Universitat Autònoma de Barcelona

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Efectes de l'estrès per calor i estratègies d'alleujament en petits remugants

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Tesis presentada por **Nabil Mehaba** y dirigida por los doctores **Ahmed AAK Salama** y **Francesc Xavier Such Martí**, del Departament de Ciència Animal i dels Aliments de la Universitat Autònoma de Barcelona.

Bellaterra, 19 de Junio del 2020

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Certifican:

Que la memoria titulada “Heat Stress Effects And Nutritional Alleviation Strategies In Small Ruminants”, presentada por **Nabil Mehaba** con la finalidad de optar al grado de Doctor en Producción Animal, ha estado realizada bajo su dirección y, considerándola acabada, autorizan su presentación para que sea juzgada por la comisión correspondiente.

Y para que conste a los efectos oportunos, firman la presente a Bellaterra, 19 de Junio del 2020.

Dr. Francesc Xavier Such Martí. Dr. Ahmed Salama.

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- **Mehaba N**, Salama AAK, Such X, Albanell E and Caja G. 2019. Lactational Responses of Heat-Stressed Dairy Goats to Dietary L-Carnitine Supplementation. *Animals*. 9:567.
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As part of my Ph.D. training, I have participated in works that has been published in the following national and international journals and symposium proceedings:

Publications in international journals:

- Coloma-García W, **Mehaba N**, Llonch P, Caja G, Such X and Salama A.A.K. 2019. Prenatal heat stress effects on gestation and postnatal behavior in kid goats. *PLoS ONE*.15(2): e0220221.
- Coloma-García W, **Mehaba N**, Such X, Caja G and Salama AAK. 2020. Impact of low ambient temperatures on physiological, productive, and metabolic variables in lactating dairy goats. *J. Dairy Sci*. (Submitted: JDS.2020-18862)
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    - Computational Tools for Bioinformatics
    - Regression Models and Methods
    - Design and Analysis of Experiments
    - Software for Data Analysis
    - Statistical Inference
    - Master of Science Thesis Manuscript (Pending)

LIST OF ABREVIATIONS

AA	Amino acid
ADF	Acid detergent fiber
ADG	Average daily gain
ALT	Alanine aminotransferase
APR	Acute phase protein
ATP	Adenosine triphosphate
ATT	Aspartate aminotransferase;
AUC	Area under curve
BBD	γ-Butyrobetaine dioxygenase
BCS	Body condition score
BHB	β-Hydroxybutyrate
BUN	Blood urea nitrogen
BW	Body weight
$BW^{0.75}$	Metabolic body weight
CAR	Carnitine
CEAAH	Ethical committee on human and animal experimentation
CON	Control
CP	Crude protein
DIM	Days in milking
DM	Dry matter
DMI	Dry matter intake
DMY	Daily milk yield
EBAL	Energy balance
EC	Epinephrine challenge
ELISA	Enzyme-linked immunosorbent assay
Exp	Experiments
FA	Fatty acid
FCM	Fat corrected milk
GLU	Glucose
GLU-CR	Glucose clearance rate
GLU-$t_{1/2}$	Glucose half lifetime
GTT	Glucose tolerance test
HPA	Hypothalamic-pituitary-adrenal axis
HPLC	High performance liquid chromatography
HS	Heat stress
HSP	Heat-shock protein
HTML	Hydroxytrimethyllysine
HTMLA	Hydroxy- trimethyllysine aldolase
IC	Insulin challenge
IGF-1	Insulin-like growth factor
IgG	Immunoglobulin G

INRA	Institut National de la Recherche Agronomique
INS-CR	Insulin clearance rate
ISBGR	Insulin stimulated blood glucose response
ITT	Insulin tolerance test
LDL	Low density lipoprotein
LSM	Least square mean
MDG	Merino de Grazalema
MF	Milk fat
MG	Murciano-Granadina
MO	Moringa oleifera
MP	Milk protein
MUFA	Mono-unsaturated fatty acid
MUN	Milk urea nitrogen
MY	Milk yield
NAD	No available data
NC	No change
NDF	Neuteral detergent fiber
NEFA	Non-esterified fatty acid
NEL	Net energy of lactation
NEM	Net energy of maintenance
NRC	National research council
P	period
PDH	Pyruvate dehydrogenase
PDI	Protein digestible in the small intestine
PDIA	Protein digestible in the intestine supplied by RUP
PFK	Phosphofructokinase 1
PPARγ	Proliferator activated receptor γ
PUFA	Poly-unsaturated fatty acid
PY	Payoya goat
RH	Relative humidity
RPB	Rumen protein balance
RR	Respiration rate
RT	Rectal temperature
RUP	Rumen undegradable protein
SCC	Somatic cell count
SEM	Standard error of the means
SFA	Short chain fatty acid
T	Treatment
T_3	Tri-iodothyronine
T_4	Thyroxine
TCA	Tricarboxylic acid cycle
TCO_2	Total carbon dioxide pressure
T_{db}	Dry bulb temperature

THI	Temperature-humidity index
TMABA	Trimethylaminobutyraldehyde
TMABA-DH	Trimethylaminobutyraldehyde dehydrogenase
TML	N6-trimethyllysine
TMLD	TML dioxygenase
TMR	Total mixed ration
TN	Thermal neutrality
TS	Total solids
TZD	Thiazolidinediones
UE_m	Fill units for sheep
UFL	Feed units for lactation
WI	Water intake

SUMMARY

In the current thesis the effects of heat stress (HS) on performance of Lacaune dairy ewes (Exp.1) as well as the response of HS Murciano-Granadina dairy goats to dietary L-carnitine (Exp. 2) and methionine (Exp. 3) were evaluated. In the 3 Exp., animals were fed a total mixed ration and milked ×2 daily. The environmental conditions were: thermal neutral (TN; THI = 59-65) and HS (day, THI = 83; night, THI = 75). Photoperiod (light-dark) was constant (12-12 h). Rectal temperature (RT), respiratory rate (RR), DMI, water intake (WI) and milk yield (MY) were recorded daily, whereas milk for composition was sampled weekly and BW was registered at the start and the end of each period. In Exp.1, ewes (n = 8) were exposed to TN or HS in a crossover design with 2 periods (21 d each). Further, ewes were administered with glucose, insulin and epinephrine to evaluate the metabolic responses. HS increased RT, RR, WI and BW loss, but reduced DMI, and milk fat and protein contents without affecting MY. Despite the reduced DMI by HS, blood NEFA did not change, but creatinine values increased. Response to the metabolic challenges indicated that HS ewes had faster uptake of glucose and greater resistance to lipolytic signals compared to TN ewes. In Exp.2 & 3 with dairy goats, the design was 4 × 4 Latin square as 2 dietary factors were added to the 2 environmental conditions. The 2 dietary conditions were control (CON) without supplementation vs. rumen protected L-carnitine (CAR, Exp. 2) or rumen protected methionine (Met, Exp. 3). In Exp. 2, HS goats experienced increased RT and RR. Additionally, HS goats suffered 26% loss in DMI, but they tended to eat longer particle sizes. CAR dramatically increased blood free-, acetyl, and total-carnitine concentrations. Despite this efficient absorption, CAR had no effect on DMI, milk production or blood metabolites in TN or HS conditions. In Exp.3, DMI for TN goats was limited to 2.0 kg/d, whereas HS goats were kept feeding *ad libitum*. Consequently, HS goats had only 9.8% (although significant) less DMI than TN. Consequently, no changes in MY were detected. Expected increments in RT and RR due to HS were detected but Met resulted in less RR in the morning and lower RT in the afternoon. In addition, Met avoided the typical BW loss under HS conditions. The profile of blood amino acids (AA) revealed less basal Met concentration, despite the comparable DMI levels. Additionally, HS goats were in shortage of glutamate, which could be related to the inflammation and immune response at the gastrointestinal level. Met supplementation spared glutamate regardless the ambient temperature. Overall, HS negatively affected the performance of dairy ewes. Metabolic adaptations of dairy ewes to HS included reduced body fat mobilization and increased muscle protein breakdown. Methionine, but not L-carnitine, had some beneficial effects on the performance of heat-stressed dairy goats. Probably some more AA in addition to methionine should be supplemented under HS conditions.

Keywords: Heat stress, dairy ewes, dairy goats, L-carnitine, methionine.

RESUMEN

En esta tesis, se estudiaron los efectos del estrés por calor (EC) sobre la producción de ovejas lecheras Lacaune (Exp.1), así como la respuesta de cabras lecheras Murciano-Granadina bajo condiciones de EC a la L-carnitina (Exp. 2) y la metionina (Exp. 3). En los 3 Exp., los animales fueron alimentados con una ración única mezclada y se ordeñaron ×2 al día. Las condiciones ambientales fueron: termo-neutralidad (TN; THI = 59-65) y EC (día, THI = 83; noche, THI = 75). El fotoperíodo (día-noche) fue constante (12-12 h). La temperatura rectal (TR), el ritmo respiratorio (RR), la IMS, el consumo de agua (CA) y la producción de leche (PL) se registraron diariamente, mientras que la leche para la composición se muestreó semanalmente y se registró el peso vivo (PV) al inicio y al final de cada período. En el Exp.1, las ovejas (n = 8) fueron expuestas a TN o EC en un diseño cruzado de 2 períodos (21 días cada uno). Además, a las ovejas se les administró glucosa, insulina y epinefrina para evaluar sus respuestas metabólicas. EC aumentó la TR, RR, CA y la pérdida de PV, pero redujo la IMS y el contenido de grasa y proteína de la leche sin afectar a PL. A pesar de la reducción de IMS por EC, los AGNE en sangre no cambiaron, y sin embargo los valores de creatinina aumentaron. La respuesta a los desafíos metabólicos indicó que las ovejas EC presentaban una rápida absorción de glucosa y una mayor resistencia a las señales lipolíticas en comparación con las ovejas TN. En los Exp.2 y 3 con cabras lecheras, el diseño fue un cuadrado latino 4 × 4, ya que se agregaron 2 factores dietéticos a las 2 condiciones ambientales. Las 2 condiciones dietéticas fueron control (CON) sin suplementación, versus L-carnitina protegida del rumen (CAR, Exp. 2) o metionina protegida del rumen (Met, Exp. 3). En Exp. 2, las cabras EC experimentaron un aumento de TR y RR. Además, las cabras EC sufrieron una pérdida del 26% en IMS, pero tendieron a comer partículas de tamaño más largo. La CAR aumentó drásticamente las concentraciones de carnitina libre, acetilo y total en sangre. A pesar de esta absorción eficiente, CAR no tuvo efecto sobre IMS, producción de leche o metabolitos en sangre en condiciones TN o EC. En el Exp.3, la IMS de las cabras TN se limitó a 2.0 kg/d, mientras que las cabras de EC se alimentaron *ad libitum*. Así pues, las cabras EC presentaron sólo un 9.8% menos IMS que TN, aunque significativo. En consecuencia, no se detectaron cambios en PL. Se observaron incrementos esperables en TR y RR debido al EC, pero Met redujo el RR por la mañana y RT en la tarde. Además, Met evitó la pérdida típica de PV en condiciones de EC. El perfil de aminoácidos en sangre (AA) reveló una menor concentración basal de Met, a pesar de los niveles comparables de IMS. Además, las cabras EC tenían poco glutamato, lo que podría estar relacionado con una inflamación y respuesta inmune a nivel gastrointestinal. La suplementación con Met ahorró glutamato, independientemente de la temperatura ambiente. En general, el EC afectó negativamente la producción de las ovejas lecheras. La adaptación metabólica de las ovejas lecheras al EC incluyó una reducción de la movilización de grasa corporal y el aumento de la degradación de las proteínas musculares. La metionina, pero no la L-carnitina, tuvo algunos efectos beneficiosos sobre el rendimiento de las cabras lecheras estresadas por el calor. Probablemente un poco más AA además de la metionina deberían ser suplementados en condiciones de EC.

Palabras claves: Estrés por calor, ovejas lecheras, cabras lecheras, L-carnitina, metionina

RESUMÉ

Cette thèse, étude les effets du stress thermique (ST) sur les performances des brebis laitières Lacaune (Exp.1) ainsi que la réponse des chèvres laitières Murciano-Granadina à la L-carnitine (Exp.2) et à la méthionine (Exp. 3) sous conditions de ST. Dans les 3 Exp, les animaux ont reçu une ration totale mélangée et traitent ×2 par jours. Les conditions environnementales étaient : thermoneutralité (TN; THI = 59-65) et ST (jour, THI = 83; nuit, THI = 75). La photopériode (jour-nuit) était constante (12-12 h). La température rectale (TR), le rythme respiratoire (RR), la MSI, la prise d'eau (PE) et la production de lait (PL) ont été enregistrés quotidiennement, tandis que le lait pour la composition a été échantillonné chaque semaine et PV a été enregistré au début et à la fin de chaque période. Dans Exp.1, les brebis (n = 8) ont été exposées au TN ou au ST avec permutation de 2 périodes (21 j chacune). En plus, les brebis ont été administrées avec du glucose, de l'insuline et de l'épinéphrine pour évaluer la réponse métabolique. Le ST a augmenté le TR, RR, PE et a réduit le PV, mais a réduit l'IMS et le contenu en matières grasses et en protéines du lait sans affecter la PL. Malgré la réduction de l'IMS par le ST, le AGNE sanguin n'a pas changé, mais les valeurs de créatinine ont augmenté. La réponse aux défis métaboliques a indiqué que les brebis ST avaient une absorption plus rapide du glucose et une plus grande résistance aux signaux lipolytiques que les brebis TN. Dans Exp.2 & 3 avec des chèvres laitières, le design expérimental était un carré latin 4 × 4 car 2 facteurs alimentaires ont été ajoutés aux 2 conditions environnementales. Les 2 conditions alimentaires étaient control (CON) sans supplémentation et une supplémentation avec la L-carnitine protégée du rumen (CAR, Exp. 2) ou avec la méthionine protégée du rumen (Met, Exp. 3). Dans Exp. 2, les chèvres ST ont demontré une augmentation du TR et RR accrues. De plus, les chèvres ST ont réduit de 26% l'IMS, mais elles avaient tendance à manger des particules plus longues. La CAR a considérablement augmenté les concentrations libres, d'acétyle et carnitine totale de sang. Malgré cette absorption efficace, la CAR n'a eu aucun effet sur l'IMS, la PL ou les métabolites sanguins dans les conditions TN ou ST. Dans Exp.3, l'IMS pour les chèvres TN était limité à 2,0 kg/j, tandis que les chèvres ST étaient nourries *ad libitum*. Par conséquent, les chèvres ST avaient seulement 9,8% (bien que significatif) de moins d'IMS que TN. Par conséquent, aucun changement dans PL n'a été détecté. Des augmentations attendues de la TR et du RR dues au ST ont été détectées, mais la Met a entraîné une diminution du RR le matin et une TR plus basse l'après-midi. De plus, Met a évité la perte de PV typique dans les conditions ST. Le profil des acides aminés du sang (AA) a révélé une concentration en Met basale inférieure, malgré des niveaux de DMI comparables. De plus, les chèvres ST manquaient de glutamate, ce qui pourrait être lié à l'inflammation et à la réponse immunitaire au niveau gastro-intestinal. La supplémentation rencontrée a épargné le glutamate quelle que soit la température ambiante. Globalement, le ST a affecté négativement la performance des brebis laitières. Les adaptations métaboliques des brebis laitières au ST comprenaient une mobilisation réduite des graisses corporelles et une dégradation accrue des protéines musculaires. La méthionine, mais pas la L-carnitine, a eu certains effets bénéfiques sur les performances des chèvres laitières soumises à un ST. Probablement un peu plus d'AA en plus de la méthionine devrait être supplémenté dans les conditions ST.

Mots-clefs: Stress thermique, brebis laitières, chèvres laitières, L-carnitine, méthionine.

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CHAPTER 1

Literature review

CHAPTER 1

1. LITERATURE REVIEW

1.1. INTRODUCTION:

The sector of small ruminants (i.e. sheep and goats) is so important such that 56% of the world ruminant domestic populations (3872 million heads) are sheep (1178 million) and goats (1000 million) (FAO, 2016) and they are distributed all over different types of ecology. Furthermore, a 60% increase in global sheep number is expected by 2050 (Foresight, 2011). Over 56% of the world's small ruminants are located in water-limiting and dry zones in developing countries, whereas temperate and humid zones account for 27% and 21%, respectively (FAO, 2016). Today's livestock producers face increased regulatory and market pressure not only to maximize animal performance but to minimize the environmental impacts of such performance, and small ruminant producers are under the same regulatory and economic pressure as other ruminant producers but lack the level of data available to other species of livestock (Dougherty et al., 2019). Furthermore, high ambient temperature has potentially several physiological adverse effects that result in a tremendous economic loss for the sheep and goat industry.

The ability of sheep and goats to cope with heat stress (**HS**) without harming their welfare and productive performance has been often overrated. It has been considered that goats are well adapted to semiarid and hot climates than cattle and sheep; however, the results of the studies carried out in native Spanish breed (Salama et al., 2014). The thermal comfort zone for temperate-region adult cattle is in the range from 5 to 15ºC as proposed by Hahn et al. (2003). However, the thermal comfort zone for Mediterranean adult goats is in the range from 13 to 20ºC as proposed by Menéndez-Buxadera et al. (2014a), and it is breed and species dependent (*Figure 1.1*) (Menéndez-Buxadera et al., 2014b). Furthermore, Appleman and Delouche (1958) observed that the heat regulatory system of Nubian goats was no longer effective when animals were continuously kept for 12 consecutive days at 40ºC, but not at 35ºC. Consequently, they concluded that the limit of heat tolerance for goats lies between 35 and 40ºC.

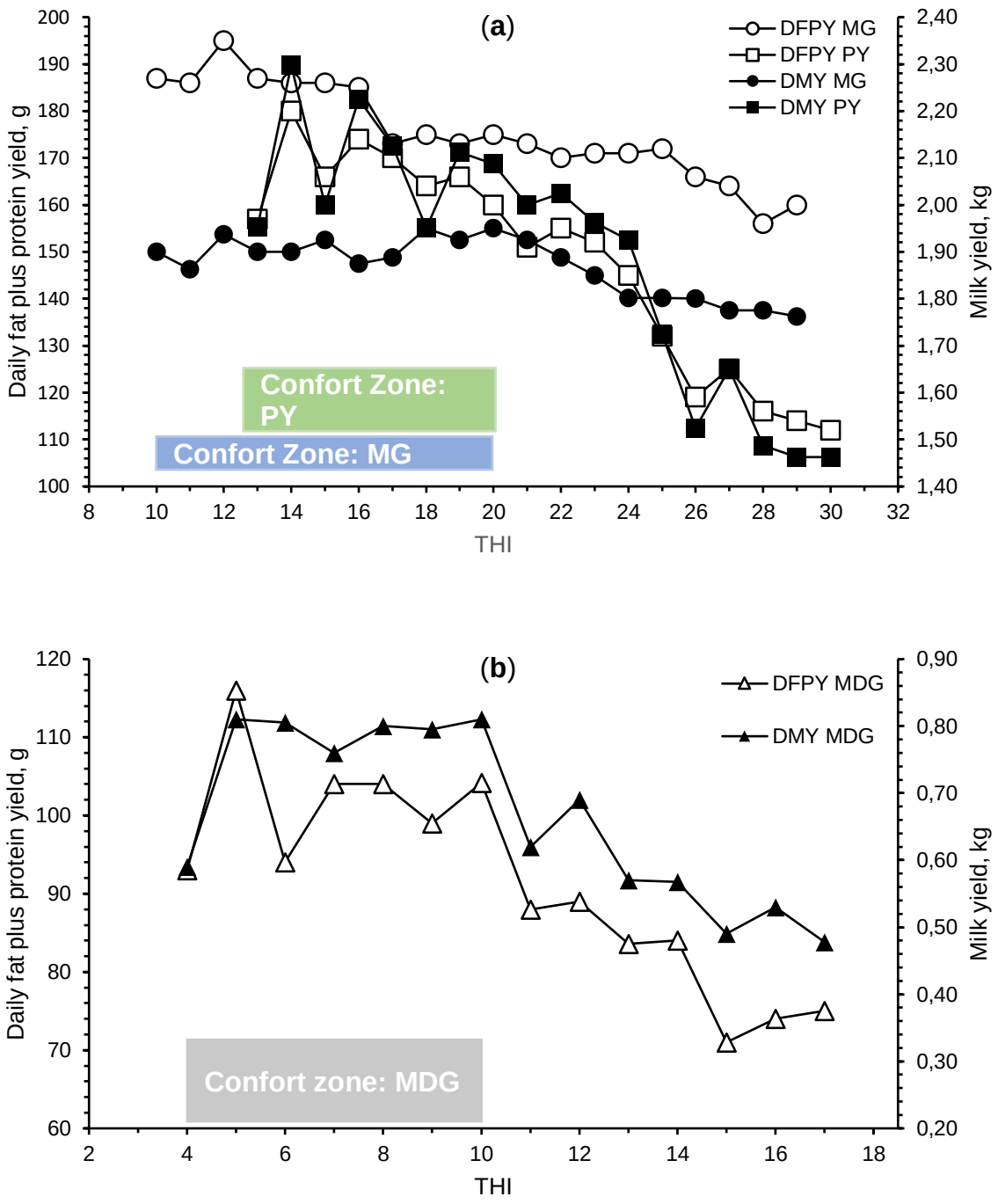

Figure 1.1: Daily milk yield (**DMY**) (Kg) and daily fat plus protein yield (**DFPY**, g) as a function of temperature humidity index (**THI**, Finocchiaro et al., 2005) values for Murciano-Granadina (MG) and Payoya (PY) goats (a) and for Merino de Grazalema (MDG) ewes (a) (Adapted from Menéndez-Buxadera et al., 2014b).

Heat balance is a complex phenomenon affected by numerous climatic factors (e.g. ambient temperature, relative humidity, wind speed, radiant heat and other

factors such as altitude, animal factors (e.g. age, genotype, hair coat characteristics, degree of acclimatization, health status, physical activity, level of performance, reproductive state, etc.) and management factors (e.g. housing, provision of shade, fans and others) (Serradilla et al., 2017). Responses to direct impacts on ruminant animals include changes in feed management (e.g. balancing feed, forage, supplements), stocking rates, genetic selection, disease risk management (e.g. vaccination or selection of resistant genotypes), climate control (e.g. shade, fans, water sprays) and timing of reproduction (Henry et al., 2018). It has been observed that the animals are exposed to stressing climatic conditions, due to high temperatures, during 45–55% of the year, generating losses of 1.9 and 3.1% of annual fat plus protein yields in Murciano-Granadina and Payoya goats, respectively (Menéndez-Buxadera et al., 2013a).

In Mediterranean climates or winter-dominant rainfall regions, where future climate predictions show no shift in rainfall distribution, the productivity of summer dominant forages and pastures will only improve at the shoulders of the production season (Cullen et al., 2012). In addition, the semi-intensive production system of the region submits lactating small ruminants to confinement and HS, due to limited ventilation of the animals and increased irradiative heat from roofs even during the night (Sitzia et al., 2015). The way forward under this shifting condition is probably to decide the animal species (non-ruminants, large or small ruminants) to rear and produce. In this regard, small ruminants appear to be more promising because of their low production cost, short generation interval, suitability to small holdings, multipurpose (meat, milk and fiber) use, ability to utilize crop residues effectively, and most importantly their tolerance to harsh climatic variables (such as low rainfall and heat stress) than cattle and other monogastrics (Akinmoladun et al., 2019).

To the best of our knowledge, reviews undertaken to develop nutritional and management strategies in small ruminants under heat stress conditions are scarce. The lack of data in the literature regarding mitigation strategies of heat stress in small ruminants led us to indirectly use references of dairy cattle.

1.2. EFFECTS OF HEAT STRESS ON SMALL RUMINANTS:

1.2.1. Physiological Responses:

Hyperthermia unlike fever represents a failure in thermoregulation (uncontrolled heat production, poor heat dissipation or an external heat load), this does not involve a thermoregulatory set point (Wrotek et al., 2011; Fecteau and White, 2014).

Physiological parameters like respiration rate and rectal temperature give an immediate response to HS, and consequently the level of animal discomfort/comfort (Marai et al., 2007). Fasoro (1999) reported normal rectal temperature between 39.2ºC and 39.8ºC for goats; while Okoruwa (2015) reported a range of 38.3 - 39.9ºC for sheep. Furthermore, rectal temperature exceeding 41.7ºC, death may occur as the animal cells begin to degenerate (Marai et al., 2007). However, heat exposure increased goats' rectal temperature from 38.6ºC to 39.7ºC (Hamzaoui et al., 2013), which are still lower than values reported by Fasoro (1999) in Nigerian breeds. Also, sheep exposed to heat showed higher rectal temperature of 39.5 - 39.8ºC in Indian sheep (Srikandakumar et al., 2003). In addition, respiration rate (breaths/min) can change frequently and it is indirectly influenced by the animal's activities (metabolism and muscle activity) (Devendra, 1987).

Reference respiratory rate for adult goats ranges between 15 and 30 breaths/min (Pugh and Baird 2012). Panting rate (breaths/min) (low: 40–60, medium: 60–80, high: 80–120, and severe HS: > 200) appears to be the most accessible and easiest method for evaluating the impact of HS on animals under extreme conditions (Silanikove, 2000). Increased respiration rate following HS has been reported in sheep 74 breaths/min (Al-Haidary et al., 2012) and goats 150 breaths/min (Hamzaoui et al., 2013).

1.2.2. Hematological, Immunological and Biochemical Responses:

The blood profile of animals is sensitive to changes in the environmental temperature and is an important indicator of physiological responses to the stressing agent (Okoruwa, 2014). When exposed to HS, goats showed an increased amount of red blood cells, packed cell volume, hemoglobin, white blood cells, neutrophil, eosinophil, lymphocyte and monocyte (Hamzaoui et al., 2013). Also, packed cell

volume, hemoglobin and red blood cells were higher under HS in sheep (Sanusi et al., 2010).

Electrolytes. The acid-base balance is a complex physiological process to maintain a stable pH in an animal's body. The vital limits of pH variation for mammals are between 7.35 and 7.45 (Houpt,1989; Constable, 1999) and regulated by a complex system of buffers (H_2CO_3 and HCO_3^-). The secretion of HCO_3^- in urine and its reabsorption suggest a large requirement and turnover of body HCO_3^- to maintain blood pH during HS (Hamzaoui et al., 2013), is a mechanism to keep blood pH within normal range.

Enzymes. Metabolic regulators are important in elucidating a picture of modulation in physiological mechanisms during stressed conditions and are best assessed by determining the enzymes governing various metabolic reactions in plasma/serum (Gupta et al., 2013). Serum level of aspartate transaminase and alanine aminotransferase is helpful in the diagnosis of the welfare of animals. The serum alanine aminotransferase value increases during HS in goats (Sharma and Kataria, 2011). Alanine aminotransferase catalyzes the transfer of an amino group from L-alanine to α-ketoglutarate, the products of this reversible transamination reaction being pyruvate and L-glutamate.

Glucose, *cholesterol*, *blood urea nitrogen*, *non-ester fatty acids* and β-*hydroxybutyrate*. Results of metabolic indicators of heat stressed small ruminants are inconsistent. HS conditions decrease glucose and cholesterol levels in goats (Ocak et al., 2009). In sheep, glucose levels increase during HS (Al-Haidary et al., 2012). Conversely, HS had no effect on glucose and blood urea nitrogen in goats (Hamzaoui et al., 2013). In HS dairy cows, plasma glucose level decreased (7%) compared to Thermal neutral (**TN**) cows (Rhoads et al., 2009).

Non-esterified fatty acids (**NEFA**) and β-hydroxybutyrate (**β-HBA**) are most indicative of the animal's energy status. The study of Eknæs et al. (2017) provides an example of this, with indoor feeding of multiparous Norwegian dairy goats from day 1 - 120 and day 201 - 230 of lactation and mountain grazing between the indoor periods. Based on computer tomography, considerable fat was mobilized in the first 2 months of lactation, with visceral fat contributing more and mobilized at twice the

rate compared with the fat of the carcass. Whereas, Hamzaoui et al. (2013) reported that a reduction in feed intake and body weight under HS was not accompanied by body fat mobilization as NEFA concentration did not vary between HS and control goats. However, exposure to HS resulted in higher NEFA in sheep (Sevi et al., 2001) and β-hydroxybutyrate concentration in goats (Salama et al., 2014). In dairy cows, HS conditions had no effect on basal NEFA levels (Wheelock et al., 2010; Rhoads et al., 2009).

The β-oxidation of NEFA produces more metabolic load than glucose oxidation, generating 1814.0 vs. 472.3 kcal energy (Wheelock et al., 2010). Therefore, glucose is preferentially utilized to sustain thermoregulation rather than milk bio-synthesis. The same reduction in plasma glucose concentration was observed in HS goats by Hamzaoui et al. (2013). Although neither diet nor environment affected post-feeding circulating NEFA, TN pair-feeded lambs had increased pre-feeding NEFA compared with HS lambs (Mahjoubi et al., 2016). However, when ruminants are in a positive energy balance, propionate is the major substrate for gluconeogenesis, and it mostly originates from starch fermentation (Huntington, 1990). Surprisingly, the HS glucose precursor supplemented lambs had decreased rectal temperature compared with the HS control lambs, despite consuming more DM (Mahjoubi et al., 2016).

Hormones. Hormones (i.e. thyroxine (**T_4**), triiodothyronine (**T_3**), prolactin, leptin, adiponectin, growth hormone, glucocorticoids, mineralocorticoids, catecholamines and antidiuretic) are involved in thermal adaptation and could be important indicators for assessment of stress in animals (Sivakumar et al., 2010). Decreased thyroid hormone level during HS is an adaptive response and affects the hypothalamic-pituitary-adrenal axis (**HPA**) to decrease thyrotropin releasing hormone (Johnson, 1985). In goats, Sivakumar et al. (2010) reported a decrease in plasma concentration of T_3 and T_4 levels. In sheep submitted to severe HS, drastically decreased levels of T_3 and T_4 was reported (Lu et al., 2019). However, exposure to HS did not result in significant changes in the thyroid hormone concentrations in sheep (Al-Haidary, 2004). A comparative study of cattle breeds showed that blood T_3 reduction is related to the capacity of heat tolerance during heat stress: breeds with higher T_3 reduction are better in maintaining rectal temperature and feed intake (Pereira et al., 2008). Therefore, reducing thyroids

hormones could be a coping mechanism to HS in order to reduce endogenous heat production (Bernabucci et al., 2010). Furthermore, an increased cortisol level during HS was reported in sheep (Barnes et al., 2004) and goats (Sivakumar et al., 2010). A possible explanation is HS can act on HPA axis activation by increasing plasma cortisol secretions to increase glucose availability and meet the increase in energy demand for thermoregulation (Matteri et al., 2000). The prolactin level increased in goats under HS (Sivakumar et al., 2010). As for the most important hormone for energy metabolism (i.e. insulin), Salama et al. (2014) reported an increase in circulating levels of insulin HS dairy goats. In addition, environment influenced circulating insulin; insulin concentration was increased in HS lambs compared to TN pair-fed lambs (Mahjoubi et al., 2016). This coping mechanism follows the line of adaptations observed in order to keep the supply glucose availability of blood glucose for energy production during HS conditions.

Immunological. The acute phase proteins (**APR**) are defined as proteins whose plasma concentrations increase or decrease classifying them into positive (i.e. C-reactive protein, serum amyloid A, haptoglobin), and negative (i.e. albumin, transferrin) acute phase proteins, respectively (Al-Dawood, 2017). The main purpose of APR is to restore the homeostasis by isolating and destroying the harmful agent and to activate the repair process (Ceciliani et al., 2002). APR are recognized as promising tools to assess welfare, health and performance in animal production (Petersen et al., 2004).

Data on the use of acute phase proteins as biomarkers and potential indicators of stress in sheep and goats are scarce. Goats exposed to HS increased circulating haptoglobin when they were metabolically challenged (Hamzaoui et al., 2013).

1.2.3. Productive Response:

The reduction in quantity and quality of milk is of important economic impact of HS in dairy animals. It has been observed that animals are exposed to high temperatures, during 45–55% of the year in the Mediterranean region, generating losses of 1.9 and 3.1% of annual fat plus protein yields in Murciano-Granadina and Payoya goats, respectively (Menéndez-Buxadera et al., 2014a).

Feed intake and water consumption. The reductions in energy intake coupled with increased maintenance costs during HS causes negative energy balance in lactating small ruminants. Interaction between stress and nutrition results in nutrient deficiency as HS is associated with marked reduction in feed intake (West, 1999). There is also a direct effect of HS on the feeding center of the hypothalamus, resulting in a hormonal response, which could also decrease metabolic rate (Johnson, 1985; West, 1999). Moreover, the maintenance requirements increased by 30% because of HS (NRC, 2007) and the energy intake would not be enough to cover the daily requirements which results in an apparent body weight loss (Hamzaoui et al., 2013). In fact, heat-stressed animals enter a bioenergetic state similar (but not to the same extent) to the negative energy balance observed in early lactation (Moore et al., 2005). But still, we think that the increase of 30% of energy maintenance is underestimating HS effects on energy metabolism, since dairy ewes and goats was in a balanced energy intake-expenditure yet still losing weight (See later). Studies showed that dry matter intake decreased following exposure to HS in sheep (Nardone et al., 1991). Dairy goats decreased their DMI by 30% (Salama et al., 2014) under HS conditions. In addition, in HS Afshari lamb, reduced DMI by approximately 14% on average compared with TN Lambs (Mahjoubi et al., 2016). However, Rhoads et al. (2009) reported that decreased DMI accounted only for about 35% of the heat stress-induced decrease in milk bio-synthesis during established lactation in lactating cows.

Sheep consume 2 kg water/kg DM at temperatures between 0 and 15ºC, and this ratio increases three times at temperatures above 20ºC (Conrad, 1985). HS goats Murciano-Granadina had greater water consumption compared with TN goats (5.50 L/d increase). The greatest values of water intake were recorded during the first week when DMI was at its lowest value (Hamzaoui et al., 2013).

Body weight changes. Hamzaoui et al. (2013) observed an apparent BW loss of 1.5 kg (−41 g/d) of HS dairy goats; in which include the inevitable variations in the digestive tract content. Therefore, leading to underestimation of BW change under HS conditions. Since its been reported in goats, HS significantly affects visceral weight (increased weight of blood, pluck, heart, spleen and kidney) rather than TN group (Hashem et al., 2013).

Milk yield and quality. Lactating goats exposed to moderate or severe (THI = 81 or 89) HS for four days lost milk yield of 3% or 13%, respectively (Sano et al., 1985). Moreover, dairy goats kept under HS in a climatic chamber reduce their milk yield by 3 - 10% (Salama et al., 2014) with impaired coagulation properties which could have an important impact on cheese industry (Salama et al., 2014). Nevertheless, Hamzaoui et al. (2013) found that dairy goats were able to maintain milk yield under HS conditions (31 – 37ºC; THI = 77 and 65, respectively). In addition, the content of protein and protein fractions in milk were reduced under HS (Hamzaoui et al., 2013). Milk fat contents did not vary between HS and TN goats (Hamzaoui et al., 2013). Although, under the same experimental conditions, Mehaba et al. (2019) observed a lower fat content in HS dairy goats compared to their TN counterpart. In dairy cows, current research indicates that HS reduces dry matter intake by 30%, milk yield decreased by 27.6% (9.6 kg) (Wheelock et al., 2010). Furthermore, the same authors observed a decrease in DMI.

Conventionally, the reduction in milk yield during the heat stress condition is attributed to the reduced feed intake and blood flow to mammary glands which has been observed in cows (West, 2003), dairy goats (Salama et al., 2014) fed ad libitum under HS. However, there is several hypothesis suggesting other mechanisms involved in milk protein reduction; a decreased protein intake and increased sweat secretion that contains protein and urea might have limited the availability of amino acids for milk protein synthesis (Salama et al., 2014). Additionally, it is suggested that decreased mammary synthesis of milk protein is the reason for low milk protein during the HS (Bernabucci et al., 2002). Moreover, results of heat-stressed bovine mammary epithelial cells in vitro suggest that HS has a direct negative effect on synthesis of protein and fat, mediated in part by coordinated changes in mRNA, and protein abundance of key networks (Salama et al., 2019).

1.3. MITIGATION STRATEGIES OF HEAT STRESS:

Among the potential approaches to alleviate HS, there are different measures that can be implemented at different levels of the production system such as:

- Physical modifications to the environment;
- Management adaptations;

- Genetic selection;
- Dietary principal components modifications.

1.3.1. Physical Modification of the Environment:

1.3.1.1. Shade:

Behavioral responses of sheep and goats under HS include bunching in the shade (Silanikove, 2000). Seeking shade is a conspicuous form of behavioral adaptation (Silanikove, 1987). If shade is not available, animals will change their posture to the vertical position in respect to the sun in order to reduce the effective area for heat exchange (Cain et al., 2006). Providing sheep and goats access to shade leads to improvements in weight gain, milk production and reproductive performance (Berger et al., 2004).

Shading is the easiest method to reduce the impact of high solar radiation, and it is applicable under extensive conditions. A well-designed shade structure reduces heat load by 30 - 50% (Muller et al., 1994). Shelters do not need to be complicated or elaborate, trees and shrubs can serve as shelters for animals from solar radiation (Onyewotu et al., 2003), and are usually the least-cost alternative.

The shed should be placed on a top of a hill if possible, opened on all sides and with wire or cable fences, the roof should be 3.5 to 4.0 m high with its long dimension east-west to prevent exposure to high sun radiation; the roof slopes should be south-north to avoid vertical sun heat (Habeeb et al., 2018). In beef cattle (Mitlohner et al., 2002), and dairy cows (West, 2003) shade has been used efficiently. For small ruminants, the adequate surface area from shade per animal is 1.86 - 2.79 m^2 for sheep to be kept loose in the shed (Habeeb et al., 2018).

1.3.1.2. Cooling Methods:

Crowding should be avoided, packing animals into a small area leads to restricts air flow and aggravates heat stress. Poor ventilation has reduced the performance of sheep (Sevi et al., 2006). In addition, fully enclosed shelters are not recommended for hot climates because of the decreased natural air velocity, therefore, it is preferred to use partially enclosed shelters (Sejian et al., 2015).

Increasing air movement promotes evaporation, makes cooling by perspiration more effective and helps removal heat dissipated by animals in the form of radiation, conduction and convection. Alteration of air temperature and velocity must be considered to alter the microclimate of an animal effectively (Da Silva, 2002). Also, spraying the roof of the barns helps in cooling of the surroundings (barn walls and roofs, fences, earth, etc…) which in turn helps keeping animal's cooler (Brouk et al., 2002).

Darcan and Güney, (2008) reported that sprayed and ventilated heat-stressed goats for 1 h/day consumed more feed (18%) and water (7%) and produced more milk (21%). Furthermore, cooling sheep and goats by spraying could reduce HS symptoms and improve animal welfare (Hamzaoui et al., 2012). Moreover, cooling dairy cows during the early postpartum improved the production performance by about 16% (i.e milk yield and FCM), indicators of metabolic status, immune response, and antioxidant capacity (Safa et al., 2019).

1.3.2. Management strategies:

A good example of management strategy is shearing animals during hot seasons. Sheared sheep evaporation becomes the most important avenue for heat dissipation, since sweating in unshorn sheep is much less important than respiratory evaporation due to the presence of a wool coat (Marai et al., 2007). However, the direct exposure of the skins to solar radiation may hurt the skin. In such case, suitable covering for the skin can be obtained by either providing shade or partial clipping of the coats.

1.3.2.1. Heat Tolerant Animal Selection:

Observations that the milk production of Holstein-Jersey dairy cattle in tropical and subtropical conditions may be 40% to 60% lower than in temperate conditions (Usman et al., 2013), raises serious concerns about resilience to future global warming. This example highlights the need to understand the role of strategies such as breeding livestock for adaptability rather than exclusively focusing on high productivity especially in the tropics. Salama et al. (2014) have showed how decreases in protein and fat in milk composition are accompanied by downregulation in the gene expression of casein, fat and lactose synthesis and

upregulation in the expression of genes related to milk cathepsins. In addition, Hamzaoui et al. (2013), using Afymetrix GeneChip Bovine in Murciano-Granadina goats in late lactation, identified 39 and 74 genes whose expression was up- and down-regulated, respectively, by HS. Carabaño et al. (2017) found a relevant signal for fat and protein yields response to heat common to the three dairy species (cattle, sheep and goats) pointing out to a region in Chromosome 6 where a gene encoding a member of the potassium channel-interacting proteins (KCNIP4) that regulate processes of defense against hypoxia and associated to hyperactivity disorders in humans. The HS elicited changes in gene expression related to transcriptional regulation and metabolic processes. Quantitative genetic analyses presented earlier have been used for the assessment of the animal's response to HS through its effect on milk production and fitness traits (Menéndez-Buxadera et al., 2014b). Moreover, the genetic component of response to climatic constraints must include traits related to thermotolerance of the animals in breeding programs of goat production (Serradilla et al., 2017). In Table 1.1, a list of genes candidate to select animals based on their heat tolerance, as it can be noticed each breed and specie developed different metabolic mechanism to cope with HS which leads to a wide range of genes involved in heat tolerance.

Table 1.1: Genes/candidate genes involved in heat tolerance (Berihulay et al., 2019).

Candidate genes	**Function**	**Breeds**	**References**
ANXA6, GPX3, GPX7, and PTGS7	Arachidonic acid metabolism	Taklimakan desert sheep	Yang et al., 2016
CPA3, CPVL, and ECE1	Renin angiotensin system	Taklimakan desert sheep	Yang et al., 2016
CALM2, CACNA2D1, KCNJ5, and COX2	Oxytocin signaling	Taklimakan desert sheep	Yang et al., 2016
RAP1A, SLC4A4, CPA3, and CPB1	Pancreatic secretion	Taklimakan desert sheep	Yang et al., 2016

OXT, AVP, MICU2, and IFT88	Regulation of homeostatic process, Reproductive physiology and response to nutrient levels/digestive system	Baraki sheep	Kim et al., 2016
UROD	Pigment biosynthetic process	Baraki sheep	Kim et al., 2016
EIF2B3	Heat stress/temperature stimuli	Baraki sheep	Kim et al., 2016
PLK3	Osmotic stress	Baraki sheep	Kim et al., 2016
TGM3	Hair follicle morphogenesis	Baraki sheep	Kim et al., 2016
MCIR, ASIP, and TYRP1	Coat color pattern	Crioula sheep	Cristina et al., 2017
HSP-70	Protect cell against thermal injury	Pelibuey and Suffolk sheep	Romero et al., 2013
HSP-70	Thermotolerant	Mexico goat	Meza-Herrera et al., 2006
ASIP, KITLG, HTT, GNA11, and *OSTM1*	Coloration	Chinese goat	Wang et al., 2016
TBX15, DGCR8, CDC25A, and *RDH16*	Body size	Chinese goat	Wang et al., 2016
FGF2, GNA13, and *PLCB1*	Thermo-tolerance (melanogenesis)	Baraki sheep	Kim et al., 2016
BMP2, BMP4, GJA3, and *GJB2*	Body size and development	Baraki sheep	Kim et al., 2016
MYH, TRHDE, and *ALDH1A3*	Energy and digestive metabolism	Baraki sheep	Kim et al., 2016
GRIA1, IL2, IL7, IL21, and *IL1R1*	Nervous autoimmune response	Baraki sheep	Kim et al., 2016
TRPM8	Regulation of body temperature	Brazilian sheep	José et al., 2017
IL10RB and IL23A	Immune response	Ugandan goat	Onzima et al., 2018

It is uncertain whether the most effective approach would be to identify phenotypes in a heat-tolerant breed that have favorable production and market traits or to select heat tolerant animals within breeds that meet market specifications (Gaughan et al., 2010). Gantner et al. (2017) found that some animals within the same breed exhibit greater tolerance to heat stress due to genotypic differences. Finding phenotypes of thermal response is still a handicap; selection criteria can

focus on increasing the tolerance threshold or the slope of decay of the considered trait (Serradilla et al., 2017). Body temperature is a good measure of heat tolerance in animals, as it represents the result of all heat gain and heat loss processes in the body. However, genetic selection for lower body temperature as a strategy to reduce the magnitude of heat stress effects in dairy cattle may be limited due to adverse association with economic indicators (Henry et al., 2018). Heritability of milk traits and genetic values of animals diminish when heat load increases (Serradilla et al., 2017). Practical example of selecting animals for heat tolerance, the SLICK haplotype (https://omia.org/OMIA001372/9913/) originally identified in Senepol cattle has already been introduced into Holsteins to improve their thermotolerance. SLICK haplotype Holstein cows are better able to regulate body temperature, and experience less-pronounced reductions in milk yield under HS (Dikmen et al., 2014).

1.3.2.2. Reproduction Strategies:

Non-lactating animals and animals for meat purposes are much less likely to experience infertility during HS due to seasonal breeding patterns that ensure that animals are not bred at the warmest time of year, and relatively low amounts of metabolic heat production as compared to lactating animals (Hansen, 2009). When exposed to continuously hot conditions, ewes that are poorly adapted to heat stress may suffer increased levels of embryonic mortality and reduced fetal growth, and mortality in lambs from heat-stressed ewes appears to be higher than in lambs produced during cooler conditions (Harle et al., 2007).

There is evidence for benefits from adaptive management strategies including stronger selection for more heat tolerant animals, ultrasonic scanning of ewes during pregnancy to target beneficial management, and, where appropriate, shifting mating time so that lambing coincides with peak forage availability and more moderate temperatures (Henry et al., 2018). Furthermore, the season of lambing will condition the lamb and kid meat quality since in an experiment to determine the effect of the slaughter temperature on meat quality in sheep and goats, reported that animals slaughtered under an ambient temperature of ~35ºC had a higher pH level and myofibrillar fragmentation index in muscles (indicator of the extent of myofibrillar protein degradation of meat post-slaughter), lower color (lightness, redness and yellowness), and expressed less juice than those slaughtered at 21ºC, which

indicates that the seasonal temperatures were the main reason for differences in meat quality (Kadim et al., 2008).

Reproduction problems during HS is not only a female problem, rams also could be affected. To avoid fertility problems during HS, the scrotal sack should be free from wool as the rams should be sheared six to eight weeks prior to breeding (Al-Dawood, 2017). Furthermore, in extreme circumstances, rams can be housed during the day and kept with the ewes at night (Gimenez and Rodning, 2007). Therefore, timed mating programs during cooler periods during summer (i.e. early morning or late evenings).

1.3.2.3. Feeding Strategies:

Small ruminants, depending on breed, have a dynamic eating behavior during warm weather conditions. For example, Fawn goats have different eating behaviors in comparison with Saanen x hair goats, which are exposed to severe heat stress and poor nutritional condition (Koluman et al., 2017). The authors indicated that Saanen goats had higher meal size and meal length and longer inter-meal interval, meal time, and eating rate within each meal, but a lower number of meals in comparison with German Improved Fawn goats. Furthermore, in extreme heat, grazing ruminants decrease their grazing time, tend to lie down to reduce their locomotion and spend more time in the shade (Silanikove, 1987; Silanikove, 2000).

Adjustments may include changes in feeding schedules (feeding at cool hours, feeding intervals). Because, during summer, the feeding behavior for most of the animal's changes and they tend to consume more feed during the cooler periods of the day (West, 1999). Therefore, feeding animals during the cooler periods of the day encourages them to maintain their normal feed intake and prevents the co-occurrence of peak metabolic and climatic heat load. Habeeb et al. (2010) showed that ewes in groups fed at 1200 and 1500 h were better than ewes fed at 0900 h in physiological and nutritional aspects. Respiration rate and temperatures of rectal, skin and ear values decreased significantly while daily feed intake, dry matter intake and water intake values increased significantly due to late of feeding time under summertime. Besides that, feeding animals at more frequent intervals helps to minimize the diurnal fluctuation in ruminal metabolites and increase feed utilization efficiency in the rumen (Soto-Navarro et al., 2000). HS goats have greater rumen

temperature values (+0.3ºC), and lower rumen pH than the TN goats in accordance with the high ambient temperature under which the HS goats were housed (Castro-Costa et al., 2015).

Adapt the working routine to the environment, sheep and goats can be handled (i.e. milking, transportation) in the early morning or late evening time (Morrison, 1983), and the afternoon work should be avoided when body temperature is already high. Improve feed presentation, wet feeds, such as silages, may increase palatability and DMI during non-seasonal production system, because the water content decreases dietary dust and favors thermoregulation (Sitzia et al., 2015). Particle size reduction is particularly beneficial during summer, as it reduces the heat production of the animals, as shown in cattle (Cannas, 2004). Furthermore, HS lactating dairy goats consumed smaller particle size compared to TN goats (Mehaba et al., 2019). To reduce feed selection, water should be added to the dry TMR and particle size should be much smaller than that usually considered optimal for dairy cows (Cannas, 2004).

1.3.3. Nutritional Strategies:

Calculated energy balance is traditionally the way of assessing diet formulation adequacy. The fact that heat-stressed goats and some extent ewes fail to enlist a ‘shift’ in energetic metabolism (despite inadequate nutrient intake) may indicate that HS directly (not mediated by feed intake) impacts energetics as stated by Ronchi et al. (1999) in dairy cows. As a matter of facts, reduced feed intake occurring in animals exposed to hot environment partly explains the biological mechanism by which HS impacts production and reproduction (Bernabucci et al., 2010).

Moreover, nutritional strategies are among the easiest and cheapest to implement. In dairy cattle, a portion of the milk production lost (35 - 50%) during HS may be potentially recovered through nutritional management (Rhoads et al., 2013). For example, in small ruminants, diets formulated for low metabolic heat increments can help to improve feed intake and performance under HS conditions.

The main purpose of feed additives, generally, is to Increase the provision of gluconeogenic precursors that may lead to a decrease in the use of amino acids (**AA**) for gluconeogenesis and, in turn, increase energy and nitrogen use efficiency

(Berg et al., 2007). Furthermore, increasing insulin sensitivity could be a good be a good strategy to alleviate HS effects on small ruminants.

1.3.3.1. Diet Principal Components Manipulation

1.3.3.1.1. Water:

Unlike feed nutrients, water does not receive adequate consideration to ensure optimal performance of ruminants, mainly to those raised under hot conditions. Furthermore, the cheapest nutrient in animal production; water metabolism under HS condition is closely linked to the thermo-regulatory requirements of the ruminant (Conte et al., 2017); yet, it is still not considered when formulating rations for HS animals. Milk contains 85% water, and in lactating requirement increases during heat stress condition as compared to non-lactating animals (Conte et al., 2017). In dairy goats, the main water out-puts are milk, urine, faeces, and various forms of evaporation, due to the needs for thermoregulation (Hamzaoui et al., 2013). The daily average rectal temperature in dehydrated goats is 0.5ºC to 0.9ºC higher than the correctly watered ones, a pointer to a reduction in evaporative heat loss (Jessen et al., 1998). In addition, high temperatures make water a valuable nutrient because it potentiates dehydration in water deprived animals and this can affect milk production.

The main physic quality of interest for water during HS is its temperature. There is a general underestimation of the positive effect on thermal balance due to a low temperature of drunken water. The rumen performs the function of a water reservoir (15% of animal body weight) for use when water is scarce (Silanikove, 2000). Castro-Costa et al. (2015) reported a rumen temperature dramatically changed by feeding (1.4 ± 0.1ºC) and drinking (-3.4 ± 0.1ºC), and 2 h were necessary to return to the fasting value. In HS condition, water offered to cows should be at low temperature. In heat stressed lactating dairy cattle, drinking water at 10ºC compared to 28ºC has positive effect on reduction of body temperature and breathing rate (Conte et al., 2017).

In general, goats are better at conserving water than sheep and possibly due to their browse diet (Silanikove, 2000). As for milk yield and quality, goat and sheep breeds differ in their capacity to cope with hot periods without water:

- Black Bedouin and Barmer goats once every four days watering regime (Silanikove, 1992).
- Desert goats raised under traditional systems may be watered only once every three to six days (Ahmed and El Kheir, 2004).
- Awassi ewes can withstand more than 6-week period of watering every two days without significant changes (Jaber et al., 2004).
- Yankasa sheep survived five days of water restriction (Igbokwe, 1993).
- Barki sheep did not endure three days without drinking (Farid et al., 1979).

The water requirements of sheep and goats increase under HS conditions; thus, it is essential that animals have a continuous access to adequate, clean, cool and fresh water. This is done by having adequate watering devices (making sure pressure is adequate to refill). The way that drinking troughs are arranged and the ease of accessibility of animals to points of water supply also affect water intake (Araujo et al., 2010).

1.3.3.1.2. Fiber:

To compensate for the reduced nutrient and energy intake caused by HS and the metabolic heat load associated with fermenting forages, nutritionists typically tend to increase the energy density of the ration using extra grains/concentrates. The combination of a 'hotter' ration and the cow's reduced ability to neutralize the rumen directly increases the risk of rumen acidosis (Conte et al., 2017). Furthermore, Castro-Costa et al. (2015) reported a decrease in rumen pH of HS goats compared to their TN counterpart. To maintain the optimal rumen function, with a level of acid detergent fiber (**ADF**) and neutral detergent fiber (**NDF**) that should not be lower than 18% and 28% on dry matter basis of the diet, respectively (West, 1999).

Once more, the breed effect in small ruminants is present in all aspects of production and management. Goats adapted to a harsh environment (desert Bedouin goats) have higher digestion capacity of high fiber diet than non-desert goats (Saanen goats). The increase in diet digestibility in heat-stressed small ruminants (i.e. goats, Hamzaoui et al., 2013) may be taken in consideration to offer a diet with high quality forages (i.e. Grass–legume mixtures generally have higher

crude protein concentration and lower fiber concentration than pure grass stands). This is because legumes usually have less fiber and favor higher intake than grasses. Increased dietary fiber intake may increase heat load and then HS in small ruminants. As shown, high fiber diets may indeed increase heat production, as demonstrated by work showing that for diets containing 100, 75 or 50% of hay, the efficiency of conversion of metabolizable energy to milk was 54, 61 and 65%, respectively (Coppock and West, 1986). However, if summer milk is produced by ewes in early lactation, dietary fiber can be reduced, and starch increased without negative effects on energy partitioning. Whereas, if mid and late lactation ewes are used, high-starch diets may impair milk production, and fat supplementation is probably more appropriate than starch supplementation (Cannas et al., 2002; Pulina et al., 2006).

West et al. (1999, 2003) reported that cows fed low fiber level (NDF = 30% of DM) during hot weather showed a higher daily milk production, lower body temperature and lower respiratory rates compared with those fed high fiber diets (NDF = 42% of DM). Furthermore, Kanjanapruthipong et al. (2010) evidenced, that under HS condition, is beneficial to reduce the level of NDF (from 21.0 to 17.4% on DM basis of dietary NDF from roughage) in the diet of the 3-week period before expected calving, with positive outcome on the postpartum metabolism and milk. Sheep are more able to convert fibrous, low-quality feedstuffs into meat and other products than cattle (Hafez, 1987). However, moderate HS decreases intake and growth in young sheep consuming a high diet containing high medium quality roughage (Marai et al., 2007).

We recommend using the nutritional recommendation for dairy cattle, until further investigation in this aspect is undertaken in small ruminants. It is worth mentioning, while formulating rations for goats and sheep the selective feeding behavior of small ruminants compared to cattle.

1.3.3.1.3. Protein:

HS small ruminants are in negative nitrogen balance, as consequence of the reduction in feed intake (O'Brian et al., 2010). The reduction in the feed intake can be counteracted by the increase of protein content of the diet. However, small ruminants like cattle should be fed the right amount of protein, because excess

dietary crude protein requires more energy for its metabolism and produces more endogenous heat, which further aggravates the HS effects (West, 2009; Conte et al., 2017). Furthermore, other than the amount of protein fed, quality of protein source should be considered under HS conditions. In a review paper, Huber et al. (1994) summarized that dairy cows fed diet containing 16.1% CP with low degradability (59% of total CP) had greater milk yield, than cows fed diet characterized by high protein content (18.5% CP) with medium degradability (65% of total CP). Also, West (2009) recommended to minimize the problem of protein intake, addition of rumen bypass protein and should be 36-40% of the total dietary protein.

1.3.3.1.4. Fat:

As previously discussed, the decrease in forage to concentrate ratio improves the efficiency of nutrients utilization in animals exposed to HS conditions. Fat supplementation increases net energy intake in HS dairy cows thanks to its higher energy density and its lower metabolic heat, in comparison with fiber or starch (Knapp and Grummer, 1991). Nevertheless, total fat must comprise fat from natural feed, oil seed and bypass fat in equal proportions (Naik, 2013). Furthermore, the effects of fat supplementation in the diet of dairy ewes on milk yield depend on the type of supplemented fat, and the level of supplementation (Caroprese et al., 2011).

For example, ruminally-protected fats in the diet lower metabolic heat increment significantly, improving the role of fats during HS (Conte et al., 2017). Moreover, fat composition plays an important role in animal metabolism, when fats, specially highly unsaturated ones, are included in the diet a reduction in growth of cellulolytic ruminal bacteria and a decrease in fiber digestion are usually observed (Mughetti et al., 2012).

The efficiency of fat supplementation to ruminants is well reported. Melo et al. (2016) informed of the supplementation of palm oil in lactating dairy cow's diet reduces effect of HS (reduce rectal temperature and respiration rate) and improve milk yield and feed efficiency. In addition, dairy goats supplemented with 4% fat (i.e. soybean oil) kept under HS increased milk fat content (Hamzaoui et al., 2012). A significant increase was recorded in live body weight and average daily gain of growing lambs fed Omega-3 (Teama and El-Tarabany, 2016).

The effects of fat supplementation in sheep were examined by Bocquier and Caja (2001). After reviewing the scientific literature, they concluded that fat supplementation did not increase milk yield in sheep and in goats and always increased milk fat content in sheep and goats. Some of the effects of fat supplementation on small ruminants are reported in Table 1.2.

Table 1.2: Effect of fat supplementation on production parameters in small ruminants under HS and TN conditions.

Reference	Species	Breed	Production stage	HS treatment	Control group	Diet supplement	Main effects
Caroprese et al., 2012	Sheep	Sarda	Late-lactation (202.1 ± 5.3 DIM)	18.7 – 30.5 ºC, 70.0% THI 64.3 – 82.1 × 44 d	Solar radiation, control	Flaxseed	• ↓: RR and Na. • NC: RT, BW, NEFA, K, Mg and, anion gap • ↑: IgG, cortisol, glucose, Na and Cl.
Caroprese et al., 2011	Sheep	Sarda	Late-lactation (202.1 ± 5.3 DIM)	18.7 – 30.5 ºC, 70.0% THI 64.3 – 82.1 × 44 d	Solar radiation, control	Flaxseed	• ↑: MY, MF, MP and milk casein, clot firmness • NC: Milk pH, clot formation, • ↓: clotting time, SCC
Caroprese et al., 2014	Sheep	Comisana	Late-lactation (202.1 ± 5.3 DIM)	21.0 – 38.0 ºC, 70.0% THI 67.9– 93.4 × 30 d	HS, control	Flaxseed	• ↓: RR and DMI. • NC: BCS and cortisol
						A. nodosum	• NC: BCS, RR, DMI and cortisol.
						Flaxseed + *A. nodosum*	• ↓: RR and, DMI • NC: BCS, and cortisol.
Teama and El-Tarabany. 2016	Goat	Baladi	Growing kids (4 -5 month)	34.4 ºC, 56.8 % THI 85.4 × 120 d	HS, control	Encapsulated Ω-3	• ↑: BW, ADG, DMI, total protein, Ig, T_3 • NC: Creatinine, T_4 • ↓: Cholesterol, triglycerides, ATT, ALT, BUN,
Hamzaoui et al., 2014	Goat	Murciano-Granadina	Mid-lactating (99 ± 1 DIM)	30.0 – 37.0 ºC, 40.0% THI 76.8 – 85.2 × 19 d	HS, control	Soybean oil	• ↑: 3.5% FCM, MF, Lact, NEFA, • NC: RR, RT, DMI, WI, BW, MY, Na, K, Cl, TCO_2, BUN, glucose, Anion gap, BHB • ↓: Blood pH.

Reference	Species	Breed	Production stage	HS treatment	Control group	Diet supplement	Main effects
Prieto et al., 2013	Sheep	Assaf	Early lactation (47 ± 1.2 DIM)	Not specified, × 63 d	Control	Sunflower oil	• NC: DMI, BW, MY, FCM, MF, Lact, SCC • ↓: MP, TS, Na:K
Ghazal et al., 2014	Goat	Saanen	Early lactation (30 ± 2 DIM)	Not specified, × 28 d	Ca salt palm oil, control	CLA: 4.5 g C18:2: cis-12,*trans*-10 + cis-9,*trans*-11	• NC: DMI, BW, MY, MP, Lact, glucose, NEFA, BUN, Insulin • ↓: MF, BHB
Castro et al., 2009	Sheep	Lacaune	Mid-Lactating (120 ± 12 DIM)	Not specified, × 28 d	Control	Sunflower oil	• ↑: digestibility of DM, OM, CP, • NC: MY, MF, MP, digestibility EENDF, ADF
						hydrogenated palm oil	• ↑: MY, MF, MP, digestibility of DM, OM, CP, • NC: digestibility of EE, NDF, ADF
Schettino et al., 2017	Goat	Saanen	Late-lactation (not specified DIM)	Not specified, × 20 d	Control	2.7% Chia seed	• NC: DMI, MY, 3.5% FCM, MF, MP, Lact, TS
						5.5 % Chia seed	• ↑: DMI • NC: MY, 3.5% FCM, MF, MP, Lact, TS

RT: rectal temperature; RR: respiration rate; DMI: dry matter intake; WI: Water intake; BW: Body weight, BCS: Body conditions score; ADG: Average daily gain; FE: feed efficiency; MY: milk yield; MF: milk fat; MP: milk protein; Lact: Lactose content; SCC: Somatic cell count; 3.5% FCM: 3.5% Fat corrected milk, TS: Total solids; A. nodosum: *Ascophyllum nodosum;* NEFA: Non-esterified fatty acids; BHB: β-hydroxybutyrate; ATT: Aspartate aminotransferase; ALT: Alanine amino transferase; BUN: Blood urea nitrogen; ↑: increase; NC: No change; ↓: decrease.

1.3.3.2. Feed Additives:

Targeted nutritional strategies to increase milk yield and improve milk composition may help to mitigate the negative consequences of HS in small ruminants. The hypothesis behind feed additives in ruminants are mainly based on increasing the provision of gluconeogenic precursors may lead to a decrease in the use of amino acids for gluconeogenesis and, in turn, increase energy and nitrogen use efficiency (Berg et al., 2007). This proposed mechanism is consistent with the decreased blood urea nitrogen (**BUN**) in gluconeogenic precursors fed lambs in both thermal regimes (Mahjoubi et al., 2016). Furthermore, lactation requires a major increase in the partitioning of AA towards the mammary gland for milk protein synthesis (31 - 46% of whole-body protein flux compared with 1 - 6% in dry, non-pregnant animals. Champredon et al., 1990; Baracos et al., 1991).

Most investigation has been focusing on the mechanisms and molecules that will help animals to spare essential amino acids that are limiting for milk production (i.e. methionine and lysine). Intravenous infusion of choline and carnitine reduced the irreversible loss of methionine by 18 to 25% in sheep, suggesting that methionine could be spared with the addition of methyl-group-containing metabolites (Lobley et al., 1996). However, in general, doses of supplementation are difficult to pin down and vary from experiment to another, making recommendations difficult to establish.

1.3.3.2.1. L-carnitine:

Carnitine synthesis is reduced by the potential inflammation induced by HS, and that carnitine (**CAR**) supplementation could cover the shortage in the production of endogenous carnitine. It is well known HS small ruminants mobilize body fat due to the reduced feed intake. Therefore, supplementation with CAR would improve the oxidation of mobilized fatty acids. L-carnitine, the biological active form of carnitine, which is synthesized in the liver, kidney, and brain from the essential amino acids' lysine and methionine, that can be considered as L-carnitine precursors (Marcovina et al., 2013). L-carnitine (δ-trimethylamino-β-hydroxybutyrate) is a quaternary hydro soluble amine with a small molecular weight that occurs naturally in microorganisms, plants, and animals (Bremer, 1983). Its concentration in animals varies according to species, tissue type (i.e. It is most accumulated in cardiac and skeletal muscles).

An excellent review of L-carnitine biochemistry is written by Vaz and Wanders (2002), in which they present further details on L-carnitine biosynthesis. Briefly, N6-trimethyllysine (TML) is first hydroxylated on the 3-position by TML dioxygenase (TMLD) to yield TML (HTML). Aldolytic cleavage of HTML yields 4-trimethylaminobutyraldehyde (TMABA) and glycine, a reaction catalysed by HTML aldolase (HTMLA). Dehydrogenation of TMABA by TMABA dehydrogenase (TMABA-DH) results in the formation of 4-N-trimethylaminobutyrate (butyrobetaine). In the last step, butyrobetaine is hydroxylated on the 3-position by γ-butyrobetaine dioxygenase (BBD) to yield carnitine (*Figure 1.2*).

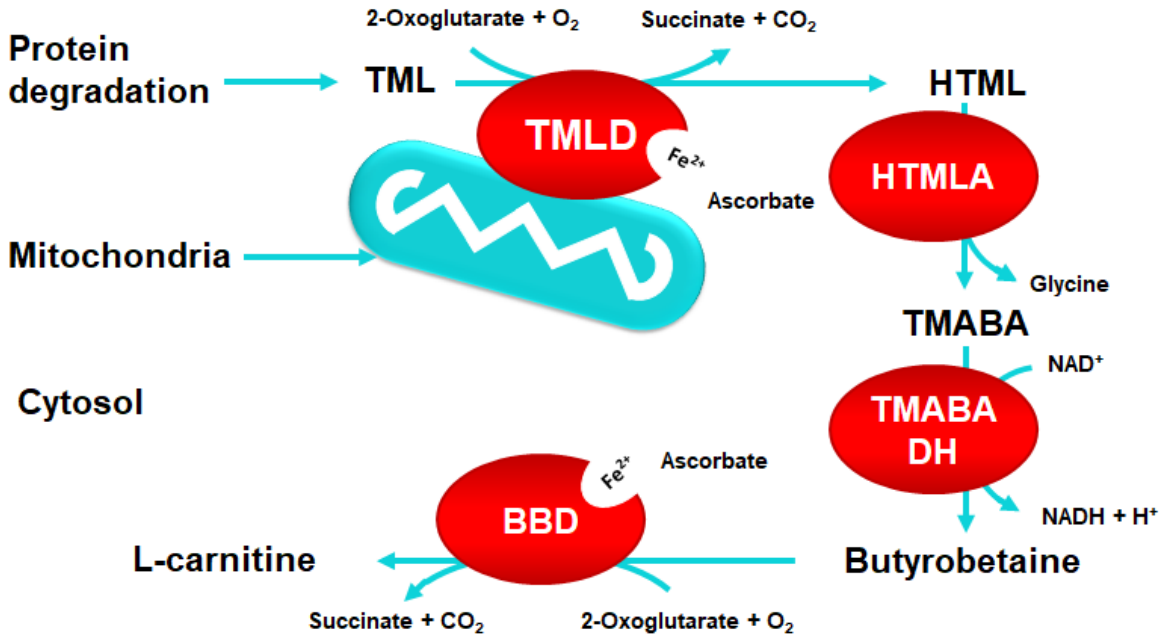

Figure 1.2: The carnitine biosynthesis pathway: Precursor, TML: N6-trimethyllysine, HTML: 3-hydroxy-N6-trimethylysine, TMABA: 4-N-trimethylaminobutyraldehyde, Butyrobetaine: 4-N-trimethylaminobutyrate (Adapted from Vaz and Wanders, 2002).

The glucose-fatty cycle or the Randle cycle (*Figure 1.3*) draws attention to competition between glucose and fatty acids for their oxidation in muscle between glucose and fatty acids for their oxidation in muscle and adipose tissue (Díaz-Ruiz et al., 2008). Moreover, it is a biochemical mechanism that controls fuel selection and adapts substrate supply and demand in normal tissues in coordination with hormones controlling substrate concentrations in the circulation.

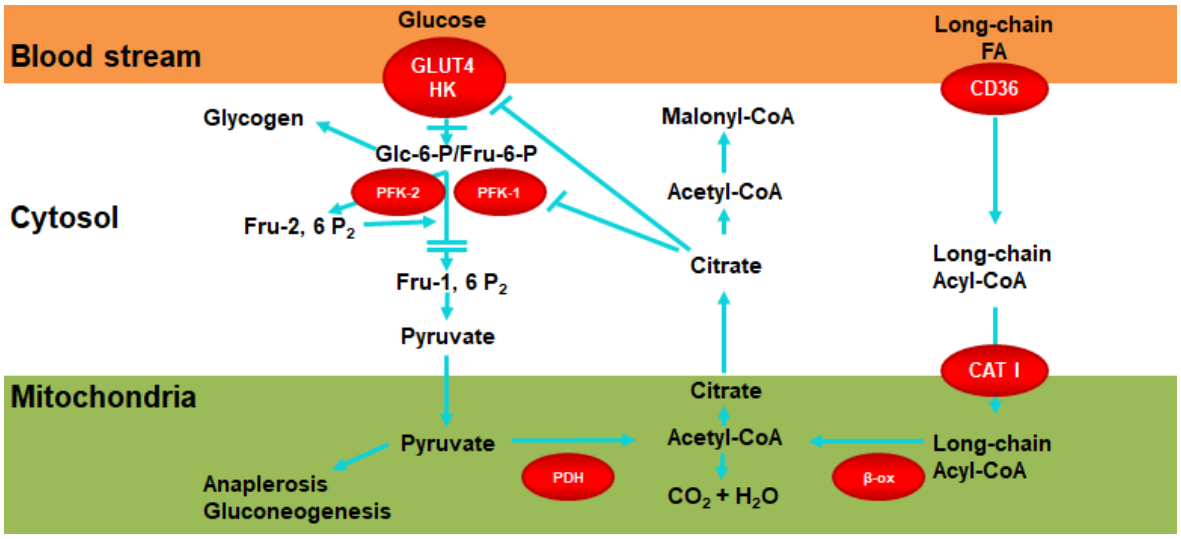

Figure 1.3: Mechanism of inhibition of glucose utilization by fatty acid oxidation (Adapted from Hue and Taegtmeyer, 2009).

Mitochondria are the “power plants” of cells and are the main location of intracellular oxidative phosphorylation and ATP synthesis. In addition, L-carnitine has two main roles in eukaryote cells that involves mitochondria: 1) A “shuttle role”, in the transfer of long-chain fatty acids from cytosol to mitochondria for subsequent β-oxidation and the production of acetyl-CoA used for energy production (by ketogenesis or in tricarboxylic acid cycle); and 2) the “buffer role” of the acyl groups (by modulation of the acyl-CoA/CoA and reduction of the acyl toxicity by excreting them as carnitine esters) see *Figure 1.4* (Hue and Taegtmeyer, 2009). There is a competition and mutual control between glucose and fatty acids for energy production at cellular level. The extent of inhibition is graded and most severe at the level of pyruvate dehydrogenase (PDH) and less severe at the level of 6-phosphofructo-1-kinase (PFK) and glucose uptake (Hue and Taegtmeyer, 2009).

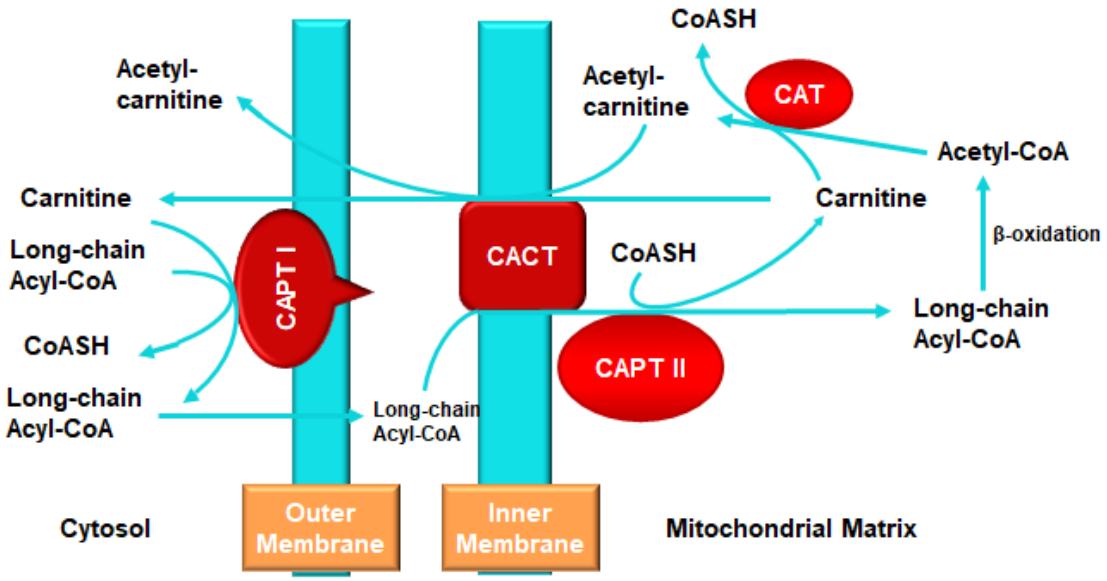

Figure 1.4: Function of carnitine in the transport of mitochondrial long-chain fatty acid oxidation and regulation of the intramitochondrial acyl-CoA/CoA ratio (Hue and Taegtmeyer, 2009).

In human, since intracellular accumulation of acyl-CoA derivatives has been implicated in the development of insulin resistance, carnitine supplementation has gained attention as a tool for the treatment of insulin resistance (Ringseis et al., 2012). Furthermore, Noland et al. (2009) clearly showed that carnitine insufficiency contributes to mitochondrial dysfunction and insulin resistance. Furthermore, L-carnitine supplementation (in its acetylated form) of 1 gram twice daily reduced blood pressure, insulin resistance (measured by euglycemic clamps), and improved adiponectin levels (a beneficial adipokine) in patients with type 2 diabetes (Ruggeneti et al., 2009).

In broilers, some authors reported that abdominal fat deposition is reduced by L-carnitine supplementation with a significant effect on daily gain and feed conversion (Rabie and Szilágyi,1998). Moreover, supplemented gilt in gestation with 0.125 g/d of L-carnitine resulted in increased live weight, carcass weight, and muscle depth of progeny at slaughter (Rooney et al., 2019).

In sheep reproduction, acetyl-L-carnitine can be used to increase the in vitro blastocyst rate of juvenile oocytes and therefore to improve juvenile in vitro embryo transfer methods (Reader et al., 2015). Moreover, Borhani et al, (2015), in growing

Afshari lambs, replacing soybean seed with canola seed and 0.11 g/d L-carnitine supplementation had no effects on feed intake, body weight gain, or feed conversion ratio. In addition, Foroozandeh et al. (2014), reported L-carnitine administration decreased NDF digestibility from and tended to decrease organic matter digestibility. Moreover, in an experiment to study the effect of fat type (i.e. soybean oil or Ca-protected fat) and 0.10 g/d of L-carnitine supplementation on the same breed, had no effects on feed intake, but improved ether extract digestibility and tended to improve growth. Moreover, the same other observed a response to L-carnitine administration for cholesterol and LDL concentration in blood depended on fat type; it significantly decreased them for the soybean oil diet compared with Ca-protected fat. However, Mehrez et al. (2015), in 0.35 g/d or 0.75 g/d of free L-carnitine supplemented Rahmani growing kids reported a decrease in feed intake compared to control, affected live body weights (i.e. lighter lambs).

The effects of 20 mg/kg BW L-carnitine subcutaneous injections on late gestating Damascus goats was a decrease in serum NEFA's. However, triglyceride and cholesterol concentration between groups were not significant. Furthermore, blood glucose concentration in L-carnitine administered goats with twin kids was higher than the controls with twin kids (Kaçar et al., 2010). Nevertheless Mehrez et al. (2015) reported in growing Rahmani lambs a decreased triglycerides concentration in 0.35 g/d and 0.75 g/d of free L-carnitine compared to control group.

LaCount et al. (1995) found no effect of L-carnitine supplementation on milk production of non-HS dairy cows. Nevertheless, feed-restricted dairy cows (with high blood NEFA levels) produced greater 3.5% fat-corrected milk when supplemented with abomasum infused L-carnitine (Carlson et al., 2006). However, in mid-lactating dairy cows supplement with 0.75 g/d of rumen-protected L-carnitine resulted in decrease in milk yield and casein yield; protein yield had a tended to decrease (Tasdemir et al., 2011). Even more conflicting, dairy cows supplemented with rumen-protected L-carnitine, from one week before calving to four weeks after parturition, produced similar milk yield to un-supplemented cows, but their milk contained greater fat and protein (Pirestani and Aghakhani, 2018). In addition, Galvis et al., 2019, supplemented peri-parturient cows (i.e. from 260 d of gestation to 20 d post-partum) with 100 g/d or 200 g/d of fumarate L-carnitine, found a

decrease in total triglycerides in fresh liver and an increase in BUN, despite of no increase in any fraction (i.e. total, free, or acylcarnitine) of blood carnitine concentration.

1.3.3.2.2. Methionine, Betaine and Choline:

Methionine is one of the limiting AA in most dairy animals' rations, rations are commonly supplemented with rumen-protected products. The most commonly used feed additive in dairy production is methionine, choline, and betaine. Moreover, the effects of supplementation with protected AA are relatively old and, in most cases, focused on dairy cows. However, there are also interesting results in small ruminants (i.e. hair producing goats). Furthermore, methionine (**Met**) was demonstrated to be the most limiting amino acid for growing sheep when microbial protein is the predominant source of absorbable AA, but its specific requirement was not well quantified (Wei et al., 2017). In stressful conditions when DM intake often decreases (e.g. periparturient period), Met supplementation improves lactation performance and reduces inflammation (Osorio et al., 2013; Batistel et al., 2017).

Methionine has three major metabolic functions: as an essential amino acid, which is required for protein synthesis; as a sulfur source, which is needed for synthesis of other sulfur-containing biochemicals; and as a methyl donor, which can provide methyl groups for use in methylation reactions (Wu and Davis, 2007).

The most cited methionine products used in research are in Table 1.3; for further information about methionine production, metabolism and use refer to Wilke (2014), who wrote review that closes with a comprehensive overview of the role and activities of global methionine manufacturers. Some current market data is also presented.

Table 1.3: Methionine products available in the market (Updated from Schwab, 2011)

Group	Product	Met content	Met bioavailability	References	Provider
Methionine analogs[2]	MetaSmart®	78%	• Rumen bioavailable: 50%	• Graulet et al., 2005	Adisseo, Antony, France
	Alimet™	88%	• Rumen bypass: 22 - 43%	• Vázquez-Añón et al., 2001.	Novus International, Inc., St. Louis, MO, USA
	Rhodimet™	88%	• Rumen bypass: NAD • Digestibility: NAD		Adisseo, Antony, France
Physically coated[3]	Met-Plus®	50 - 65%	• Bioavailability: 33 – 40%	• Olley et al., 2004	Nisso America, Inc.
	Mepron™ M85	85%	• Rumen bypass: 70% • Bioavailability: 40%	• Schwab, 2011 • Ordway, 2005	Evonik-Degussa Corporation, Germany
pH-sensitive protection	Smartamine™	75 - 80%	• Rumen bypass: 85% • Bioavailability: 88%	• Baldi and Pinotti, 2006 • Robert and Williams, 1997	Adisseo, Antony, France
	METHIOPLUS™	55%	• Rumen bypass: 68.5% • Digestibility: 91.0%	• Abdi-Benemar et al., 2016	Kemin Industries, Des Moines, IA, USA
	MetiPEARL™	48%	• Rumen bypass1 66% • Digestibility1 90%		Kemin Industries, Des Moines, IA, USA

NAD: No available data; [1]Data provided by the manufacturer; [2]lipid-protected, [3]carbohydrate-protected product.

Several studies aimed to determine Met and Lysine (**Lys**) requirements for ruminants among them Rulquin and Verité (1993), from the data of 57 works in which with 164 diets two or more levels of Lys and Met were used. These authors conclude that the optimal doses-productive response ratio is when the contributions

of Lys and Met are respectively 7.3% and 2.5% PDI (digestible protein in the intestine) of the INRA system (2018). Furthermore, under 6.8% and 2.0% PDI, of Lys and Met respectively, the protein content in milk drastically decrease. Moreover, in an experiment to determine the methionine requirements of growing lambs (36 kg of BW) it was found that the mean Met requirement was estimated to be 1.28 ± 0.11 g/d of supplementation. The total post-ruminal minimum requirement of Met would be 2.02 g/day when the basal passage of Met (0.74 g/day) was summed (Wei et al., 2017). Evidence of the efficiency of methionine supplementation are widely spread. For instance, it was observed that the administration by perfusion of Met under TN conditions can increase the synthesis of milk protein in lactating goats (Lin et al., 2009). Moreover, other *in vitro* studies with mammary epithelial cell suggest that adding 57 µg/ml (0.382 mmol/L) of Met can significantly increase the expression of the as1-casein protein gene (Liu et al., 2007), which has Important role in the capacity of milk to transport calcium phosphate and determine milk technological properties.

Han et al. (2015), in an in-vitro study, showed that Met (60 mg/L) increased the viability and attenuated morphological damage in hyperthermia-treated bovine mammary epithelial cells. Moreover, they also exhibited a certain amount of heat shock protein (**HSP70**) reserve after methionine pretreatment for 24 h, and the expression level of the HSP70 gene and protein further increased, the results evidenced that Met has cytoprotective effects on hyperthermia-induced damage in bovine mammary epithelial. At cellular level, methionine supplementation induces an up-regulation of proteins related to mitochondrial functions such as TCA cycle, electron transport chain and respiration, combined with an enhancement of mitochondrial pyruvate uptake and TCA cycle activity (Tripodi et al., 2018). Though, studies in small ruminants are scarce, Sevi et al. (1998) informed on the effects of supplementing 3.5 g/d or 7.0 g/d (with 35% of L-Met) of rumen-protected Met on mid-lactating Comisana ewes observed an increase in the ratio between long-chain and short- chain fatty acids in milk fatty acid profile. Moreover, supplementation of 2.5 g/d but not 5.0 g/d of rumen-protected Met (85% of L-Met) to mid-lactating dairy goats improved milk yield, fat-corrected milk, energy-corrected milk, and milk protein values in dairy goats (Flores et al., 2009). Studies on the effect of supplementing rumen-protected methionine are rare, a recent study on dairy cows supplemented

with 0.11% DM rumen-protected Met during a HS challenge did not report any change in blood biomarkers (Pate et al., 2019).

Choline, the beta-hydroxy-ethyl-trimethyl-ammonium ion, is a strong base containing a trimethylated quaternary nitrogen. Choline occurs widely in biological materials as the compound itself, as acetylcholine and as various phospholipids (Kuksis and Mookerjea, 1978). Choline is not considered a vitamin in a traditional sense because it is not a part of an enzyme system and is required in grams rather in mg amounts as a true vitamin (NRC, 2001), but just in some aspects, as a component of phospholipids and as acetyl-choline it plays an important role in animal metabolism.

Furthermore, choline has three major metabolic functions: as a component of phosphatidylcholine in goats; phosphatidylcholine contributes approximately 80% of phospholipids (Emmanuel and Kennelly, 1984); as a precursor of neurotransmitter acetylcholine; and as a precursor of betaine and Met, which acts as a source of labile methyl groups for methylation reactions (NRC, 1993). De novo synthesis of choline occurs through the sequential methylation of phosphatidylethanolamine, with the methyl groups being supplied by S-adenosyl-L-methionine (Mato et al., 1994). Puchala et al. (1998) have observed a linear response in the growth rate and in the conversion rate of kids supplemented with rumen protected choline at a dose of 8-6 g/kg DM ingested, concluding that the choline can also be limiting in goat rations.

Furthermore, results obtained of the effects of rumen-protected choline supplementation to transition cows suggested that a greater choline availability can improve not only milk production efficiency, but also lipid and methyl group metabolism in transition dairy cows (Baldi and Pinotti, 2006). Moreover, Myers et al. (2019), observed a reduction in hepatic triglycerides deposition and an increase circulating lipoprotein and triglycerides in dairy cows supplemented different level of rumen-protected choline. Nevertheless, D'Ambrosio et al. (2007), reported choline administration was associated with greater milk yield, despite no change in DMI.

Betaine (tri-methylglycine) is a natural compound present in bacteria, plant and animal cells. Betaine is a non-toxic amino acid derivative found widely distributed in

nature (Kettunen et al., 2001). Mammals utilize betaine for two major functions. The first is as a methyl donor (via S-adenosyl-methionine), thereby sparing methionine and increasing the available substrates for protein synthesis (Matthews et al., 2001). Second, when not catabolized, betaine is used as an organic osmo-protectant (Huang et al., 2007). Evidence of betaine effects are well documented, betaine supplementation (0.125%) increased IGF-1, free thyronine, free thyroxine and insulin levels all significantly (Huang et al., 2006). Furthermore, growing lambs supplemented with 2 g/kg of betaine had significantly reduced fat thickness, while liveweight and muscle area did not differ (Fernández et al., 1998). Moreover, under HS betaine supplementation (0, 2 and 4 g/d) have dose-dependent physiological responses in sheep exposed to HS (36 - 43ºC) and TN conditions (22ºC) (DiGiacomo, 2011). In addition, rectal temperatures were lower in sheep supplemented 2 g/d and higher in those supplemented 4 g/d compared with control (DiGiacomo et al., 2012).

To investigate post-ruminal choline supply during a feed restriction-induced negative energy balance mid-lactating cows was supplemented with increasing doses of choline (6.25 g/d to 25 g/d), it was observed that betaine and carnitine were greater with feed restriction and further increased with increasing doses of choline supplementation. Data suggest that enhanced supply of choline during negative energy balance decreases entry of homocysteine to the trans-sulfuration pathway, potentially favoring re-methylation to Met by acquiring a methyl group from betaine (Coleman et al., 2019). This could be a strategy for HS small ruminants since they present a negative energy balance. The link between choline supplementation and milk response has mainly been attributed to the metabolic interchangeability of choline and methionine, in the sense that both can furnish labile methyl groups (D'Ambrosio et al., 2007). What is more, there is an associative effect of AA, the effect of supplementing a lonely AA is different from supplementing a combination of AA. Approximatively one-third of the methionine methyl group is transferred to choline in studies with lactating dairy goats *Figure 1.5* (Emmanuel and Kennelly, 1984). Furthermore, more than 30% of Met absorbed is used to synthetize choline in dairy cows. In this way Met will help spare choline and vice-versa.

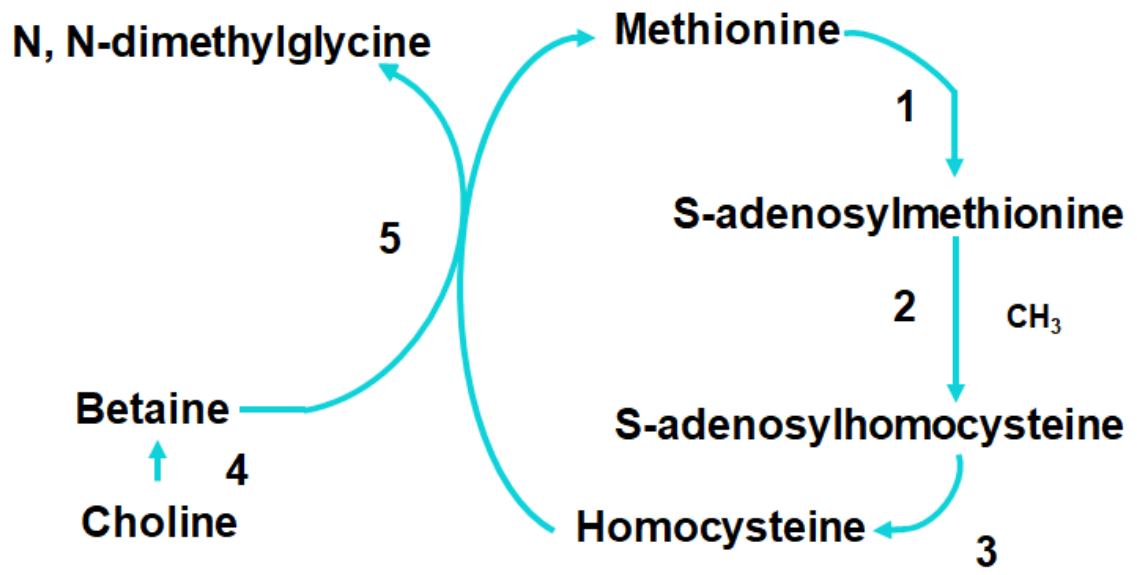

Figure 1.5: Metabolism of methionine, choline and betaine (Mehler, 1986). Numerals indicate the following enzymes: 1) methionine adenosyl-transferase; 2) various enzymes; 3) S-adenosylhomocysteine hydro- lase; 4) choline dehydrogenase; and 5) betaine- homocysteine methyltransferase.

In catfish production, sparing of methionine by betaine or choline, which are methyl group sources is demonstrated in supplementation experiment with different levels of choline, betaine and Met. It was concluded that in the absence of sufficient methionine, choline or betaine can spare a portion of methionine requirement. In the absence of sufficient choline, methionine cannot spare choline requirement. Further, in the absence of sufficient choline, betaine can spare at least a portion of choline requirement (Wu and Davis, 2005).

1.3.3.2.3. Lysine:

The decrease in DMI in HS conditions could affect also Lys intake as an essential AA for milk production. Lysine after Met is the second limiting AA in dairy production. Huber et al. (1994) reported that the increase of Lys supplementation allowed the increase of milk yield (+11%). Moreover, cows under shaded environment and fed high lysine diet produced slightly more milk than those fed low lysine and receiving evaporative cooling plus shade, suggesting that dietary protein quality compensated for lack of cooling under hot weather (Huber et al., 1994). Moreover, Mabjeesh et al. (2000), reported a milk and protein yields and DMI were higher in early than in

late lactation, but Lysine and methionine infusion (Lys: 370 mg/h + Met: 84 mg/h) did not affect these variables. These studies demonstrate the importance of a correct Lys intake during HS conditions.

Sun et al. (2007) in an investigation on the effects of supplementation of various sources of Met and Lys on nutrient digestion, N utilization, and duodenal AA flows in growing goats supplemented goats with 2 g/d of methionine plus 2.4 g/d of lysine, they informed of no difference in DMI and digestibility, an increase in plasma Met, Lys and total essential AA. Furthermore, Sevi et al. (1998) informed on the effects of supplementing 10.5 g/d or 21.0 g/d of rumen-protected Lys (with 35% of L-lysine) on mid-lactating Comisana ewes; they observed an increase in the ratio between long-chain and short-chain fatty acids in milk fatty acid profile.

An interesting aspect of methionine metabolism is that regardless of stage of lactation, the absolute and fractional oxidation rates of Lys by the mammary gland increased in response to lysine and methionine infusion (Mabjeesh et al., 2000). However, when corrected for Lys oxidation, net uptake of Lys by the gland was less than milk protein Lys secretion. Suggesting that when Lys is in excess of requirements, the mammary gland appears to dispose of the extra supply via the oxidative mechanism (Mabjeesh et al., 2000). Therefore, it is more stressing for dairy small ruminants under HS conditions a shortage of Lys that its excess.

1.3.3.2.4. Other Amino Acids:

In an excellent review presented by Torre and Caja (1998), concluded that the use of methionine and lysine is due to their commercial availability, and the nonuse of other forms is firstly due to their non essentiality followed by their high rumen degradability. Nevertheless, 20 years later we still stumble against the same questions in dairy small ruminants' production.

Between the non-essential AA glutamine plays a central role in interorgan N transfer and regulation of intermediary metabolism (Häussinger et al., 1994). Glutamine is the most abundant AA in the body (ranging from 2 to 15 mM; Le Boucher et al., 1997). Glutamine is a mediator of the cytokine chain reaction and is involved in transcription factors important for heat shock protein 70 expression (Hamiel et al., 2009). As for its effects, Caroprese et al. (2013) observed that rumen-

protected glutamine is beneficial in dairy cows exposed to in hot climate by sustaining cow immune reactions in terms of a strengthening of cell-mediated immune response, which is weakened in heat-stressed cows (Lacetera et al., 2005). Furthermore, glutamine infusion in sheep with nutritionally induced metabolic acidosis with glutamine infusion reduced partial pressure of O_2 and plasma glucose; some improvement in overall mobilization of NEFA (Odongo et al., 2009). Under HS conditions, glutamine incorporation in the diet of dairy animals improved a delayed-type hypersensitivity to mitogens and antigen-specific humoral responses, influencing IL-10 secretion (Caroprese et al., 2013).

For other non-essential AA, few experiment are available; for example, intravenous infusion of lactating dairy cows under moderate HS conditions with a mix of the two first limiting AA and branched chain AA (L-methionine (12 g), L-lysine (21 g), L-leucine (35 g), L-isoleucine (15 g), and L-valine (15 g) per d)) had no effects on milk yield, DMI, milk protein yield whereas, milk protein content increased by supplementation. Furthermore, an increase in body temperature in infused cows compared to their control counterpart was observed (Kassube et al., 2017). Furthermore, despite the fact that histidine has been identified as possibly third limiting for sheep (Schwab, 2011), no information is available of the supplementation or balancing of diets for this AA.

1.3.3.2.5. Propylene glycol:

Hamzaoui et al. (2014) hypothesized that supplementation with propylene glycol would increase blood glucose and spare amino acids for milk protein synthesis rather than glucose production. The propylene glycol increased blood glucose and insulin, but decreased DMI, blood NEFA and β-HBA, resulting in lower milk fat with no change in milk protein content. Moreover, Mahjoubi et al. (2016), observed that post-feeding glucose concentration was increased in HS lambs supplemented with glucogenic precursor (glycerol, 330 g/kg), mono propylene glycol (94.5 g/kg), calcium propionate (70.5 g/kg), niacin (470 mg/kg), and cobalt sulfate (185 mg/kg) and colloidal silica as the carrier, compared their TN counterpart. Furthermore, the effects of the intra-ruminal dosing of a glucogenic mixture including 70% glycerol, 20% propylene glycol and 10% water was studied on thirty late lactation dairy ewes of Sarda breed (Porcu et al., 2018). The administration of the glucogenic mixture

increased plasma osmolarity and blood volume. Moreover, it increased plasma content of glycerol, glucose and insulin while decreasing plasma level of NEFA and urea. Although, milk yield and milk lactose content were decreased by the glucogenic treatment, whereas milk protein and casein contents were increased (Porcu et al., 2018). In addition, insulinemia is commonly improved by the administration of propylene glycol in sheep (Chiofalo et al., 2005) during the post-partum period.

1.3.3.2.6. Insulin Sensitizers:

Other dietary strategies should be directed towards alleviating insulin resistance, for example, the use of antidiabetic agents. Supplementing diet ingredients or pharmaceuticals that enhance insulin sensitivity may be an effective tactic to improve the likelihood of surviving an otherwise lethal heat load (Rhoads et al., 2013).

From the therapeutic standpoint, the Thiazolidinediones (**TZD**) are potent insulin sensitizers in muscle, liver, and adipocytes and enhance β-cell function (DeFronzo, 2009). TZD could be a could strategy to enhance insulin since evidence on its efficacy in in dairy cows under TN conditions is reported; administered cows with 0, 2.0, or 4.0 mg of TZD/kg of body weight (BW) by intrajugular infusion once daily from 21 d before expected parturition until parturition, altered expression and plasma concentrations of leptin, and increased expression of Peroxisome proliferator-activated receptor (**PPARγ**) in adipose tissue (Schoenberg et al., 2011). However, injection of 8 mg/kg of BW of 2,4-thiazolidinedione to lactating dairy goats resulted in decrease in NEFA, β-HBA, and fatty acids available in plasma while glucose increased in TZD compared to the control group (Jaaf et al., 2019). Whereas, treated dairy goats in mid-late lactation with TZD did not decreased in NEFA, did not increase milk fat yield but tended to prevent milk fat decrease after induction of mammary infection (Rosa et al., 2017). Moreover, lack of activation of PPARγ by TZD is also supported by findings from a recent study carried out in sheep with induction of milk fat depression by conjugated linoleic acid. Treatment with 2,4-TZD did not alleviate the negative effect on milk fat synthesis (Sandri et al., 2018).

Other components of the TZD family is pioglitazone, single intravenous or oral dose of PGT (10 mg/kg) was administered to male sheep, resulted in maximal

concentration reached after a longer maximal time in sheep compared to that reported for humans. Nevertheless, pioglitazone has a marked post-ruminal absorption which results in a noticeable bioavailability in sheep (Ghoreishi et al., 2012). It is still of interest investigate its application to small ruminants under HS conditions as alleviation strategy.

1.3.3.2.7. Minerals:

Possible solutions may be some of the antidiabetic minerals such as chromium, zinc, and vanadium. However, results may be more equivocal; in part, this may reflect variability in absorption of minerals, since their absorption and utilization may depend on incorporation into organic molecules (Kegley et al., 1997). In a succinct review by DiGiacomo et al. (2014), reported the potential use of minerals as feed additives for HS nutritional strategies, succinctly:

Organic Selenium. Eid et al. (2016) reported that Se acts in strengthening the immune system of animals, being a component of selenoenzyme glutathione peroxidase, which metabolizes hydrogen peroxide and lipid hydroperoxides, neutralizing free radicals and promoting improvements in animals' immunity.

Vitamin E. is considered to be necessary to protect the newborn against oxidative stress, whereas vitamin A is required for growth and development. A dietary supplementation with 50 mg/kg of vitamin E and 0.3 mg/kg of selenium during summer improved sheep reproductive performance and lamb growth. It also had a beneficial effect on blood metabolites, protein metabolism and thyroxin concentration (El-Shahat and Abdel Monem, 2011).

Chromium. When organic forms of chromium have been used, there have been consistent improvements in insulin sensitivity in ruminants fed dietary chromium (DiGiacomo et al., 2014). For example, results for plasma glucose concentrations at 30 and 60 min after glucose infusion were lower in the kids fed 1.5 mg Cr diet than the kids fed control diet (Emami et al., 2015). Furthermore, calves supplemented with chromium in summer had a reduced insulin response (~30%) to a glucose infusion compared with control animals, suggesting improved efficiency of insulin (Yari et al., 2010). Moreover, DMI, milk fat and protein yield increased

while serum insulin and NEFA concentrations decreased in chromium-supplemented, early-lactation dairy cows during summer (Nikkhah et al., 2011).

Zinc. Sasaki et al. (2002) showed that insulin promoted adipocyte proliferation in ovine preadipocyte cells. In addition, at relatively high levels, zinc exerts potent insulin-mimetic effects, and so dietary zinc has been proposed as a means of manipulating adipogenesis and marbling in cattle (Kawachi, 2006).

Vanadium. has been reported to possess insulin-mimetic activity on various types of cells and so has been suggested as a means of increasing adipogenesis (Kawachi, 2006). Nevertheless, concerns about toxicity and contamination of the food chain should be raised.

1.3.3.2.8. Plant Extracts and Others:

Ruminal microbial populations may have potential to adapt over time to effects of some bioactive additives. Thus, findings may differ between studies. Furthermore, under HS, feeding strategies capable of increasing digestive efficiency, such as live yeast supplementation, may increase nutrient flow to the small intestine and dairy small ruminants' performance. For these consideration supplementations modifying ruminal activity are advised in HS conditions.

The use of algae to mitigate HS in some region of the world (i.e. Brazil, north of France, etc…) led to mitigate the deleterious thermo-physiological effects of HS (i.e. low body temperature and respiration rate). De-Lima et al. (2019), reported the inclusion of Gracilaria birdiae in the diet of lactating goats does not influence milk production but does contribute to the attenuation of the deleterious effects of HS. The physiological mechanisms that may be related to the beneficial effects of Gracilaria birdiae involve the antioxidants present in the algae composition. Souza et al. (2011) observed a positive correlation between the phenolic compounds (gallic acid and apigenin) in Gracilaria and the antioxidant potential. Abdoun et al. (2014) evaluating the inclusion of 5% of DM Ulva lactuca in the diet of lambs and by Yates et al. (2010) with the inclusion of 1% commercial extracts of the brown macroalgae *Ascophyllum nodosum* in the diet of goats observed no effects on voluntary feed intake, and a reduction RR, agreeing with the results of De-Lima et al. (2019) in HS dairy goats. Furthermore, Kellogg et al. (2006) concluded that supplementation with

0.25% commercial extract of *Ascophyllum nodosum* (Tasco™, Acadian Agritech, Dartmouth, Nova Scotia, Canada) macroalgae was beneficial for the production of cow milk under moderate HS.

Ghanem et al. (2008) found that vitamin C supplementations to sheep and goats are effective in alleviating HS. Furthermore, vitamin E and C supplementations decreased rectal temperature and respiration rate (Sivakumar et al., 2010), and alleviated HS in goats (Kobeisy, 1997). Moreover, after the increase in plasma and milk niacin concentrations in a linear manner, after the supplementation of increasing doses of rumen-protected niacin supplementation (0 to 12g/d) to dairy cows, increased water intake during both TN and HS and hair coat temperature during TN was observed; whereas, core body temperature was unaffected (Rungruang et al., 2014).

As for plant extract, Branciari et al. (2015) demonstrated that feeding rosemary dehydrated leaves to lactating Sarda ewes positively affected the antioxidant capacity of milk. Kholif et al. (2016) included lemongrass or rosemary herbs at 0 or 10 g/d in the diet of early lactating Damascus goats in a 12 wk study and concluded an increasing milk yield and improving the profile of milk FA.

In sheep, curcumin inhibits the expression of IL-8, a chemokine stimulating neutrophilic activity, and Bcl2A1, an anti-apoptotic factor (Farinacci et al., 2009). Furthermore, the addition of cumin seed extract (1.27% of DM) significantly enhanced milk production by 13% in supplemented goat (Miri et al., 2013). In addition, goats which were fed with 1g/L cumin seed extract produced milk with higher recovery of linoleic acid and linolenic acid (Heidarian et al., 2015). Moreover, diet containing cumin powder (1.25%) was found to be remarkably beneficial in streptozotocin induced diabetic rats as indicated by reduction in hyperglycemia and glucosuria, improved body weights, lowered blood urea level and reduced excretions of urea and creatinine by diabetic animals (Lee, 2005). Morsy et al. (2018) conducted a similar study with Damascus goats in early lactation consuming diets providing 0 or 10 g/day of dried mustard or cumin seed. The seeds resulted in increases milk yield, DMI, concentration and the molar percentage of propionate in ruminal fluid, milk and milk levels of unsaturated FA and conjugated linoleic acid and decreased saturated FA.

Canaes et al. (2017) included citral oil, the primary essential oil in lemongrass (Cymbopogon citratus), at 0, 0.08, 0.16, or 0.24 ml/kg BW in a 50% concentrate diet fed to mid-lactating Saanen goats; reported fat-corrected milk yield tended to decrease linearly with increasing citral oil level. Babiker et al. (2017) noted enhancements of numerous variables from feeding Moringa oleifera (MO) leaves, to lactating Aardi goats of Saudi Arabia (90 d DIM) milk yield and milk fat was greater for the MO leaf diet, there were improvements in many antioxidant status indicators of serum and milk.

Combination of single component additives could be considered as a mitigation strategy in HS small ruminants. For example, mixed solution, a commercial product specially designed for HSed ruminants. Colombo et al. (2019), supplemented an immunomodulatory feed (Dried Saccharomyces cerevisiae, dried Trichoderma longibrachiatum fermentation product, niacin, vitamin B12, riboflavin-5-phosphate, d-calcium pantothenate, choline chloride, biotin, thiamine monohydrate, pyridoxine hydrochloride, menodione dimethylpyrimidinol bisulfate, folic acid, calcium aluminosilicate, sodium aluminosilicate, diatomaceous earth, calcium carbonate, rice hulls, and mineral oil) to resulted in less vaginal temperature during severe HS condition

CHAPTER 2

Hypothesis and Objectives

CHAPTER 2

2. HYPOTHESIS AND OBJECTIVES:

This thesis is included in a large research project aimed to evaluate the impact of thermal stress on dairy small ruminants. Detailed responses of dairy goats to heat stress were already evaluated in our laboratory (Hamzaoui et al., 2013; Salama et al., 2014; Contreras-Jodar et al., 2018, 2019; Coloma-Garcia et al., 2020; Salama et al., 2020). However, little is known in case of dairy ewes. Therefore, the first goal of the current thesis was to evaluate the effects of heat stress on the lactational performance and metabolism of dairy ewes. The second main objective was to test whether some dietary supplements would alleviate the impact of heat stress on dairy goats.

Specifically, the hypotheses and objectives were:

2.1. Evaluate the response of dairy ewes to heat stress (Chapter 3)

Hypothesis: Exposing dairy ewes to controlled heat stress conditions would help in understanding better the effects of heat stress and would be useful in establishing future strategies to alleviate its effects.

Specific objectives: Evaluate the effects of heat stress on:

- Physiological traits (rectal temperature and respiratory rate).
- Productive variables (feed intake, water consumption, body weight change, and milk yield and composition).
- Blood metabolism variables (acid-base balance status, insulin, glucose, NEFA, BHB, triglycerides cholesterol, creatinine, urea, and albumin).

2.2. The use of dietary supplements to improve the performance of dairy goats in heat stress conditions (Chapters 4 and 5)

Hypothesis: Heat-stressed goats experience altered fat metabolism and are in shortage of essential AA as they use these AA to cover their energy needs. Consequently, we hypothesized that supplementation of dairy goats with L-carnitine (Chapter 4) and rumen-protected methionine (Chapter 5) would increase the utilization of fatty acids and available AA for milk synthesis, respectively.

Specific objectives: Evaluate the effects L-carnitine (Chapter 4) and methionine (Chapter 5) in heat-stressed goats on:

- Rectal temperatures and respiratory rates.
- Feed intake, water consumption and body weight.
- Milk yield and milk composition.
- Milk fatty acid profile (Chapter 5 only).
- Acid-base balance status.
- Energy metabolism indicators: insulin, glucose, NEFA, BHB, triglycerides, and cholesterol.
- Protein metabolism indicators; creatinine and urea.
- Blood amino acid profile (Chapter 5 only).

CHAPTER 3
Lactational and physiological responses of dairy ewes to controlled heat stress conditions

CHAPTER 3

3. LACTATIONAL, PHYSIOLOGICAL, AND METABOLIC RESPONSES OF DAIRY EWES TO CONTROLLED HEAT STRESS CONDITIONS[1]

3.1. ABSTRACT

Heat stress (**HS**) has a significant economic impact on the global dairy industry. However, the mechanisms by which HS negatively affects metabolism and milk synthesis in dairy ewes are not well defined. This study evaluated the production and metabolic variables in dairy ewes under controlled HS conditions. Eight Lacaune ewes (75.5 ± 3.2 kg body weight; 165 ± 4 days of lactation; 2.31 ± 0.04 kg milk per day) were submitted to thermoneutral (**TN**) or HS conditions in a crossover design (2 periods, 21 d each, 6 d transition). Conditions (day-night, 12-12 h; relative humidity; temperature-humidity index, THI) were: TN (20 - 15°C; 50 ± 5%; THI = 65 - 59) and HS (35 - 28°C; 45 ± 5%; THI = 83 - 75). Ewes were fed ad libitum and milked twice daily. Rectal temperature (**RT**), respiratory rate (**RR**), feed intake, water consumption, and milk yield were daily recorded. Milk and blood samples were collected weekly. Additionally, TN and HS ewes were exposed to glucose tolerance test, insulin tolerance test, and epinephrine challenge. Heat stress reduced feed intake (–11%), and increased RT (+0.77ºC), RR (+90 breaths/min) and water consumption (+28%). Despite the reduced feed intake, HS ewes produced similar milk to TN ewes, but their milk contained lower fat (–1.7 points) and protein (–0.86 points). Further, HS milk tended to contain more somatic cells (+0.23 log points). Blood creatinine was greater in HS compared to TN, but no differences in blood glucose, non-esterified fatty acids or urea were detected. When glucose was infused, TN and HS had similar insulin response, but higher glucose response (+85%) was detected in HS ewes. Epinephrine infusion resulted in lower non-esterified fatty acids response (–215%) in HS than TN ewes. In conclusion, HS decreased feed intake, but milk production was not affected. The tendency of increased milk somatic cells might have negative impact on milk coagulation characteristics. Heat stress caused metabolic adaptations that included increased

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body muscle degradation and reduced adipose tissue mobilization. These adaptations allowed ewes to spare glucose and to avoid reductions in milk yield.

3.2. INTRODUCTION

Sheep production is one of the most important agricultural activities in the Mediterranean area and several regions in the world. The European Mediterranean countries account for more than 13% of the world sheep population, and 28% of the global dairy sheep production is concentrated in this region (FAOSTAT, 2018). Sheep milk is mainly used for cheese production and any negative impact on milk yield or milk quality will be reflected in cheese yield, and consequently reduced benefits for farmers. Predictions relative to climate change effects consider the Mediterranean basin as one of the regions where larger increases in temperatures are expected (Pasqui and Di Giuseppe, 2019).

Sheep thermoneutral zone is claimed to be between 5 and 25ºC (Curtis, 1983). Another study on the relationship between production traits and climatic variables indicated a comfort zone between 11 and 21ºC of average daily temperature, within which ewes could express their highest production capacity (Ramon et al., 2016). Heat stress (**HS**) negatively affects milk yield and its components in dairy cows (Baumgard et al., 2011) and dairy goats (Salama et al., 2014). With regard to dairy sheep, most of the available studies evaluated the impact of HS on milk production by comparing between seasons (Finocchiaro et al., 2005; Peana et al., 2007; Ramon et al., 2016). These studies detected reductions in milk yield and milk components during summer. However, in these previous mentioned studies comparing seasons, the effect of HS was often confounded with that of different feeding patterns across seasons. To the best of our knowledge, no published studies have evaluated the detailed responses of lactating dairy ewes to HS under controlled climatic and feeding conditions.

With regard to metabolism under HS, available results in dairy cows (Wheelock et al., 2010; Baumgard et al., 2011) and goats (Hamzaoui et al., 2013; Salama et al., 2014; Mehaba et al., 2019) indicated homeorhetic adaptations to suppress lipid mobilization, despite the fact that HS animals are under unfavorable energy balance. Studies comparing between heat-stressed and thermo-neutral pair-fed

cows showed that the lack of body lipid mobilization induced by HS is due to greater blood levels of insulin accompanied by changes in the expression of gluconeogenic genes in liver (Wheelock et al., 2010; Baumgard et al., 2011). Furthermore, heat-stressed dairy cows (Baumgard and Rhoads, 2013) and goats (Salama et al., 2014; Mehaba et al. 2019) suffer increased protein catabolism, and some of these mobilized AA could be used for glucose synthesis. Whether similar metabolic changes occur in heat-stressed lactating dairy ewes is not known.

The identification of HS animals and understanding the physiological mechanisms by which HS reduces milk production is critical for developing novel approaches to maintain production or minimize the reduction in productivity during HS conditions. Compared to dairy cows and goats, little attention has been paid to get comprehensive detailed productive and metabolic responses to HS in lactating dairy ewes. Therefore, the objectives of the present study were to evaluate the impact of HS on productive variables (milk yield and milk composition) and to measure the metabolic and physiological responses of dairy ewes to HS under controlled climatic chamber conditions.

3.3. MATERIALS AND METHODS

3.3.1. 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 Lacaune dairy ewes (75.5 ± 3.2 kg BW; 165 ± 4 DIM; 2.31 ± 0.04 L/d of milk yield) with healthy and symmetrical udders from the experimental farm of the UAB were used. Animals were allocated individually in pens (1.8 × 0.90 × 0.80 m) with separate feed and water throughout the experiment. Ewes were blocked in 2 balanced groups according to milk yield and milk composition. The experimental design was a crossover with 2 periods of 21 days each, and 2 climatic treatments that differed in the temperature-humidity index (**THI**) values. With the aim to have the same wool length in all animals, ewes were shorn before the beginning of the experiment to have 2-cm long fleece.

Before the experimental periods, ewes were adapted to the experimental conditions for 14 d. Afterwards, ewe groups were randomly assigned to 2 treatments: thermo-neutral conditions (**TN**; 15 to 20 ºC, 50 ± 5% humidity, THI = 59 to 65) or HS (12-h day at 35ºC day and 45 ± 5% humidity; THI = 83; and 12-h night at 28ºC and 45 ± 5% humidity; THI = 75). After the first period, 6 d of transition was allowed, during which all ewes remained at TN conditions. Ewes were switched to the opposite treatment in the second period. Photoperiod was 12-h light (0800 to 2000 h):12-h dark (2000 to 0800 h) and data of environmental temperature and humidity were recorded using data loggers (Opus 10; Lufft Mess- und Regeltechnik GmbH, Fellbach, Germany). The THI were calculated according to NRC (1971):

$$THI = (1.8 \times T_{db} + 32) - [(0.55 - 0.0055 \times RH) \times (1.8 \times T_{db} - 26)]$$

Where T_{db} is the dry bulb temperature (°C) and RH is the relative humidity (%).

Throughout the experiment, (February to May), the ambient temperature of TN ewes was maintained at 15 to 20°C with the help of an electric heather equipped with a thermostat (3.5 kW; General Electric, Barcelona, Spain) when needed. The HS ewes 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 m3/h air turnover was maintained throughout the experiment.

Ewes were milked twice daily (0800 and 1700 h). Milking was performed at a vacuum of 40 kPa, 120 pulses/min, and 50% pulsation ratio. The milking routine included manual cluster attachment and machine milking. Teat dipping with an iodine solution (P3-ioshield, Ecolab Hispano-Portuguesa, Barcelona, Spain) was done at the end of milking. All ewes were individually fed a TMR (Table 3.1) once daily (0900 h) and orts were recorded daily. The diet was formulated to meet or exceed the predicted requirements (INRA, 2018) of energy, protein, minerals, and vitamins. Intake was adjusted at 20% leftover based on the previous day intake. Clean water was permanently available at ambient temperature.

Table 3.1. Ingredients, chemical composition, and nutritive value of the total mixed ration offered to the dairy ewes.

Item	Value
Ingredients (% as fed)	
Alfalfa hay	60.0
Concentrate	40.0
Cracked oat grain	5.0
Cracked corn grain	4.0
Brewing barley	10.0
Soybean hull	45.0
Soybean meal, 44%	5.0
Rapeseed meal	10.0
Corn gluten feed	10.0
Soybean oil	5.0
Cane molasses	2.0
Salt (NaCl)	0.5
Vitamin and mineral complex for goats	1.0
Dicalcium phosphate	2.5
Chemical composition (% of DM)	
Dry matter	87.9
Organic Matter	87.8
Crude protein	17.0
Ether extract	3.73
Neutral detergent fiber	26.5
Acid detergent fiber	17.6
Acid detergent lignin	3.02
Ash	0.09
Nutritive value[1]	
UFL[2], /kg	0.92
NEL[3], Mcal/kg	1.62
PDI[4], g/kg	91.2
PDIA[5], g/kg	42.8
RPB[6], g/kg	34.3
Ca_{abs}, g/kg	3.04
P_{abs}, g/kg	5.38

[1] Calculated according to Institut National de la Recherche Agronomique (INRA, 2018). [2] Feed units for lactation. [3] Net energy for lactation (1 UFL = 1.7 Mcal of NEL). [4] Protein digested in the small intestine from food and microbial synthesis origins. [5] Protein digested in the small intestine supplied by food rumen undegradable protein. [6] Rumen protein balance, represents RUP, microbial protein and endogenous protein.

3.3.2. Sampling, Measurements and Analyses

Rectal temperature (**RT**) and respiratory rate (**RR**) were registered 3 times daily (0800, 1200, and 1700 h). The RR was determined by counting flank movements for 12s and multiplying by 5. The RT was measured using a standard digital

thermometer (model Accu-vet, ST714AC, Tecnovet S.L, Barcelona, Spain; reading range 32.0 to 42.0ºC and accuracy ± 0.10ºC).

Feed intake and water consumption of each ewe was measured daily throughout the experiment by recording the amount offered and refused. Feed samples were collected before the beginning of each experimental period and were ground through a 1-mm stainless steel screen, and then analyzed for DM, ADF, NDF, and ash contents according to AOAC (2003). The Dumas method (AOAC, 2003) with a Leco analyzer (Leco Corp., St. Joseph, MI) was used for CP determinations (N × 6.25). The chemical composition and nutritive value of ration ingredients are shown in Table 3.1.

Ewes were weighed weekly in 2 consecutive days after milking and before feeding to measure the change in body weight. Weighing was performed using an electronic scale (Tru-test A6500, Auckland, New Zealand). Additionally, BW values were used to calculate net energy balance using the following equation: energy balance = net energy intake – (NEM + NEL).

The NEM was calculated using the following equation: NEM = (0.0345 × BW0.75; INRA, 2018). Maintenance costs were increased by 30% for HS ewes as recommended (NRC, 2001). The NEL was calculated by using the following equation: NEL = [0.2224 + 0.0071 (fat, g/kg) + 0.0043 × (protein, g/kg)] × 0.686 × milk yield (INRA, 2018).

Milk yield at each milking was weighed by electronic scale (Mobba industrial, Barcelona, Spain) and registered daily. Milk samples from each ewe (from both a.m. and p.m. milking) were collected twice per week. Milk samples were stored at 4°C with a preservative (Bronopol, Broad Spectrum Micro-tabs II, D&F Control Systems, San Ramon, CA) until analysis. Samples were analyzed for fat, protein (N × 6.38), lactose, and SCC in the Dairy Herd Improvement Laboratory of Catalonia (ALLIC, Cabrils, Barcelona, Spain) using an automatic cell counter (Fossomatic 5000, Foss Electric, Hillerød, Denmark). A milk aliquot was stored at –20ºC to determine milk osmolarity using a Fiske 110 osmometer (Fiske Associations, Norwood, MA).

Two 10-mL blood samples were collected from the jugular vein using heparinized (170 IU) or spray-coated K2-EDTA Vacutainer tubes (BD, Belliver Industrial Estate,

Plymouth, UK) at d 3, 10 and 17 of each period. Blood was sampled at 0800 h before the morning milking and feeding. Blood samples remained on ice until plasma was obtained by centrifugation at 2,000 × g for 15 min at 4°C and stored at –20ºC until the analyses of glucose, non-esterified fatty acids (**NEFA**), BHB, cholesterol, and albumin. The NEFA was analyzed by the ACS-ACOD colorimetric enzymatic test method (Wako Chemicals, Neuss, Germany). The BHB was determined by kinetic enzymatic method using commercial kit (Ranbut, Randox, UK). Cholesterol was analyzed by the enzymatic method (cholesterol esterase/peroxidase) using an Olympus AU400 analyzer (Olympus Europa Holding GmbH, Hamburg, Germany).

Additional blood samples (approximately 0.3 mL) were collected using insulin syringes (1 mL; BD Micro-Fine; BD Medical-Diabetes Care, Franklin Lakes, NJ) and immediately analyzed for major ions and metabolites. A single drop of blood was applied to disposable cartridges containing biochemical and silicon chip technology (i-STAT CHEM8+; Abbott Point of Care Inc., Princeton, NJ). Then, the cartridge was inserted into an i-STAT hand-held analyzer, and the results of glucose, BUN, creatinine, hematocrit, hemoglobin, Cl, Na, K, Ca ions, total CO2 concentration, and anion gap were obtained.

On d 15 of the second period, each ewe was fitted with indwelling jugular silicone rubber catheters (Nutricath Silicone, 60 cm length and 14-gauge, Vygon, Valencia, Spain). On d 17, 19, and 21 glucose tolerance test (**GTT**), insulin tolerance test, and epinephrine challenge were done, respectively. Dextrose (D9434, Sigma-Aldrich, St-Louis, Missouri), insulin (bovine insulin form pancreas, I6634, Sigma-Aldrich) and epinephrine (E4250, Sigma-Aldrich) were diluted in sterile 0.9% NaCl solution (Vitulia 0.9%, Laboratorios ERN S.A., Barcelona, Spain). All solutions were sterilized by filtration through 0.22 µm polyether-sulfone filters (Millex GP, Millipore) using 1 filter for each 200 mL solution and kept at 4ºC until infusion.

At the day of each metabolic test, 3 pre-challenge blood samples (–20, –10, 0 min) were collected followed by an intravenous bolus dose of glucose (0.25 g/kg BW), insulin (4.6 µg/kg BW), and epinephrine (2 µg/kg BW). Glucose, insulin and epinephrine were administered at room temperature within a period of 10s and immediately followed by a 1 ml heparinized-saline (1:10 vol/vol). Thereafter, blood samples were collected at 5, 10, 20, 30, 45, 60, 90, and 120 min into vacuum tubes

and immediately placed in ice. After each blood sampling, the catheters were flushed with heparinized 0.9% saline solution (500 IU/mL; Clexane 4000 UI, Sanofi Aventis, Paris, France). Plasma was harvested by centrifugation for 15 min at 2,000 × g at 4ºC and stored at –20 ºC for further analysis.

Plasma insulin concentration was analyzed by ELISA immunoassay sandwich type for quantitative determination of ovine insulin in plasma (Mercodia, Diagnostics, Uppsala, Sweden). Reacted ELISA plates were read for insulin in an automatic reader (iEMS Reader MF V.2.9-0, Lab systems España, Barcelona, Spain) at optical density of 450 nm. Glucose was analyzed by the hexokinase method (OSR 6121, Reagent System Olympus, Beckman Coulter, Krefeld, Ireland). Values of NEFA and BHB were determined as previously indicated.

Area under the curve (**AUC**) of metabolite responses to GTT, insulin tolerance test and epinephrine challenge was calculated by the trapezoidal method after the correction for the baseline values. Plasma baseline data were obtained by averaging the corresponding values at min –20, –10 and 0. Values of peaks or nadirs of each metabolite after infusions were recorded. Glucose clearance rate and glucose half-life during the GTT were calculated according to Kerestes et al. (2009) using these formulas:

$$\text{Glucose clearance rate (\%/min)} = \frac{\ln(\text{glucose t5}) - \ln(\text{glucose t60})}{\text{t60-t5}} \times 100$$

$$\text{Glucose half-life (min)} = \frac{0.693}{\text{glucose clearance rate}} \times 100$$

In case of the insulin tolerance test, insulin-stimulated blood glucose response was calculated as indicated by Kerestes et al. (2009) as follows:

$$\text{Insulin stimulated glucose response (\%)} = \frac{\text{glucose t0} - \text{glucose t30}}{\text{glucose t0}} \times 100$$

3.3.3. Statistical Analyses

Data were analyzed by the MIXED procedure for repeated measurements of SAS v. 9.4 (SAS Institute Inc., Cary, NC). The statistical mixed model contained the effects of environmental conditions (TN and HS), period (1 and 2), the sampling time, and the environmental conditions × period, environmental conditions ×

sampling time, period × sampling time interactions as fixed effects, as well as the random effects of the animal and the residual error. The model considered the possible carryover effects of previous HS periods through the environmental conditions × period interaction. For the data of RT and RR measured at 0900, 1200 and 1700 h, a fixed factor of the hour of day was added to the model. Data of performances (i.e., DMI, water consumption, and milk yield) and physiological indicators (i.e., RT and RR) were analyzed on a weekly basis. For the basal levels of physiological challenge parameters, a t-test for independent means were used. Differences between least squares means were determined with the PDIFF test of SAS. Significance was declared at $P < 0.05$ and tendency at $P < 0.10$ unless otherwise indicated. Results were presented as LS means ± SEM and their differences separated by the PDIFF option of SAS.

3.4. RESULTS AND DISCUSSION

3.4.1. Body temperature and respiratory rate

The RT and RR were greater ($P < 0.001$) in HS than TN ewes (Table 3.2). Overall, RT of HS ewes increased by 0.77ºC. Furthermore, RT increased in HS ewes throughout the day in accordance with increment in the ambient temperature from 28ºC during night to 35ºC during the day. However, the differences were not significant between the 0800 and 1700 h measurements in TN ewes. The RR results followed the same pattern as those of RT. Heat-stressed ewes had 214% increase in RR (+90 breaths/min) on average compared to TN ewes. Ewes under HS conditions increased RR by 34% in the evening compared to the morning measurement values in agreement with the increment in ambient temperature throughout the day from 28 to 35ºC.

In sheep, heat loss via the increase in RR is the principal way of heat dissipation because sweating is prevented by the presence of wool coat (Marai et al., 2007). It has been estimated that ~65 % of body heat losses in sheep is through the respiratory tract during hyperthermia (Hales and Brown, 1974). In the present study, ewes were shorn before the experiment (2-cm fleece length), and some heat dissipation by sweating could have also occurred in addition to evaporation by panting. Period had significant effect on RT (Table 3.2), which could be due to

differences in the stage of lactation and metabolic heat production variation. However, there was no significant treatment × period interaction, indicating that the response to heat treatments was consistent over periods

Table 3.2: Body temperature and respiration rate of dairy ewes under thermo-neutral (TN; n = 8) and heat stress (HS; n = 8) conditions. Values are LSM and SE of the means (SEM).

Item	TN	HS	SEM	P-value		
				T	Period	T × P[1]
Body temperature, ° C						
0800h[2]	38.80	39.38[b]	0.08	0.001	0.063	-
1200h[3]	38.83	39.67[a]	0.08	0.001	0.300	-
1700h[3]	38.97	39.85[a]	0.08	0.001	0.049	-
Average	38.86	39.63	0.04	0.001	0.007	0.656
Respiration rate, breaths/min						
0800h	39	115[c]	3	0.001	0.579	-
1200h	41	127[b]	3	0.001	0.177	-
1700h	46	154[a]	3	0.001	0.194	-
Average	42	132	2	0.001	0.725	0.367

[a, b, c] Values within a column with different superscripts differ significantly ($P < 0.05$). [1]Interaction of treatment (T) × period (P). [2]HS conditions before changing from night (28 °C and 45% humidity; THI = 75) to day (35°C and 45% humidity; THI = 83) conditions. [3]During the day (35°C and 45% humidity; THI = 83) conditions.

3.4.2. Feed intake, Water Consumption, and Body weight Change

On average, DMI decreased ($P < 0.001$) in HS ewes by 11% throughout the experiment (Table 3.3). This lower feed intake induced slight negative energy balance in HS compared to TN ewes (Table 3.3). Reduction in appetite under HS is a primarily result of the elevated body temperature. There is a direct effect of HS on the feeding center of the hypothalamus, resulting in a hormonal response, which also decrease metabolic rate (West, 1999). The reduced feed intake may be also related to an increase in gut fill due to an increase in water consumption (Silanikove, 1992). In addition to fermentation, rumen seems to serve as a water reservoir during high heat load (Silanikove, 1992). The decrease in DMI observed in our HS ewes (–11%) is lower than that reported in dairy cows (–30%; Wheelock et al., 2010) and goats (–21%; Hamzaoui et al., 2013) heat-stressed to similar extent as in the present study. Based on DMI results, it seems that dairy ewes suffer less HS compared to dairy cows and goats.

The HS ewes had greater water consumption compared with TN ewes (+2.0 L/d; $P < 0.001$), which represents 28% more water consumption compared to TN ewes. Conrad (1985) observed that sheep consume 2 kg water/kg DM at temperatures between 0 and 15 ºC, and this ratio increases 3 times at temperatures above 20ºC. Furthermore, Marai et al. (2007) reported that water consumption increases by up to 50% under high ambient temperatures. Obviously, the increase in water consumption in the present study was mainly to meet the increment of water requirements for heat dissipation by evaporation.

Table 3.3: Productive parameters and milk composition of dairy ewes under thermo-neutral (TN; n = 8) or heat stress (HS; n = 8) conditions. Values are LSM and SE of the means (SEM).

Item	TN	HS	SEM	*P*-value		
				T	Period	T × P[1]
DMI, kg/d	2.69	2.39	0.08	0.019	0.001	0.812
Water Consumption, L/d	7.14	9.14	0.31	0.001	0.003	0.159
BW Change, kg/21d	4.50	–0.38	0.41	0.002	0.226	0.729
Milk yield, kg/d	1.65	1.83	0.15	0.434	0.352	0.883
FCM[2], L/d	1.64	1.64	0.08	0.985	0.337	0.772
Efficiency[3]	0.62	0.70	0.05	0.334	0.354	0.425
EBAL[4], UFL/d	0.45	–0.03	0.09	0.003	0.005	0.886
Milk composition						
Fat, %	6.81	5.74	0.13	0.001	0.980	0.180
Protein, %	6.37	5.51	0.13	0.001	0.177	0.049
Lactose, %	4.43	4.74	0.09	0.028	0.427	0.184
MUN[5], mg/L	457	464	17	0.795	0.089	0.003
Fat : protein ratio	1.07	1.05	0.03	0.539	0.643	0.274
SCC[6], log10/mL	4.40	4.63	0.08	0.066	0.125	0.679
Fat yield, g/d	108	101	4.0	0.305	0.219	0.933
Protein yield, g/d	97.7	94.7	5.2	0.680	0.216	0.812
Lactose yield, g/d	69.4	81.9	7.3	0.250	0.292	0.548
Milk osmolality, mOsm	298	302	1	0.001	0.084	0.008

[1]Interaction of treatment (T) × period (P). [2]6.5% FCM = L of milk yield × [0.37 + 0.09 × (fat%)] (Pulina et al, 2005). [3]Efficiency = 6.5% FCM/DMI. [4]Energy balance: Energy intake – [maintenance requirements ($0.0345 \times BW^{0.75}$) + Lactation requirement (0.686 × L of milk yield × [0.0071 × (fat%) + 0.0043 × (protein %) + 0.2224]]); INRA, 2018). [5]MUN = milk urea nitrogen. [6]SCC = Somatic cell count.

Ewes under HS conditions lost BW compared to TN that gained weight (Table 3.3; $P < 0.01$). On average, the HS ewes lost 18 g/d, whereas TN ewes gained 214 g/d. This loss in BW in HS ewes agrees with the slight negative energy balance detected compared with TN ewes (Table 3.3). Under HS conditions, maintenance

requirements increase because of the increment in energy expended for heat dissipation and the production of high amounts of heat shock proteins. In addition, HS cows (Ronchi et al., 1999) and goats (Salama et al., 2014) mobilize muscles and use some AA for gluconeogenesis. Our HS ewes also degraded muscles as indicated by greater blood creatinine levels compared to TN ewes (see later).

3.4.3. Milk yield and Composition

Both TN and HS ewes had similar values of milk yield and FCM (Table 3.3) despite the reduced DMI, and increased RT and RT. Ewes in the present experiment were in the second half of lactation, and milk yield response to HS would have been different if ewes were in earlier stage of lactation. Abdalla et al. (1993) also reported that crossbred (Finn × Dorset × Rambouillet) early lactating ewes do not suffer milk yield losses under controlled HS conditions (constant 35°C, 55%, THI = 86). Additionally, Hamzaoui et al. (2013) under HS controlled conditions observed that heat-stressed late lactating goats produce similar milk yield to goats under TN conditions. Nevertheless, in the studies evaluating the effect of HS by comparing among seasons, milk yield of mid-lactating was reduced by 15% when maximum ambient temperatures were higher than 21−24ºC in Sarda ewes (Peana et al., 2007). Furthermore, late-lactating Comisana ewes experience a reduction in milk yield after exposure to temperatures over 35ºC, even for short periods (Sevi et al., 2001). Evaluation the impact of HS by the comparison between seasons includes the inevitable variations due to different feeding and photoperiod conditions, and these variations were not present in the current study.

Compared to TN, HS decreased milk fat and protein contents by 13 to 16 % (Table 3.3). Similarly, Abdalla et al. (1993) reported that milk fat and protein contents are depressed in HS non-dairy ewes as compared to TN. In addition, Ramon et al. (2016) reported in Manchega breed a reduction in fat and protein yields during summer. In the present study, lactose content was increased in HS ewes compared to TN ($P < 0.05$) in accordance with the numerical increment in milk yield. Heat stress tended also to increase ($P < 0.10$) milk SCC by 5% in HS ewes compared to TN ewes. However, Caroprese et al. (2011) reported no change in milk SCC in ewes exposed to solar radiation compared to ewes maintained in shade. It is well known that milk coagulation is strongly affected by milk composition and SCC (Albenzio et

al., 2004). Therefore, worsened coagulation properties of HS milk might be expected. Despite the reduced fat and protein contents by HS, fat, protein, and lactose yields were not affected, which might be caused by the numerical increment in milk yield by HS. Consequently, the mammary synthetic capacity was not impaired by HS in our ewes, which is in contrast to what recently observed in bovine (Salama et al., 2019).

Milk osmolality was increased ($P < 0.001$) by HS (Table 3.3). Nevertheless, several authors reported a decreased milk osmolality in heat-stressed goats (Olsson and Dahlborn, 1989) and ewes (Thompson et al., 1981). In the current study, milk osmolality increased by only 1% in HS ewes, and values were within the normal physiological range reported for sheep milk (304 to 378 mOsm/L; Büttel et al., 2008). Additionally, the treatment × period interaction was significant, which indicates inconsistent response of milk osmolality to HS throughout periods. All together makes the detected significant difference in milk osmolality due to HS marginally relevant.

3.4.4. Blood variables

There were no differences between treatments in blood electrolytes (Table 3.4). Nevertheless, HS ewes tended ($P < 0.10$) to have increased blood Cl concentration compared to TN ewes. Usually, high ambient temperatures result in a reduction in Na, K, Ca and P, and an increase in Cl concentrations (Hamzaoui et al., 2013; Mehaba et al., 2019). Similar to our results, HS had no effect on blood Na and K concentrations in late lactating dairy goats, but Cl levels increased (Hamzaoui et al., 2013). The increment in Cl (anions with acidifying effect) might be occurred to avoid alkalosis resulting from panting. Blood total CO_2 concentration dropped ($P < 0.05$) by HS, which is similar to what was observed in dairy goats (Hamzaoui et al., 2013) as a result of panting and greater removal of blood CO_2 under HS conditions.

No differences were observed between TN and HS ewes with regard to blood glucose, cholesterol, NEFA, or BHB values (Table 3.4). Similarly, heat-stressed late lactating dairy goats (Hamzaoui et al., 2013) and early-lactating ewes (Abdalla et al., 1993) are able to keep similar blood glucose levels to TN animals. Possible explanations of how HS ewes were able to keep blood glucose values are discussed hereafter. The lack of a NEFA response to HS despite the unfavorable energy

balance was also reported in dairy goats (Hamzaoui et al., 2013; Mehaba et al., 2019) and dairy cows (Baumgard et al., 2011). Metabolic tests reported in the following section might help in understanding why HS resisted the mobilization of body fat reserves. Nevertheless, Abdalla et al. (1993) detected increased blood NEFA levels in early lactating non-dairy ewes exposed to 35ºC compared to control ewes at 20ºC. Hemoglobin and hematocrit values did not vary between TN and HS ewes. Similarly, packed cell volume and hemoglobin were unaffected in HS goats (Hamzaoui et al., 2013), although some studies showed increases (Okoruwa, 2014) or decreases (Singh et al., 2016) in sheep exposed to high ambient temperatures. Discrepancy among studies could be explained by differences in the specie, breed, aptitude (dairy vs. non-dairy), stage of lactation, and heat stress intensity and duration.

Table 3.4: Blood variables of dairy ewes under thermo-neutral (TN; n = 8) or heat stress (HS = 8) conditions. Values are LSM and SE of the means (SEM).

Item	TN	HS	SEM	*P*-value		
				T	Period	T × P[1]
Acid-base balance						
Na, mmol/L	146.4	145.9	0.3	0.264	0.018	0.772
K, mmol/L	4.28	4.41	0.06	0.154	0.610	0.619
iCa, mmol/L	1.31	1.33	0.01	0.224	0.650	0.001
Cl, mmol/L	105.8	107.1	0.5	0.098	0.613	0.693
Total CO_2,[2] mmHg	26.3	24.3	0.6	0.023	0.362	0.687
Anion Gap[3], mmol/L	19.6	19.9	0.4	0.564	0.088	0.629
Energy metabolism						
Glucose, g/dL	60.7	58.7	1.8	0.488	0.798	0.774
NEFA, mmol/L	0.13	0.13	0.02	0.869	0.057	0.444
β-HBA, mmol/L	0.45	0.44	0.04	0.909	0.820	0.157
Cholesterol, mg/dL	107	99	10	0.556	0.981	0.176
Hematocrit, % PCV[4]	22.1	20.8	0.6	0.130	0.179	0.240
Hemoglobin,[3] mmol/L	7.5	7.1	0.2	0.134	0.193	0.226
Protein metabolism						
Creatinine, mg/dL	0.6	0.8	0.02	0.001	0.988	0.099
BUN[5], mg/dL	20.8	21.6	0.51	0.245	0.172	0.006
Albumin, g/dL	3.4	3.4	0.1	0.480	0.020	0.450

[1]Interaction of treatment (T) × period (P). [2]Total pressure of CO_2. [3]Calculated values by the i-STAT device software (Abbott Point of Care Inc., Princeton, NJ). [4]PCV = packed cell volume. [5]Blood Urea Nitrogen.

There was an increase ($P < 0.001$) in blood creatinine in HS ewes compared to TN ewes, which might indicate muscle degradation. Although our HS dairy ewes

experienced lower DMI and had less favorable energy balance (Table 3.3), they did not use body fat reserves (lack of change in blood NEFA as shown in Table 3.4), but they mobilized body protein presumably to use some glucogenic AA for glucose synthesis (blood glucose values did not change). Heat-stressed dairy goats also experience increased blood creatinine levels compared to TN animals (Salama et al., 2014; Mehaba et al., 2019).

The reduced DMI (and consequently protein intake) would result in decreased BUN as reported in HS goats compared to TN goats (Hamzaoui et al., 2013; Mehaba et al., 2019). Nevertheless, values of BUN and albumin were not affected by HS in the current study. Studies comparing HS and TN pair-fed cows (TN animals with similar intake as HS animals) showed that HS per se increase BUN values (Wheelock et al., 2010). Those authors hypothesized that the increment in BUN is due to AA deamination resulting from muscle degradation (this assumption is supported by greater blood creatinine in the current study). Alternatively, greater BUN in HS compared to TN animals when DMI level is similar (Lamp et al., 2015) could be explained by the fact that HS reduces kidney perfusion and enhances plasma water loss (also into milk resulting in diluted milk as observed in the current study), which leads to the accumulation of urea in plasma. Thus, it seems that the extent of decrease in protein intake in our study (–11%) was not enough to overcome the favorable conditions of HS on urea accumulation, which resulted in similar BUN values between TN and HS ewes (in fact BUN was numerically increased by 4% in HS ewes). The goat studies that detected reduced blood BUN by HS (Hamzaoui et al., 2013; Mehaba et al., 2019) reported that DMI decreases by 21 to 26% due to HS, which is 2 times the reduction in DMI observed in our ewes.

3.4.5. Responses to the Metabolic Tests

3.4.5.1. Glucose tolerance test

Results of the GTT as indicator of insulin sensitivity are shown in *Figure 3.1* and Table 3.5. The basal glucose levels were similar in TN and HS ewes (Table 3.5) in agreement with the no differences in glucose levels indicated in Table 3.4. Glucose peaked at 5 min in both treatments, but the peak was much greater ($P < 0.05$) in HS (+511%) than in TN (+252%) ewes (*Figure 3.1A*). Blood glucose levels gradually

decreased in both treatments and returned to the basal values by 60 min after glucose administration. No differences over time-points were detected between groups from min 10 to 90.

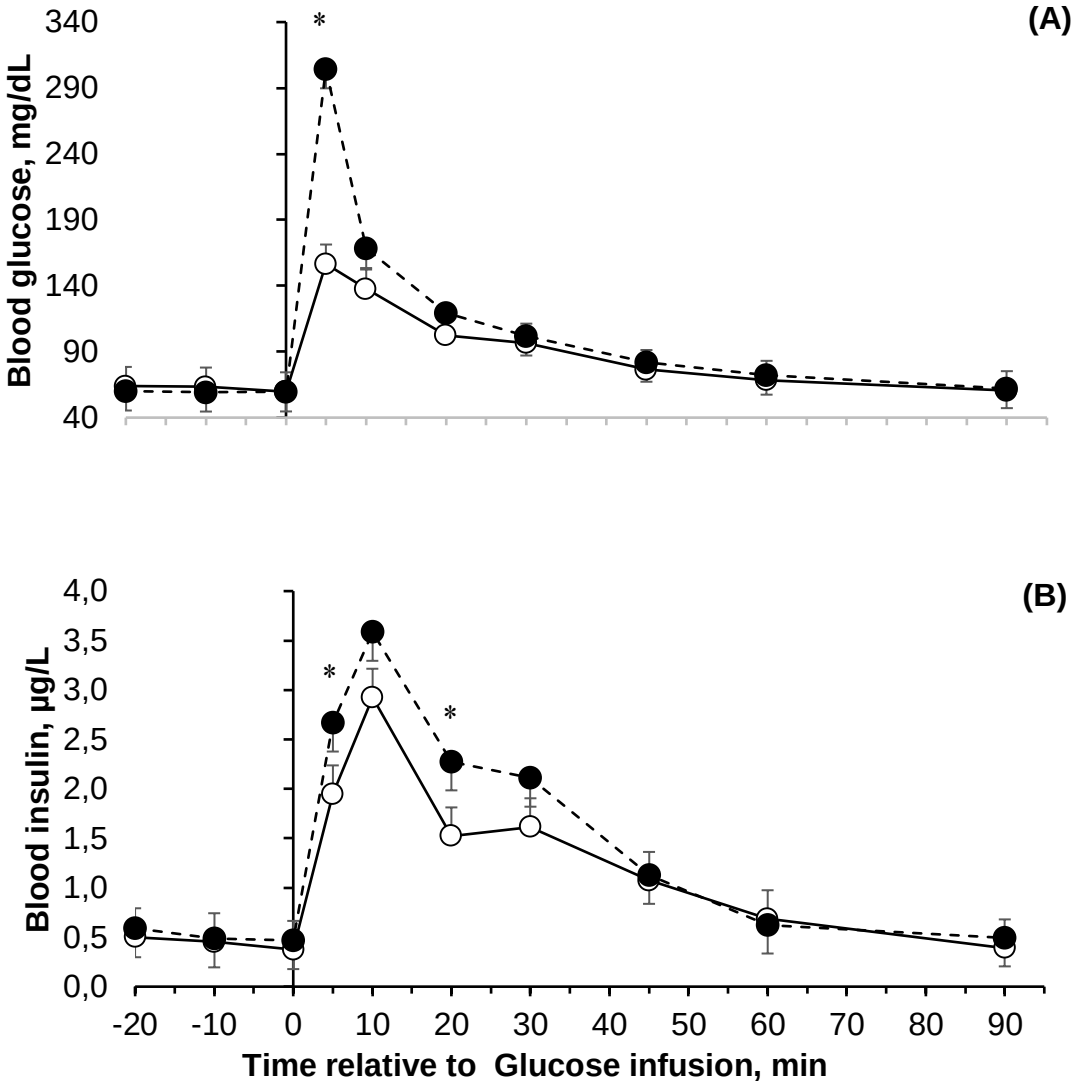

Figure 3.1: Plasma glucose (A) and insulin (B) response to glucose tolerance test of Lacaune dairy ewes under thermo-neutral (TN; ○; n = 4) or heat stress (HS; ●; n = 4) conditions. Values are least square means with SEM indicated by vertical bars. * indicates a difference at $P < 0.05$ between TN and HS treatments.

Basal plasma insulin values did not differ between TN and HS ewes (*Figure 3.1B*; Table 3.5). Similarly, there are no differences in basal blood insulin values between TN and HS in non-lactating Suffolk ewes (Achmadi et al., 1993) and heifers (Itoh et al., 1998). As a response to the glucose infusion, plasma insulin levels of both TN and HS ewes peaked at min 10, but no differences in peak values was observed

between TN and HS ewes. After the peak, insulin values decreased gradually and reached the basal levels at min 90. The HS ewes had greater ($P < 0.05$) insulin values than TN ewes only at 5 and 20 min, and no differences were detected between groups at the remaining time points (*Figure 3.1B*).

As shown in Table 3.5, the mean glucose AUC tended ($P < 0.10$) to be greater at 45 and 90 min in HS ewes compared to TN ewes. However, the insulin AUC values were similar in both groups. Wheelock et al. (2010) found also that lactating heat-stressed cows have greater glucose response after GTT than TN cows fed ad libitum without differences in the insulin response. With similar insulin secretion kinetics after glucose administration, HS ewes had greater immediate glucose level than TN ewes, which might be interpreted as decreased insulin sensitivity in HS ewes. This greater available glucose in HS ewes was rapidly uptaken, resulting in increased ($P < 0.01$) glucose clearance rate and decreased ($P < 0.01$) glucose half-life under HS conditions. Glucose is the most efficiently utilized energy source compared to other substrates as fat or protein (Baldwin et al., 1980), which might explain its fast disappearance in HS conditions. Due to the apparent lack of NEFA availability as a fuel substrate, it seems that heat-stressed animals increase both their production of, and reliance on, glucose as a fuel. Because insulin response to GTT increased only numerically ($P > 0.20$) by HS (Table 3.5), the faster uptake of glucose in HS ewes might have been caused not only by insulin, but also through a non-insulin-mediated glucose uptake process. In fact, the whole body utilization of glucose can also occur through noninsulin-dependent pathways in sheep (Janes et al., 1985) and dairy cows (Rose et al., 1997).

Table 3.5. Glucose and insulin kinetics in response to glucose tolerance test in dairy ewes under thermo-neutral (TN; n = 4) or heat stress (HS; n = 4). Values are means and SE of the means (SEM).

Item	TN	HS	SEM	*P*
Glucose				
Basal, mg/dL	62.7	59.6	1.6	0.186
Peak, mg/dL	94	245	28	0.028
CR_{60},[1] %/min	1.7	2.9	0.2	0.010
AUC[2], mg/L × min				
45 min	1258	2333	318	0.071
90 min	1321	2457	375	0.093
$GLU_{-t1/2}$,[3] min	41.7	25.1	2.45	0.004
Insulin				
Basal, µg/L	0.44	0.51	0.08	0.562
Peak, µg/L	2.92	3.58	1.11	0.433
CR_{60}[1], %/min	3.1	3.6	0.79	0.664
AUC[2], µg/L × min				
45 min	32.7	44.4	18.3	0.258
90 min	35.4	46.5	5.8	0.245

[1]Clearance rate form the peak to 60 min. [2] Area under the curve corrected for the basal levels. [3]Glucose half-life.

3.4.5.2. Insulin tolerance test.

Results of the insulin tolerance test as indicator of insulin responsiveness are shown in *Figure 3.2* and Table 3.6. Basal glucose levels did not vary between TN and HS ewes and averaged 59.3 ± 1.8 mg/dL. Plasma glucose concentration in both groups decreased to a nadir at 30 min post-infusion. Thereafter, blood glucose values elevated, and by 120 min they returned to the basal values in HS ewes, but not in TN (60.9 ± 3.5 vs. 50.9 ± 3.4 mg/dL for TN and HS ewes, respectively; $P < 0.05$), indicating prolonged effect of insulin in TN compared to HS ewes (*Figure 3.2*).

However, taking all time points into consideration, insulin AUC and insulin-stimulated blood glucose response did not vary between TN and HS ewes (Table 3.6), which indicates unchanged insulin responsiveness. This result agrees with the findings of Achmadi et al. (1993) who found that glucose amount needed to keep euglycemia when insulin is infused do not vary between TN and HS meat ewes. Given the fact that blood glucose did not return to the basal levels by min 120 in TN ewes (*Figure 3.2*), it is possible that differences in insulin responsiveness kinetics would have been detected if sampling time was extended beyond 120 min.

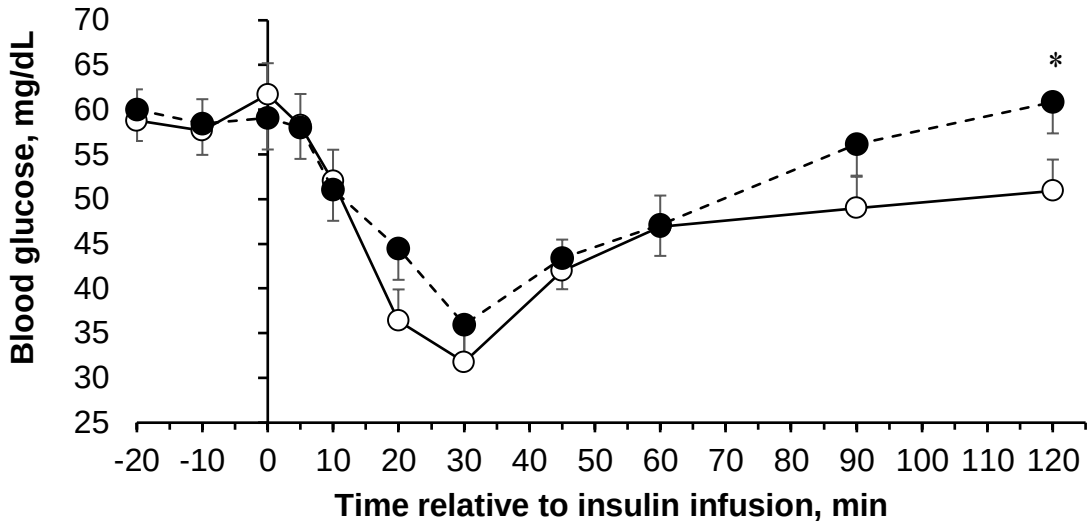

Figure 3.2: Plasma glucose response to insulin challenge of Lacaune dairy ewes under thermo-neutral (TN; ○; n = 4) or heat stress (HS; ●; n = 4) conditions. Values are means with SE indicated by vertical bars. * indicates a difference at $P < 0.05$ between TN and HS treatments.

Table 3.6: Blood glucose, non-esterified fatty acids (NEFA), and β-hydroxybutyrate (BHB) kinetics in response to insulin tolerance test in dairy ewes under thermo-neutral (TN; n = 4) or heat stress (HS; n = 4) conditions. Values are means and SE of the means (SEM).

Item	TN	HS	SEM	*P*
Glucose				
Basal, mg/dL	59.2	59.4	1.8	0.934
Nadir, mg/dL	31.7	34.0	2.5	0.542
AUC_{120}[1], mg/dL × min	-522	-386	87	0.313
ISBGR[2], %	0.47	0.40	0.04	0.258
NEFA				
Basal, mmol/L	0.26	0.34	0.05	0.304
Nadir, mmol/L	0.19	0.15	0.05	0.242
AUC_{120}, mmol/L × min	1.85	-1.54	0.72	0.017
BHB				
Basal, mmol/L	0.43	0.44	0.06	0.881
Nadir, mmol/L	0.36	0.42	0.05	0.486
AUC_{120}, mmol/L × min	0.18	1.88	1.01	0.301

[1] Area under the curve from 0 to 120 min corrected for the basal levels. [2]Insulin stimulated blood glucose response. For calculation see Materials and Methods.

Neither plasma NEFA nor BHB concentrations were affected by the insulin infusion, although NEFA AUC was lower in HS compared to TN ewes (Table 3.6).

The lower NEFA AUC levels in HS ewes might be related to the more dependence on glucose as energy source and the absence of body fat mobilization, despite the reduction in DMI and BW under HS. More details on NEFA kinetics under HS are presented in the epinephrine challenge test.

3.4.5.3. Epinephrine challenge.

Plasma glucose peak in response to the epinephrine challenge was similar between TN and HS ewes (Table 3.7), but HS ewes had an earlier peak (5 min) compared with TN ewes (10 min) as shown in *Figure 3.3A*. At 10 min, TN ewes had greater ($P < 0.05$) blood glucose (114 mg/dL) than HS ewes (99 mg/dL). Similar glucose AUC between TN and HS ewes suggests equal liver sensitivity to epinephrine with regard to breaking down glycogen and releasing glucose.

Blood NEFA levels (*Figure 3.3B*) were lower in HS ewes compared to TN ewes from 30 min post epinephrine infusion until the last time point (120 min). Nevertheless, there was an increase in BHB plasma concentration in HS ewes compared to TN ewes ($P < 0.05$) after the intravenous infusion of epinephrine. The blunted lipolytic response to epinephrine challenge in HS ewes occurred although these ewes suffered a slight calculated NEBAL (–0.03 UFL/d) and lost BW (–0.38 kg/period), all of which are parameters typically associated with elevated circulating NEFA levels. Baumgard et al. (2011) demonstrated that heat-stressed cows have a blunted (compared with pair-fed TN controls) NEFA response to an epinephrine challenge. Epinephrine has been reported to be elevated during HS, especially during the early phase (acute) of hyperthermia (Silanikove et al., 2000). This elevation could modify the density and affinity of the adrenergic receptors (Mirit et al., 2000), which could explain the altered lipolytic response in HS ewes compared to TN ewes.

Alternatively, the released NEFA in response to epinephrine administration could have long time of clearance in TN ewes, whereas in case of HS ewes, NEFA were rapidly uptaken by liver and converted into ketone bodies. This would explain the greater ($P < 0.05$) AUC of BHB (Table 3.7) in HS compared to TN ewes. Salama et al. (2014) proposed that liver of HS goats is able to uptake NEFA faster and converts them to BHB (utilized as energy source for body tissues to spare glucose) compared

to TN goats. This also could have occurred in our HS ewes, resulting in greater BHB levels compared to TN ewes.

Table 3.7: Glucose, non-esterified fatty acids (NEFA) and β-hydroxybutyrate (β-BHB) kinetics in response to epinephrine challenge in dairy ewes under thermo-neutral (TN; n = 4) or heat stress (HS; n = 4) conditions. Values are means and SE of the means (SEM).

Item	**TN**	**HS**	**SEM**	***P***
Glucose				
Basal, mg/dL	62.9	61.3	1.5	0.478
Peak, mg/dL	114	104	16	0.682
AUC_{120},[1] mg/dL × min	900	881	152	0.933
CR[2] (%/min)	1.2	1.0	0.1	0.466
$t_{1/2}$,[3] min	62.3	74.4	8.7	0.369
NEFA				
Basal, mmol/L	0.27	0.28	0.04	0.920
Peak, mmol/L	0.44	0.39	0.12	0.578
AUC_{120}, [1] mmol/L × min	1.82	-2.10	0.98	0.030
BHB				
Basal, mmol/L	0.47	0.46	0.06	0.925
Peak, mmol/L	0.49	0.57	0.13	0.441
AUC_{120}, [1] mmol/L × min	-1.17	1.42	0.71	0.047

[1]Area under the curve from 0 to 120 min corrected for the basal levels. [2]Glucose clearance rate. [3]Glucose half-life.

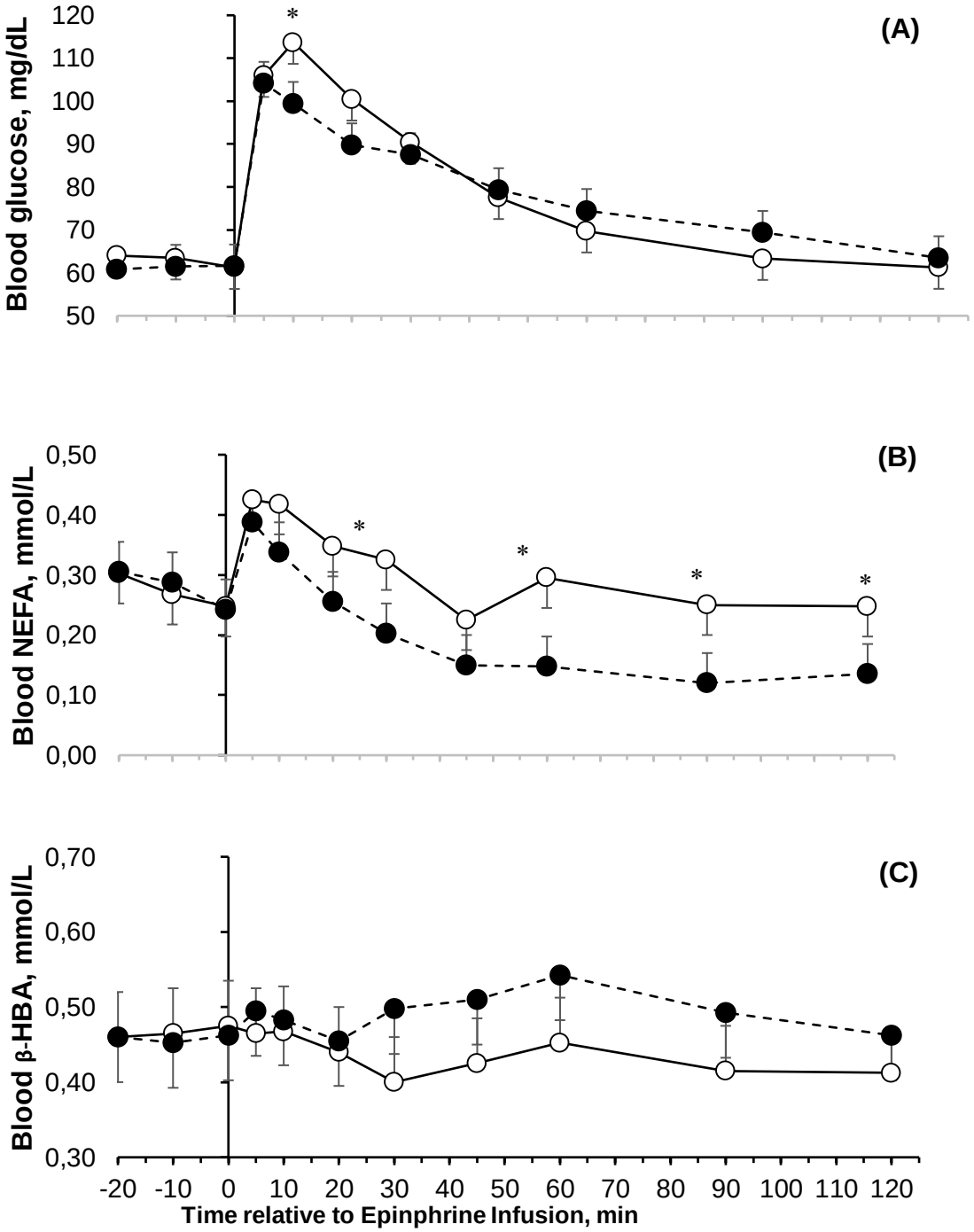

Figure 3.3: Plasma glucose (A), NEFA (B) and β-HBA (C) response to epinephrine challenge of Lacaune dairy ewes under thermo-neutral (TN; ○; n = 4) or heat stress (HS; ●; n = 4) conditions. Values are least square means with SE indicated by vertical bars. * indicates a difference at $P < 0.05$ between TN and HS treatments.

3.5. CONCLUSIONS

In the current study we comprehensively measured the physiological, metabolic and productive response of dairy ewes to controlled heat stress conditions. Heat stress reduced feed intake, but ewes were able to maintain milk yield as well as the yields of fat, protein and lactose. The tendency of increased milk SCC by HS might have negative impact on milk coagulation properties for cheese production. Heat-stressed ewes did not mobilize body fat reserves, but degraded muscles as indicated by the greater blood creatinine levels. Heat-stressed ewes tended to have more available glucose in the blood after glucose was administered, and it is likely that this glucose was uptaken through insulin- and noninsulin-mediated pathways. Further, adipose tissue of HS ewes became more resistant to the lipolytic signals. Overall, these metabolic adaptations allowed ewes to spare glucose and to avoid reductions in milk yield.

CHAPTER 4

Lactational responses of heat stressed dairy goats to dietary L-carnitine supplementation

CHAPTER 4

4. LACTATIONAL RESPONSES OF HEAT-STRESSED DAIRY GOATS TO DIETARY L-CARNITINE SUPPLEMENTATION[2]

4.1. ABSTRACT

Heat stress causes significant losses in milk production, and nutritional strategies are needed to alleviate its effects. Endogenous carnitine synthesis is also reduced by heat stress (**HS**). Carnitine plays a central role in fatty acid oxidation and buffers the toxic effects of acyl groups. We hypothesized that carnitine supplementation would make up for any carnitine deficiencies during HS and improve lipid metabolism. The objective was to evaluate rumen-protected L-carnitine (**CAR**) supplementation in dairy goats under thermo-neutral (**TN**) or HS conditions. Four Murciano-Granadina dairy goats were used in a four × four Latin square design. Goats were allocated to one of four treatments in a two × two factorial arrangement. Factors were 1) diet: control (**CON**) or supplementation with CAR (1 g/d); and 2) ambient conditions: TN (15 to 20ºC) or HS (0900 to 2100 h at 35ºC, 2100 to 0900 h at 28ºC). Blood free-, acetyl-, and total-carnitine concentrations increased almost three times by supplementation. Despite this efficient absorption, CAR had no effect on feed intake, milk production or blood metabolites in TN or HS conditions. Heat stress increased rectal temperature and respiratory rate. Additionally, HS goats experienced 26% loss in feed intake, but they tended to eat longer particle sizes. Compared to TN, heat-stressed goats lost more subcutaneous fat (difference in fat thickness measured before and after each period = −0.72 vs. +0.64 mm). In conclusion, supplemented L-carnitine was efficiently absorbed, but it had no lactational effects on performance of goats under thermo-neutral or heat stress conditions.

4.2. INTRODUCTION

Heat stress (**HS**) causes significant losses in milk yield and milk components in dairy animals (Baumgard and Rhoads, 2013; Salama et al., 2016). Goats are

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considered more tolerant to HS compared to dairy cows because of their greater sweating rate and lower body weight (**BW**):surface ratio, allowing greater heat dissipation (Salama et al., 2014). However, lactating dairy goats exhibit several changes in performance due to HS, including reductions in feed intake, milk yield, milk fat, and milk protein (Salama et al., 2014; Hamzaoui et al., 2013).

The HS compromises animal performance by directly altering metabolism and the hierarchy of nutrient utilization (West, 2003). For instance, heat-stressed (**HSed**) goats challenged with epinephrine have lower blood concentrations of non-esterified fatty acids (**NEFA**) compared to goats under thermal neutral (**TN**) conditions, indicating that lipid tissue of HSed goats becomes less sensitive to lipolytic signals (Salama et al., 2014). Additionally, blood NEFA and ß-hydroxybutyrate (**BHB**) do not vary between HS and TN goats (Salama et al., 2014) or cows (Baumgard and Rhoads, 2013), although they experience negative energy balance. These findings might indicate that HSed animals are not able to use body fat reserves to cover their energy needs. Recently, blood transcriptomics of HSed goats showed the downregulation of several anti-inflammatory pathways, indicating that HSed goats could be in an inflammation status (Contreras-Jodar et al., 2018). Inflammation impairs peroxisome proliferator-activated receptor-regulated fatty acid oxidation and carnitine synthesis from lysine (Palomer et al., 2013). Thus, HSed animals may need more carnitine compared to TN animals.

L-carnitine (CAR) has two main roles in eukaryote cells: 1) A “shuttle role”, in the transfer of long-chain fatty acids from cytosol to mitochondria for subsequent β-oxidation and the production of acetyl-CoA used for energy production (by ketogenesis or in TCA cycle); and 2) the “buffer role” of the acyl groups (by modulation of the acyl-CoA/CoA and reduction of the acyl toxicity by excreting them as carnitine esters) (Vaz and Wanders, 2002).

Heat stress blocks fat mobilization in dairy cows (Baumgard and Rhoads, 2013; Salama et al., 2016) and apparently it does the same in dairy goats (Salama et al., 2014). However, Hamzaoui et al. (2013) exposed goats to HS for four weeks and reported that blood NEFA levels are greater in HS in the first week than TN, but for the remaining weeks no differences in NEFA are detected. This finding might indicate that HSed goats start to mobilize lipid, but as they are not able to use these

fatty acids (presumably because synthesis of carnitine is reduced as indicated above) they stop mobilizing lipid tissue despite the decreased energy intake. Randle. (1998) reported that the provision of free fatty acids promotes fatty acid oxidation and storage, inhibits glucose oxidation and may promote glucose storage if glycogen reserves are incomplete. Therefore, we hypothesized that CAR supplementation would help in the utilization of fatty acids and improve energy metabolism. The objective of the current study was to determine the influence of L-carnitine supplementation on the physiological and lactational responses in dairy goats under heat stress conditions. To date and to the best of our knowledge, no information is available on the effects L-carnitine supplementation in heat-stressed dairy goats.

4.3. 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 Autònoma de Barcelona (CEEAH reference 11/1430) and the codes of recommendations for the welfare of livestock of the Ministry of Agriculture, Food and Environment of Spain.

4.3.1. Animals, Treatments, and Management Conditions

Four multiparous Murciano-Granadina dairy goats (134 ± 2 days in milk; 2.48 ± 0.02 L/d milk yield; 46.1 ± 0.5 kg BW) with healthy and symmetrical udders from the herd of the experimental farm of the Universitat Autònoma de Barcelona were used. The design was 4 × 4 Latin square with 16 d periods. The first 11 d were for adaptation, while the last 5 d were the measurement period. Dietary L-carnitine supplementation increases carnitine levels in blood, milk, liver, and muscles within 1 wk in dairy cows (Carlson et al., 2007). Consequently, we considered the 16 d experimental periods enough time to study the possible CAR effects.

Goats were allocated to one of 4 treatments in 2 × 2 factorial arrangements. Factors were: 1) Diet: control without supplementation (CON), or supplementation with 5 g of rumen-protected CAR (20% pure L-carnitine; CarnEon20 Rumin-Pro, Kaesler Animal Nutrition, Cuxhaven, Germany); and 2) ambient conditions: TN (15 to 20ºC and 40% to 70% relative humidity throughout the day) or HS (from 0900 to

2100 h at 35ºC and from 2100 to 0900 h at 28 °C with 45 ± 5% relative humidity). This resulted in 4 treatment combinations: TN-CON, TN-CAR, HS-CON, and HS-CAR.

The temperature humidity index (THI) values were calculated according to NRC (NRC, 1971):

$$THI = (1.8 \times T_{db} + 32) - [(0.55 - 0.0055 \times RH) \times (1.8 \times T_{db} - 26.8)]$$

where T_{db} is the dry bulb temperature (°C) and RH is the relative humidity (%). The THI values for TN varied between 59 and 65 throughout the day, whereas for HS, the THI values were 84 and 75 for the day and night, respectively. These THI values used in the current experiment for HSed goats are classified as “alert” (80 ≤ THI < 85) and “normal” (THI < 80) stress levels during the day and night, respectively (Silanikove and Kolumna, 2015). The photoperiod was maintained constant 12–12 h (day, 0900 to 2100 h; night, 2100 to 0900 h) in both TN and HS conditions. Data of environmental temperature and humidity were recorded every 10 min by using 2 data loggers (Opus 10, Lufft, Fellbach, Germany).

Throughout the experiment, (mid-March to mid-June), the TN goats were kept indoors, and the temperature was maintained at 15 to 20ºC with the help of an electric heater equipped with a thermostat (3.5 kW; General Electric, Barcelona, Spain) when necessary. Temperature and relative humidity averaged 17.4 ± 0.5ºC and 62% ± 5% (THI = 63) for the TN goats. 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.

Goats had a 4 wk pre-experimental period under TN conditions for the adaptation to the diet and to the experimental conditions before applying the ambient conditions. When goats were switched from TN to HS conditions, the temperature increased in 2 steps (1 d at 25ºC and 1 d at 30ºC, 45 ± 5% humidity), but no transition was applied for the change from HS to TN.

Goats were milked twice daily (0800 and 1700 h) using a portable milking machine set at 42 kPa, 90 pulses/min, and 66% pulsation ratio, provided with

recording jars (5 L ± 5%). 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).

The 5 g of CAR supplement was daily weighed by an electronic scale (Mobba Barcelona, Barcelona, Spain), mixed with 50 g crushed barley, and offered individually before the morning milking. The CON goats were also fed 50 g crushed barley without CAR supplementation. The CAR product contained 11.6% crude protein and 50.8% ether extract on dry matter (DM) basis. The 5 g daily dose of the commercial CAR supplement provided 1 g pure L-carnitine. The CAR dose (1 g) in the current study is equivalent to 0.056 g/kg $BW^{0.75}$, which is similar to the dosage used by LaCount et al. (1996) in dairy cows (0.054 g/kg $BW^{0.75}$), although LaCount et al. (1996) used rumen-unprotected L-carnitine. We chose to use this similar dose despite the fact that our product is rumen-protected because our HSed goats presumably need more carnitine as mentioned in the introduction.

The daily total mixed ration was distributed individually to each goat once daily after the morning milking and adjusted at 30% leftover based on the previous day intake. The ration was formulated to cover requirements according to the Institut National de la Recherche Agronomique (INRA, 2018) and consisted of (as fed) alfalfa hay 60.4%, ground barley grain 15%, beet pulp 9.1%, crashed 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 chemical composition and nutritive value of the ration are shown in Table 4.1. Mineral and vitamin blocks were freely available to each goat (Na, 36.74%; Ca, 0.32%; Mg, 1.09%; Zn, 5 g/kg; Mn, 1.5 g/kg; S, 912 mg/kg; Fe, 304 mg/kg; I, 75 mg/kg; Co, 50 mg/kg; Se, 25 mg/kg; Ovi bloc, Sal Cupido, Barcelona, Spain). Clean water was permanently available at ambient temperature.

Table 4.1: Chemical composition and nutritive value of the ration expressed on dry matter (DM) basis.

Item	Total mixed ration
Component, %	
Dry matter	88.2
Organic matter	88.1
Crude protein	17.7
Ether extract	1.79
Neutral detergent fiber	39.3
Acid detergent fiber	28.6
Nutritive value[1]	
UEL,[2] /kg	1.08
UFL,[3] /kg	0.76
PDI,[4] g/kg	94.2
PDIA,[5] g/kg	45.5
RPB,[6] g/kg	31.6
Ca_{abs}, g/kg	2.73
P_{abs}, g/kg	0.84

[1] Calculated according to the Institut National de la Recherche Agronomique (INRA, 2018). [2] Fill units for dairy goats (1 UEL = 1 kg DM of reference grass). [3] Net energy for lactation (1 UFL = 1.76 Mcal of NE_L). [4] Protein digestible in the intestine from dietary and microbial origin. [5] Protein digestible in the intestine from dietary origin. [6] Rumen protein balance.

4.3.2. Sample Collection, Analyses, and Measurements

Rectal temperatures and respiratory rates were daily recorded at 0800, 1200, and 1700 h. Rectal temperature was measured by a digital clinical thermometer (Model ICO Technology "mini color", Barcelona, Spain; range, 32.0 to 43.9ºC; accuracy, ± 0.1ºC), whereas number of inhalations and exhalations counted during 60 s indicated the respiratory rate.

Feed intake was recorded daily throughout the experiment. A feed sample was collected before the beginning of the experimental period and was ground through a 1 mm stainless steel screen, and then analyzed for DM, acid detergent fiber, neutral detergent fiber, and ash according to the official analytical methods (AOAC, 2003). The Dumas method (AOAC, 2003) with a Leco analyzer (Leco Corporation, St. Joseph, MI) was used for crude protein determinations.

A sample of 5% total orts was collected daily during the measurement period and mixed together to make a composite of orts per each goat and period. Particle size of the ration and orts were measured according to Heinrichs and Kononoff. (2002). The Penn State Particle Separator (DSE 2013-186, PA) was used. The

average particle size was calculated using a spreadsheet downloaded from the Penn State Extention Website (https://extension.psu.edu/penn-state-particle-separator).

Milk yield of individual goats was weighed at each milking throughout the experiment using an electronic scale (Mobba Barcelona). Milk composition was evaluated for 2 d (d 14 and 15 of each period). A composite milk sample from the morning and afternoon milking's (approximately 100 mL) was collected and preserved with an antimicrobial tablet (Bronopol, Broad Spectrum Microtabs II, D&F Control Systems, San Ramon, CA) at 4ºC until analysis. Refrigerated milk samples were sent to the Laboratori Interprofessional Lleter de Catalunya (ALLIC, Cabrils, Barcelona, Spain) for the analyses of major components (total solids, fat, protein, and lactose) using medium infrared spectrophotometry (MilkoScan FT2, Foss, DK-3400 Hillerød, Denmark), and somatic cell count using an automatic cell counter (Fossomatic 5000, Foss Electric, Hillerød, Denmark) previously calibrated for goat milk.

Goats were weighed before feeding at the beginning and the end of each experimental period by a scale (model Tru-Test AG500 Digital Indicator, Auckland, New Zealand; accuracy, ± 0.5 kg). The scale was calibrated by a 5 kg weight before every weighing. The subcutaneous fat thickness was measured by ultrasonography according to Teixeira et al. (2008). The ultrasound images were taken using a VET 180 Plus ultrasound (Sonosonite, Bothell, WA) with a 5 MHz probe (veterinary model). When the ultrasound images were taken at the breastbone, goats were restrained in dorsal recumbency on a table. The fat thickness was measured in a perpendicular position to the ventral midline at the level of the third and fourth sternebrae. Images were then processed using an image analysis program (ImageJ v.1.48, National Institutes of Health; available at www.imagej.nih.gov/ij/download.html). Three measurements of the distance between the skin and the sternum were done for each goat.

Blood samples were taken at the last day of each period from the jugular vein into 10 mL vacutainers with K2-EDTA (BD Diagnostics, Franklin Lakes, NJ) before feeding. Plasma was obtained by the centrifugation of blood for 15 min at 1500 × g and stored at –30ºC until the analysis of NEFA, BHB, triglycerides, and cholesterol.

The NEFA were determined by colorimetric enzymatic test ACSACOD method using a commercial kit (Wako Chemicals, Neuss, Germany). The BHB was determined by kinetic enzymatic method using commercial kit (Ranbut, Randox, UK). Cholesterol was analyzed by the enzymatic method (cholesterol esterase/peroxidase), whereas triglycerides were analyzed with enzymatic method (glycerol phosphate oxidase) using an Olympus analyzer (Olympus AU400, Dusseldorf, Germany). Blood carnitine fractions (free carnitine, acetyl carnitine, and total carnitine) were determined using a quasi-solid phase extraction without derivatization reactions by means of normal-phase liquid chromatography and electro spray ionization tandem mass spectrometry (Applied Biosystems, Darmstadt, Germany) according to Hirche et al. (2009).

At the end of each period, additional blood samples (approximately 0.5 mL) were collected by insulin syringes (1 mL; BD Micro-Fine, BD Medical-Diabetes Care, Franklin Lakes, NJ), before feeding and immediately analyzed for major ions and metabolites. A single drop of blood was applied to disposable cartridges containing biochemical and silicon chip technology (i-STAT Chem8+, Abbott Point of Care, Princeton, NJ). Then, the cartridge was inserted into an i-STAT handheld analyzer, and glucose, urea, Cl^-, Na^{2+}, K^+, ionized Ca, total CO_2 concentration, anion gap, hematocrit, hemoglobin, creatinine, and base excess were obtained.

4.3.3. Statistical Analyses

Data were analyzed by the PROC MIXED for repeated measurements of SAS v. 9.1.3 (SAS Institute Inc., Cary, NC). The statistical mixed model contained the fixed effects of the temperature (TN and HS), dietary supplementation (CON and CAR), experimental day, period; the random effect of the animal; the interactions of temperature × supplementation, temperature × period, supplementation × period; and the residual error. The model considered the possible carryover effects of previous HS periods through the temperature × period interaction. For the data of rectal temperature and respiratory rate measured at 0900, 1200, and 1700 h, a fixed factor of the hour of day was added to the model. For the data of blood metabolites, and changes of BW and fat thickness, the PROC MIXED was used without repeated measures, and consequently the day effect was removed from the model. Differences between least square means were determined with the PDIFF test of

SAS. Significance was declared at $P < 0.05$ and tendency at $P < 0.10$ unless otherwise indicated.

4.4. RESULTS AND DISCUSSION

4.4.1. Carnitine Concentrations in Blood

Blood basal concentrations (samples taken before feeding) of L-carnitine fractions are shown in Table 4.2. All L-carnitine fractions (i.e., free-, acetyl-, and total-carnitine) in plasma increased ($P < 0.01$) almost three times by CAR supplementation in both TN and HS conditions. LaCount et al. (1995) reported that carnitine concentrations in plasma and liver increase when rumen-unprotected L-carnitine is administered into either the rumen or abomasum of dairy cows, indicating that both sites of administration are equally effective for increasing carnitine concentrations in tissues. LaCount et al. (1996) also reported a linear increase in plasma and milk carnitine concentrations as dietary carnitine concentration increased. Additionally, Carlson et al. (2007) supplemented periparturient dairy cows with 0.046, 0.382, and 0.763 g L-carnitine /kg $BW^{0.75}$ in the diet and detected that total carnitine concentration in plasma increased four to 10 times by the two higher doses.

Table 4.2: Least squares means for L-carnitine fraction concentrations (µmol/L) in plasma of dairy goats under thermo-neutral (TN) or heat stress (HS) conditions. In each ambient temperature, goats were fed a control diet (CON) or supplemented with 1g L-carnitine (CAR).

L-Carnitine	TN		HS		SEM	Effect[1] (*P* <)		
	CON	CAR	CON	CAR		T	C	T × C
Free	20.42	61.23	18.42	50.50	4.06	0.13	0.01	0.30
Acetyl-carnitine	6.79	17.49	6.90	21.24	2.08	0.35	0.01	0.38
Total	26.78	78.30	24.90	71.30	4.32	0.32	0.01	0.56

[1]Effects of temperature (T), CAR supplementation (C), and their interaction (T × C).

In a preliminary work, we measured the in situ degradability of the CAR product and observed that 72.8% of CAR dry matter disappeared at 16 h. Despite this high disappearance in the rumen, CAR in the current study was efficiently absorbed, as indicated by the elevated levels of carnitine in plasma (Table 4.2). This relatively high solubility does not necessarily mean that the L-carnitine was degraded in the

rumen. In fact, rumen microorganisms are not able to completely degrade the solubilized L-carnitine, as dietary supplementation with rumen-unprotected carnitine resulted in elevated blood levels of carnitine in dairy cows (Carlson et al., 2007; Silanikove and Koluman, 2015; Greenwood et al., 2001).

Free L-carnitine represented approximately 75% of total L-carnitine in the plasma of our goats (Table 4.2). Free L-carnitine concentrations numerically decreased (−14% on average; $P < 0.13$) by HS, and it seems that this free carnitine was transformed to acetyl-carnitine (more than 16% of total carnitine was presented as acetyl-carnitine in the HS group). Similarly, Thomson et al. (1979) reported that dairy goats exposed to cold stress experienced decreased blood carnitine levels with lower loss in the milk, which resulted in saving 52 μmoles/d of carnitine that most probably was used for the increment in fatty acid oxidation. Overall, CAR supplemented in the current experiment was absorbed efficiently and was available in blood for metabolism in both TN and HS goats.

4.4.2. Rectal Temperature and Respiratory Rate

The HS goats showed greater ($P < 0.001$) rectal temperatures and respiratory rates than TN goats at 0800, 1200, and 1700 h (Table 4.3). Compared to TN goats, HSed goats experienced an increment in rectal temperatures (0.65 to 1.25ºC) and respiratory rates (53 to 91 breaths/min), with the highest values recorded at 1700 h when the heat load was at its maximal level. This agrees with the results of Sivakumar et al. (2010), Hamzaoui et al. (2013), and Contreras-Jodar et al. (2018), where goats exposed to HS experience high rectal temperatures and respiratory rates. The increment in respiratory rate under HS conditions is a known mechanism for dissipating heat load by pulmonary evaporation. The supplementation with CAR had no effect on rectal temperature or respiratory rate throughout the day.

Table 4.3: Least squares means for respiratory rate and body temperature of dairy goats under thermoneutral (TN) and heat stress (HS) conditions. In each ambient temperature, goats were fed a control diet (CON) or supplemented with 1g L-carnitine (CAR).

Item	TN		HS		SEM	Effect[1] (*P* <)		
	CON	CAR	CON	CAR		T	C	T × C
Rectal temperature, ºC								
0800 h	38.5	38.5	39.1	39.2	0.05	0.001	0.290	0.146
1200 h	38.6	38.5	39.7	39.7	0.05	0.001	0.520	0.863
1700 h	38.7	38.6	39.9	39.9	0.05	0.001	0.222	0.648
Average	38.6	38.5	39.6	39.6	0.04	0.001	0.762	0.464
Respiratory rate, breaths/min								
0800 h	36	35	88	88	3.0	0.001	0.939	0.933
1200 h	36	36	126	121	3.0	0.001	0.455	0.282
1700 h	40	39	133	127	3.0	0.001	0.247	0.144
Average	37	37	116	112	2.4	0.001	0.430	0.542

[1]Effects of temperature (T), CAR supplementation (C), and their interaction (T × C). [a-c] Values within the same column for each parameter with different superscripts differ (P < 0.05).

4.4.3. Feed Intake and Feed Sorting

Average DM intake decreased in HS animals by 26% throughout the experimental period (1.90 ± 0.10 kg/d vs. 2.58 ± 0.10 kg/d; *P* < 0.001; Table 4.4). Goats in the current study were in mid lactation and the reduction of DM intake by HS was greater than that previously observed in HS dairy goats during late lactation (Hamzaoui et al., 2013). On the other hand, CAR supplementation did not affect DM intake, which is in accordance with the results found in cows supplemented with 0.046 or 0.382 g L-carnitine /kg $BW^{0.75}$ (Carlson et al., 2007). However, when cows were fed a high dose of L-carnitine (0.763 g/kg $BW^{0.75}$), DM intake decreased, plausibly because of increased hepatic ATP production (Carlson et al., 2007).

Heat-stressed goats tended (*P* < 0.06) to eat longer particle size compared to TN goats, as the average particle length of their orts decreased by 27% (6.2 ± 0.64 mm vs. 4.5 ± 0.64 mm for TN and HS, respectively; Table 4.4). Feed sorting against short particles (that contain greater energy content than longer particles) in addition to the fact that feed intake is reduced by HS would exacerbate challenges associated with reduced energy intake. Castro-Costa et al. (2015) reported that HS decreases rumen pH in dairy goats eating the same amount of food as TN goats.

Although we did not measure rumen pH in the current study, we speculate that our HS goats ate longer particles to manage possible low pH in the rumen. Additional work is needed to explore the relationship between rumen pH and feed sorting behavior under HS conditions. The increased feed sorting for long particles (presumably forage particles) is in contrast to the common nutritional practice of reducing ration forage content during HS (Salama et al., 2016). No effect of CAR supplementation on the orts particle size was observed.

4.4.4. Milk Yield and Composition

As shown in Table 4.4, milk yield tended ($P < 0.06$) to decrease by 11% in goats exposed to HS (1.63 ± 0.09 kg/d) compared to TN goats (1.84 ± 0.09 kg/d). This reduction was similar to what observed by Hamzaoui et al. (2014) in HSed dairy goats at mid lactation. The consequences of high ambient temperature in lactating dairy ruminants are well known (Baumgards and Rhoads, 2013; Salama et al., 2016, West, 2003), which include increased body temperature, reduced DM intake, and consequently, altered milk yield and composition. Milk composition was also affected by high ambient temperature (Table 4.4); the HS goats producing milk with lower fat ($P < 0.08$), protein ($P < 0.05$), and lactose ($P < 0.01$) contents than TN goats.

We hypothesized that carnitine synthesis is reduced by the potential inflammation induced by HS, and that CAR supplementation would cover the shortage in the production of endogenous carnitine. Additionally, if the HSed goats mobilize body fat due to the reduced feed intake, supplemented CAR would improve the oxidation of mobilized fatty acids. This could improve the efficiency of energy use and reduce the adverse effect of HS on milk production. However, CAR supplementation did not affect milk yield or milk composition (Table 4), indicating that CAR supplementation has no beneficial effects on milk production of HSed goats. Similarly, LaCount et al. (1995) found no effect of CAR supplementation on milk production of non-heat-stressed dairy cows. Nevertheless, feed-restricted dairy cows (with high blood NEFA levels) produced greater 3.5% fat corrected milk when supplemented with abomasum infused L-carnitine (Carlson et al., 2006). Furthermore, dairy cows supplemented with rumen-protected L-carnitine, from one week before calving to four weeks after parturition, produced similar milk yield to un-

supplemented cows, but their milk contained greater fat and protein (Pirestani and Aghakhani, 2018). These studies indicate that carnitine supplementation may be required during the situations of DM intake depression that cause body fat mobilization and elevated blood NEFA (e.g., transition period or feed restriction). Although HS in the current study reduced feed intake by 26%, HS and TN goats had similar blood NEFA levels (see the blood metabolites section), which may explain why CAR did not improve the performance of HSed goats.

Table 4.4: Least squares means for feed intake, average particle size of the orts, and milk production of dairy goats under thermo-neutral (TN) or heat stress (HS) conditions. In each ambient temperature, goats were fed a control diet (CON) or supplemented with 1g L-carnitine (CAR).

Item	TN		HS		SEM	Effect[1] (*P* <)		
	CON	CAR	CON	CAR		T	C	T × C
DMI, kg/d	2.60	2.56	1.85	1.95	0.16	0.007	0.976	0.859
Orts particle size, mm	5.31	7.92	3.99	5.02	1.10	0.057	0.238	0.674
Milk yield, kg/d	1.90	1.80	1.59	1.69	0.14	0.059	0.955	0.730
FCM, L/d[2]	2.28	2.15	1.81	1.90	0.19	0.028	0.880	0.765
Milk composition, %								
TS	8.89	8.91	8.37	8.46	0.19	0.005	0.926	0.961
Fat	4.33	4.21	4.02	3.96	0.20	0.076	0.729	0.984
Protein	3.51	3.54	3.14	3.22	0.18	0.049	0.951	0.989
Lactose	4.64	4.65	4.47	4.47	0.06	0.006	0.991	0.788
Fat yield, g/d	85.8	80.7	66.5	69.3	7.8	0.015	0.864	0.844
Protein yield, g/d	69.1	64.6	50.3	54.5	6.0	0.008	0.991	0.737
SCC, Log	5.97	6.00	6.30	6.22	0.23	0.276	0.873	0.842

[1]Effects of temperature (T), CAR supplementation (C), and their interaction (T × C). [2]Fat corrected milk at 3.5%; FCM = L × [0.432 + 0.162 × (fat %)], being L liters of milk yield.

4.4.5. Body Weight and Subcutaneous Fat Assessment

Changes in BW and subcutaneous (s.c.) fat were expressed as the difference between the values at the start and the end of each experimental period (*Figure 4.1*). On average, HSed goats lost 146 g/d of BW, whereas TN goats gained 139 g/d, agreeing with the results of Hamzaoui et al. (2013). A portion of the BW changes of TN and HS goats included the inevitable variations in the digestive tract content (reduced feed intake in HS), which were unknown in our data. Supplementation with L-carnitine did not affect the overall BW variation. This agrees with the results found in growing sheep in which L-carnitine do not affect average daily gain (Chapa et al., 2001).

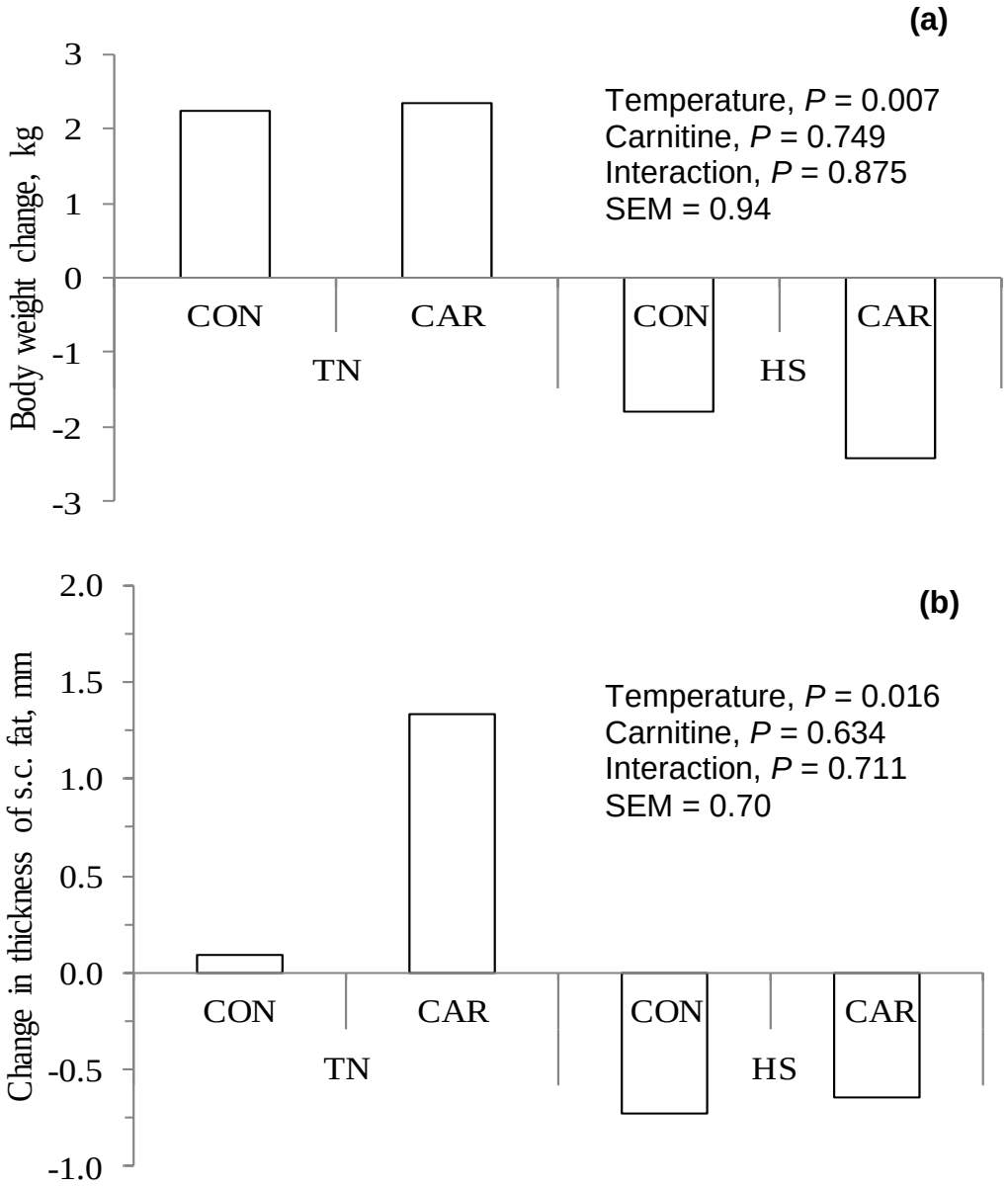

Figure 4.1. Changes in body weight (**a**) and subcutaneous fat thickness (**b**), measured as the difference between values at the start and the end of each experimental period in dairy goats under thermoneutral (**TN**) or heat stress (**HS**) conditions. In each ambient temperature, goats were fed a control diet (**CON**) or supplemented with 5 g CAR containing 1 g pure L-carnitine.

The values of ultrasound fat measurements (*Figure 4.1*) revealed that HSed goats had lower s.c. fat thickness compared to TN (−0.72 vs. +0.64 mm; $P < 0.05$). L-carnitine supplementation did not affect s.c. fat mobilization in accordance with

the results of Hajilou et al. (2014), who detected no changes in s.c. fat in finishing bulls supplemented with L-carnitine. In addition, BW variation and ultrasound measurements were positively correlated (r = 0.54), indicating that HSed goats in negative energy balance would mobilize body fat. Mendizabal et al. (2007) indicated that, for the processes of storage and mobilization of fat reserves in adult goats, the s.c. fat was particularly active and appears to be highly specialized for lipid accumulation and mobilization.

4.4.6. Blood Metabolites

Blood metabolites of TN and HSed goats with and without L-carnitine supplementation are shown in Table 4.5. L-carnitine supplementation did not affect (P > 0.10) blood urea or glucose levels. Previous reports revealed that carnitine supplementation increases insulin secretion and hepatic glucose output by incrementing the flux of metabolites through pyruvate carboxylase (Carlson et al., 2007; Owen et al., 2001). In agreement with this notion, Greenwood et al. (2001) observed that blood glucose levels increase by 3% in growing steers supplemented with carnitine. Nevertheless, blood glucose in our goats was not affected by CAR supplementation.

Supplementation with L-carnitine did not affect blood NEFA, BHB, cholesterol, or triglycerides concentrations (Table 4.5). Dairy cows in early lactation have decreased blood NEFA and cholesterol concentrations when supplemented with 10 g/d of protected carnitine (Scholz et al., 2014). In addition, Citil et al. (2009) reported that feed supplementation with L-carnitine decreases serum triglycerides and cholesterol in lactating ewes. Nevertheless, and agreeing with our findings, Carlson et al. (2006) reported that plasma NEFA is not altered in mid-lactation dairy cows infused with L-carnitine.

Table 4.5: Least squares means for blood metabolites in dairy goats under thermo-neutral (TN) or heat stress (HS) conditions. In each ambient temperature, goats were fed a control diet (CON) or supplemented with 1g L-carnitine (CAR).

Item	TN		HS		SEM	Effect[1] (*P* <)		
	CON	CAR	CON	CAR		T	C	T × C
Na, mmol/L	148.0	147.6	147.0	147.0	0.52	0.349	0.881	0.299
K, mmol/L	3.47	3.47	3.84	3.87	0.15	0.042	0.754	0.243
Ionized Ca, mmol/L	1.28	1.27	1.27	1.31	0.02	0.066	0.378	0.310
Cl, mmol/L	103.8	103.6	107.9	107.8	0.67	0.007	0.109	0.497
TCO_2, mmol/L	25.7	25.9	20.9	21.2	0.69	0.009	0.678	0.871
Anion gap	23.0	22.7	22.8	23.1	0.54	0.707	0.247	0.869
Hematocrit, % PCV	17.6	18.6	17.7	18.4	0.92	0.770	0.609	0.899
Hemoglobin, g/dL	5.98	6.31	6.05	6.26	0.31	0.778	0.599	0.891
Glucose, mg/dL	59.1	59.6	59.9	58.6	1.71	0.743	0.962	0.733
Urea, mg/dL	23.2	24.5	18.3	19.8	1.85	0.008	0.710	0.971
Creatinine, mg/dL	0.47	0.52	0.57	0.54	0.02	0.007	0.906	0.211
Triglycerides, mg/dL	17.6	18.0	17.1	17.1	1.3	0.878	0.660	0.501
Cholesterol, mg/dL	74.7	76.2	85.5	79.9	5.8	0.219	0.911	0.733
NEFA/mmol/L	0.08	0.13	0.15	0.12	0.02	0.291	0.333	0.276
β-HB, mmol/L	0.72	0.70	0.80	0.85	0.11	0.187	0.972	0.932

[1]Effects of temperature (T), CAR supplementation (C), and their interaction (T × C).

The decrease ($P < 0.01$) in blood urea concentration by HS could be explained by their lower DM intake and, consequently, reduced N intake. Despite the reduced feed intake (Table 4.4), HSed goats were able to keep similar glucose levels to TN goats. Creatinine levels increased ($P < 0.01$) with HS, which might indicate increased muscle degradation. It is possible that some glucogenic AA produced from muscle degradation were used for gluconeogenesis, resulting in keeping similar blood glucose levels. Additionally, Salama et al. (2014) reported that HSed goats secrete lower insulin when glucose is infused compared to TN goats, which might be an adaptation to HS and could explain unaltered glucose levels in HS goats.

Values of total blood CO_2 were lower in HS compared to TN goats, which agrees with previous studies (Hamzaoui et al., 2013; Sivakumar et al., 2010). The greater respiratory rate observed in HSed goats (Table 4.3) contributed to washing off of CO_2, and consequently a lower concentration of CO_2 in blood. The Cl^- and K^+ blood concentrations were greater ($P < 0.05$) in HS than in TN goats. The blood ionized Ca concentration also tended ($P < 0.10$) to be greater in HS (1.29 mmol/L) compared

to TN goats (1.26 mmol/L). Similarly, Srikandakumar and Johnson (2004) reported greater concentrations of K, Cl, and Ca in blood of heat-stressed cows. Collier et al. (2006) reported that K requirements in HSed dairy cows increase by as much as 12% because sweat is high in K. In the current study, blood K in HSed goats increased by 11% to meet K requirements despite the reduced mineral intake. However, our goats had free access to mineral-vitamin blocks, which might have allowed them to obtain minerals as needed.

As indicated above, s.c. fat thickness measured by ultrasonography was decreased by HS (*Figure 4.1*), indicating that goats would have mobilized body fat reserves. However, both blood NEFA and β-HB did not change by HS in the current study (Table 4.5) and previous studies done in dairy goats (Salama et al., 2014) and dairy cows (Baumgard and Rhoads, 2013). It is possible that there was some degree of body fat mobilization, but NEFA were rapidly taken up by the mammary gland for fat synthesis (milk fat was less affected by HS than milk protein as shown in Table 4.4) and were not transformed to ketone bodies by the liver. We detected no correlation between blood NEFA and β-HB in TN (r = 0.02; *P* = 0.953) or HS (r = 0.18; *P* = 0.478) goats.

Finally, and from the viewpoint of animal welfare, the level of HS used in the current study was moderate according to the established levels of HS in dairy goats (Silanikove and Koluman, 2015). Furthermore, we used the minimum possible number of goats (n = 4; Latin square four × four) and the shortest time for each period (16 days) to test the effects of L-carnitine. As stated previously, our hypothesis was solid and we expected positive effects of L-carnitine on HS goats, but we were not able to detect such effects. This will avoid unnecessary exposure of animals to HS in the future to test the effects of L-carnitine.

4.5. CONCLUSIONS

Heat stress negatively affected the lactational performance of dairy goats. Additionally, heat stress altered feeding behavior as heat-stressed goats tended to consume longer feed particles as an attempt to keep stable rumen pH. Supplementation of thermo-neutral and heat-stressed lactating dairy goats with rumen protected L-carnitine dramatically increased blood carnitine fractions (free-,

acetyl-, and total-carnitine). Despite the effective absorption of carnitine, no productive benefits or physiological changes were observed in dairy goats under thermo-neutral or heat stress conditions. Evidence was not obtained to support the hypothesis that carnitine supplementation is needed under heat stress conditions.

CHAPTER 5

Effects of rumen-protected methionine on physiological indicators and lactation performance of dairy goats under heat stress conditions

CHAPTER 5

5. EFFECTS OF RUMEN-PROTECTED METHIONINE ON PHYSIOLOGICAL INDICATORS AND LACTATION PERFORMANCE OF DAIRY GOATS UNDER HEAT STRESS CONDITIONS

5.1. ABSTRACT

Heat stress (**HS**) results in a marked decrease in DM intake (**DMI**), and hence less amino acids (**AA**) are available for milk. Methionine (Met) is one of the limiting AA in dairy animals, and when supplemented to HS mammary cell cultures, milk synthesis improves. The objective was to evaluate the response of HS dairy goats to feed supplementation with rumen-protected Met. Dairy goats (n = 8) were used in 4×4 Latin square (4 periods; 21 d each). Factors were: 1) thermal neutral (**TN**) (15 to 20°C), 2) HS (12 h/d at 35 ± 0.5°C and 12 h/d at 27.9 ± 0.07°C), 3) control diet (Con), and 4) diet supplemented with 2.6 g/d rumen-protected Met. Rectal temperature (**RT**) and respiratory rate (**RR**) were measured daily at 0800, 1200 and 1700 h. DMI, water consumption and milk yield were also daily recorded. Milk samples were collected weekly for composition and fatty acid profile. Blood samples were collected at day 17 of each period for the determination of blood metabolites. Data were analyzed by Proc Mixed of SAS for repeated measurements. Compared to TN, HS goats had greater RT (+1.01C), RR (+99 breaths/min) and water consumption (+1.93 L/d). HS × Met interaction ($P < 0.05$ to 0.10) was detected for RT and RR, where HS goats respired less (–5 to 6 breaths/min) at 0800 and 1200 h, and had lower RT (–0.11ºC) at 1700 h. By design, DMI was only 9.8% (albeit significant) less in HS than HS goats. At this comparable DMI, milk yield was similar between TN and HS goats. However, HS goats experienced reduced milk fat and protein contents, but there were no effects on milk component yields. Desaturation indices of milk fatty acids were reduced by HS. Blood insulin and BHB values were increased by HS, whereas NEFA decreased. Dietary Met did not affect metabolic or productive variables, except BW gain that increased by Met. In conclusion, dietary Met did not improve milk production performance. The observed apparent effect of Met on thermoregulation deserves more evaluation. Metabolic changes due to HS included greater blood β-HBA which may spare glucose. Probably more AA in addition to Met should be supplemented to improve performance in HS.

5.2. INTRODUCTION

Heat stress (**HS**) is a major challenge for dairy animals, and in dairy goats it reduces DM intake, decreases milk yield, and alters milk composition, especially milk protein content (Hamzaoui et al., 2013; Contreras-Jodar et al., 2018). Low protein content in milk produced from HS cows and goats is not only caused by reduced DM intake, but also is related to decreased amino acids (**AA**) available for protein synthesis because AA are likely used for other biochemical processes, such as gluconeogenesis and the synthesis of high amounts of heat shock proteins (Collier et al., 2008; Salama et al., 2014, 2016).

Several studies have been carried out in lactating goats to identify the limiting AA for milk production, and the most frequently reported AA are methionine (**Met**) and lysine (Flores et al., 2009). Met supplemented in a rumen-protected form is efficiently absorbed from the gastrointestinal tract, and results in improved milk yield, fat-corrected milk, and milk protein content in dairy goats (Flores et al., 2009), dairy ewes (Goulas et al., 2003) and dairy cows (Zhou et al., 2016). Met could improve the energy balance during lactation by 2 mechanisms: first as essential amino acid and integral part of proteins, Met preserves the lean mass, and second because of their functions related to carnitine synthesis and as methyl group donor, which could improve fat utilization at the cellular level (Pissios et al., 2013).

In stressful conditions when DM intake often decreases (e.g. periparturient period), Met supplementation improves lactation performance and reduces inflammation (Osorio et al., 2013; Batistel et al., 2017). Furthermore, supplementation of heat-stressed bovine mammary cells with Met increases the mRNA of genes related to heat shock proteins, milk synthesis transcription and translation, insulin signaling, AA transportation, and cell proliferation (Salama et al., 2019). In dairy cows under HS conditions, rumen-protected Met supplementation maintains milk protein and fat concentrations (Pate et al., 2020). To our knowledge, no studies were carried out to compare the response to Met supplementation under thermal neutral (**TN**) and HS conditions in dairy goats.

Our hypothesis was that goats supplemented with Met would improve lactational performance in heat stress conditions compared with goats without Met

supplementation. The objective was therefore to study whether supplementation of rumen protected Met to dairy goats under controlled HS conditions would affect physiological variables, plasma amino acid blood profile, milk yield, and milk composition.

5.3. MATERIALS AND METHODS

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). The study was conducted at the Autonomous University of Barcelona's Veterinary Faculty experimental farm, located at 41º30´20″N and 2º05′46″E (elevation, 162 m).

5.3.1. Animals, Treatments, and Management Conditions

Eight multiparous, mid-lactating Murciano-Granadina dairy goats (43.3 ± 1.5 kg BW; 89.6 ± 0.3 DIM and; 2.40 ± 0.06 L/d) with healthy and symmetrical udders from the herd of the experimental farm of the Universitat Autònoma de Barcelona were used. The design was 4 × 4 Latin square with 21-d periods and 7-d washout periods between each period. Goats were allocated to one of 4 treatments in 2 × 2 factorial arrangements. Factors were: 1) Diet: control without supplementation (**Con**), or supplementation with 2.6 g of rumen-protected Methionine (Met; Smartamine M, Adisseo NA, Alpharetta) and 2) Ambient conditions: thermo-neutral (TN; 15 to 20ºC; temperature humidity index (**THI**) = 59 to 65) or HS (from 0900 to 2100 h at 35ºC and from 2100 to 0900 h at 28ºC with 45 ± 5% relative humidity; THI = 84 and 75, during the day and night, respectively). This resulted in 4 treatment combinations: TN-Con, TN- Met, HS-Con, and HS-Met.

The THI values were calculated according to NRC (1971) as follows:

$$THI = (1.8 \times T_{db} + 32) - [(0.55 - 0.0055 \times RH) \times (1.8 \times T_{db} - 26.8)]$$

Where T_{db} is the dry bulb temperature (ºC) and RH is the relative humidity (%).

The THI values used in the current experiment for HS goats are classified as "alert" ($80 \leq THI < 85$) and "normal" ($THI < 80$) stress levels during the day and night, respectively (Silanikove and Kolumna, 2015). The photoperiod was maintained

constant 12–12 h (day, 0900 to 2100 h; night, 2100 to 0900 h) in both TN and HS conditions. Data of environmental temperature and humidity were recorded every 10 min by using 2 data loggers (Opus 10, Lufft, Fellbach, Germany).

Throughout the experiment, (late-December to early-May), the TN goats were kept indoors, and the temperature was maintained at 15 to 20ºC with the help of an electric heater equipped with a thermostat (3.5 kW; General Electric, Barcelona, Spain) when necessary. Temperature and relative humidity averaged 19.4 ± 0.5ºC and 58 ± 5% (THI = 63) for the TN goats. 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.

Goats had a 4 wk pre-experimental period under TN conditions for the adaptation to the diet and to the experimental conditions before applying the ambient conditions. When goats were switched from TN to HS conditions, the temperature increased in 2 steps (1 d at 25ºC and 1 d at 30ºC, 45 ± 5% humidity), but no transition was applied for the change from HS to TN.

Throughout the experiment, goats were milked twice daily (0800 and 1700 h), and yields were recorded for each milking. Milking was performed at a vacuum of 40 kPa, 120 pulses/min, and 50% pulsation ratio. The milking routine included manual cluster attachment, teat dipping with an iodine solution (P3-ioshield, Ecolab Hispano-Portuguesa, Barcelona, Spain) was done at the end of milking. Goats were individually fed a total mixed ration (**TMR**) once daily after the morning milking (0900 h), and orts were recorded daily. Diet ingredients are shown in Table 5.1. Feed offer was limited to 2.0 kg DM for both TN and HS goats. This DM amount was calculated to cover nutrient requirements of the expected fat corrected milk (**FCM**) of HS goats according to INRA (2018).

Table 5.1: Ingredients, chemical composition, and nutritive value (DM basis) of the total mixed ration offered to Murciano-Granadina dairy goats under HS and TN conditions.

Item	Value
Ingredients (% as fed)	
Alfalfa hay	60.0
Concentrate	40.0
Cracked oat grain	5.0
Cracked corn grain	4.0
Brewing barley	10.0
Soybean hull	45.0
Soybean meal, 44%	5.0
Rapeseed meal	10.0
Corn gluten feed	10.0
Soybean oil	5.0
Cane molasse	2.0
Salt (NaCl)	0.5
Vitamins minerals for goats[1]	1.0
Bicalcique phosphate	2.5
Chemical composition (% of DM)	
Dry matter	86.5
Organic Matter	86.4
Crude protein	15.9
Ether extract	2.56
Neutral detergent fiber	27.7
Acid detergent fiber	19.6
Acid detergent lignin	3.00
Ash	0.08
Nutrient supply[2]	
UE_L[3], /kg	1.00
UFL[4], /kg	0.89
NE_L, Mcal/kg	1.57
PDI[5], g/kg	89.4
PDIA[6], g/kg	41.0
RPB[7], g/kg	28.6
Ca_{abs}, g/kg	5.38
P_{abs}, g/kg	3.04

[1] Calculated according to Institut National de la Recherche Agronomique (INRA, 2018). [2] Feed units for lactation. [3] Net energy for lactation (1 UFL = 1.7 Mcal of NEL). [4] Protein digested in the small intestine from food and microbial synthesis origins. [5] Protein digested in the small intestine supplied by food rumen undegradable protein. [6] Rumen protein balance, represents RUP, microbial protein and endogenous protein.

Our previous experiments on the same goat breed under similar experimental conditions (Mehaba et al., 2019; Salama et al., 2020) indicated expected FCM of 1.9 to 2.2 kg under HS conditions. The reason why we limited the feed offer to only 2.0 kg DM was to reduce differences in DM intake as much as possible between TN and HS goats.

This allowed us to test the effects of Met supplementation under different ambient temperature, but with comparable feed intake levels (avoid differences in the amounts of AA supplemented from the basal diet). Clean water was permanently available at ambient temperature. Mineral and vitamin blocks were freely available for each goat (Ovi bloc, Sal Cupido, Barcelona, Spain). The two environmental conditions began on the first morning of the first day of each period and was stopped on the next morning after the last feeding on the last day of the second period.

Smartamine contains 80% DL-Met, physically protected by a pH-sensitive coating, which is considered to have a methionine bioavailability of 80 % (Schwab, 2011), and 85 % rumen protection measured in situ at 3 h (Mbanzamihigo et al., 1997). Thus, every 2.6 g of Smartamine supplemented in the current experiment provide the goat with 2 g methionine. Met doses were daily weighed by an electronic scale (Sartorius ED224S, Sartorius, AG, Germany), mixed with 50 g crushed corn, and offered individually before the morning milking. Con goats were fed 50 g crushed corn without Met supplementation. The dose of Met used in the current experiment (0.10% of DM) is slightly higher than that used in previous studies demonstrating beneficial effects of Met on production performance and health (i.e. 0.09%) in dairy cows (Zhou et al., 2016) and goats (Alonso-Mélendez et al., 2016).

5.3.2. Measurements, Sampling, and Analyses

Body Temperature and Respiration Rate. Rectal temperature and respiration rate were recorded three times daily (0800, 1200, and 1700 h). Respiration rate were determined by counting flank movements for 12 s and multiplying by 5. Rectal temperatures were measured using a standard digital thermometer (model Accu-vet, ST714AC, Tecnovet S.L, Barcelona, Spain, reading range, 32.0 – 42.0ºC; and accuracy ±0.10ºC).

Milk Yield and Composition. Milk yield of individual goats was weighed at each milking using an electronic scale (Mobba Barcelona). Milk samples from each goat (from both the morning and evening milking) were collected twice per week for two consecutive days during each period. Milk samples were stored at 4ºC with a preservative (Bronopol, Broad Spectrum Micro-tabs II, D&F Control Systems, San Ramon, CA) until analysis. Samples were analyzed for fat, total protein (N × 6.38), lactose, solid no-fat, milk urea nitrogen and SCC in the Dairy Herd Improvement Laboratory of Catalonia (ALLIC, Cabrils, Barcelona, Spain) using an automatic cell counter (Fossomatic 5000, Foss Electric, Hillerød, Denmark).

Milk fatty acids profile. An additional milk samples were taken each week to analyze fatty acids (**FA**) profile. Milk was immediately cooled, and the fat fraction was separated by centrifugation for 30 min at 6,000 × g and 4ºC, and then stored at −20ºC. Milk FA were analyzed after extraction of milk fat samples and methylation (Palmquist and Jenkins, 2003) to avoid migration of conjugated double bonds of unsaturated FA. Briefly, milk fat samples (60 to 70 mg) were dissolved in 1 mL of benzene, and alkaline transesterification was completed by using 2 mL of 0.5 M sodium methoxide in methanol (10 min at 50ºC). A second methylation was done with 3 mL of 100 mL/L of methanolic HCl (10 min at 80ºC). After addition of 1 mL of heptane and 7.5 mL of 60 g/L of K_2CO_3 and centrifugation, the top solvent layers were transferred to a tube, 1 g of Na^2SO_4 was added, and the samples were centrifuged at 6,000 × *g* and 4ºC. The clear layers containing the FA methyl esters (**FAME**) were transferred to 1 mL autosampler vials and sent to be analyzed at Servei d'Anàlisi Quimic of the UAB. Separation and quantification of the FAME was carried out by using a gas chromatograph (HP 6890, Agilent Technologies, Palo Alto, CA) equipped with a flame-ionization detector and capillary column (DB23, 60 m × 0.25 mm i.d. with a 0.25 µm capillary thickness; Agilent Technologies., Santa Clara, USA). The initial temperature of 70ºC (for 1 min) was increased to 225ºC (for 15 min) at a rate of 1ºC/min. Individual FA were identified by comparison of retention times with those of pure standards (Supleco 37 FAME, Sigma-Aldrich Química, Madrid, Spain) and expressed as percentages of the total FA detected as FAME.

Feed and Water Intake. Feed intake and water intake of each dairy goats was determined (accuracy: ± 20 g) daily throughout the experiment. Saw dust trays were

put below the water troughs and weighted twice daily to consider water wastes. Feed samples were collected before the beginning of each experimental period and were ground through a 1 mm stainless steel screen, and then analyzed for DM, ADF, NDF, and ash content according to analytical standard methods (AOAC International, 2003). The Dumas method (AOAC International, 2003) with a Leco analyzer (Leco Corp., St. Joseph, MI) was used for N determinations and CP was calculated as percentage of N × 6.25. The chemical composition and nutritive value of ration are shown in Table 5.1. Furthermore, to calculate energy balance, in UFL/d (feed units for lactation, 1 UFL = 1.7 Mcal of NEL) maintenance requirements were increased by 25% during the HS conditions as recommended (NRC, 2001).

Body Weight. Before the beginning of the experiment, all goats were weighed for two consecutive days to establish the starting Body weight (**BW**). During each experimental period, goats were weighed weekly, and for two consecutive days. Weighing was performed using an electronic scale (Tru-test A6500, Auckland, New Zealand).

Blood Measures. At d 17 of each period, 2 blood samples (10 mL each) were collected from the jugular vein using heparinized (170 IU) or spray-coated K2-EDTA Vacutainer tubes (BD, Belliver Industrial Estate, Plymouth, UK) before the morning milking and feeding. Blood samples remained on ice until plasma was harvested following centrifugation at 2,000 × *g* for 15 min at 4ºC and plasma transferred to 0.5 mL Eppendorf tubes and stored at −20ºC for non-esterified fatty acids (**NEFA**), β-hydroxybutyrate (**β-HBA**), triglycerides cholesterol, and albumin analyses. NEFA was analyzed by the ACS-ACOD colorimetric enzymatic test method (Wako Chemicals, Neuss, Germany). The β-HBA was determined by kinetic enzymatic method using commercial kit (Ranbut, Randox, UK). Cholesterol was analyzed by the enzymatic method (cholesterol esterase/peroxidase), whereas triglycerides were analyzed with enzymatic method (glycerol phosphate oxidase) using Olympus AU400 analyzer (Olympus Europa Holding GmbH, Hamburg, Germany). Insulin was determined by ELISA sandwich type (Ovine Insulin, Mercodia, Uppsala, Sweden). The stopped ELISA plates were read in an automatic reader (iEMS Reader MF V.2.9–0, Labsystems España, Barcelona, Spain) at 450 nm.

Additional blood samples (approximately 0.3 mL) were collected using insulin syringes (1 mL; BD Micro-Fine; BD Medical-Diabetes Care, Franklin Lakes, NJ) and immediately analyzed for major ions and metabolites. A single drop of blood was applied into disposable cartridges containing biochemical and silicon chip technology (i-STAT CHEM8+; Abbott Point of Care Inc., Princeton, NJ). Then, the cartridge was inserted into an i-STAT hand-held analyzer, and the results of glucose, urea, creatinine, hematocrit, hemoglobin, Cl^-, Na^+, K^+, Ca ions, total CO_2 concentration, and anion gap were obtained.

Plasma amino acids profile. Plasma AAs were measured by HPLC following a modified protocol (Castellanos et al., 2016). Shortly, at the day of assay, 100 µL of thawed serum were transferred to a 1.5 mL micro-centrifuge tube and 10 µL of 100 mM DTT prepared in milliQ water were added. The solution was vortexed for 30 s and an equivalent volume of 10% sulfosalicylic acid (110 µL, filtered at 0.22 µm, and with GABA at 125 µM as internal standard) was added to precipitate the proteins, and immediately followed by mixing for 2 min. The samples were centrifuged at 14,000 × g for 10 min at 4ºC and the supernatant was collected (Sharma et al., 2014). Pure AAs were used as standards and GABA was used as internal control (Sigma, St. Louis, MO, USA). Standards were prepared at 1 mM stock solutions in 0.1 M HCl and was serially diluted from 500 µM to 7.81 µM to perform the standard curve. For derivatization, the AccQ Fluor (Waters, Milford, MA, USA) method was used following the instructions provided by the manufacturer. A total of 10 µL standard solution or supernatant of deproteinized serum sample was transferred into a 1.5 mL micro-centrifuge tube, buffered with 70 µL Waters AccQFluor Borate Buffer, derivatized by addition of 20 µL of AccQ-Fluor reagent, vortexed, and heated for 10 min at 55ºC. Samples were kept in refrigerator and vortexed prior analysis.

HPLC was performed using an Elite LaCHrom (Hitachi, Tokyo, Japan) equipped with an UV detector (Hitachi L-24200, Tokyo, Japan) with a Novapak C18 column (300 mm × 3.9 mm) from Waters. The flow rate was 1.0 mL/min and the column temperature was kept at 38ºC. The injection volume was 10 µL and the detection wavelength was set at 254 nm. The solvent system consisted of two eluents: (A) AccQ >Tag eluent A concentrate (10%, v/v) and water (90%, v/v) and (B) 60% acetonitrile/40% water. The following gradient elution was used: 0 to 0.50 min, 100%

A; 0.5 min, 98% A to 2% B; 20 min, 93% A to 7% B; 32 min, 91.5% A to 8.5% B; 40 min, 82% A to 18% B; 47.5 min, 79% A to 21% B; 55 min, 60% A to 40% B; 60 min, 100% B. EZChrom Elite system V3.1.7 software (Agilent, Santa Clara, CA, USA) was used for system control and data acquisition.

5.3.3. Statistical Analyses

Data were analyzed by the MIXED procedure for repeated measurements of SAS v. 9.4 (SAS Institute Inc., Cary, NC). The statistical mixed model contained the environmental conditions (TN, HS), diet (Con, Met), period (1, 2, 3, 4), the sampling time, and the environmental conditions × period, environmental conditions × sampling time, period × sampling time interactions as fixed effects, as well as the random effects of the animal and the residual error. The model considered the possible carryover effects of previous HS periods through the temperature × period interaction. For the data of rectal temperature and respiratory rate measured at 0900h, 1200h and 1700 h, a fixed factor of the hour of day was added to the model. Data of performances (i.e., intake, water, and milk yield) and physiological indicators (i.e., rectal temperature and respiratory rate) were analyzed on a weekly basis. Differences between least squares means were determined with the PDIFF test of SAS. Significance was declared at $P < 0.05$ and tendency at $P < 0.10$ unless otherwise indicated. Results were presented as LS means ± SEM and their differences separated by the PDIFF option of SAS.

5.4. RESULTS AND DISCUSSION

5.4.1. Body temperature and respiration rate

Body temperature and respiration rate data are shown in Table 5.2. Overall average of body temperature was +1.03ºC greater ($P < 0.001$) in HS compared to TN goats. Furthermore, in both temperature conditions, body temperature increased throughout the day, being the morning measurement the lowest then increasing to reach the highest values at the evening. These results are in accordance with the findings in sheep (Marai et al., 1997b) and dairy goats (Hamzaoui et al., 2013). For respiration rate, HS goats had 411% increase compared to TN goats on average, corresponding to 100 breaths/min. Met supplementation decreased ($P = 0.03$) respiration rate at 0800 h in HS conditions.

Table 5.2: Body temperature and respiration rate of dairy goats under thermo-neutral (TN) or heat stress (HS) conditions. In each ambient temperature, goats were fed a control diet (Con) or supplemented with rumen protected methionine (Met). Values are least squares means and SE of the means (SEM).

Item	TN		HS		SEM	Effect[1] (*P-value*)		
	Con	Met	Con	Met		T	Diet	T × D
Body temperature, ºC								
0800h[2]	38.44[c]	38.43[c]	39.27[c]	39.21[c]	0.04	0.001	0.413	0.286
1200h[3]	38.59[b]	38.60[b]	39.72[b]	39.63[b]	0.04	0.001	0.284	0.116
1700h[3]	38.78[a]	38.78[a]	40.01[a]	39.90[a]	0.04	0.001	0.157	0.054
Average	38.60	38.60	39.67	39.58	0.04	0.001	0.193	0.212
Respiration Rate, Breaths/min								
0800h	28[b]	28[b]	98[c]	92[c]	2	0.001	0.098	0.030
1200h	32[a]	32[a]	140[b]	135[b]	2	0.001	0.209	0.074
1700h	35[a]	36[a]	162[a]	161[a]	2	0.001	0.948	0.830
Average	32	32	133	130	2	0.001	0.248	0.234

[a, b, c] Values within a column with different superscripts differ significantly ($P < 0.05$). [1] Effects of temperature (T), Met supplementation (D), and their interaction (T × D). [2] In HS conditions before changing from night (28 °C and 45% humidity; THI = 75) until day (35°C and 45% humidity; THI = 83) conditions. [3]During the day conditions (35°C and 45% humidity; THI = 83 in HS; and 15-20°C and 45% humidity; THI = 59 to 65 in TN conditions).

Additionally, there was temperature × Met interaction at 0800 ($P < 0.05$) and 1200 h ($P = 0.074$), where respiration rate was not affected by Met supplementation under TN conditions, whereas the increment under HS conditions was lower in Met-supplemented goats at 0800 (–6 breaths/min) and 1200 h (–5 breaths/min). Thus, Met-supplemented goats needed fewer breaths/min to keep similar rectal temperature at 0800 and 1200 h or even less temperature at 1700 h (HS × Met; $P = 0.054$). It seems that Met supplementation serves to control body temperature after several hours of exposure to high temperatures. The biological relevance of these results must be considered with caution. In contrast to our findings, Kassube et al. (2017) observed increased body temperatures and respiration rates due to the intravenous infusion of a mixture of Met, Lys, and branched chain amino acids (**BCAA**) to HS dairy cows.

5.4.2. Feed intake and body weight change

Although we aimed at keeping similar levels of feed intake, HS depressed DMI (Table 5.3) by 0.2 kg/d (–9.8 %; $P < 0.05$) compared to TN goats. However, this

decrease in DMI due to HS is much lower than the 21 to 28% DMI losses reported in the same goat breed under similar HS conditions compared to TN goats fed ad libitum (Hamzaoui et al., 2013; Mehaba et al., 2019; Salama et al., 2020). Water consumption, on the other hand, increased (+48%; $P < 0.01$) in HS compared to TN goats to dissipate heat by evaporation (sweating and panting).

Met supplementation had no effects on DMI or water consumption. Effects of Met supplementation on DMI are inconsistent. Robinson et al. (2000) reported a significant decrease in DMI when 16 g/d methionine was infused abomasally in cows fed a corn and timothy silage diet under TN conditions. However, Varvikko et al. (1999) did not observe any effects on DMI in response to an abomasal infusion of 40 g/d methionine when cows were fed a grass silage diet. Similarly, Baldwin et al. (1993), in experiments with lambs and ewes, demonstrated that neither lactating ewes nor lambs responded to 0.2% of DM rumen protected Met supplementation in diets. Similar to our results, Rodriguez-Guerrero et al. (2018) observed no effects of Met supplementation on DMI or digestibility in growing lambs. Furthermore, Madsen et al. (2005) reported that supplementation with RP lysine (**Lys**) or Met had no effect on feed intake in early or late lactating goats under TN conditions.

Table 5.3: Productive variables of dairy goats under thermo-neutral (TN) or heat stress (HS) conditions. In each ambient temperature, goats were fed a control diet (Con) or supplemented with rumen protected methionine (Met). Values are least squares means and SE of the means (SEM).

Item	TN		HS		SEM	Effect[1] (*P-value*)		
	Con	Met	Con	Met		T	Diet	T × D
DMI, kg/d	2.07	2.00	1.80	1.88	0.08	0.024	0.977	0.338
Water intake, kg/d	4.69	4.41	6.32	6.74	0.54	0.002	0.898	0.519
BW change, kg/21d	0.10	0.29	-0.37	0.04	0.17	0.049	0.099	0.512
Milk yield, kg/d	2.03	1.98	2.04	2.17	0.16	0.539	0.789	0.560
FCM[2], kg/d	2.43	2.46	2.21	2.39	0.09	0.137	0.260	0.422
EBAL[3], UFL/d	0.19	0.14	–0.13	–0.14	0.05	0.001	0.374	0.522
Milk composition								
Fat, %	4.90	4.97	4.11	4.36	0.11	0.001	0.152	0.426
Protein, %	3.60	3.60	3.23	3.25	0.11	0.003	0.887	0.920
Lactose, %	4.47	4.43	4.33	4.35	0.05	0.052	0.804	0.908
MUN[4], mg/L	111	112	118	120	7	0.318	0.841	0.965
SCC[5], log	5.54	5.71	5.75	5.82	0.19	0.410	0.559	0.788
Fat/Protein ratio	1.37	1.38	1.28	1.35	0.03	0.061	0.194	0.367
Components yield								
Fat yield, g/d	99.9	102.4	83.2	94.2	6.6	0.074	0.318	0.525
Protein yield, g/d	73.2	73.3	65.2	69.9	4.9	0.255	0.629	0.646
Lactose yield, g/d	91.4	89.7	88.2	94.6	6.4	0.896	0.720	0.537

[1] Effects of temperature (T), Met supplementation (D), and their interaction (T × D). [2]FCM = Fat corrected milk at 3.5% = kg of milk yield × [0.432 + 0.162 × (fat%)]. [3]EBAL = Energy balance. [4]MUN = milk urea nitrogen. [5]SCC = Somatic cell count.

Met-supplemented goats tended to have greater ($P < 0.10$) greater BW gain. In fact, HS goats lost 370 g in the 21-d experimental period, and this loss disappeared when Met was supplemented (Table 5.3). Dietary Met also resulted in more BW gain under TN conditions, but to lower extent compared to HS treatment. The improvement of BW in Met-supplemented goats might be explained by the well documented positive effect of Met on overall performance during the stressful situations (e.g. transition period in dairy cows). These positive effects include improvements in liver function, and reduced inflammation and oxidative stress (Osorio et al., 2013; Zhou et al, 2016; Batistel et al., 2017). In dairy goats, HS has been shown to increase cell oxidative stress and inflammation (Contreras-Jodar et al., 2018), and dietary Met might alleviate these effects. The difference in BW gain (5 vs. –18 g/d for TN-Con and HS-Con goats, respectively) was significant, but less than differences between TN and HS reported in previous dairy goat studies

(Hamzaoui et al., 2013; Mehaba et al., 2019) due to the fact that DMI of TN goats in the current study was limited. Heat stress also causes BW losses in ewes (Caroprese et al., 2014) and cows (Baumgard and Rhoads, 2013).

5.4.3. Milk yield and milk composition

Heat stress did not affect milk yield or fat corrected milk compared to TN (Table 5.3). Previous studies comparing TN and HS goats fed ad libitum in a similar stage of lactation showed 11 to 20% losses in milk yield due to HS (Mehaba et al., 2019; Salama et al., 2020). In the current study, however, our TN goats ate only 0.2 kg DM more (albeit significant), which could explain similar milk yield. Historically, reduced feed intake has been considered as the main reason for decreased milk production in HS conditions. However, studies in dairy cows (Rhoads et al., 2009; Wheelock et al., 2010) used the pair-feeding model (both TN and HS animal eating similar but not identical DM) to avoid the confusion between HS effects and feed intake level. Rhoads et al. (2009) reported a 40 and 21% reduction in milk yield for cows in HS and TN-pair fed, respectively. Wheelock et al. (2010) also reported a 28 and 14% reduction in milk yield for HS and TN-pair fed, respectively, which means that the reduction of feed intake only accounts for half of the heat-stressed induced losses in milk production. Nevertheless, in the current study no milk yield losses were detected when DMI level was comparable, which might indicate that greater losses detected in the studies of goats mentioned previously (Mehaba et al., 2019; Salama et al., 2020) would be mainly due to the reduced feed intake (26 to 28%). The calculated energy balance values in HS goats were slightly negative, whereas TN goats were in a slightly positive energy balance. Thus, the ingested energy by HS goats together with the slight BW loss (18 g/d in HS without Met) seemed to be enough to cover milk yield requirements, although 25% extra maintenance energy requirements (NRC, 2001) were considered for the energy balance calculation under HS conditions. It should be kept in mind that energy balance values are an estimation because nutritive values of the diet in Table 5.1 (UFL, PDI, PDIA, etc...) are calculated from INRA (2018) tables.

Heat stress decreased ($P < 0.01$) milk fat and protein contents and tended to decrease ($P = 0.052$) lactose content (Table 5.3). Consequently, milk fat yield tended to decrease ($P < 0.10$) and milk protein yield decreased numerically ($P =$

0.255) by HS. The negative effect of HS on milk composition has been reported in dairy goats (Hamzaoui et al., 2013; Contreras-Jodar et al., 2018; Mehaba et al., 2019), ewes (Caroprese et al., 2014) and cows (Baumgard and Rhoads, 2013). Heat stress has a direct negative effect on synthesis of milk fat and protein, mediated in part by coordinated changes in mRNA, microRNA, and protein abundance of key networks (Salama et al., 2019).

Met supplementation had no effects on milk yield or milk composition, except milk fat that was numerically increased (P = 0.152) in TN (+0.07 points) and HS (+0.25 points) conditions (Table 5.3). Batistel et al. (2017) reported increased milk fat content and milk fat yield for cows supplemented with Met compared with control cows during the stressful periparturient period. Similar to our results, rumen-protected Met supplementation had no effect on milk yield or milk components in non-heat-stressed goats (Al-Qaisi and Titi, 2014; Policak-Milas et al., 2007). Nevertheless, Flores et al. (2009) found that milk yield and milk protein content were increased when 2.5 g/d of rumen protected Met with 85% of methionine content was supplemented to lactating goats. In addition, Madsen et al. (2005) showed that supplementing rumen protected Met had a positive effect on milk yield in early lactation goats when methionine and lysine were given in combination. Moreover, Antongiovanni et al., (2002) reported an increase in milk production and fat corrected milk in supplemented 5 g/d rumen protected Met with 95% of methionine content to dairy sheep. In dairy cows, supplementation with Met significantly improves milk protein percentage and yield in dairy cows (Zhou et al., 2016). Rulquin et al. (2006) reported an increase in milk protein yield (+41 g/d) when Smartamine (13.3 g of Met equivalent/d) is supplemented to dairy cows.

5.4.4. Milk fatty acid (FA) profile

As shown in Table 5.4, rumen protected Met supplementation did not affect individual FA in both environmental conditions. Because of the low rumen degradability of the supplemented Met, changes in rumen fermentation (FA biohydrogenation or microbial growth) that would impact milk FA profile are not expected. Similarly, Pate et al. (2020) reported that Met supplementation has no effect on the percentage of de novo or performed FA in milk of TN or HS cows.

Heat stress, on the other hand, decreased C12:0 ($P < 0.05$), C14:0 ($P = 0.162$), C16:0 ($P = 0.185$), and C16:1 ($P < 0.01$) that are totally or partially originated from de novo synthesis. De novo FA are synthesized from acetate and butyrate produced from feed fermentation in the rumen. Thus, HS-induced decrease of these de novo FA may be partially explained by the reduced feed intake (Table 5.3), and consequently suppressed ruminal fermentation with less supply of acetate and butyrate needed for milk fat synthesis. However, the level of DMI in TN goats was only 200 g over that of HS goats, which might not be enough to induce marked changes in rumen fermentation related to the level of intake. Alternatively, high ambient temperature could directly affect rumen microbes and fermentation (discussed later). Besides, heat stress has been reported to reduce the mammary cell synthetic capacity of milk components, including milk fat (Salama et al., 2019). All together makes it logical to have reduced milk de novo FA synthesized by the mammary cells under HS conditions. On the other hand, some of the preformed FA taken up by the mammary gland from food or body tissues (C18, C20 and C24) were increased ($P < 0.05$) by HS. Similar to our findings, Hammami et al. (2015) applied correlation analysis between THI values and milk FA profile and reported that the increment in THI is associated with a decrease in milk short- and medium-chain FA, and an increase in long-chain FA.

It is interesting to mention that the ratios of C14:1 to C14:0, C16:1 to C16:0 and C18:1 to C18:0 were lower ($P < 0.05$) in HS compared to TN goats, regardless Met supplementation (Table 5.4). This implies that the activity of Δ9-desaturase decreased by heat stress. This disagrees with the findings of Liu et al. (2017) who reported no change in the desaturation activity by HS in dairy cows.

Odd FA (C11:0, C13:0, C15:0 and C17:0) are predominantly originated from rumen micro-organisms lipids in addition to small amounts from de novo synthesis from propionate in mammary cells (Vlaeminck et al., 2006). In the current study, the concentration of C11:0 ($P < 0.05$), C13:0 ($P < 0.05$), C15:0 ($P = 0.261$) were reduced by HS, but C17:0 ($P < 0.01$) increased (Table 5.4). Similar to our results, triacylglycerol groups containing a FA with odd number of carbons show a significant reduction in heat-stressed dairy cows (Liu et al., 2017).

Table 5.4: Milk fatty acids (g/100 g of milk fat) of dairy goats under thermo-neutral (TN) or heat stress (HS) conditions. In each ambient temperature, goats were fed a control diet (Con) or supplemented with rumen protected methionine (Met). Values are least squares means and SE of the means (SEM).

Item	TN		HS		SEM	Effect[1] (*P-value*)		
	Con	Met	Con	Met		T	Diet	T × D
C8:0	2.91	2.82	2.92	2.92	0.11	0.629	0.687	0.646
C10:0	11.2	10.9	10.7	10.8	0.34	0.374	0.704	0.481
C11:0	0.12	0.10	0.08	0.07	0.01	0.015	0.241	0.687
C12:0	5.45	5.28	4.76	4.85	0.22	0.020	0.841	0.562
C13:0	0.09	0.08	0.07	0.07	0.01	0.036	0.188	0.535
C14:0	11.0	11.0	10.6	10.6	0.26	0.162	0.999	0.950
C14:1	0.18	0.18	0.12	0.12	0.01	0.001	0.741	0.738
C15:0	0.92	0.87	0.87	0.86	0.03	0.261	0.276	0.569
C16:0	28.9	29.2	28.0	28.0	0.72	0.185	0.838	0.837
C16:1	0.71	0.70	0.56	0.57	0.04	0.004	0.965	0.896
C17:0	0.67	0.67	0.73	0.72	0.02	0.004	0.888	0.924
C18:0	10.8	11.1	13.7	14.0	0.49	0.001	0.556	0.990
C18:1n9c + C18:1n9t	22.2	22.4	21.8	21.7	0.92	0.546	0.966	0.876
C18:1n7	0.63	0.62	0.61	0.61	0.01	0.171	0.634	0.782
C18:2n6c	2.90	2.79	2.86	2.84	0.09	0.934	0.460	0.606
C18:3n6	0.05	0.05	0.25	0.25	0.01	0.001	0.638	0.807
C18:3n3	0.67	0.65	0.60	0.60	0.02	0.008	0.425	0.558
C20:0	0.26	0.25	0.27	0.29	0.01	0.016	0.607	0.326
C20:4n6	0.15	0.14	0.15	0.15	0.01	0.813	0.754	0.955
C20:5n3	0.06	0.07	0.05	0.05	0.004	0.505	0.783	0.894
C22:0	0.11	0.10	0.11	0.11	0.004	0.392	0.373	0.574
C24:0	0.13	0.13	0.15	0.14	0.01	0.027	0.834	0.915
Saturation degree								
SFA[2]	72.4	72.4	73.0	73.4	1.0	0.438	0.845	0.825
MUFA[3]	23.7	23.9	23.2	22.9	0.9	0.422	0.930	0.779
PUFA[4]	3.86	3.71	3.73	3.68	0.10	0.479	0.348	0.619
Summation by source								
<C16	30.6	30.0	29.0	29.2	0.7	0.130	0.766	0.566
C16	29.2	28.8	28.0	28.0	0.8	0.219	0.842	0.759
>C16	38.0	38.3	40.4	40.3	0.8	0.014	0.902	0.803
Desaturation index								
C14:1 to C14:0	0.016	0.016	0.012	0.012	0.001	0.001	0.771	0.808
C16:1 to C16:0	0.025	0.025	0.021	0.021	0.002	0.023	0.973	0.837
C18:1 to C18:0	2.13	2.11	1.66	1.62	0.13	0.001	0.824	0.943

[1] Effects of temperature (T), Met supplementation (D), and their interaction (T × D). [2]SFA: Short Chain Fatty Acid. [3]MUFA: Mono-Unsaturated Fatty Acid. [4]PUFA: Poly-Unsaturated Fatty Acid.

It is not clear why C17:0 is the only odd FA that increased in our HS goats, but it is worth mentioning that the diet can contain minor concentrations of C17:0 as

previously reported in dairy cows diets (Baumann et al., 2016), which could interfere with the endogenous production of C17:0 by rumen microbes. The lower concentration of odd FA could be related to alterations in rumen microbiota. Profiling the rumen microbiota by 16sRNA gene cloning confirmed that HS induces significant changes in microbial diversity in heifers (Tajima et al., 2007). Additionally, we previously showed that HS goats eating the same amounts of DM as TN goats experience lower mean daily rumen pH (Castro-Costa et al., 2015), which could affect the microbial population.

5.4.5. Blood metabolic indicators

As shown in Table 5.5, Met supplementation had no effects on acid-base indicators. Results on the effect of methionine supplementation on blood electrolytes are scarce, which makes discussion and comparison with other studies difficult. Levels of K tended (P = 0.105) to increase, whereas values of Na, *i*Ca, and Cl increased ($P < 0.05$) due to HS (Table 5.5). Further, HS resulted in lower ($P < 0.001$) total CO_2 blood pressure. Previous results in heat-stressed dairy goats (Hamzaoui et al., 2013; Mehaba et al., 2019) and cows (Srikandakumar and Johnson, 2004; Cowley et al., 2015) showed generally similar trends to the data reported in the current study. The decrease in total CO_2 blood pressure by HS was expected as HS goats experienced greater respiration rate compared to TN goats (Table 5.2) and high amounts of CO_2 were removed.

None of the protein metabolism indicators (creatinine, urea, and albumin) was affected by ambient temperature conditions or Met supplementation (Table 5.5). Nevertheless, Muramatsu et al. (1989) reported that N balance of goats is improved by supplementing with rumen protected Met. In addition, Piccioli-Cappelli et al. (2016) detected a decrease in BUN levels and increased blood levels of albumin in goats supplemented with rumen-protected Met at early lactation. Similar to the effects of HS observed in the present study, Kamiya et al., (2006) reported that blood albumin values do not vary between TN and HS cows regardless the feed intake of TN cows (ad libitum or pair-fed). Blood urea did not differ between TN and HS goats, as difference in DMI (and consequently protein intake) was not markedly different (only 9.8 % less in HS). On the other hand, previous experiments in goats (Hamzaoui et al., 2013; Mehaba et al., 2019) showed lower blood urea values in HS

than in TN because HS animals have 21 to 26 % lower DM intake than TN goats. Comparison between HS cows and TN-pair fed cows indicate numerically greater (Kamiya et al., 2006), tendency of being greater (Gao et al., 2017) or significantly greater (Wheelock et al., 2010; Cowley et al., 2015) blood urea values in HS conditions due to higher protein catabolism and the use of AA as gluconeogenic substrates.

Table 5.5: Blood metabolic indicators of dairy goats under thermo-neutral (TN) or heat stress (HS) conditions. In each ambient temperature, goats were fed a control diet (Con) or supplemented with rumen protected methionine (Met). Values are least squares means and SE of the means (SEM).

Item	TN		HS		SEM	Effect[1] (P-value)		
	Con	Met	Con	Met		T	Diet	T × D
Acid-base balance								
Na, mmol/L	144	143	145	145	0.6	0.018	0.342	0.566
K, mmol/L	3.71	3.63	3.89	3.99	0.16	0.105	0.969	0.563
iCa, mmol/L	1.21	1.20	1.26	1.25	0.02	0.010	0.739	0.894
Cl, mmol/L	105	104	110	110	1.1	0.001	0.597	0.751
TCO_2,[2] mmHg	26.5	25.9	22.0	22.1	0.9	0.001	0.792	0.693
Anion Gap[3], mmol/L	17.3	17.5	17.8	17.9	0.4	0.225	0.599	0.861
Energy metabolism								
Insulin, µg/L	0.22	0.16	0.26	0.28	0.04	0.047	0.591	0.351
Glucose, g/dL	56.9	55.8	57.2	57.3	1.1	0.426	0.468	0.466
NEFA, mmol/L	0.28	0.16	0.08	0.08	0.06	0.051	0.398	0.389
β-HBA, mmol/L	0.50	0.55	0.73	0.62	0.06	0.028	0.640	0.234
Triglycerides, mg/dL	14.75	14.56	14.18	15.86	1.24	0.772	0.550	0.456
Cholesterol, mg/dL	84.0	84.2	84.2	87.5	5.3	0.745	0.743	0.773
Hematocrit, % PCV[5]	18.0	18.3	17.8	17.5	0.5	0.361	0.998	0.646
Hemoglobin[3], mmol/L	6.13	6.20	6.04	5.95	0.19	0.373	0.974	0.667
Protein metabolism								
Creatinine, mg/dL	0.49	0.53	0.53	0.54	0.04	0.558	0.557	0.769
BUN[4], mg/dL	19.8	20.8	21.1	19.7	1.3	0.887	0.877	0.369
Albumin, g/dL	3.34	3.37	3.38	3.35	0.10	0.922	0.980	0.768

[1] Effects of temperature (T), Met supplementation (D), and their interaction (T × D). [2]TCO_2: Total pressure of CO_2. [3]Calculated values by the i-STAT device software (Abbott Point of Care Inc., Princeton, NJ). [4]BUN: Blood Urea Nitrogen. [5]PCV: packed cell volume.

Plasma creatinine and 3-methylhistidine are specific biomarkers of muscle degradation. 3-methylhistidine is elevated in HS dairy cows (Kamiya et al., 2006) and creatinine is also increased in HS goats (Mehaba et al., 2019) compared to TN animals fed ad libitum. However, when TN animals had comparable feed intake level

to HS animals, 3-methylhistidine in dairy cows (Kamiya et al., 2006) and creatinine in dairy goats (the current study) did not vary. Other pair-fed studies in dairy cows detected increased blood creatinine by HS due to body muscle mobilization (Cowley et al., 2015).

Methionine supplementation had no effect on blood variables related to energy metabolism, including insulin (Table 5.5). Calculated energy balance (Table 5.3) was slightly positive (TN goats) or slightly negative (HS goats), which might explain the absence of significant effect of Met on energy metabolism. When Met is supplemented to transition dairy cows (significant negative energy balance), blood levels of cholesterol increase (Batistel et al., 2017), indicating an amelioration in liver function during the postpartum period. In addition, late pregnant Saanen goats supplemented with rumen protected Met have lower concentrations of NEFA and β-HBA compared with control goats (Piccioli-Cappelli et al., 2016). With regard to insulin levels, Osorio et al. (2013) reported no change in blood insulin concentration in lactating dairy cows supplemented with Met, which agrees with insulin results in the current study. Nevertheless, another study performed with lactating dairy cows reported greater insulin concentration in response to rumen protected Met (Blum et al., 1999).

Heat stress increased ($P < 0.05$) levels of insulin and β-HBA and decreased ($P = 0.051$) values of NEFA (Table 5.5). However, values of blood glucose, triglycerides, and cholesterol were similar between TN and HS goats. In the previous studies comparing both TN and HS animals fed ad libitum values of blood insulin, glucose, NEFA, and β-HBA are similar in dairy goats (Hamzaoui et al., 2013; Mehaba et al., 2019) and cows (Kassube et al., 2017). However, when TN pair-fed and HS cows are compared (Rhoads et al., 2009; Wheelock et al., 2010; Cowley et al., 2015; Gao et al., 2017), HS animals experience greater blood insulin and lower NEFA, but similar blood glucose, which is similar to what observed in the present experiment. Increased plasma NEFA level is a known mechanism to spare glucose, which would have occurred in TN goats to support milk synthesis. The reason why TN goats increased NEFA but not β-HBA in the current experiment is not clear, as elevation of blood NEFA is linked to augmented ketone bodies production. Cowley

et al. (2015) detected increased blood NEFA values in intake-restricted TN dairy cows without changes in β-HBA levels.

On the other hand, the lack of a NEFA increment in HS goats occurred despite the fact that HS is reported to increase blood cortisol and epinephrine (Beede and Collier, 1986), and this lipolytic hormonal milieu would lead to adipose tissue mobilization. However, lipid tissue in HS goats (Salama et al., 2014), ewes (current thesis), and cows (Baumgard et al., 2006) has been demonstrated to become less sensitive to lipolytic signals, which explain the lack of NEFA increment in HS goats. Additionally, the increased basal insulin level might be an additional explanation of why NEFA did not increase in HS conditions as insulin is a potent antilipolytic hormone (Vernon, 1992). Salama et al. (2014) showed the possibility that lower NEFA levels in HS animals is a consequence of fast uptake of NEFA for energy production through the synthesis of ketone bodies. Our findings support this hypothesis, as blood NEFA decreased, but β-HBA values were elevated by HS, which could be a glucose-sparing mechanism in HS conditions.

5.4.6. Plasma amino acids profile

Basal blood AA concentration values are shown in Table 5.6. The efficiency of Smartamine in providing Met is confirmed in several studies in dairy cows (Südekum et al., 2004; Rulquin et al., 2006) and goats (Flores et al., 2009). Therefore, the objective in the current study was not to test whether Met is absorbed from Smartamine. Rather, we aimed at detecting what AA are deficient under HS conditions, as studies in goats are scarce, and therefore we measured the blood basal AA levels.

Levels of Met, Glu, Met as percentage of essential AA (**EAA**) and percentage of total AA, and Lys as percentage of EAA and percentage of total AA were lowered ($P < 0.05$ to 0.10) by HS (Table 5.6). Nevertheless, HS resulted in greater ($P < 0.05$) values of His and Pro. The fact that blood Met was lower under HS conditions indicates that it is an important AA, and dairy goats need it under such conditions. Met supplementation increased ($P < 0.01$) Met concentration as % of EAA. In fact, Met was used by HS to alleviate some negative effects of HS, as Met-supplemented goats kept similar RT at 0800 h with less respiration rate ($P < 0.10$; Table 5.2) and suffered less BW loss ($P < 0.10$; Table 5.3).

Table 5.6: Plasma amino acids concentrations (µmol/L) of dairy goats under thermo-neutral (TN) or heat stress (HS) conditions. In each ambient temperature, goats were fed a control diet (Con) or supplemented with rumen protected methionine (Met). Values are least squares means and SE of the means (SEM).

Item	TN		HS		SEM	Effect[1] (P-value)		
	Con	Met	Con	Met		T	Diet	T × D
Essential AA (EAA)								
Arg	269	235	269	259	16	0.476	0.185	0.444
His	81.7	62.8	83.1	91.5	6.3	0.028	0.421	0.038
Ile	184	161	172	177	11	0.857	0.405	0.189
Leu	193	158	187	190	13	0.333	0.240	0.174
Lys	302	245	242	245	23	0.204	0.241	0.202
Met	59.9	59.1	48.9	51.3	3.6	0.018	0.824	0.665
Cys	243	221	223	238	14	0.888	0.811	0.199
Phe	71.7	66.6	70.1	74.5	5.5	0.573	0.959	0.401
Thr	60.3	81.5	88.9	85.8	12.3	0.195	0.334	0.465
Trp	67.0	68.2	69.4	65.7	6.1	0.997	0.834	0.687
Val	345	277	302	312	22	0.854	0.208	0.096
Non-essential AA (NEAA)								
Ala	228	217	225	237	14	0.540	0.994	0.433
Asn / Ser	236	234	247	249	21	0.525	0.991	0.912
Gln	448	421	426	447	20	0.919	0.874	0.235
Glu	88.0	97.6	76.8	82.9	4.2	0.006	0.076	0.679
Gly	1353	1299	1175	1327	100	0.465	0.636	0.313
Pro	169	162	189	191	11	0.042	0.790	0.668
Tyr	102	88.1	84.4	88.2	7.3	0.258	0.515	0.253
Citrulline	210	201	183	198	13	0.269	0.815	0.369
Ornithine	97.9	83.5	101	107	10.4	0.214	0.662	0.352
BCAA[2]	721	597	661	679	42	0.792	0.217	0.106
EAA	1645	1396	1535	1551	94	0.816	0.231	0.171
NEAA	2621	2515	2423	2624	151	0.768	0.757	0.318
Total AA	4266	3912	3958	4174	230	0.922	0.770	0.228
Met, % of EAA	3.69	4.27	2.98	3.32	0.14	0.001	0.003	0.399
Met, % of TAA	1.40	1.49	1.16	1.21	0.06	0.001	0.199	0.761
Lys, % of EAA	18.0	17.9	15.9	16.2	0.9	0.055	0.925	0.848
Lys, % of TAA	6.89	6.16	6.14	5.87	0.29	0.091	0.103	0.436

[1]Effects of temperature (T), Met supplementation (D), and their interaction (T × D). [2]BCAA: Branched-chain AA (Leu, Ile and, Val).

It is interesting to note that HS goats were also in shortage of Glu. Glutamic acid is not considered EAA, but its supplementation under HS conditions improved BW gain in broiler chickens (Olubodun et al., 2015; Porto et al., 2015). Glutamic acid is a major oxidative fuel for the intestine (Burrin and Stoll, 2009) and immune cells (Huang et al., 2003). Further, Glu has been shown to have a protective effect on

intestinal mucosa, maintaining intestinal barrier function (Wang et al., 2014). In HS conditions, gut barrier function is impaired (Koch et al., 2019) and the secretion of gut-derived toxins is increased (Contreras-Jodar et al., 2019), which causes the activation of the innate immune system and systemic inflammation. Both Glu and Gln are extensively used to support the immune function locally in the intestine (Newsholme, 2001). So, it is possible that Glu is needed during HS to maintain the gut barrier function and meet the greater requirements for immune response, which explains its lower levels in HS goats.

The fact that Met supplementation tended ($P < 0.10$) to improve blood Glu levels by 8 to 11% (Table 5.6) agrees with the increment of 12% in Glu levels observed when dairy cows are supplemented with Met (Whiting et al., 1972). Methionine is catabolized by the portal-drained viscera in ruminants (Lobley et al., 2003). In addition, Wu (1998) reported that net intestinal utilization of Met can account for up to 52% of Met supplied. So, it seems that Met supplementation in the current study saved the usage of Glu by the gastro-intestinal tract, which resulted in greater Glu levels available in blood.

Several studies indicated that HS increases blood creatinine levels which would be due to muscle protein mobilization in cows (Schneider et al., 1988; Cowley et al., 2015) and goats (Mehaba et al., 2019) to provide AA to produce energy, although in the current study blood creatinine levels were not affected by HS. Proline and Gly make up for a significant proportion of AA in muscle collagen, which might explain the greater levels of Pro observed in HS goats (Table 5.6).

Rumen protected Met supplementation decreased His levels in TN goats, but not in HS goats (significant temperature x diet interaction; $P < 0.05$). Conversely, Sun et al. (2007) reported no change in plasma His concentration in supplemented lipid-coated Zn-Met (1.8 g/d) or Mn-Lys (2.3 g/d) chelates compared to control goats under TN conditions (21ºC). Additionally, there was ambient temperature x Met supplementation interaction, where Met supplementation tended to decrease Val ($P < 0.10$) and BCAA (Ile, Leu, and Val; $P = 0.106$) in TN, but not HS goats. Linton et al. (1968) hypothesized that any synthesis involving methionine should also utilize some valine, which might explain the reduced blood Val (and consequently BCAA) levels when Met was supplemented in TN conditions. It is unclear why the same did

not occur in HS conditions, but it could be related to differences in the level of β-HBA between TN and HS goats (greater blood β-HBA in HS as shown in Table 5.5). Wu (2013) indicated that both acetoacetate and β-HBA inhibit the transamination of Val, and the addition of pyruvate prevents this inhibitory effect of ketone bodies.

5.5. CONCLUSIONS

Contrary to our hypothesis, methionine supplementation did not improve milk production performance. However, Met supplementation avoided body weight loss in HS conditions. Further, Met-supplemented HS goats experienced less respiratory rate with lower body temperature at the time of the day when the heat load was maximal. This apparent effect of Met on thermoregulation in goats should be carefully evaluated in the future. At comparable feed intake level, heat stress did not result in significant changes in milk yield. However, milk fat, protein and lactose contents were reduced by HS. The elevation in blood β-HBA by HS may spare glucose. Heat-stressed goats were in shortage of glutamate, which could be related to the inflammation and immune response at the gastrointestinal level. Methionine supplementation spared glutamate regardless the ambient temperature. Probably more AA in addition to Met should be supplemented to improve the lactational performance under HS conditions

CHAPTER 6

Conclusions and Implications

CHAPTER 6

6. CONCLUSIONS AND IMPLICATIONS

6.1. CONCLUSIONS

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

6.1.1. Exposure of dairy ewes to heat stress resulted in:

- Increased rectal temperature (+0.77ºC on average) and respiration rate (+90 breaths/min on average).
- Body weight losses (–380 g/d, on average).
- Unaffected milk yield, but decreased milk fat (–16%) and milk protein (–14%) contents.
- No change in blood glucose levels, despite the reduced feed intake (–11%).
- No mobilization of body fat reserves as the adipose tissue became more resistant to the lipolytic signals.
- Increased muscle protein mobilization as indicated by greater blood creatinine levels.
- Greater availability of glucose with faster uptake when glucose was administered. This fast uptake is very likely to occur through noninsulin-mediated pathways.

6.1.2. Comparison between thermo-neutral and heat-stressed goats with and without dietary rumen-protected L-carnitine revealed that:

- Heat stress negatively affected the lactational performance of dairy goats.
- Heat stress altered feeding behavior as heat-stressed goats tended to consume longer feed particles as an attempt to keep stable rumen pH.
- L-carnitine dramatically increased blood carnitine fractions (free-, acetyl-, and total-carnitine) regardless the ambient temperature.
- Although absorbed efficiently, L-carnitine had no productive or physiological benefits under thermo-neutral or heat stress conditions.

6.1.3. Supplementation of rumen-protected methionine to thermal-neutral or heat-stressed dairy goats under comparable feed intake level resulted in:

- No effect of temperature on milk yield, but milk fat, protein and lactose contents were reduced by heat stress.
- Less respiratory rate with lower body temperature at the time of the day when the heat load was maximal in heat-stressed goats with methionine.
- Avoiding the typical body weight loss in heat stress conditions when methionine was fed.
- No improvement in milk yield or milk composition when methionine was supplemented in both temperature conditions.
- Reduced blood basal methionine levels in heat-stressed goats, although feed intake level was comparable, which could indicate high demands of methionine in high ambient temperatures.
- Shortage of blood glutamate in heat stress, which could be related to the inflammation and immune response at the gastrointestinal level.
- Methionine supplementation spared glutamate regardless the ambient temperature.

6.2. IMPLICATIONS

- Evaluation of different intensities of heat stress at different stages of lactation is warranted in dairy ewes.
- The impact of heat stress on milk somatic cell count of dairy ewes and possibly on milk coagulation properties should be explored in the future.
- The role of methionine in thermoregulation deserves more attention.
- Improved body weight when methionine was supplemented could be related improved intestinal microbiota and hence enchanted nutrient absorption. Consequently, the relationship between methionine and intestinal microbiota under heat stress conditions deserves more research.
- The use of a mixture of feed additives (e.g. specific amino acids, glucose precursors, etc.) rather than a single feed additive would be a good strategy to reduce the impact of heat stress

CHAPTER 7

References

CHAPTER 7

7. REFERENCES

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