Running head: RESPONSES OF DAIRY EWES TO HEAT STRESS

Heat stress affects some physiological and productive variables and alters metabolism in dairy ewes

Nabil Mehaba[†], Wellington Coloma-Garcia[‡], Xavier Such, Gerardo Caja, and Ahmed A. K. Salama[*]

Group of Research in Ruminants (G2R), Department of Animal and Food Sciences, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain

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 thermo-neutral (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

[†] Current addresss: Tests and Trials Ltd., Ignacio Luzán, 24, 2240, Monzón, Huesca, Spain
[‡] Current address: Facultad de Medicina Veterinaria, Universidad Agraria del Ecuador (UAE), Guayaquil, Ecuador
[*] Corresponding author: ahmed.salama@uab.cat

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. Overall, HS decreased feed intake, but milk production was not affected. Heat stress caused metabolic adaptations that included increased body muscle degradation and reduced adipose tissue mobilization. These adaptations allowed ewes to spare glucose and to avoid reductions in milk yield.

Key words: milk production, metabolism, heat stress, dairy ewes

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 alterations in milk yield or milk quality will impact cheese yield. 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).

46 The sheep thermo-neutral zone is claimed to be between 5 and 25ºC (Curtis, 1983).
47 Another study on the relationship between production traits and climatic variables indicated a
48 comfort zone between 11 and 21ºC of average daily temperature (Ramon et al., 2016). Heat
49 stress (**HS**) negatively affects milk yield and its components in Holstein dairy cows (Baumgard
50 et al., 2011) and Murciano-Granadina dairy goats (Salama et al., 2014). With regard to dairy
51 sheep, most of the available studies evaluated the impact of HS on milk production by comparing
52 between seasons (Finocchiaro et al., 2005; Peana et al., 2007; Ramon et al., 2016). These studies
53 detected reductions in milk yield and milk components during summer. However, in studies
54 comparing seasons, the effect of HS was often confounded with that of different feeding patterns
55 across seasons. To the best of our knowledge, no published studies have evaluated the detailed
56 responses of lactating dairy ewes to HS under controlled climatic and feeding conditions.
57 With regard to metabolism under HS, available results in dairy cows (Wheelock et al.,
58 2010; Baumgard et al., 2011) and goats (Hamzaoui et al., 2013; Salama et al., 2014; Mehaba et
59 al., 2019) indicated homeorhetic adaptations to suppress lipid mobilization, despite reduced feed
60 intake and increased maintenance requirements. Studies comparing between heat-stressed and
61 thermo-neutral pair-fed cows showed that the lack of body lipid mobilization induced by HS is
62 because of greater blood insulin accompanied by changes in the expression of gluconeogenic
63 genes in liver (Wheelock et al., 2010; Baumgard et al., 2011). Furthermore, heat-stressed dairy
64 cows (Baumgard and Rhoads, 2013) and goats (Salama et al., 2014; Mehaba et al. 2019)
65 experience increased protein catabolism, and some of these mobilized AA could be used for
66 glucose synthesis. Whether similar metabolic changes occur in heat-stressed lactating dairy ewes
67 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 minimize production losses during HS conditions. Furthermore, little attention has been paid to evaluate comprehensively the 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.

MATERIALS AND METHODS

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 (Ref. 3142), 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 were used. Animals were allocated individually in 1.62 m^2-pens (1.8 × 0.90 m) with separate feed and water throughout the experiment. Ewes were blocked in two balanced groups according to milk yield and milk composition. The experimental design was a crossover with two periods of 21 days each, and two 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.

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% relative humidity, THI = 59 to 65) or HS (12-h day at 35ºC day and 45 ± 5% relative humidity; THI = 83; and 12-h night at 28ºC and 45 ± 5% relative 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). The THI were calculated according to NRC (1971).

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 as described by Hamzaoui et al (2013). Ewes were machine-milked twice daily (0800 and 1700 h). All TN and HS ewes were individually fed ad libitum a TMR (Table 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. Clean water was permanently available at ambient temperature.

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

Feed intake and water consumption was measured daily throughout the experiment. Feed samples were collected before the beginning of each experimental period and were analyzed for

DM, ADF, NDF, CP and ash contents according to AOAC (2003). The chemical composition and nutritive value of ration ingredients are shown in Table 1.

Ewes were weighed weekly using an electronic scale (Tru-test A6500, Auckland, New Zealand) in 2 consecutive days after milking and before feeding. The net energy balance was calculated using the following equation: energy balance = net energy intake – (NE_M + NE_L). The NE_M was calculated using the following equation: $NE_M = (0.0345 \times BW^{0.75}$; INRA, 2018). Maintenance costs were increased by 30% for HS ewes as recommended (NRC, 2001). The NE_L was calculated by using the following equation: NE_L = [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 at each milking were collected twice per week. Milk samples were analyzed for fat, protein (N × 6.38), lactose, and SCC as previously described (Mehaba et al., 2019). A milk aliquot was stored at –20ºC to determine milk osmolality using a Fiske 110 osmometer (Fiske Associations, Norwood, MA).

Two 10-mL blood samples were collected at 0800 h (before milking and feeding) from the jugular vein using heparinized and K_2-EDTA vacutainer tubes (BD, Belliver Industrial Estate, Plymouth, UK) at d 3, 10 and 17 of each period. 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 (Mehaba et al., 2019). Additional blood samples (approximately 0.3 mL) were collected and immediately applied to disposable cartridges (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 CO_2 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**; 0.25 g/kg BW), insulin tolerance test (4.6 µg/kg BW), and epinephrine challenge (2 µg/kg BW) 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; Merck Life Science S.L.U., Madrid, Spain) using 1 filter for each 200 mL solution and kept at 4ºC.

On the day of each metabolic test, 3 pre-challenge blood samples (–20, –10, 0 min) were collected followed by an intravenous bolus dose of the corresponding metabolite. Thereafter, blood samples were collected at 5, 10, 20, 30, 45, 60, 90, and 120 min. 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). Glucose was analyzed by the hexokinase method (OSR 6121, Reagent System Olympus, Beckman Coulter, Krefeld, Ireland). Values of NEFA and BHB were determined as indicated by Mehaba et al. (2019).

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.

158 Glucose clearance rate and glucose half-life during the GTT were calculated according to
159 Kerestes et al. (2009). For insulin tolerance test, insulin-stimulated blood glucose response was
160 calculated as indicated by Kerestes et al. (2009).
161
162 ***Statistical Analyses***
163 Data were analyzed by the MIXED procedure for repeated measurements of SAS v. 9.4
164 (SAS Institute Inc., Cary, NC). The statistical mixed model contained the effects of
165 environmental conditions (TN and HS), period (1 and 2), the sampling time, and the
166 environmental conditions × period, environmental conditions × sampling time, period ×
167 sampling time interactions as fixed effects, as well as the random effects of the animal and the
168 residual error. For the data of RT and RR measured at 0900, 1200 and 1700 h, a fixed factor of
169 the hour of day was added to the model. Data of performances (i.e., DMI, water consumption,
170 and milk yield) and physiological indicators (i.e., RT and RR) were analyzed on a weekly basis.
171 For the basal levels of physiological challenge parameters a t-test for independent means were
172 used. Differences between least squares means were determined with the PDIFF option of SAS.
173
174 **RESULTS AND DISCUSSION**
175
176 ***Rectal Temperature and Respiratory Rate***
177 The RT was greater (+ 77ºC on average; $P < 0.001$) in HS than TN ewes (Table 2).
178 Furthermore, RT increased ($P < 0.05$) in HS ewes from 39.38ºC at 0800 to 39.85ºC at 1700 in
179 accordance with increment in the ambient temperature from 28ºC during night to 35ºC during the
180 day. However, RT did not vary between 0800 and 1700 h in TN ewes. Heat-stressed ewes had
181 214% increase in RR (+90 breaths/min; $P < 0.001$) on average compared with that for TN ewes.

182 The HS ewes increased ($P < 0.05$) RR by 34% in the evening (154 breaths/min) compared with
183 that for the morning measurement values (115 breaths/min). The increased RR in HS ewes was
184 to dissipate heat, as ~65 % of body heat is lost through the respiratory tract in sheep under
185 hyperthermia (Hales and Brown, 1974). Our ewes were shorn before the experiment (2-cm fleece
186 length), and some heat dissipation by sweating could have also occurred in addition to
187 evaporation by panting.
188
189 ***Feed intake and Body Weight Change***
190 The DMI decreased ($P < 0.001$) in HS ewes by 11% throughout the experiment (Table 2).
191 This lower DMI induced slight negative energy balance in HS compared to TN ewes (Table 2).
192 Reduction in appetite under HS is a primarily result of the elevated body temperature. The
193 reduced DMI may be also related to an increase in gut fill as a result of an increase in water
194 consumption (Silanikove, 1992). The rumen seems to serve as a water reservoir during high heat
195 load (Silanikove, 1992). The decrease in DMI observed in our HS ewes is lower than those
196 values reported in Holstein dairy cows (–30%; Wheelock et al., 2010) and Murciano-Granadina
197 goats (–21%; Hamzaoui et al., 2013) heat-stressed to similar extent as in the present study. In
198 addition, our HS ewes did not experience significant milk yield losses (see later). Consequently,
199 it seems that dairy ewes experience less productive losses under HS conditions compared to
200 dairy cows and goats. This assumption should be confirmed by carrying out comparative studies
201 using animals from the different species at similar stage of lactation and feeding conditions. The
202 HS ewes had greater water consumption compared with TN ewes (+2.0 L/d; $P < 0.001$).
203 Obviously, the increase in water consumption in the present study was mainly to meet the
204 increment of water requirements for heat dissipation by evaporation.

The HS ewes lost BW, whereas TN ewes gained weight (Table 2; $P < 0.01$). On average, 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 that for TN ewes (Table 2). 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 Holstein cows (Ronchi et al., 1999) and Murciano-Granadina 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 with that for TN ewes (see later).

Milk yield and Milk Composition

Despite the reduced DMI and increased RT and RR, HS ewes produced similar milk yield and FCM compared with that for TN ewes (Table 2). Our ewes were in their 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% relative humidity, THI = 86). Additionally, Hamzaoui et al. (2013) under controlled climatic conditions observed that HS Murciano-Granadina goats in late lactation produce similar milk yield to TN goats. Nevertheless, in the studies evaluating the effect of HS by comparing among seasons, milk yield of mid-lactating Sarda ewes was reduced by 15% when maximum ambient temperature is higher than 24ºC (Peana et al., 2007). Further, late-lactating Comisana ewes experience a reduction in milk yield at temperatures > 35ºC (Sevi et al., 2001). Evaluating the impact of HS by the comparison between seasons includes the inevitable variations related to different feeding and photoperiod conditions, and these variations were not present in our study.

Compared to TN, HS decreased milk fat and protein contents by 13 to 16 %, respectively (Table 2). Similarly, Abdalla et al. (1993) reported that milk fat and protein contents are depressed by HS in crossbred ewes. In addition, Ramon et al. (2016) reported a reduction in fat and protein yields during summer in Manchega ewes. In the present study, lactose content was increased in HS ewes compared with that for TN ($P < 0.05$) in accordance with the numerical increment in milk yield. Despite the reduced fat and protein contents by HS, fat, protein, and lactose yields were not affected (Table 2), 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 mammary cells (Salama et al., 2019).

Heat stress tended to increase ($P < 0.10$) milk SCC by 5% in HS ewes compared with that for TN ewes. It is not clear why milk SCC increased by HS, but stressful conditions such as sudden change in milking regimen (Salama et al., 2003) and social isolation (Stelwagen et al., 2000) result in increased milk SCC. The $\log_{10}$ of SCC values in our TN and HS ewes (4.40 to 4.63) are well below the reported thresholds of mammary inflammation in sheep (5.48 to 6.00; Murphy et al., 2018). This indicates that the increment of milk SCC in HS ewes is unlikely related to mammary inflammation. In contrast to our results, Caroprese et al. (2011) reported no change in milk SCC in ewes exposed to solar radiation compared to ewes maintained in shade.

Milk osmolality increased ($P < 0.001$) by HS (Table 2). Nevertheless, a decreased milk osmolality was observed in heat-stressed goats (Olsson and Dahlborn, 1989) and Friesland ewes (Thompson et al., 1981), which is related to over-hydration and hemodilution. Milk osmolality is under strict control, and is isotonic with plasma. Lactose is the major osmotic component of milk, and the increase in milk lactose content in HS ewes might explain the small (+1%), albeit

significant, increment in milk osmolality. In the current study, values of milk osmolality (298 to 302 mOsm/kg) were close to the normal osmolality reported for sheep milk (294 mOsm/kg; Thompson et al., 1981). 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 caused by HS marginally relevant.

Blood Metabolites

There were no differences between treatments in blood electrolytes (Table 3). Nevertheless, HS ewes tended ($P < 0.10$) to have increased blood Cl concentration compared with that for TN ewes. Usually, high ambient temperatures result in a reduction in blood 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 Murciano-Granadina goats, but Cl levels increased (Hamzaoui et al., 2013). Blood total CO_2 concentration dropped ($P < 0.05$) by HS as a result of panting and greater removal of blood CO_2.

No differences were observed between TN and HS ewes with regard to blood glucose, cholesterol, NEFA, or BHB values (Table 3). Similarly, heat-stressed late lactating Murciano-Granadina dairy goats (Hamzaoui et al., 2013) and early-lactating crossbred 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 negative energy balance was also reported in Murciano-Granadina dairy goats (Hamzaoui et al., 2013; Mehaba et al., 2019) and Holstein dairy cows (Baumgard et al., 2011). Nevertheless, Abdalla et al. (1993) detected increased blood NEFA levels in early lactating crossbred 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 Murciano-Granadina goats (Hamzaoui et al., 2013), although some studies showed increases (Okoruwa, 2014) or decreases (Singh et al., 2016) in HS sheep. 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.

Values of BUN and albumin were not affected by HS (Table 3). However, there was an increase ($P < 0.001$) in blood creatinine in HS ewes compared with that for TN ewes, which might indicate muscle degradation. Although our HS dairy ewes experienced lower DMI and had slightly negative energy balance (Table 2), they did not use body fat reserves (no change in blood NEFA as shown in Table 3). However, they mobilized body protein presumably to use some glucogenic AA for glucose synthesis. Heat-stressed Murciano-Grnadina dairy goats also experience increased blood creatinine levels compared to TN animals (Mehaba et al., 2019).

Responses to the Metabolic Tests

Glucose Tolerance Test. Results of the GTT as indicator of insulin sensitivity are shown in Figure 1 and Table 4. The basal glucose levels were not different in TN and HS ewes. Glucose peaked at 5 min in both treatments, but the peak was greater ($P < 0.05$) in HS (+511%) than in TN (+252%) ewes (Figure 1a). Blood glucose levels gradually decreased in both treatments and returned to the basal values by 60 min after glucose administration.

Basal plasma insulin values did not differ between TN and HS ewes (Figure 1b; Table 4), although DMI was depressed by HS. Maintaining blood insulin levels during HS is crucial for the activation of the cellular stress response (Li et al., 2006). Further, insulin is a potent lipogenic and antilipolytic hormone (Baumgard and Rhoads, 2013). Consequently, maintaining normal

297 insulin levels during HS might explain the absence of significant differences in blood NEFA
298 concentrations between TN and HS ewes (Table 2). As a response to the glucose infusion,
299 plasma insulin levels of both TN and HS ewes peaked at min 10, but no differences in peak
300 values were observed between groups. After the peak, insulin values decreased gradually and
301 reached the basal levels at min 90. The HS ewes had greater ($P < 0.05$) insulin values than TN
302 ewes only at 5 and 20 min (Figure 1b).

303 Mean glucose AUC tended ($P < 10$) to be greater at 45 and 90 min in HS ewes compared
304 with that for TN ewes (Table 4). However, the insulin AUC values did not differ between
305 groups. Wheelock et al. (2010) also found that lactating HS cows have greater glucose response
306 after GTT than TN cows fed ad libitum without differences in the insulin response. With similar
307 insulin secretion kinetics during GTT, HS ewes had greater immediate glucose level than TN
308 ewes, which might be interpreted as decreased insulin sensitivity in HS ewes. This greater
309 available glucose in HS ewes was rapidly uptaken, resulting in increased ($P < 0.01$) glucose
310 clearance rate and decreased ($P < 0.01$) glucose half-life (Table 4). Glucose is the most
311 efficiently utilized energy source compared to other substrates as fat or protein (Baldwin et al.,
312 1980), which might explain its fast disappearance in HS conditions. Because of the apparent lack
313 of NEFA availability as a fuel substrate, it seems that heat-stressed animals increase both their
314 production of, and reliance on, glucose as a fuel. As insulin response to GTT did not vary
315 between TN and HS ewes (Table 4), the faster uptake of glucose in HS ewes might have been
316 caused not only by insulin, but also through a non-insulin-mediated glucose uptake process. In
317 fact, the whole body utilization of glucose can also occur through noninsulin-dependent
318 pathways in crossbred wethers (Janes et al., 1985) and Holstein cows (Rose et al., 1997).

Insulin Tolerance Test. Results of the insulin tolerance test as indicator of insulin responsiveness are shown in Figure 2 and Table 4. 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; $P < 0.05$). This result indicates prolonged effect of insulin in TN compared to HS ewes (Figure 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 4), which might be interpreted as 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 Suffolk ewes. Given the fact that blood glucose did not return to the basal levels by min 120 in TN ewes (Figure 2), it is possible that differences in insulin responsiveness kinetics would have been detected if sampling time was extended beyond 120 min. Neither plasma NEFA nor BHB concentrations were affected by the insulin infusion, although NEFA AUC was lower in HS compared with that for TN ewes (Table 4). The lower NEFA AUC levels in HS ewes might be related to the increased dependence on glucose as energy source and the decreased body fat mobilization.

Epinephrine Challenge. Plasma glucose peak in response to the epinephrine challenge was similar between groups (Table 4), but HS ewes had an earlier peak (5 min) than TN ewes (10 min) as shown in Figure 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.

342 Blood NEFA levels (Figure 3b) were lower in HS ewes than TN ewes from 30 min after
343 infusion until min 120. The blunted lipolytic response to epinephrine in HS ewes occurred
344 despite the reduced DMI, which is typically associated with elevated circulating NEFA levels.
345 Baumgard et al. (2011) also demonstrated that HS Holstein cows have reduced NEFA response
346 to epinephrine administration. Epinephrine has been reported to be elevated during HS,
347 especially during the early phase (acute) of hyperthermia (Silanikove et al., 2000). This elevation
348 could modify the density and affinity of the adrenergic receptors (Mirit et al., 2000), which could
349 explain the increased resistance of adipose tissue to lipolytic signals in HS ewes.
350 Alternatively, the released NEFA in HS ewes were rapidly uptaken by liver and
351 converted into ketone bodies. This would explain the greater ($P < 0.05$) AUC of BHB (Table 4)
352 in HS compared with that for TN ewes. Salama et al. (2014) proposed that liver of HS Murciano-
353 Granadina goats is able to uptake NEFA faster and converts them to BHB (utilized as energy
354 source for body tissues to spare glucose) compared to TN goats.
355
356 **CONCLUSIONS**
357 Heat stress reduced feed intake, but Lacaune dairy ewes were able to maintain milk yield
358 as well as the yields of fat, protein and lactose. Heat-stressed ewes did not mobilize body fat
359 reserves, but degraded muscles as indicated by the greater blood creatinine levels. Heat-stressed
360 ewes tended to have more available glucose in the blood after glucose was administered, and it is
361 likely that this glucose was uptaken through insulin- and noninsulin-mediated pathways. Further,
362 adipose tissue of HS ewes became more resistant to the lipolytic signals. Overall, these metabolic
363 adaptations allowed dairy ewes to spare glucose and to avoid reductions in milk yield.
364

ACKNOWLEDGMENTS

This work is part of 2 research projects funded by the Spanish Ministry of Economy and Competitiveness (AGL2013-44061-R and RTA205-00035-C03-02). Nabil Mehaba received a scholarship from Agency for Management of University and Research Grants (AGAUR) of the Catalan Government (Reference 2017FI_B 00303). The authors are also grateful to the team of SGCE (Servei de Granges i Camps Experimentals) of the UAB for the care of the animals.

REFERENCES

Abdalla, E. B., E. A. Kotby, and H. D. Johnson. 1993. Physiological responses to heat-induced hyperthermia of pregnant and lactating ewes. Small Rumin. Res. 11:125–34.

Achmadi, J., T. Yanagisawa, H. Sano, and Y. Terashima. 1993. Pancreatic insulin secretory response and insulin action in heat-exposed sheep given a concentrate or roughage diet. Dom. Anim. Endocrinol. 10:279–287.

AOAC International. 2003. Official Methods of Analysis of AOAC International. Vol. I. 17th ed. AOAC International, Gaithersburg, MD.

Baldwin, R. L., N. E. Smith, J. Taylor, and M. Sharp. 1980. Manipulating metabolic parameters to improve growth rate and milk secretion. J. Anim. Sci. 51:1416–1428.

Baumgard, L. H., and R. P. Rhoads. 2013. Effects of heat stress on postabsorptive metabolism and energetics. Ann. Rev. Anim. Biosci. 1:311–337.

Baumgard, L. H., J. B. Wheelock, S. R. Sanders, C. E. Moore, and H. B. Green. 2011. Post-absorptive carbohydrate adaptations to heat stress and monensin supplementation in lactating Holstein cows. J. Dairy Sci. 94:5620–5633.

Caroprese, M., M. Albenzio, A. Bruno, V. Fedele, A. Santillo, and A. Sevi. 2011. Effect of solar radiation and flaxseed supplementation on milk production and fatty acid profile of lactating

ewes under high ambient temperature. J. Dairy Sci. 94:3856–3867.

Curtis, S. E. 1983. Environmental management in animal agriculture. 2nd ed. Iowa State University, Ames, Iowa.

FAO. (2018). FAOSTAT. http://www.fao.org/faostat/en/#data/QA.

Finocchiaro, R., J. B. C. H. M. van Kaam, B. Portolano, and I. Misztal. 2005. Effect of heat stress on production of Mediterranean dairy sheep. J. Dairy Sci. 88:1855–1864.

Hales, J. R., and G. D. Brown. 1974. Net energetic and thermoregulatory efficiency during panting in the sheep. Comp. Biochem. Physiol. A Comp. Physiol. 49(3A):413-422.

Hamzaoui, S., A. A. K. Salama, E. Albanell, X. Such, and G. Caja. 2013. Physiological responses and lactational performances of late-lactation dairy goats under heat stress conditions. J. Dairy Sci. 96:6355–6365.

INRA. 2018. INRA Feeding System for Ruminants. Wageningen Acad. Publ., Wageningen, the Netherlands.

Janes, A. N., T. E. Weekes, and D. G. Armstrong. 1985. Insulin action and glucose metabolism in sheep fed on dried-grass or ground maize-based diets. Br. J. Nutr. 54:459–471.

Kerestes, M., V. Faigl, M. Kulcsar, O. Balogh, J. Foldi, H. Febel, Y. Chilliard, and G. Huszenicza. 2009. Periparturient insulin secretion and whole-body insulin responsiveness in dairy cows showing various forms of ketone pattern with or without puerperal metritis. Domest. Anim. Endocrinol. 37:250–261.

Li, G., I. S. Ali, and R. W. Currie. 2006. Insulin induces myocardial protection and HSP70 localization to plasma membranes in rat hearts. Am. J. Physiol. Heart Circ. Physiol. 291:H1709–721.

Mehaba, N., A. A. K. Salama, X. Such, E. Albanell, and G. Caja. 2019. Lactational responses of

412 heat-stressed dairy goats to dietary L-carnitine supplementation. Animals. 9:567. doi:
413 10.3390/ani9080567.
414 Mirit, E., C. Gross, Y. Hasin, A. Palmon, and M. Horowitz. 2000. Changes in cardiac mechanics
415 with heat acclimation: adrenergic signaling and SR-Ca regulatory proteins. Am. J. Physiol.
416 Regulatory Integrative Comp. Physiol. 279:77–85.
417 Murphy, T. W., W. C. Stewart, and J. B. Taylor. 2018. Factors affecting ewe somatic cell count
418 and its relationship with lamb weaning weight in extensively managed flocks. Transl. Anim.
419 Sci. 2(Suppl. 1):S159–S162.
420 NRC. 1971. A Guide to Environmental Research on Animals. National Academy of Science,
421 Washington, DC.
422 NRC. 2001. Nutrient requirements of dairy cattle. 7th ed. National Academy of Science,
423 Washington, DC.
424 Okoruwa, M. I. 2014. Effect of heat stress on thermoregulatory, live body weight and
425 physiological responses of dwarf goats in southern Nigeria. Europ. Sci. J. 10:255–264.
426 Olsson, K., and K. Dahlborn 1989. Fluid balance during heat stress in lactating goats. Q. J. Exp.
427 Physiol. 74:645–659.
428 Pasqui, M., and E. Di Giuseppe. 2019. Climate change, future warming, and adaptation in
429 Europe. Anim. Front. 9:6–11.
430 Peana, I., G. Fois, and A. Cannas. 2007. Effects of heat stress and diet on milk production and
431 feed and energy intake of Sarda ewes. Ital. J. Anim. Sci. 6:577–579.
432 Pulina, G., N. Macciotta, and A. Nudda. 2005. Milk composition and feeding in the Italian dairy
433 sheep. Ital. J. Anim. Sci. 4:5–14.
434 Ramon, M., C. Diaz, M. D. Perez-Guzman, and M. J. Carabaño. 2016. Effect of exposure to

435 adverse climatic conditions on production in Manchega dairy sheep. J. Dairy Sci. 99:5764–
436 5779.
437 Ronchi, B., U. Bernabucci, N. Lacetera, A. Verini Supplizi, and A. Nardone. 1999. Distinct and
438 common effects of heat stress and restricted feeding on metabolic status in Holstein heifers.
439 Zoot. Nutri. Anim. 25:11–20.
440 Rose, M. T., Y. Obara, F. Itoh, H. Hashimoto, and Y. Takahashi. 1997. Non-insulin- and insulin-
441 mediated glucose uptake in dairy cows. J. Dairy Res. 64:341–353.
442 Salama, A.A.K., G. Caja, E. Albanell, X. Such, R. Casals, and J. Plaixats. 2003. Effects of
443 dietary supplements of zinc methionine on milk production, udder health and zinc
444 metabolism in dairy goats. J. Dairy Res. 70:9–17.
445 Salama, A. A. K., G. Caja, S. Hamzaoui, B. Badaoui, A. Castro-Costa, D. A. E. Façanha, M. M.
446 Guilhermino, and R. Bozzi. 2014. Different levels of response to heat stress in dairy goats.
447 Small Rumin. Res. 121:73–79.
448 Salama, A. A. K., M. Duque, L. Wang, K. Shahzad, M. Olivera, and J. J. Loor. 2019. Enhanced
449 supply of methionine or arginine alters mechanistic target. J. Dairy Sci. 102:2469–2480.
450 Sevi, A., G. Annicchiarico, M. Albenzio, L. Taibi, A. Muscio, and S. Dell'Aquila. 2001. Effects
451 of solar radiation and feeding time on behavior, immune response and production of
452 lactating ewes under high ambient temperature. J. Dairy Sci. 84:629–640.
453 Silanikove, N. 1992. Effects of water scarcity and hot environment on appetite and digestion in
454 ruminants: A review. Livest. Prod. Sci. 30:175–194.
455 Silanikove, N. 2000. Effects of heat stress on the welfare of extensively managed domestic
456 ruminants. Livest. Prod. Sci. 67:1–18.
457 Singh, K. M., S. Singh, I. Ganguly, A. Ganguly, R. K. Nachiappan, A. Chopra, and H. K. Narula

458 2016. Evaluation of Indian sheep breeds of arid zone under heat stress condition. Small
459 Rumin. Res. 141:113–117.
460 Stelwagen, K. H. Hopster, J. T. N. Van Der Werf, and H. J. Blokhuis. 2000. Effects of isolation
461 stress on mammary tight junctions in lactating dairy cows. J. Dairy Sci. 83:48–51.
462 Thompson, G. E., P. E. Hartmann, J. A. Goode, and K. S. Lindsay. 1981. Some effects of acute
463 fasting and climatic stresses upon milk secretion in Friesland sheep. Comp. Bioch. Phys.
464 70A:13–16.
465 Wheelock, J. B., R. P. Rhoads, M. J. Vanbaale, S. R. Sanders, and L. H. Baumgard. 2010.
466 Effects of heat stress on energetic metabolism in lactating Holstein cows. J. Dairy Sci.
467 93:644–655.

468 **Table 1.** Ingredients, chemical composition, and nutritive value of the total mixed ration offered

469 to the dairy ewes.

Item	Value
Ingredients (% as fed)	
Alfalfa hay	60.0
Cracked oat grain	2.0
Cracked corn grain	1.6
Brewing barley	4.0
Soybean hull	18.0
Soybean meal, 44%	2.0
Rapeseed meal	4.0
Corn gluten feed	4.0
Soybean oil	2.0
Cane molasses	0.8
Salt (NaCl)	0.2
Vitamin and mineral complex for goats	0.4
Dicalcium phosphate	1.0
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
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

470 [1] Calculated according to Institut National de la Recherche Agronomique (INRA, 2018).

471 [2] Feed units for lactation.

472 [3] Net energy for lactation (1 UFL = 1.7 Mcal of NE_L).

473 [4] Protein digested in the small intestine from food and microbial synthesis origins.

474 [5] Protein digested in the small intestine supplied by food rumen undegradable protein.

475 [6] Rumen protein balance, represents RUP, microbial protein and endogenous protein.

476 **Table 2.** Rectal temperature, respiratory rate, feed intake, body weight and milk production
477 variables of dairy ewes under thermo-neutral (TN; n = 8) or heat stress (HS; n = 8) conditions.
478 Values are least squares means and standard error of the mean (SEM).

Item	TN	HS	SEM	*P*-value		
				Treatment	Period	T × P[1]
Rectal temperature, ºC	38.86	39.63	0.04	0.001	0.007	0.656
Respiratory rate, breaths/min	42	132	2	0.001	0.725	0.367
DM intake, 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
Fat-corrected milk[2], kg/d	1.64	1.64	0.08	0.985	0.337	0.772
Energy balance[3], 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
Fat : protein ratio	1.07	1.05	0.03	0.539	0.643	0.274
SCC, 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/kg	298	302	1	0.001	0.084	0.008

479 [1]Interaction of treatment (T) × period (P).

480 [2]$FCM_{6.5\%}$ = kg of milk yield × (0.37 + 0.09 × fat%) according to Pulina et al. (2005).

481 [3]Energy balance = Energy intake – [0.0345 × $BW^{0.75}$] + [0.686 × milk yield, L × (0.0071 × fat, g/L +
482 0.0043 × protein, g/L + 0.2224)] according to INRA (2018).

Table 3. Blood metabolites of dairy ewes under thermo-neutral (TN; n = 8) or heat stress (HS; n = 8) conditions. Values are least squares means and standard error of the mean (SEM).

Item	TN	HS	SEM	*P*-value		
				Treatment	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
Ca, 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, mmHg	26.3	24.3	0.6	0.023	0.362	0.687
Anion Gap, 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
Non esterified fatty acids, mmol/L	0.13	0.13	0.02	0.869	0.057	0.444
ß-hydroxybutyrate, 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	22.1	20.8	0.6	0.130	0.179	0.240
Hemoglobin, mmol/L	7.5	7.1	0.2	0.134	0.193	0.226
Protein metabolism						
Creatinine, mg/dL	0.59	0.81	0.02	0.001	0.988	0.099
Blood urea N, 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).

Table 4. Metabolite kinetics in response to glucose tolerance test, insulin tolerance test, and epinephrine challenge in dairy ewes under thermo-neutral (TN; n = 4) or heat stress (HS; n = 4). Values are least squares means and standard error of the mean (SEM).

Item	TN	HS	SEM	*P*-value
Glucose tolerance test				
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
$AUC_{45\ min}$,[2] mg/L × min	1258	2333	318	0.071
$AUC_{90\ min}$, mg/L × min	1321	2457	375	0.093
GLU-$t_{1/2}$,[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
$AUC_{45\ min}$, µg /L × min	32.7	44.4	18.3	0.258
$AUC_{90\ min}$, µg /L × min	35.4	46.5	5.8	0.245
Insulin tolerance test				
Glucose				
Basal, mg/dL	59.2	59.4	1.8	0.934
Nadir, mg/dL	31.7	34.0	2.5	0.542
$AUC_{120\ min}$, mg/dL × min	-522	-386	87	0.313
ISBGR[4], %	0.47	0.40	0.04	0.258
Non-esterified fatty acids				
Basal, mmol/L	0.26	0.34	0.05	0.304
Nadir, mmol/L	0.19	0.15	0.05	0.242
$AUC^2_{120\ min}$, mmol/L × min	1.85	-1.54	0.72	0.017
ß-hydroxybutyrate				
Basal, mmol/L	0.43	0.44	0.06	0.881
Nadir, mmol/L	0.36	0.42	0.05	0.486
$AUC^2_{120\ min}$, mmol/L × min	0.18	1.88	1.01	0.301
Epinephrine challenge				
Glucose				
Basal, mg/dL	62.9	61.3	1.5	0.478
Peak, mg/dL	114	104	16	0.682
$AUC_{120\ min}$, mg/dL × min	900	881	152	0.933
Non-esterified fatty acids				
Basal, mmol/L	0.27	0.28	0.04	0.920
Peak, mmol/L	0.44	0.39	0.12	0.578
$AUC_{120\ min}$, mmol/L × min	1.82	-2.10	0.98	0.030
ß-hydroxybutyrate				
Basal, mmol/L	0.47	0.46	0.06	0.925
Peak, mmol/L	0.49	0.57	0.13	0.441
$AUC_{120\ min}$, mmol/L × min	-1.17	1.42	0.71	0.047

[1]Clearance rate form the peak to 60 min $= \frac{\ln(\text{glucose at 5 min}) - \ln(\text{glucose at 60 min})}{\text{min 60} - \text{min 5}} \times 100$

[2] Area under the curve corrected for the basal levels.

[3] Glucose half life $= \frac{0.693}{\text{glucose clearance rate}} \times 100$

[4] Insulin stimulated blood glucose response $= \frac{\text{glucose at 0 min} - \text{glucose at 30 min}}{\text{glucose at 0 min}} \times 100$

493 **Figure legend:**

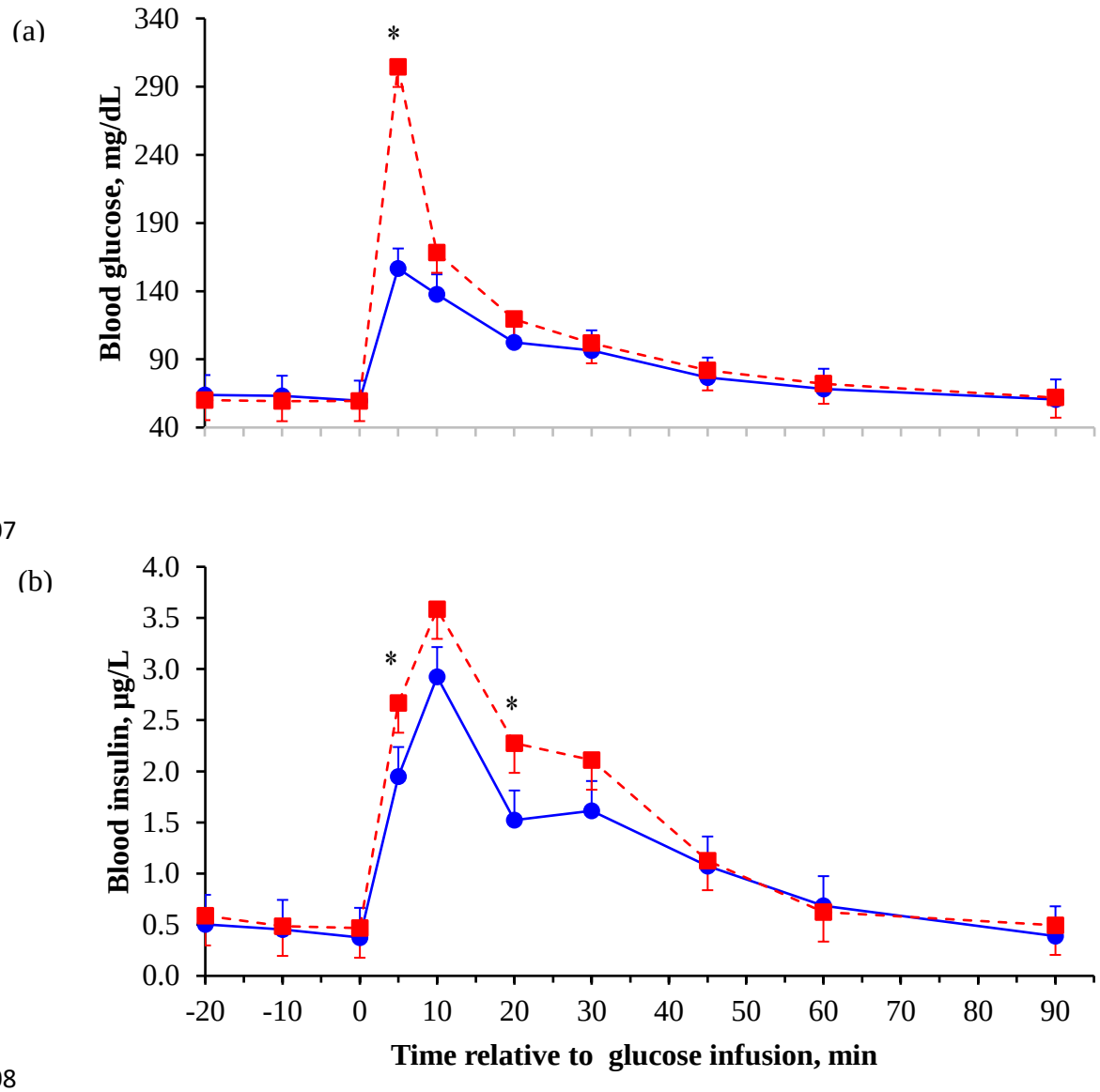
494 **Figure 1.** Plasma glucose (**A**) and insulin (**B**) response to glucose tolerance test of Lacaune dairy
495 ewes under thermo-neutral (TN; ●) or heat stress (HS; ■) conditions. Values are least square
496 means with SEM indicated by vertical bars. * indicates a difference at $P < 0.05$ between TN and
497 HS treatments.

498 **Figure 2.** Plasma glucose response to insulin tolerance test of Lacaune dairy ewes under thermo-
499 neutral (TN; ●) or heat stress (HS; ■) conditions. Values are means with SE indicated by vertical
500 bars. * indicates a difference at $P < 0.05$ between TN and HS treatments.

501 **Figure 3.** Plasma glucose (**A**), NEFA (**B**) and BHBA (**C**) response to epinephrine challenge of
502 Lacaune dairy ewes under thermo-neutral (TN; ●) or heat stress (HS; ■) conditions. Values are
503 least square means with SE indicated by vertical bars. * indicates a difference at $P < 0.05$
504 between TN and HS treatments.

505 **Figure 1.**

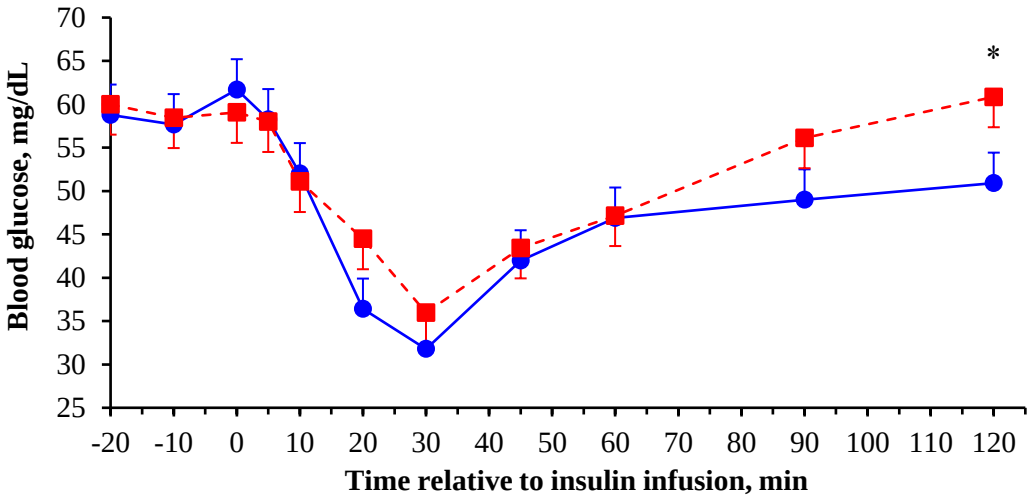
506



507

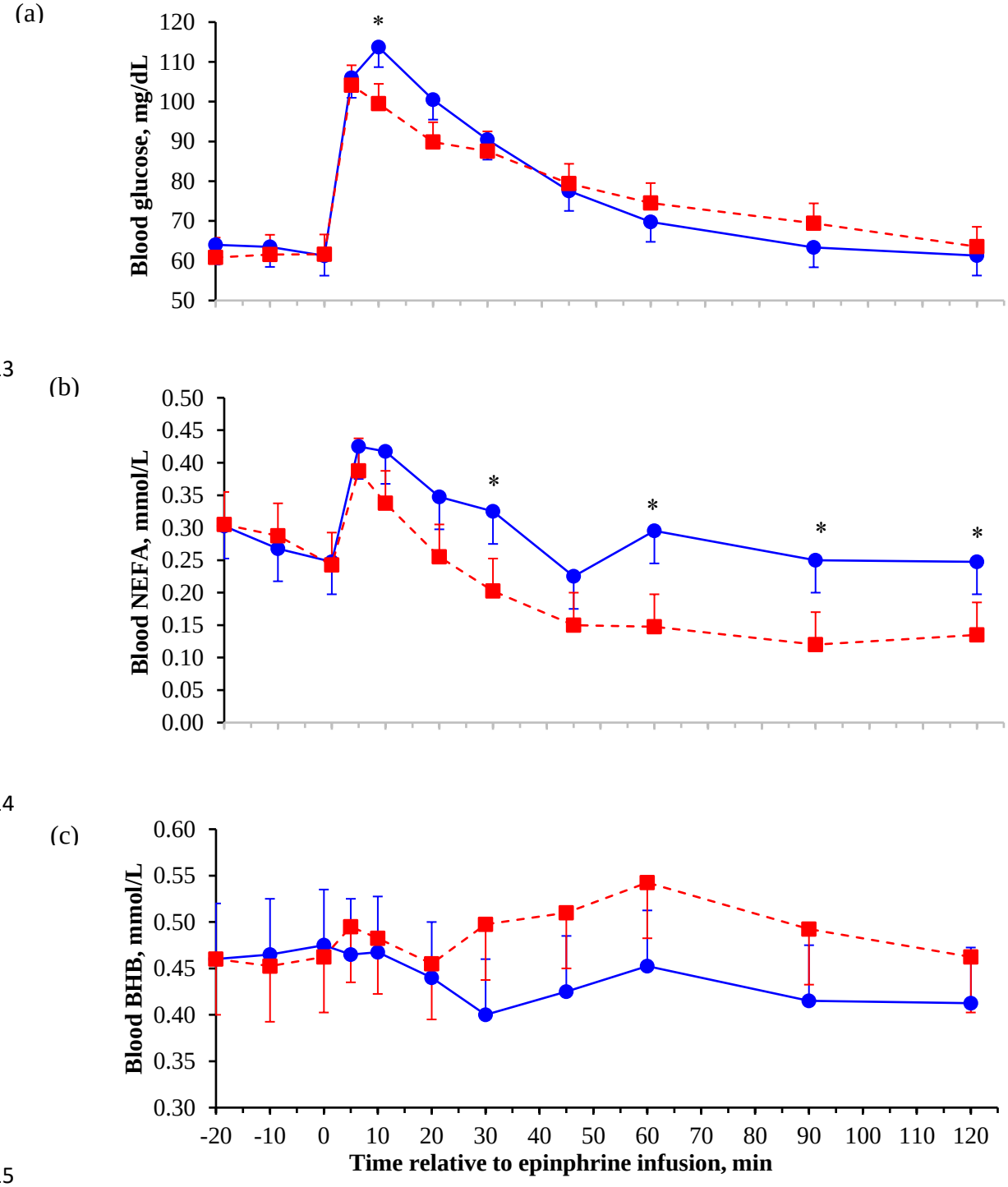
508

509 **Figure 2.**



510

Figure 3.


(a)
Blood glucose, mg/dL
120
110
100
90
80
70
60
50
*
(b)
Blood NEFA, mmol/L
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
*
*
*
*
(c)
Blood BHB, mmol/L
0.60
0.55
0.50
0.45
0.40
0.35
0.30
-20
-10
0
10
20
30
40
50
60
70
80
90
100
110
120
Time relative to epinphrine infusion, min