for a long time.

Resident somatic cells have a long storage period (milking interval) in the udder and during this time cells ingest fat globules and casein (Paape *et al.*, 1975), resulting in reduced phagocytic and bactericidal activities, and consequently, the mammary gland immunity is impaired (Rainard and Riollet, 2006). Therefore, the immediate entrance of new blood cells to the mammary gland is primordial to face infections. The recruitment of new somatic cells (indicated by time at which SCC increased with regard to 0 h) was faster in TN than in HS halves. Compared to 0 h values in LPS halves, log10 SCC increased at 2 h by 0.54 units in TN halves ($P < 0.001$) and remained higher than the 0 h values until 48 h ($P < 0.01$) and 72 h ($P < 0.10$). On the other hand, log10 SCC in HS-LPS did not increase from 0 to 2 h (only +0.05; $P = 0.758$), but increased at 4 h onwards ($P < 0.01$).

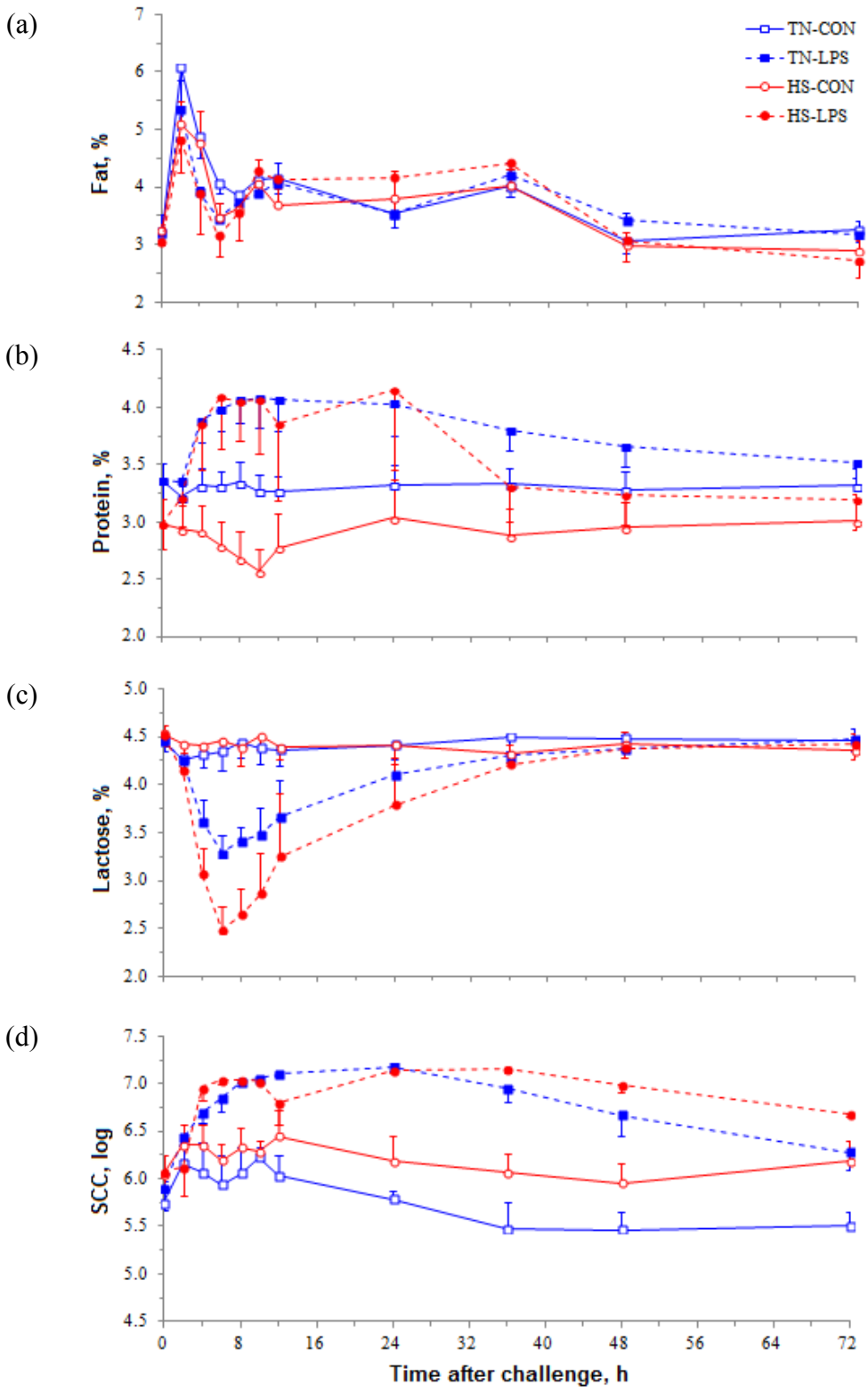

Figure 2. Hourly means and SEM for milk fat (a), protein (b), lactose (C), and SCC (d) following intramammary injection of endotoxin in dairy goats maintained under thermal neutral (TN) or heat stress (HS) conditions. For each TN and HS goat, one half-udder was administered with 10 µg E. coli lipopolysaccharide (LPS), whereas the other half was injected with saline as the control (CON).

This result can indicate a faster immune response in TN goats with earlier entrance of new cells with more phagocytic capacity. It is also possible that the phagocytic capacity of the new recruited cells from blood is lower in HS than in TN goats because Contreras-Jodar *et al.* (2018) showed that the phagosome pathway was downregulated by HS. Additionally, Lecchi et al. (2016) found that high temperatures reduce the phagocytosis capacity of blood neutrophils. Thus, under HS conditions the increment in SCC after stimulation by LPS is delayed and could be with cells that have reduced phagocytic capacity, which might increase the susceptibility of heat-stressed animals to mastitis.

5.4.3. Effect of heat stress on milk metabolome

A representative ^{1}H NMR of goat's milk spectrum with some of the most common detectable metabolites is illustrated in Figure 3.

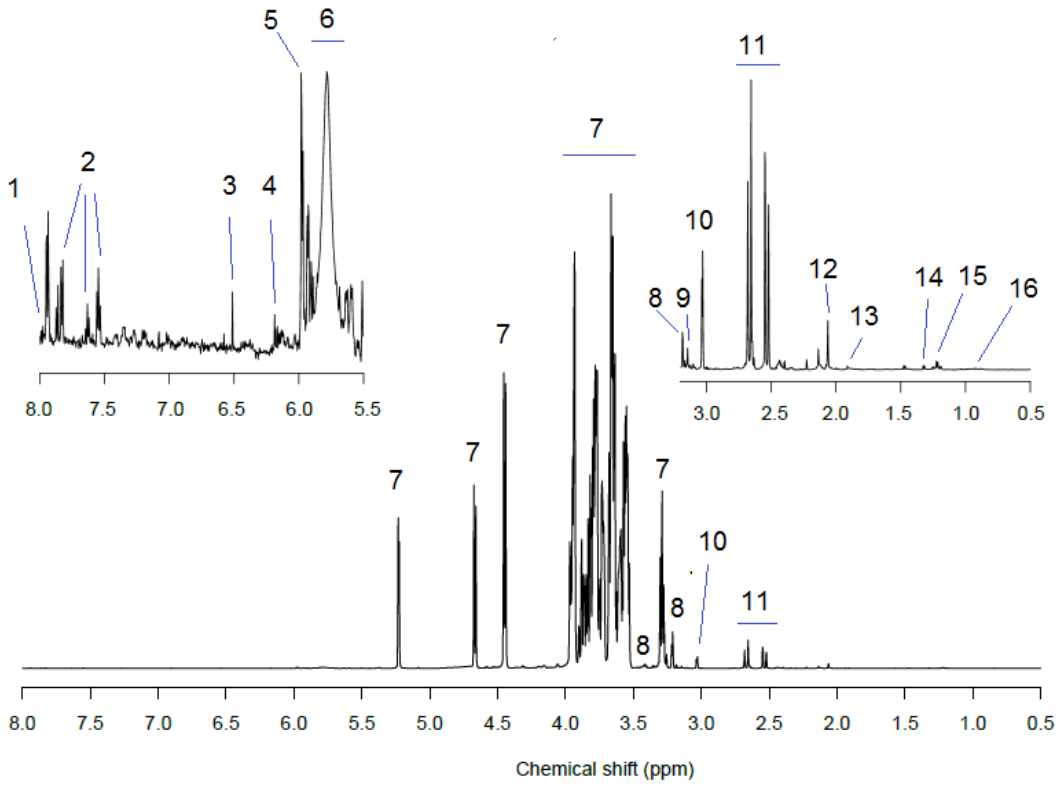

Figure 3. Representative 600 MHz 1H NMR spectrum of goat milk. Numbers indicates the following metabolites: 1: 3-metylhistidine; 2: hippurate; 3: fumarate; 4: orotate; 5: maleate; 6: urea; 7: lactose; 8: carnitine; 9: creatinine; 10: choline; 11: citrate; 12: N-acetylcarbohydrates; 13: acetate; 14: lactate; 15: ß-hydroxybutyrate; 16: butyrate. Spectral regions from δ 8.0 to 5.5 and from δ 3.0 to 0.5 were zoomed for a properly view.

The metabolomic profile was analysed using milk of HS and TN goats from the CON udder halves at 0 h. A PCA was initially applied to all data, and no outliers were detected based on the principals of Hotelling's T^2 (95% interval of confidence). Consequently, PLS-DA was applied to identify the key metabolites responsible of the differences in milk metabolome between HS and TN dairy goats. The PLS-DA scores plot showed a clear separation between HS and TN datasets (Figure 4). The cross-validation (first 2 components) gave $R^2_x = 0.36$, $R^2_y = 0.60$ and $Q^2 = 0.24$. The R^2 and Q^2 values in the model were significantly higher than in the random model ($P < 0.01$), although Q^2 did not reach the acceptable value for a recommended biological model (i.e. ≥ 0.40; Westerhuis *et al*., 2008) probably because of the small sample size used in the present comparison (4 goats TN vs. 4 goats HS). However, the principal objective of the current study was to determine whether the response of the mammary gland to inflammation varies according to ambient temperature (i.e. TN vs. HS). This objective was achieved by the comparison of both udder halves (one control and one treated with LPS) within the same animal (see the following section), which eliminates the variation due to animal, and makes it easier to detect differences with fewer animals.

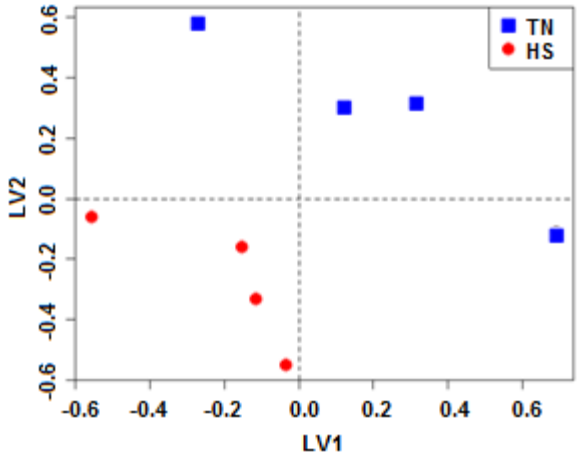

Figure 4. Partial Least Squares-Discriminant Analysis (PLS-DA) scores plot of 1H NMR metabolomics spectra of milk produced from udder halves of goats maintained under thermo-neutral (TN) or heat stress (HS) or conditions. Milk samples were collected before the administration of LPS.

The top-ranking chemical shifts responsible for discriminating milk metabolome between TN and HS goats were δ 2.55, 2.52 and 2.65 ppm. These chemical shifts

corresponded to citrate, which was greater in milk of HS goats ($\log_2$ FC = 2.89; P = 0.003). A recent report agrees with our results, where citrate has been also described as a biomarker for HS in cow's milk (Tian *et al.*, 2016).

Citrate is a normal constituent present in the milk of many animals in significant concentrations (Faulkner and Peaker, 1982). The mammary epithelium of lactating goats was found to be impermeable to citrate in both directions (Linzell *et al.*, 1976). Therefore, citrate in milk does not pass from blood but it is synthesized in the mammary gland. Citrate forms one of the main buffer systems that regulate the equilibrium between Ca^{2+} and H^{+} ions and plays an important role in the mineral equilibrium of milk. This makes citrate having a marked effect on the coagulation of milk protein (Faulkner and Peaker, 1982; Sundekilde *et al*., 2013). Alteration of citrate concentrations changes the amount of free Ca^{2+} in the soluble phase of milk, which in turn, affects the precipitation of milk proteins. Thus, greater milk concentrations of citrate could be related to impaired milk coagulation during cheese making. In fact, we detected negative effects of HS on milk coagulation properties in dairy goats (Salama *et al.*, 2014).

Citrate is produced in the mitochondria from oxaloacetate and acetyl-CoA by the enzyme citrate synthase as part of the TCA cycle, and plays a central role in cellular energy metabolism (Williams and O'Neill, 2018). When cells are exposed to acute or chronic stress, molecular mechanisms are activated to re-establish the mitochondrial function (Figure 5).

In case of heat stress, some genes that encode a diverse set of heat shock proteins are strongly upregulated in mammary gland to protect the cell form unfolded or misfolded proteins within the cell (Salama *et al*., 2019). As shown in Figure 5, heat shock response activates NF-κB signalling, which modifies mitochondrial function (Kapila *et al*., 2016). The NF-κB targets genes that break the TCA cycle and upregulates the citrate carrier. The citrate carrier exports citrate from the mitochondria to the cytosol. Once in the cytosol, citrate is broken down by ATP-citrate lyase into acetyl-CoA and oxaloacetate (Infantino *et al.*, 2011). The export of citrate has been linked to the production of several pro-inflammatory mediators such as nitrogen oxide, reactive oxygen species and prostaglandin E250, molecules that activate macrophages and dendritic cells (Williams and O'Neill, 2018) during chronic inflammation situations (Iacobazzi *et al.*, 2013) such as heat stress.

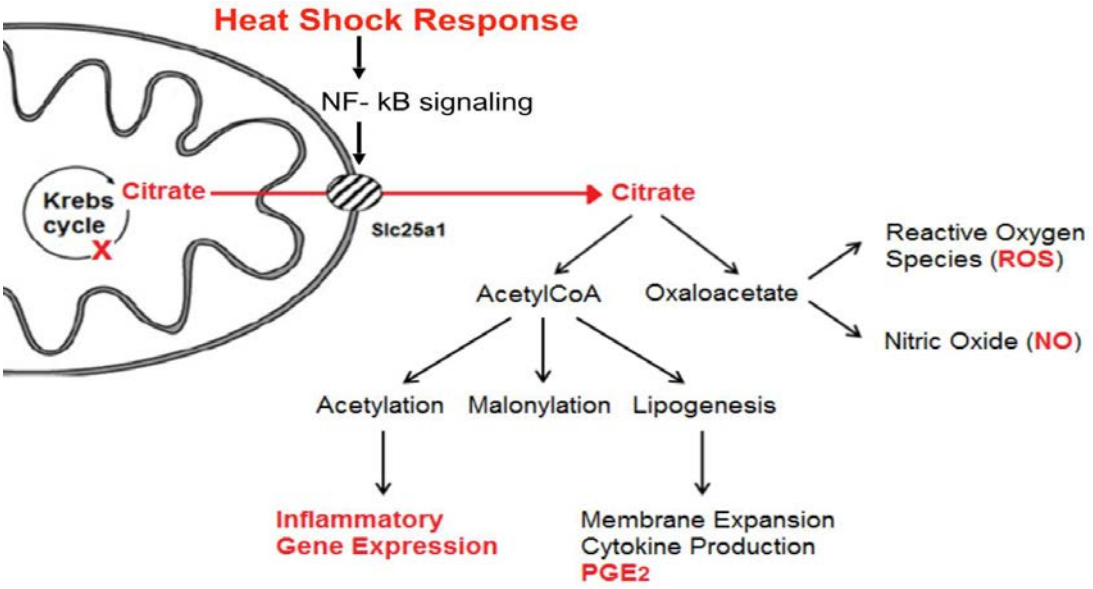

Figure 5. Schematic model indicating how the heat shock activates the transcription factor NF-kB, which consequently upregulates the mitochondrial citrate transporter (Slc25a1). Then, citrate is transported from the mitochondria to the cytosol in order to synthesize the pro-inflammatory mediators and to modulate gene expression under heat stress conditions.

5.4.4. Effect of intramammary LPS challenge on milk metabolome under thermal-neutral and heat stress conditions

Milk metabolomic profile of CON and LPS udder halves in TN (TN-CON vs. TN-LPS) and HS (HS-CON vs. HS-LPS) goats were analysed at 0, 4, 6, 12, and 24 h. The PCA revealed that no outliers were found in the fitted model. The PLS-DA scores plot showed stronger separation between time points in HS-LPS compared to TN-LPS halves because of overlapping between initial and final time points in case of TN-LPS treatment. The comparisons 0 vs 6 h and 0 vs 12 h are shown in Figure 6 for both TN-LPS and HS-LPS goats. The best cross-validated PLS-DA model for TN-LPS udders at 6 h post-challenge was obtained using the first 2 latent variables ($R^2_x = 0.74$, $R^2_y = 0.11$ and $Q^2 = 0.68$; Figure 6a), whereas TN-LPS udders at 12 h was not predictive and was given only for one latent variable ($R^2_x = 0.60$ and $Q^2 = -0.50$; Figure 6b). On the other hand, a significant regression with 2 latent variables were observed in PLS-DA model for HS-LPS at 6 h ($R^2_x = 0.51$, $R^2_y = 0.28$ and $Q^2 = 0.75$; Figure 6c) and was still significant at 12 h post-challenge ($R^2_x = 0.80$, $R^2_y = 0.07$ and $Q^2 = 0.78$; Figure 6d). This difference between TN and HS in response to LPS over time can be explained by

the fact that metabolites were generally less affected and restored earlier in TN than in HS conditions as discussed above.

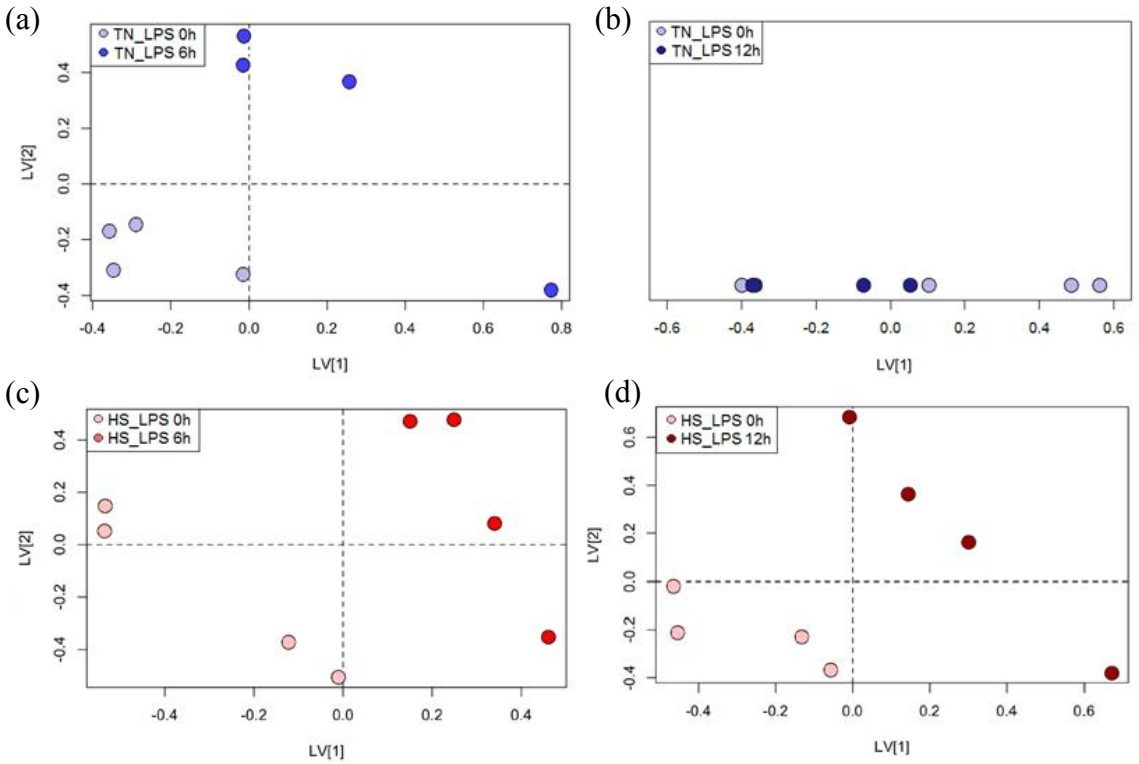

Figure 6. Partial Least Squares-Discriminant Analysis (PLS-DA) scores plot of 1H NMR-milk metabolomics spectra in dairy goats maintained under thermal neutral (TN) or heat stress (HS). For each TN and HS goat, one half-udder was administered with 10 μg E. coli lipopolysaccharide (LPS) or saline as the control (CON). The comparisons at different time points were as follows: a) TN-LPS at 0 h vs. at 6 h, b) TN-LPS at 0 h vs. at 12 h, c) HS-LPS at 0 h vs. at 6 h, and d) HS-LPS at 0 h vs. at 12 h.

The top-ranking chemical shifts responsible for discriminating time points after LPS administration were lactose (δ 3.84), choline (δ 3.19), phosphocholine (δ 4.16), N-acetylcarbohydrates (δ 2.05), L-lactate (δ 1.32), and ß-hydroxybutyrate (δ 1.20), with different change profile over time between TN and HS goats (Figure 7).

Lactose detected by ^{1}H NMR metabolomics decreased after LPS infusion, which agrees with changes observed in this component by the chemical analysis of milk (Figure 2c).

Generally, metabolites did not change ($P > 0.15$) over time in udder halves without LPS treatment (TN-CON and TN-LPS). Choline levels in milk increased in HS-LPS udders throughout time, reaching its maximum values at 12 ($P < 0.05$) and 24 h ($P < 0.01$) post-challenge (Figure 7a). On the contrary, phosphocholine (Figure 7b) decreased its levels in the HS-LPS udders at 6 h ($P < 0.05$) and tended to be different at 12 and 24 h ($P < 0.10$). Both choline and phosphocholine levels in milk of TN-LPS followed similar pattern over time as in HS-LPS, but the change was numerical ($P > 0.34$).

Although choline can be synthesized in the liver and released into blood, a correlation between plasma free choline and milk choline was not observed in cows (Artegoitia *et al.*, 2014), suggesting that at least part of the choline found in milk is synthesized in the mammary gland. Differences in milk choline after LPS treatment in the current study might be related to a cholinergic signalling in the mammary gland immune system induced by LPS. Monocytes and macrophages recognize pathogen-associated molecular patterns through pattern recognition receptors (like TLR4) located on the cell surfaces. Activation of the TLR4 receptors by LPS triggers the cholinergic system needed for the regulation of pro-inflammatory cytokine production and chemokine expression to fight against both pathogen invasion and endotoxins (Slawinska *et al.*, 2016). Furthermore, when lymphocytes are activated, T cells synthesize and directly release acetylcholine without storage in order to activate B cells, dendritic cells and macrophages (Fujii *et al.*, 2017). Additionally, for macrophage activation choline metabolism and the expression of *CTL1* (choline transporter) are upregulated, using the acetylcholine as a cell-cell signalling for macrophage polarization (Snider, 2017). This might be the reason why choline has been described as a biomarker of inflammation in breastfeeding women, where a strong positive correlation was detected between choline and serum C-reactive protein levels (Ozarda *et al.*, 2014).

As shown in Figure 7, an increase ($P < 0.05$) in milk N-acetylcarbohydrates levels were detected after LPS injection in both TN and HS conditions. Values were not restored even at 24 h post-challenge. N-acetylcarbohydrates in goat's milk are oligosaccharides synthesized and secreted by the mammary epithelial cell that confer unique health benefits to both the neonate and the dam (Meyrand *et al.*, 2013). The N-acetyl carbohydrates have anti-adhesive effect for bacterial, viral and protozoa pathogens, preventing them from binding to the dam's mammary tissue and to the

neonate gastrointestinal cells. It seems that N-acetylcarbohydrates increased in the current study to boost the mammary immune system against the simulated infection by LPS.

L-lactate was increased by LPS injection significantly in the TN, but only numerically in the HS udder halves. L-lactate peaked at 6 h post-challenge ($P < 0.05$) and then returned progressively to its basal level in TN-LPS udder halves. On the other hand, the increase of L-lactate in HS was only numerical and peaked at 4 ($P = 0.39$) to 6 h ($P = 0.22$) post-challenge (Figure 7). The LPS induces formation of L-lactate by acute conversion of epithelial cell metabolism from mainly mitochondrial-oxidative to principally glycolytic, which allows the diversion of metabolic resources normally used to synthesize milk to support the immune system (Davis *et al.*, 2004). Increment in milk L-lactate after LPS challenge or natural infection has been reported in dairy cows (Davis *et al.*, 2004).

Milk ß-hydroxybutyrate (BHBA) increased at 4 ($P < 0.05$) and 6 h ($P < 0.01$) after LPS injection in HS, but not in TN conditions (Figure 7). Although heat-stressed dairy goats suffer negative energy balance, blood levels of non-esterified fatty acids do not change, but blood BHBA values increase consistently (Hamzaoui *et al.*, 2013; Salama *et al.*, 2014). The BHBA could be used as a source of energy to spare glucose under heat stress conditions (Salama *et al.*, 2014). For the immune cells, although glucose is the most quantitatively important fuel, there are other fuels such as glutamine, fatty acids, and ketone bodies (Wolowcuz *et al.*, 2008). In the current study, we observed that changes in energy sources by heat stress might have resulted in a shift in the fuel source of immune cells. Immune cells in TN goats mainly used glucose as energy source and metabolized it to L-lactate, whereas under HS conditions BHBA together with glucose were important energy sources (Figure 7.1 and 7.2).

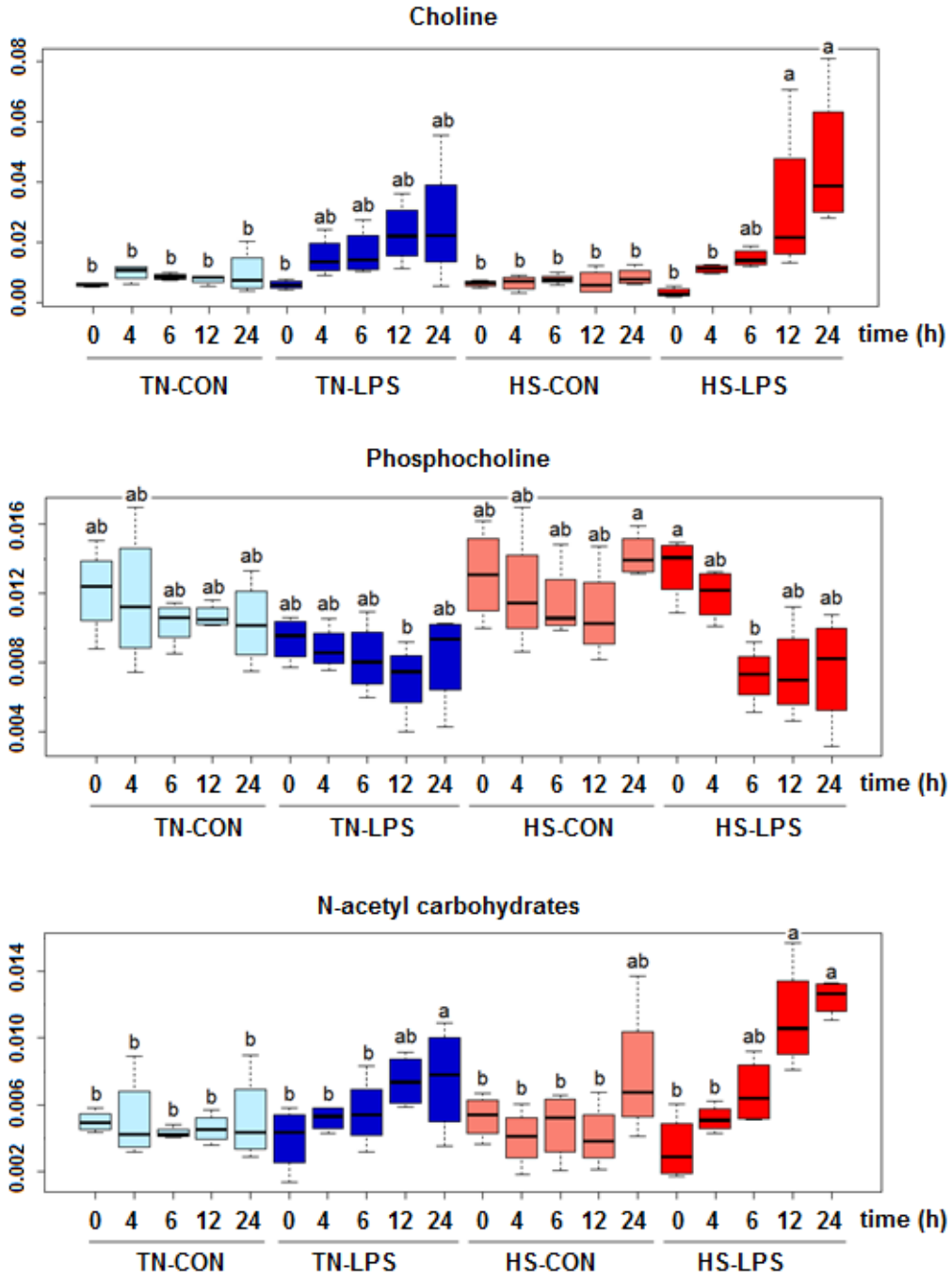

Figure 7.1. Hourly means and SEM for milk metabolites detected by 1H NMR-based metabolomics. The metabolomic profile was evaluated following intramammary injection of endotoxin in dairy goats maintained under thermal neutral (TN) or heat stress (HS). For each TN and HS goat, one half-udder was administered with 10 μg E. coli LPS (LPS), whereas the other half was injected with saline as the control (CON). This resulted in 4 treatment combinations: TN-CON, TN-LPS, HS-CON, and HS-LPS.

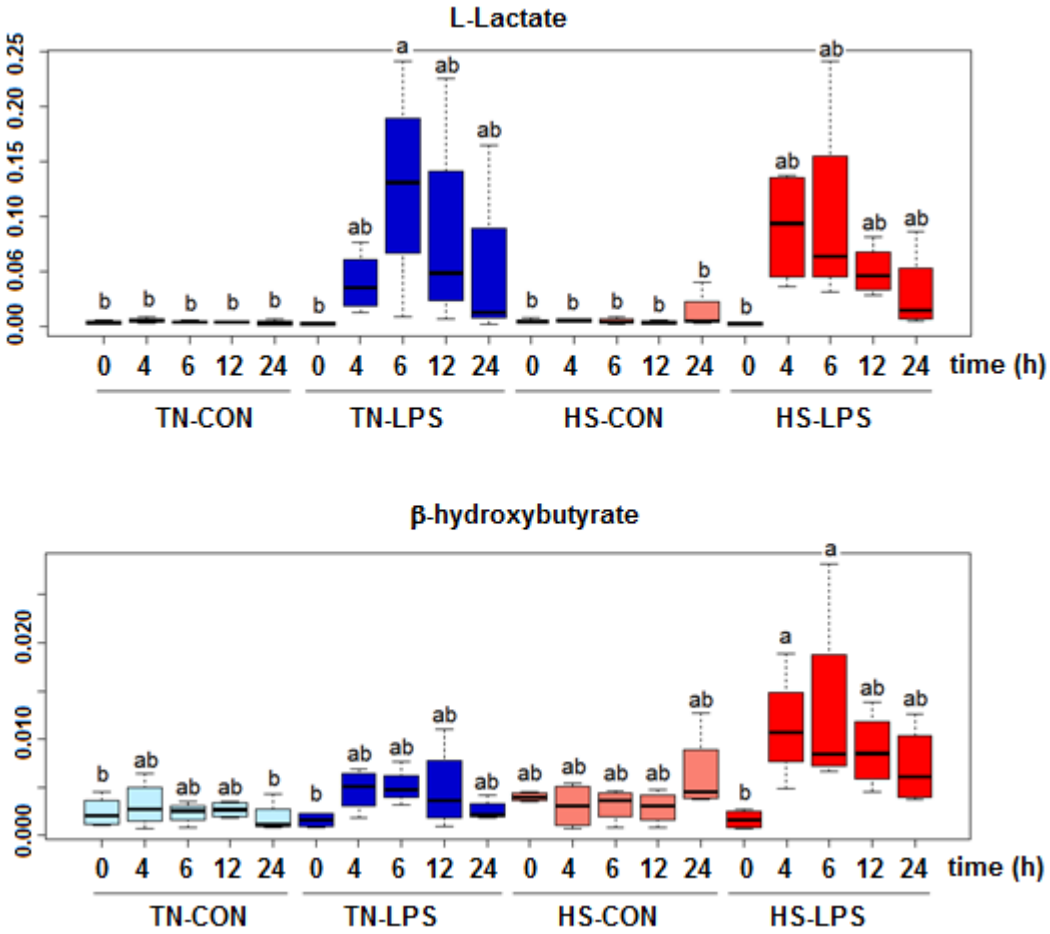

Figure 7.2. Hourly means and SEM for milk metabolites detected by 1H NMR-based metabolomics. The metabolomic profile was evaluated following intramammary injection of endotoxin in dairy goats maintained under thermal neutral (TN) or heat stress (HS). For each TN and HS goat, one half-udder was administered with 10 μg E. coli LPS (LPS), whereas the other half was injected with saline as the control (CON). This resulted in 4 treatment combinations: TN-CON, TN-LPS, HS-CON, and HS-LPS.

5.5. CONCLUSIONS

Heat stress caused marked changes in thermophysiological traits in dairy goats, including greater rectal temperature and respiratory rate, alongside decreased feed intake and milk yield. Administration of LPS in the mammary gland resulted in a systemic febrile response, which was detectable in TN goats, but was masked by the greater body temperatures normally observed in HS goats. Somatic cell recruitment

after intramammary endotoxin challenge was delayed in heat-stressed goats. Milk metabolome changed throughout time after LPS administration, with values restored earlier in TN compared to HS goats. Milk citrate increased by heat stress as part of the heat shock response, enabling the synthesis of pro-inflammatory mediators. Inflammation markers included choline, phosphocholine, N-acetylcarbohydrates, L-lactate and ß-hydroxybutyrate. However, the importance of these markers varied between TN and HS indicating different mammary immune response. Overall, heat stress negatively affected the mammary immune response, which could make HS animals more prone to mastitis.

CHAPTER 6

Conclusions

CHAPTER 6
CONCLUSIONS

The conclusions obtained in the different experiments carried out in this doctoral thesis are:

6.1. Specific conclusions

6.1.1. Thermophysiological responses of dairy goats to chronic heat stress

- Differences in rectal temperature (0.5 to 1.3ºC) and respiratory rate (1.5 to 3.3-fold) between HS and TN goats were maximal during the first 3 d of the experiment and reduced thereafter, but remained significant thereafter.
- Chronic heat stress at mid-lactation reduced feed intake (–27 to –35%), milk yield (–8%), milk fat (–9 to –12%), milk protein (–13 to –16%) and lactose (–5%), compared to thermal-neutral dairy goats, whereas water intake increased markedly (1.7-fold). Additionally, the HS goats lost weight (–115 g/d, on average).

6.1.2. Thermophysiological responses of dairy goats to chronic heat stress and intramammary LPS challenge

- The local treatment with LPS in one udder-half resulted in a systemic increment of rectal temperature. In both TN and HS conditions, rectal temperatures of the goats after the challenge were greater than before the challenge. On the following day and onwards, rectal temperatures returned to basal values.
- The increments of rectal temperature by heat stress or by LPS were similar in the first 12 h after the challenge. Therefore, changes in body temperature due to infections under heat-stressed conditions are hard to be detected.
- Respiratory rate did not change in thermal-neutral goats when challenged with LPS, but increased 2.5-fold in heat-stressed goats due to the increment in ambient temperature.
- Milk fat was not affected by intramammary LPS challenge, but milk protein increased by 17%. The increment in milk protein caused by LPS injection was

more marked in HS than in TN goats because of greater leakiness of mammary tight-junctions.

- Somatic cell recruitment after intramammary endotoxin challenge was delayed in heat-stressed goats.

6.1.3. Immune system response to chronic heat stress conditions

- Blood transcriptomics revealed 143 differentially expressed genes (55 genes were up-regulated, whereas 88 were down-regulated) in chronic heat-stressed dairy goats.

- The functional analyses using the 143 differentially expressed genes revealed that 31 biological pathways (3 upregulated and 28 down-regulated) were impacted by HS in blood. The upregulated pathways were involved in apoptosis and cell death (i.e., pyrimidine metabolism, purine metabolism, drug metabolism–cytochrome P450). Some of the 28 downregulated pathways were mainly related to immune cell proliferation and migration, and lipid and tissue repair metabolism.

- Heat stress resulted in alterations in the functionality of immune cells, which would make the immune system of heat-stressed goats less capable of fending-off diseases.

6.1.4. Biomarkers for chronic heat stress: metabolomic urinalysis

- Heat stress caused physiological changes that were reflected in their ^{1}H NMR metabolomic urinary profile. Thus, 15 differentially excreted metabolites in urine were described (7 overexcreted and 8 underexcreted) as consequence of heat stress in dairy goats.

- The overexcreted metabolites in urine were mainly related to gut-derived toxic compounds generated by an abnormal overgrowth of gastrointestinal microbiota under HS conditions that compromised the integrity and increased the permeability of the gastrointestinal tract.

- The use of hippurate and other phenylalanine derivatives from microbiota dysbiosis are proposed as candidate urinary biomarkers to identify heat-stressed goats in practice.

6.1.5. Biomarker for chronic heat stress: milk metabolomics

- Heat stress caused physiological changes that were translated into their ^{1}H NMR metabolomic milk profile. Milk citrate increased by HS as part of the heat shock response, enabling the synthesis of pro-inflammatory mediators and it is proposed as candidate biomarker for HS in milk.

- When mammary glands of dairy goats were challenged with LPS, changes in the milk metabolome were observed throughout time. Thus, the TN goats restored their basal metabolome earlier than HS goats. Inflammation markers included choline, phosphocholine, N-acetylcarbohydrates, lactose, L-lactate and ß-hydroxybutyrate. However, the importance of these markers varied between TN and HS indicating different mammary immune response.

- Heat stress negatively affected the mammary immune response, which could make heat-stressed animals more prone to mastitis.

6.2. Implications

Omic techniques offer a holistic approach to better understand the physiological and metabolic changes induced by HS in dairy goats and shed light towards establishing new alleviation strategies. Cellular proteostasis and apoptosis, microbiota dysbiosis and impaired immunocompetence are key mechanisms by which performance is reduced in heat-stressed dairy animals. More research related to these mechanisms will reveal novel useful markers of heat stress and help in finding new solutions to reduce its negative impacts on animal productivity and welfare.

CHAPTER 7

References

CHAPTER 7
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COLOPHON

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