

Effects of different feeding time and frequency on metabolic conditions and milk production in heat-stressed dairy cows

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Abstract The aim of this paper was to evaluate the effects of three different feeding management (FM) schedules on physiological markers of heat stress (HS), metabolic conditions, milk yield and quality during the hot season in dairy cows. The study involved 27 mid-lactating cows, subdivided in three homogeneous groups differing in feeding time and frequency: total mixed ration (TMR) delivered once daily in the morning (M); twice daily, half in the morning and half in the evening (ME); once daily in the evening (E). During the trial, blood samples were collected in the morning (a.m.) and in the evening (p.m.), breathing rate (BR), rectal temperature (RT), and milk yield were recorded and individual milk samples were collected. Microclimate data indicated that cows were subjected to mild-moderate HS. During the hotter days, cows receiving M treatment showed higher values of RT (38.97 °C vs 38.68 °C and 38.62 °C, in ME and E) and BR (71.44 vs 66.52 and 65.26 breaths min⁻¹, in ME and E), a.m. plasma glucose was lower in M (3.69 vs 3.83 and 3.83 mmolL⁻¹, in ME and E) and a.m. plasma urea was lower in E (4.82 vs 5.48 and 5.35 mmolL⁻¹, in M and ME). Milk yield was unaffected by FM, as well as milk composition and cheese-making properties. Only milk protein content and yield were higher in M (3.42 vs 3.36 and 3.27 g

100 mL⁻¹; and 1.11 vs 1.08 and 1.02 kg day⁻¹, for ME and E). Our results on cow physiology indicate that M seems a less suitable FM to match cow welfare during the summer season.

Keywords Dairy cow · Feeding management · Heat stress · Metabolic conditions

Introduction

The degree of heat stress (HS) experienced by dairy cattle is commonly estimated by the temperature-humidity index (THI) a useful composite climatic welfare index for heat load, which can be calculated with formulas differing in their ability to quantify HS across climates (Bohmanova et al. 2007; Dikmen and Hansen 2009).

Dairy animals have a zone of thermal comfort, where the level of metabolic heat production is unaffected by temperature changes (Silanikove 2000). Body temperature (BT) has a circadian rhythm with a mean value of 38.3 °C and a range of excursion of 1.4 °C for mature cows in thermoneutral conditions (Piccione et al. 2003; Shehab-El-Deen et al. 2010). Similarly, Shehab-El-Deen et al. (2010) discussed the circadian rhythm and the range of excursion of the breathing rate (BR).

However, responses during HS vary according to physiological stage (Abeni et al. 2007; Calamari et al. 2007). HS has been shown to impair the productivity (West 2003), reproductive efficiency (Jordan 2003; Flamenbaum and Galon 2010; Walsh et al. 2011) and welfare of dairy cattle (Silanikove 2000) in hot humid climates. These negative effects were also observed in temperate areas such as Italy (Bernabucci et al. 2002a), with negative effects on milk characteristics (Bernabucci and Calamari 1998) and milk cheese-making properties (Calamari and Mariani 1998).

During periods of high environmental temperatures coupled with high relative humidity, dairy cows produce and accumulate a large quantity of metabolic heat, because of

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compromised (non-evaporative and evaporative) cooling capability during the hotter hours of the day (Silanikove 2000). As a consequence, particularly in high-yielding heat-stressed cows that cannot dissipate sufficient body heat, BT and BR increase and are associated with decreased dry matter intake (Aharoni et al. 2005). Furthermore, milk yield decreases and milk quality impairs as consequence of the cumulative effects of heat stress on feed intake, metabolism, and physiology of dairy cattle, reducing profitability for dairy farmers (Collier et al. 1982).

One of the primary objectives of dairy producers during the summer season is to decrease heat load to maintain dry matter intake (DMI) and milk yield. Several nutritional strategies have been developed to account for reduced DMI (Drackley et al. 2003; Adin et al. 2008, 2009; Wang et al. 2010) and the modified nutrient requirement of dairy cows during hot weather (Arieli et al. 2004; Calamari et al. 2011), but few studies have tested the response of different feeding management (FM) strategies. Some strategies for managing heat load include feeding schedules to decrease metabolic heat production and stimulate DMI (Aharoni et al. 2005; Brosh et al. 1998). Because heat production increases during and after feeding, the effects of evening feeding instead of morning feeding was tested in lactating dairy cows. Shifting the greater part of feed intake to the night hours resulted in lower energy expenditure during daytime and higher efficiency of energy utilization for milk production (Aharoni et al. 2005). Altering feeding time and/or feed amount have been shown to be beneficial in reducing HS in cattle in hot environments, but positive responses in meat (Mader and Davis 2004) and milk (Aharoni et al. 2005) production were not always obtained. Ominski et al. (2002) found that evening feeding did not decrease production losses associated with short-term, moderate HS. Nikkiah et al. (2008) reported that changing feeding time altered feeding behavior and 24-h patterns of blood metabolites, but had no effect on milk yield and protein content.

More recent results of Mäntysaari et al. (2006) confirmed that FM (five times vs once a day) had no effect on milk yield or composition. In this latter study, however, feeding strategies were not tested under thermal stress conditions. The results of Kudrna (2003) during winter showed that dairy cows may be fed once a day without any unfavorable impact on production performance and health. On the contrary, in an experiment on the effect of feeding frequency using total mixed ration (TMR) carried out by Kudrna et al. (2001) during summer, the results showed higher milk yield performance in cows offered feeds three times a day rather than twice.

HS has been reported to affect blood metabolites (Bernabucci et al. 2002b; Abeni et al. 2007; Calamari et al. 2007; O'Brien et al. 2010); however, the 24-h patterns of such metabolites under HS conditions in high-yielding dairy cows are unclear. Additionally, recent findings suggest different physiological and

metabolic adaptations during reduced DMI in HS compared with other situation where DMI is reduced, such as early postpartum (Bernabucci et al. 2010).

There appears to be little work addressing the effects of FM (frequency and time of feed delivery) on the physiology and performance of mid–high yielding dairy cows during HS periods. We hypothesized that delivery of TMR at different times during HS periods, altering the feeding behavior during 24 h, may induce variation of some blood metabolites between morning and evening. These changes could affect the availability and utilization of nutrients, influencing the metabolic stress experienced by dairy cows.

Therefore, the objective of the present study was to evaluate how different FM, either number and time of feed delivery, might affect physiological response and performances during the summer season in mid-lactating dairy cows.

Materials and methods

Animal and management conditions

The research protocol and animal care were in accordance with EC Council Directive guidelines for animals used for experimental and other scientific purposes (European Community 1986). All cows were inspected daily and records of all health-related problems and incidents that occurred throughout the trial, or any treatment administered were kept.

The Italian Friesian dairy cows involved in this study were raised in a free stall barn at the experimental farm “Vittorio Tadini”, located near Piacenza (45°01'N, 9°40'E; altitude 68 m a.s.l.), Italy. Cows were housed in pens, consisting of a resting area with cubicles, self-locking head gates at the feed bunk, with free access to fresh potable water and an unshaded hard court paddock outside. The pens were not equipped with cooling systems. Cows were group-fed and the daily amount of TMR offered and orts collected were recorded. The animals were fed according to their requirements for maintenance and milk production. The diet consisted of alfalfa hay and haylage, corn silage, cereals, concentrates, and vitamin and mineral premixes (Table 1). To ensure cows had ad libitum access to the TMR, the amount offered to the cows was assessed on a daily basis with the aim of producing a 3 % to 8 % refusal.

Cows were milked in an auto-tandem milking parlour twice daily (0400 and 1500 hours).

Experimental design

Twenty-seven healthy dairy cows, selected from the herd, were blocked into three homogeneous groups (nine cows each) according to parity (2.33 ± 1.50 ; 2.44 ± 2.07 , and 1.88 ± 1.17), energy corrected milk yield (ECM: 34.9 ± 5.6 ; $35.8 \pm$

Table 1 Average composition, chemical analysis and nutritive value of total mixed ration (TMR) basal diet fed to dairy cows during the trial. DM Dry matter, NDF neutral detergent fiber, NE_l net energy for lactation

Item	% of DM
Ingredient	
Corn silage	35.1
Alfalfa hay	13.3
Alfalfa haylage	10.0
Cereals ^a	19.1
Proteic concentrate ^b	15.0
Fibrous concentrate ^c	7.5
Nutrient	
Crude protein	16.3
Ether extract	2.8
NDF	34.2
NDF from forages	25.1
Starch	21.6
NE _l ^d , Mcal kg ⁻¹ DM	1.53

^a Cereal concentrate mix was based on barley, wheat by-products, corn meal, and corn by-products

^b Proteic concentrate was based on roasted soybean meal, dried brewers grains, linseed cake, beep-pulp, and corn gluten meal; 1 kg was integrated with: 200 mg Mn; 10 mg Fe; 15 mg Cu; 3 mg Co; 10 mg I; 400 mg Zn; 0.1 mg Se; 80,000 IU vitamin A; 4,000 IU vitamin D₃; 40 mg vitamin E (alpha-tocopherol 91 %); 10 mg vitamin B₁; 12 mg vitamin B₂; 18 mg vitamin B₆; 0.02 mg vitamin B₁₂; 100 mg niacin; and 500 mg DL methionine. Macro minerals were supplied using CaCO₃; NaCaMgH(PO₄)₂; NaHCO₃; NaCl; MgO and S

^c Fibrous concentrate was based on wheat middlings, beep-pulp, coconut meal, sunflower meal, and sugar cane molasses

^d Calculated according to NRC (2001)

8.6; and 34.0±11.6 kg day⁻¹) and days in milk (DIM; 126.7±45.8; 116.7±46.8; and 105.8±46.0 d) in group A, B, and C, respectively. The primiparous: multiparous (≥2 lactation) ratio was 4:5, 5:4, and 5:4 in group A, B, and C, respectively.

The study was conducted as a randomized complete block design, in which all cows were exposed to the same environmental temperature and humidity. They received the same diet, differing only in the time (morning and/or evening) and number (a single distribution or two distributions) of the daily TMR delivery during the treatment period.

Three periods were identified: period 1 (P1; pre-experimental period) from 7 June to 3 July; period 2 (P2; experimental period), from 4 July to 20 August, characterized by higher THI; and period 3 (P3; post-experimental period), from 21 August to 5 September. During P1 and P3, dairy cows were fed ad libitum the same TMR delivered once daily in the morning (at 0730 hours). During P2, the three groups were assigned randomly to one of three treatments consisting of different FM system: TMR delivered ad

libitum once daily in the morning (group A, treatment M; at 0730 hours); TMR delivered ad libitum twice daily, half in the morning and half in the evening (group B, treatment ME; at 0730 and 1800 hours); TMR delivered ad libitum once daily, but in the evening (group C, treatment E; at 1800 hours). Fresh TMRs were prepared twice daily and offered to the cows based on the treatment schedule.

Response variables were recorded on two sampling point in P1 (control T₁ and T₂), four in P2 (T₃, T₄, T₅, and T₆) and one in P3 (T₇) (Fig. 1). At each sampling point, data from rectal temperature (RT), BR, and milk yield were recorded; while blood and milk samples were collected.

Measurements

Microclimatic conditions

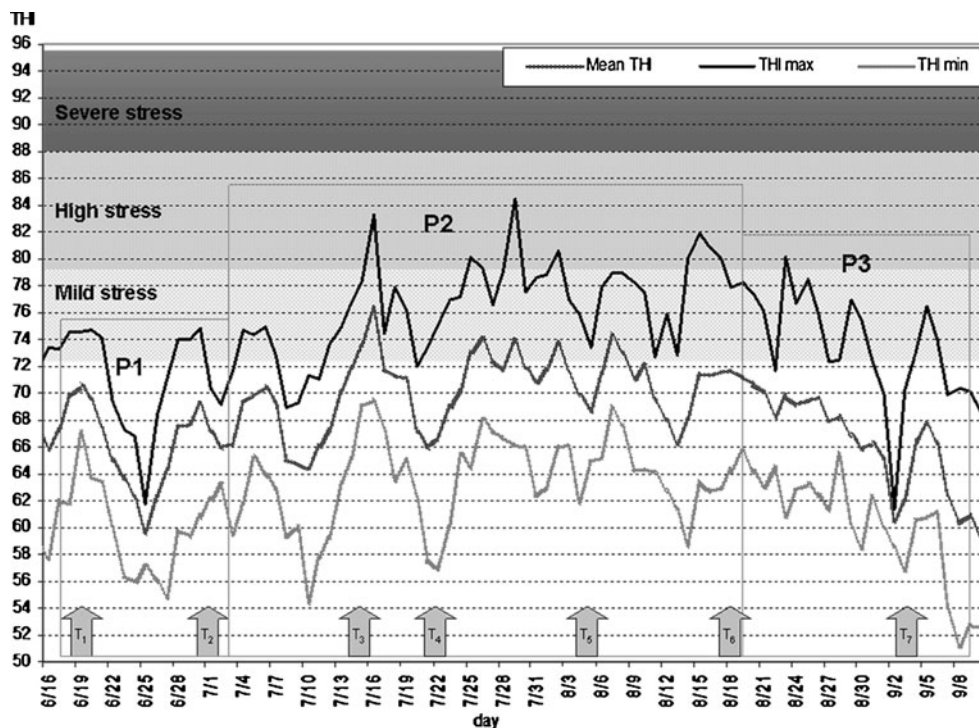
Temperature and relative humidity of the inside barn were recorded daily during the study period using two electronic probes (Gemini Data Logger, Chichester, UK) connected to a data logger programmed to record every 10 min. Mean, minimum and maximum daily values for air temperature and humidity were recorded throughout the trial. Data were used to compute a composite climatic welfare index, the THI, according to Kelly and Bond (1971), as cited in Ingraham et al. (1979). Mean, minimum and maximum daily values for THI were calculated throughout the trial and HS was estimated according to Armstrong (1994).

Feed samples and feed intake

Representative samples of hay, silage, and concentrates were taken weekly during the study and pooled on a period basis for the analyses. The TMR and refusal samples were collected weekly to determine their dry matter (DM). Refusals were weighed daily and removed before feeding (0700 hours in M and ME; and 1730 hours in E). Daily feed intake (kg DMI cow⁻¹ day⁻¹) was calculated by subtracting the weighed refusal (DM basis) from the total fresh TMR offered (DM basis), divided per nine animals. Then, for each week, a mean daily feed intake was calculated. The DM content of the TMR and refusal for that week was applied to estimate the daily DMI on a weekly basis.

Feedstuffs were analyzed for DM, crude protein (CP), ether extract, ash, neutral detergent fibre (NDF), and starch content (AOAC 1990); NDF was analyzed with a heat stable amylase with correction for residual ash, according to the method of Mertens (1997), without sodium sulfite and using an Ankom 2200 (Macedon, NY) for extraction and filtering. The net energy for lactation (NE_l) content of feedstuffs was estimated according to NRC (2001). Energy balance was estimated according to NRC (2001) and nitrogen balance by the equations proposed by Jonker et al. (1998) as modified by Calamari et al. (2006).

Fig. 1 Daily mean, minimum and maximum temperature-humidity index (THI) and the potential for heat stress during the trial. The *arrows* on each figure indicate the sampling points



Rectal temperature and breathing rate

Rectal temperature and BR were used as indicators of HS and were recorded just before the evening blood sampling (1700 hours). For measurement of RT, a digital thermometer was used. The BR of cows was registered by counting flank movements during two 30 s cycles.

Blood sampling and analysis

At each sampling point, blood samples were taken from all cows at 0700 hours (before TMR distribution) and immediately after the afternoon milking (1700 hours). The samples were collected by venipuncture from the jugular vein into a 10-mL Li-heparin treated tubes (Vacuette, containing 18 IU Li-heparin mL⁻¹, Kremsmünster, Austria), immediately placed into an ice-bath and centrifuged (3,500 g for 15 min at 10 °C) within 1 h after collection. The plasma was collected and stored at -20 °C until analysis.

In each plasma sample, the concentrations of metabolites were analyzed at 37 °C by an automated clinical analyzer (Monarch, Instrumentation Laboratory, Lexington, MA). Analysis for glucose, urea, creatinine, total cholesterol, and triglycerides were conducted using commercial kits (Instrumentation Laboratory), non-esterified fatty acids (NEFA) using Wako Chemicals kits (Wako, Neuss, Germany), and β-hydroxybutyric acid (BHBA) using Ranbut kit (Randox, Crumlin, Co. Antrim, UK). All measurements were carried out according to the manufacturer's instructions, and adapted to the clinical analyzer's operative procedures as described by Bertoni et al. (1998).

Milk sampling and analysis

At each sampling point, during the p.m. milking, milk yield was measured and individual samples were collected from each cow. Fat, protein, and lactose contents were determined on fresh samples by the infrared technique (Milkoscan 133B, Foss Electric, Hillerød, Denmark). Somatic cell count (SCC) was determined by an optofluorometric method (Fossomatic 5000, Foss Electric); pH using a pHmeter, and titratable acidity (TA) was measured using an automatic titration system (MicroTT 2050 Titrator, Crison, Barcelona, Spain). Natural creaming (NC) of milk fat, an important step in cheese-making process for long-ripening cheese, was assessed employing the method suggested by Speroni and Bertoni (1984), described briefly as follows. Milk was analyzed for fat content, then 10 mL were allowed to cream in a plastic tube at 15 °C for 180 min. The surface layer of fat was removed, and 2 mL collected from the bottom of the tube was analyzed for fat content. NC was expressed as the percentage of the removed fat (initial fat content - final fat content) on the initial fat content, and calculated using: $NC (\%) = (\text{initial fat } \% - \text{final fat } \%) / \text{initial fat } \% \times 100$.

Furthermore, milk rennet coagulation properties were assessed as clotting time (r), curd firming rate (k_{20}), and curd firmness (a_{30}), using Formagraph (Foss Electric), where 10 mL milk was heated to 35 °C, and 200 μL rennet (Hansen standard 190 with 63 % chymosin and 37 % pepsin, Pacovis Amrein, Bern, Switzerland) diluted to 1.6 % (w/w) in distilled water was added to milk.

Yield of energy-corrected milk (ECM) was calculated according to the formula of Smith et al. (2002).

Statistical analysis

Results were analyzed using the MIXED models procedure (SAS 1999) according to Littell et al. (1998). Sources of variation included FM (three levels), time (sampling time within P2), and FM×time interaction. The random variable was cow within treatment. Values from plasma analysis were processed separately according to sampling moment of the day (morning or afternoon). The data from SCC were log transformed before their submission to statistical analysis because they were not distributed normally ($W=0.37$), according to the Shapiro-Wilk test (SAS 1999). Data collected during P1 (mean of the T_1 and T_2 sampling point) were used as covariate for each variable. Data were evaluated using a mixed model, taking into account the repeated measures in time on each cow; five covariance structures were tested for each item: simple, compound symmetry, first-order autoregressive, first order ante dependence, and unstructured (Littell et al. 1998) to choose (according to the Akaike's Information Criteria) the one that best fit the data (SAS 1999).

Results are presented in tables as least square means and standard error of the mean (SEM) of the P2 period (control T_3 , T_4 , T_5 and T_6). Orthogonal contrasts were made between any treatment mean during the last two control points of the P2 period (T_{5-6} : hotter phase) as follows: (1) M vs ME, (2) M vs E, and (3) ME vs E to evaluate the effects of feeding management. Differences were considered significant when $P<0.05$; a trend was considered when $P<0.10$.

Results

Microclimatic conditions

The course of daily minimum, mean and maximum THI during the trial period is shown in Fig. 1. The microclimatic conditions indicate that, during P2 and P3, the cows experienced maximum daily THI above 72 (mild HS).

For the duration of P1 and P3, the mean daily THI was below the upper critical value of 72. During P2, daily mean THI values were greater than 72 (for 19 % of the days) and cows were exposed to maximum THI above 79 (for 21 % of the days).

Diet characteristics and feed intake

Chemical and nutritive characteristics of the TMR used throughout the experiment are reported in Table 1.

The mean feed intake checked on a group basis during P2 was 23.05, 24.52, and 23.30 kg DM head⁻¹ day⁻¹ in M, ME, and E respectively (SEM = 0.09, $P<0.05$). The NDF content of the diets was greater than the minimum suggested by the NRC (2001), with an adequate proportion of NDF from forage to maintain proper ruminal fluid pH. The energy

balance during the experimental period was -2.2 (SEM= 1.1); +1.2 (SEM=0.8); and +1.2 (SEM=1.0) Mcal day⁻¹ in M, ME, and E, respectively. The estimated N balance was positive in ME (16 g day⁻¹; SEM=8.0) and E (24 g day⁻¹; SEM=14.0) feeding management; conversely, in M treatment it was slightly negative (-8 g day⁻¹; SEM=8.0).

Rectal temperature and breathing rate

The mean values of RT and BR during P2 (average T_{3-6} values) are shown in Table 2. Besides a time effect ($P<0.001$), an effect of FM was detected for both RT and BR, being values higher in M compared with ME and E cows ($P<0.05$).

Orthogonal contrasts showed significant differences for RT only, as these values were higher in group M (38.97 °C) compared to ME (38.68 °C; $P<0.05$) and E groups (38.62 °C; $P<0.01$).

The trend of acclimation during the trial relative to RT and BR of the cows is depicted in Fig. 2. During P2, the values increased gradually and, as expected, the highest RT and BR values were registered at T_5 and T_6 , respectively. The RT was greater in M compared with ME ($P>0.05$ at T_4 and $P<0.05$ at T_6), and E ($P<0.05$ at T_4 and $P<0.01$ at T_5), and reached a maximum mean value of 39.02 °C at T_5 . As regards BR, during P2, values were higher in group M compared with ME ($P>0.05$ at T_3 and $P<0.001$ at T_4) and E ($P<0.05$ at T_4 and T_5). Group M showed a quick increase of BR values from T_3 to T_6 , and reaching a maximum mean value of 70.89 breaths min⁻¹ at T_6 ; conversely, BR increased in group ME and E only at T_5 and T_6 .

During P3, a reduction in RT and BR (on average 38.47 °C and 52.6 breaths min⁻¹, respectively) was observed and no differences were found between groups.

Blood: energy and protein metabolism

Least square means of plasma metabolites related to energy and protein metabolism during the entire P2 (average T_{3-6} values) are shown in Table 3. The FM had no significant effect on plasma metabolite concentration in the morning samples during the whole P2 period; on the contrary, it affected ($P<0.05$) BHBA, urea and creatinine concentration in the evening samples. Both time and FM×time interaction affected plasma cholesterol and creatinine (a.m. sampling), and glucose, NEFA, and urea concentration in the a.m. and p.m. sampling.

Orthogonal contrasts used to determine FM effect during the hotter phase (T_{5-6}), showed significant differences between groups. The FM affected the concentration of plasma metabolites as follows: glucose lower in M than ME and E in the a.m. samples (3.686 vs 3.835 and 3.832 mmolL⁻¹, respectively; $P<0.05$); BHBA higher in ME than M (0.404 vs 0.337 mmolL⁻¹; $P<0.05$) and E (0.341 mmolL⁻¹; $P>0.05$) in the a.m. samples, and lower in E than M and ME (0.346 vs

Table 2 Least squares means of the physiological variables observed during the entire experimental period (P2, mean of T₃₋₆)

Variable	Feeding management ^c			SEM ^d	P value ^e		
	M	ME	E		FM	T	FM x T
Rectal temperature (°C)	38.70 b*	38.48 a*	38.44 a*	0.067	0.026	<.0001	0.103
Breathing rate (n°/60 s)	65.78 Bb*	56.64 Aa*	57.58 ABa*	2.230	0.016	<.0001	–

*A, B: $P < 0.01$; a,b: $P < 0.05$

^c Dietary treatment were: TMR offered in the morning (M), in the morning and in the evening (ME), or in the evening (E)

^d The highest standard error of the mean

^e Probabilities of significance for feeding management (FM), time (T) and their interaction

0.455 and 0.459 mmolL⁻¹, respectively; $P < 0.01$) in the p.m. samples; cholesterol higher in ME than M in the a.m. samples (6.784 vs 6.331 mmolL⁻¹). Total cholesterol (a.m. samples) had a trend to be higher in ME, but no difference was observed (at that time) for triglycerides and NEFA.

The behavior of the plasma metabolites related to energy metabolism (glucose and NEFA contents) during the trial in the a.m. and p.m. sampling is depicted in Fig. 3.

Cows fed the diet during the morning (M) had a.m. plasma glucose values (Fig. 3a) similar to those fed in the evening (E), but higher compared to the ME cows at T₄ (3.833 vs 3.650 mmolL⁻¹; $P < 0.05$); afterwards the value decreased, and at T₅ it was significantly lower than E (3.684 vs 3.904 mmolL⁻¹; $P < 0.05$), whereas at T₆ it was lower than ME (3.671 vs 3.867 mmolL⁻¹; $P < 0.05$); during P3 glucose concentration increased in all groups without significant differences. The lowest plasma glucose levels in the p.m. sampling were observed during P2 in the E group compared to ME and M groups at T₃ (3.488 vs 3.632 and 3.621 mmolL⁻¹, respectively; $P < 0.05$) and in the ME and E groups compared to M group (3.494 and 3.473 vs 3.707 mmolL⁻¹; $P < 0.05$) at T₄, after which levels remained low in all groups without significant differences until P3.

Within the experimental period (P2), at control time T₄, plasma NEFA values in a.m. sampling resulted higher in ME schedule (0.064 mmolL⁻¹) when compared to M (0.046 mmolL⁻¹; $P > 0.05$) and E (0.032 mmolL⁻¹; $P < 0.01$). In the p.m. sampling (Fig. 3d) differences among groups were found only at T₃, NEFA values being lower in E (0.026 mmolL⁻¹) than in M and ME cows (0.044 and 0.040 mmolL⁻¹; $P < 0.05$).

Orthogonal contrasts of plasma variables related to protein metabolism, used to determine FM effect during the hotter phase (T₅₋₆), showed an FM effect: urea lower ($P < 0.05$) in E than M and ME in both the a.m. (4.821 vs 5.484 and 5.349 mmolL⁻¹, respectively), and p.m. samples (5.119 vs 5.591 and 5.546 mmolL⁻¹, respectively); and creatinine lower in M compared to ME and E (87.45 vs 91.14 and 91.25 μmolL⁻¹, respectively; $P < 0.01$) in the p.m. samples.

Changes in plasma urea in a.m. and p.m. samples are reported in Fig. 4. The main differences among FM were observed at T₅ (both samplings), when E cows had lower ($P < 0.01$) values than M and ME.

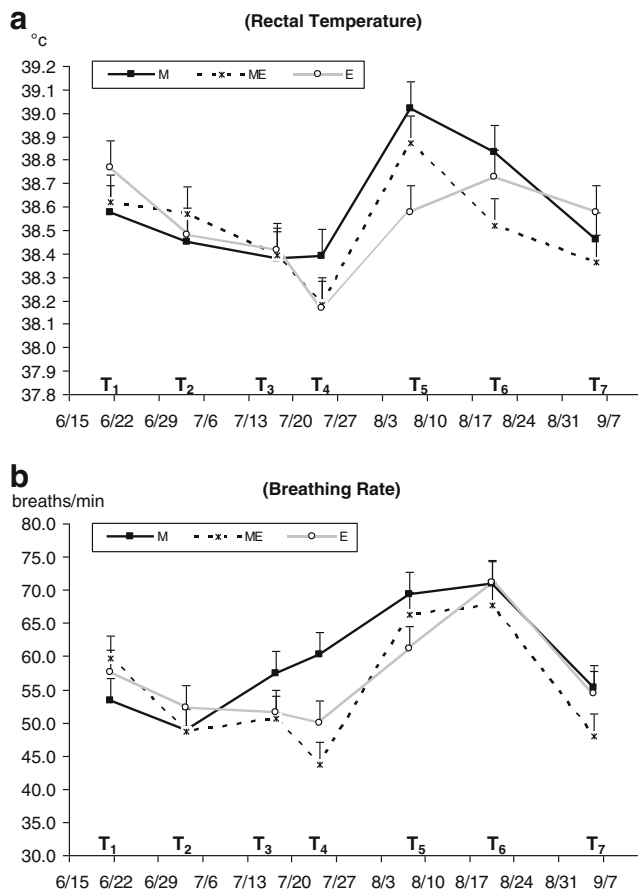


Fig. 2 Rectal temperature (RT; a) and breathing rate (BR; b) recorded at each sampling point: T₁–T₂ pre-experimental period (P1); T₃–T₆ experimental period (P2) with different feeding management being total mixed ration (TMR) distributed in the morning (group M), in the morning and in the evening (group ME) or in the evening (group E); and T₇ post-experimental period (P3). Values are least squares means (not covaried data); vertical bars represent SEM

Table 3 Least squares means of some plasma metabolites observed during the entire experimental period (P2, mean of T₃₋₆). NEFA Non-esterified fatty acids, BHBA β-hydroxybutyric acid

Variable	Feeding management ^c			SEM ^d	P value ^e		
	M	ME	E		FM	T	FM x T
Glucose, mmol/L ^f	3.775	3.827	3.829	0.034	–	0.004	0.002
Glucose, mmol/L ^g	3.544	3.518	3.467	0.042	–	0.001	0.005
Cholesterol, mmol/L ^f	6.463	6.776	6.616	0.157	–	<.0001	0.021
Triglyceride, mmol/L ^f	0.070	0.071	0.071	0.004	–	0.104	–
NEFA, mmol/L ^f	0.045	0.054	0.043	0.005	–	–	0.049
NEFA, mmol/L ^g	0.041	0.046	0.044	0.003	–	<.0001	0.034
BHBA, mmol/L ^f	0.323	0.376	0.341	0.023	–	–	–
BHBA, mmol/L ^g	0.477 B*	0.453 B*	0.383 A*	0.018	0.002	–	–
Urea, mmol/L ^f	5.563	5.419	5.081	0.142	0.068	<.0001	<.0001
Urea, mmol/L ^g	5.638 bAB*	5.762 Bb*	5.307 aA*	0.108	0.018	<.0001	0.006
Creatinine, μmol/L ^f	89.43	92.38	90.13	1.040	–	0.002	<.0001
Creatinine, μmol/L ^g	87.83 Aa*	90.37 ABb*	91.22 Bb*	0.737	0.009	<.0001	–

*A,B: $P < 0.01$; a,b: $P < 0.05$

^c Dietary treatments were: TMR offered in the morning (M), in the morning and in the evening (ME), or in the evening (E)

^d Highest standard error of the mean

^e Probabilities of significance for feeding management (FM), time (T) and their interaction

^f a.m. sampling point

^g p.m. sampling point

Milk yield and renneting properties

Table 4 reports results on milk yield, milk components relative to the p.m. milking, and coagulation features obtained from the three feeding schedule during the entire P2 (average T₃₋₆ values).

Time affected almost all milk parameters. Feeding management (average values of T₃₋₆) had no effect on milk yield, SCC, and milk components; ECM was numerically ($P > 0.05$) higher in M and ME cows than E cows, and milk protein (content and yield) values were significantly higher ($P < 0.05$) in M compared to ME and E cows.

Orthogonal contrasts (mean values of T₅₋₆) evidenced a trend for higher ECM value in ME than E (32.46 vs 30.39 kg; $P > 0.05$), while for protein content showed significant differences between groups, values being higher in M milk (3.50 g 100 mL⁻¹) than ME and E milk (3.38 and 3.31 g 100 mL⁻¹; $P < 0.05$); whereas protein yield was significantly lower in E (0.99 kg day⁻¹) than ME (1.05 kg day⁻¹; $P > 0.05$) and M (1.07 kg day⁻¹; $P < 0.05$) cows.

Cheese-making features (natural creaming, pH, titratable acidity, and the clotting features r , k_{20} , and a_{30}) were not affected by FM, but interactions between FM and time were evidenced only for natural creaming and clotting features. However, the orthogonal contrasts did not underline significant effects during T₅₋₆ sampling time.

Discussion

According to Armstrong (1994), cows in our study were subjected to mild to high HS during P2. In the present study, it was not possible to record individual feed intake; however, DMI on a group basis evidenced higher values in ME cows. Aharoni et al. (2005) reported higher DMI in cows fed during the day compared to night hours. Brosh et al. (1998) demonstrated how eating during the cooler hours of the night was beneficial to the cows during the hot season, because the postprandial heat loads was not added to the environmental heat load of the hotter hours. This was especially true for animals on a high-energy diet, the general situation of lactating dairy cows. Nikkhah et al. (2008) observed how changing the feed delivery time from 0900 to 2100 h increased DMI within 3 h after feeding, but it did not affect daily DMI. One possible explanation of our observation could be related to the availability of fresh feed as, after feed delivery, or to the typical behavior at the return from milking, which stimulates feeding activity (DeVries et al. 2003). Additionally, the availability of the feed over time permits to dairy cows to eat whenever they want and could encourage animals to spend more time eating, distributing intake over the course of the day and maintaining a stable rumen microbial population (Nocek and Braund 1985).

The general homeostatic response to HS in dairy cattle includes increased respiration rates, panting, drooling,

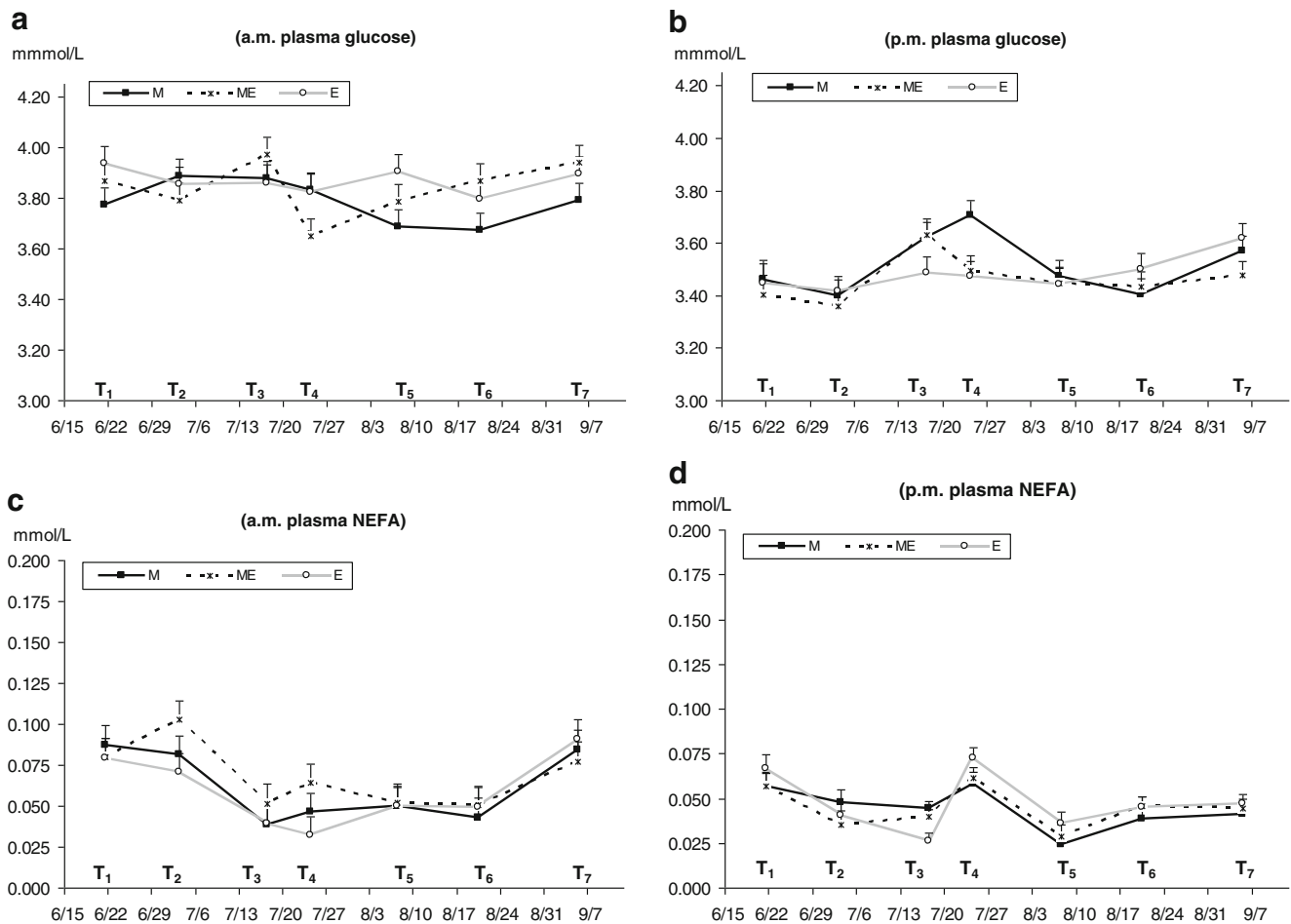


Fig. 3 Plasma glucose (**a** morning samples, **b** evening samples) and non-esterified fatty acids (NEFA) (**c** morning samples, **d** evening samples) content recorded at each sampling point: T₁–T₂ pre-experimental period (P1); T₃–T₆ experimental period (P2) with

different feeding management being TMR distributed in the morning (group M), in the morning and in the evening (group ME) or in the evening (group E); and T₇ post-experimental period (P3). Values are least squares means (not covaried data); vertical bars SEM

reduced heart rates, decreased feed intake and milk yield. The RT can be used as indicator of physiological response to thermal stress in dairy cows, because it is nearly constant under normal conditions and a rise is associated with reduced performance. In order to maintain BT within a normal range, cows dissipate heat by vaporization of water from the body surface and lungs.

From our results, BR was higher in the M group throughout P2. During the hotter period of the current trial, only a trend to differ between M and E was reported as a result of the lower number of observation in a phase where all the cows were exposed to a high heat load, so that the ANOVA did not achieve higher significance. In their analysis of HS indicators, Brown-Brandl et al. (2005) concluded that BR is the most appropriate indicator of thermal stress. The time of BR assessment in the present study underlined how, in the hottest part of the day, cows with the M feeding schedule probably reached a peak of endogenous metabolic heat production at that time, as confirmed also by RT. The

increase in BR can be associated with the attempt to dissipate this metabolic heat excess. Differently, Ominski et al. (2002) found that evening feeding did not result in different BR or vaginal temperature compared to a morning feeding schedule.

Both RT and BR can be used as indicators of HS, so that estimates of meteorological variables associated with RT should prove valuable in relating environmental conditions to the magnitude of hyperthermia experienced by heat-stressed cows (Dikmen and Hansen 2009). Evening and ME feeding schedules appeared to be beneficial in lowering mean RT and BR, and in maintaining DMI, so may be a valuable method to alleviate the thermoregulatory limitations of a hot climate.

A negative effect of THI on plasma glucose was observed in a previous study in heat-stressed cows (Abeni et al. 2007). This result can be explained by different factors: decreased energy intake, as consequence of the reduction in DMI; increased cost of thermoregulation, and negative

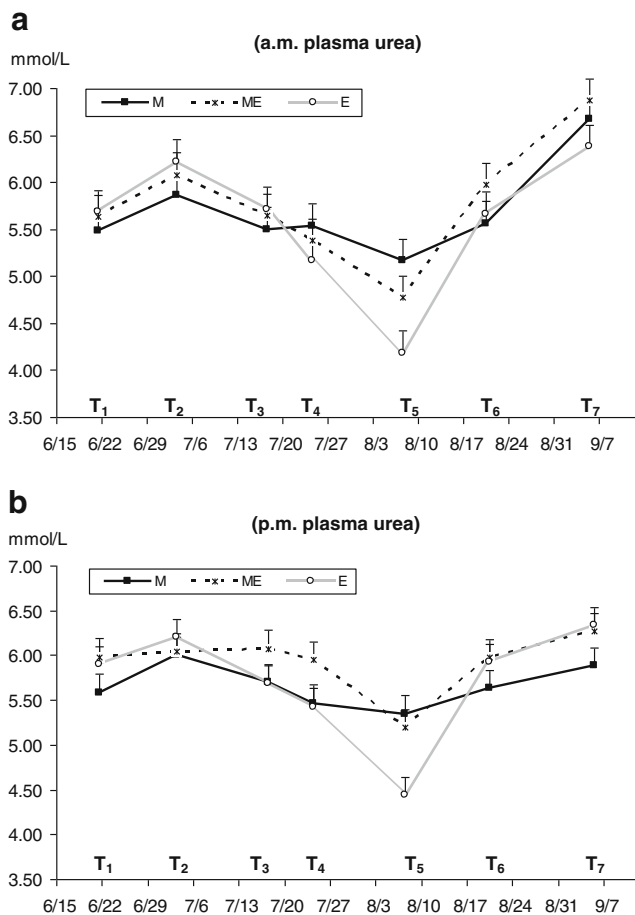


Fig. 4 Plasma urea (**a** morning samples, **b** evening samples) content recorded at each sampling point: T₁–T₂ pre-experimental period (P1); T₃–T₆ experimental period (P2) with different feeding management being TMR distributed in the morning (group M), in the morning and in the evening (group ME) or in the evening (group E); and T₇ post-experimental period (P3). Values are least squares means (not covaried data); vertical bars SEM

effect of heat on gluconeogenesis, as an endocrine acclimation to hot conditions. Differences in plasma glucose concentration in the FM schedule during the hotter period (T₅–T₆) must be discussed considering the time relative to TMR delivery. Shehab-El-Deen et al. (2010) reported how the circadian rhythm of plasma glucose during HS was characterized by lower values in the hottest hour of the day. In p.m. samples from our trial, no difference was detected among cows receiving the FM schedule, while a.m. sampling time evidenced lower plasma glucose in cows fed according to the M system than in the other two FM groups. The apparent discrepancy between the a.m. and p.m. results for plasma glucose probably relies on the interaction of the experimental treatment (FM) with the circadian rhythm for this metabolite (Giannetto and Piccione 2009; Shehab-El-Deen et al. 2010).

Nikkhah et al. (2008) reported effects of feed delivery time on the 24-h patterns of blood metabolites—essentially due to the greater feed intake during the 3 h after feed delivery of cows

fed at 2100 hours—but daily average of glucose, urea, BHBA, and NEFA in peripheral blood were not affected by feeding schedule. The lower values of plasma NEFA in the morning samples during HS period (in P2 compared to P1 and P3) agrees with recent findings (Bernabucci et al. 2010; Calamari et al. 2011). Bernabucci et al. (2010) clearly showed that acclimation to HS involves hormonal and metabolic changes, as a decline in anabolic hormones and an increase in the catabolic ones, that alter energy balance. As response to reduced feed intake and consequent negative energy balance, insulin levels decrease allowing for adipose lipolysis and increase of circulating NEFAs. Conversely, heat-stressed cows, despite inadequate nutrient intake, exhibit increased basal insulin levels and lack of increase of NEFA levels. In the current study, the reduction of NEFA during HS was not affected by feeding management.

An interesting result was that on plasma urea, with lowest values in E both in the a.m. and p.m. samples. Plasma urea concentration is the result of the balance between urea uptake (from the gastrointestinal tract and from liver metabolism) and excretion (urine, milk). Obitsu et al. (2011) reported higher plasma urea concentration in heat-stressed cows as a result of a reduced plasma urea clearance from the gastrointestinal tract associated with a reduced DMI (see also Kennedy and Milligan 1980). We did not monitor meal size and frequency; however, one possible hypothesis to explain the lower plasma urea in E could rely on a better use and recycling of this molecule in the digestive tract due to different meal patterns throughout the 24 h.

Recently, O'Brien et al. (2010) discussed how the heat-induced increase in plasma urea may be considered the result of inefficient rumen microbial nitrogen incorporation, because HS can alter rumen fermentation patterns (Schneider et al. 1988). The lower plasma urea concentration in E seems to suggest that FM schedule may improve nitrogen utilization in the rumen, probably as consequence of greater DMI during nighttime, when adverse effects from HS are reduced. In this case, we exclude a role of a different muscle proteolysis, because we reported low plasma creatinine just in M, where plasma urea was higher than E. In studies by Abeni et al. (2007) and Obitsu et al. (2011), plasma creatinine was higher in HS conditions than under no HS. The current study evidences an effect of FM on this metabolite during a HS period, particularly in the p.m. samples, where the M schedule had lower values than the other two groups.

Aharoni et al. (1999) reported how milk protein content was unaffected by heat load, but the study of Cincović et al. (2010) showed a significantly lower heat-induced milk yield, milk fat and protein percent in the middle of lactation. Ominski et al. (2002) found that evening feeding did not result in different milk yield and composition, except for a reduction in milk fat content when compared to morning feeding schedule. In Nikkhah et al. (2008), the feeding schedule did not affect milk yield and milk protein content, but there were trends for increased milk fat content and milk fat yield in multiparous cows

Table 4 Least squares means of milk yield and milk characteristics observed during the entire experimental period (P2, mean of T₃₋₆). ECM Energy corrected milk yield, SCC somatic cell count

Variable	Feeding management ^c			SEM ^d	P value ^e		
	M	ME	E		FM	T	FM x T
Milk yield, kg /day	33.10	32.31	31.71	0.591	–	<0.001	–
ECM ^d , kg /day	33.16	33.03	31.30	0.675	–	0.007	–
Fat, g /100 mL	3.419	3.567	3.475	0.144	–	<0.001	–
Fat yield, kg /day	1.134	1.159	1.072	0.046	–	0.151	–
Protein, g /100 mL	3.425 b*	3.364 ab*	3.271 a*	0.040	0.035	<0.001	0.013
Protein yield, kg /day	1.115 Bb*	1.083 b*	1.024 aA*	0.017	0.003	<0.001	–
Fat/protein ratio	1.003	1.073	1.051	0.039	–	–	–
Lactose, g /100 mL	5.227	5.248	5.203	0.026	–	–	–
SCC, log (n° /10 ³ mL)	1.713	1.683	1.800	0.111	–	0.004	–
Natural creaming, %	49.61	49.00	51.76	2.020	–	–	0.001
pH	6.695	6.683	6.687	0.009	–	<0.001	–
Titratable acidity, °SH/50 mL	3.362	3.370	3.407	0.041	–	<0.001	–
r, min	23.82	22.24	22.98	0.664	–	0.041	0.018
k ₂₀ , min	16.46	14.04	17.39	1.169	–	–	0.018
a ₃₀ , mm	12.34	15.36	12.20	1.557	–	–	0.011

*A,B: $P < 0.01$; a,b: $P < 0.05$

^c Dietary treatments were: TMR offered in the morning (M), in the morning and in the evening (ME), or in the evening (E)

^d The highest standard error of the mean

^e Probabilities of significance for feeding management (FM), time (T) and their interaction

fed in the evening. In the present study, fat content of the p.m. milking and milk fat yield were not affected by FM, and the fat-to-protein ratio, an indirect signal of possible changes in feeding and rumen activity, also did not differ among different FM regimes in HS.

Our results on milk composition, even if relative to the p.m. milking only, agree with those from Mäntysaari et al. (2006), confirming, with the exception only of milk protein content, how feeding frequency seems not to affect, milk composition per se, although it affects feeding efficiency generally.

In our previous works (Calamari and Mariani 1998; Calamari et al. 1997), we reported the negative effect of HS on those features of special interest for cheese-making activity in Italy, particularly for products that rely for their quality on milk processing (natural creaming and clotting) and successive cheese ripening. In the current study, the titratable acidity was around the minimum value of the optimal range and poor rennet clotting properties were recorded. The impairment of these characteristics makes the milk less suitable for the production of hard cheese with a long ripening time. These poor characteristics were observed in all three groups, without effect of FM.

Bernabucci et al. (2002a) demonstrated that HS decreases milk α_s - and β -casein concentration, also resulting in an increased percentage of whey protein of the total milk protein level, suggesting that HS can affect protein synthesis

in the mammary gland. The lower protein content of E milk (p.m. milking) seems not to agree with a better nitrogen utilization as evidenced by lower plasma urea levels; however, the hypothesis of a reduced mammary protein synthesis had no adverse effect on clotting properties of E milk, considering the important role of casein fractions in the milk clotting process (Calamari and Mariani 1998).

Conclusion

Considering the three different feeding strategies evaluated in the present study to counteract HS, we can conclude that M—the most common feeding schedule used in dairy farm in Italy—seems the least suitable method to match cow welfare. Compared with those fed in the evening or either in the morning and evening, cows under the M regime experienced greater HS, with greater increase of RT and BR.

Cows receiving the diet once a day in the morning (M) showed more pronounced alteration of blood variables related to energy metabolism, with impairment of energy balance. Blood variables related to protein metabolism were also affected by FM, with lower urea values in cows fed in the evening. Although milk yield and milk characteristics were not affected by FM, some metabolic variations were observed and the estimated energy and nitrogen balance

suggested a favorable effect of fresh feed availability during the evening on physiological aspects of animal welfare

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