ABSTRACT

Dairy cows during their first and second lactation have different milk yield, body development, feed intake, and metabolic and endocrine statuses. However, large diurnal variations can also exist in terms of biomarkers and hormones related to feeding behavior and energy metabolism. Thus, we investigated the diurnal patterns of the main metabolic plasma analytes and hormones in the same cows during their first and second lactations in different stages of the lactation cycle. Eight Holstein dairy cows were monitored during their first and second lactation, during which they were reared under the same conditions. Blood samples were collected before the morning feeding (0 h) and after 1, 2, 3, 4.5, 6, 9, and 12 h on scheduled days between −21 d relative to calving (DRC) and 120 DRC for the assessment of some metabolic biomarkers and hormones. Data were analyzed using the GLIMMIX procedure of SAS (SAS Institute Inc.). Regardless of parity and stage of lactation, glucose, urea, β-hydroxybutyrate, and insulin peaked a few hours after the morning feeding, whereas nonesterified fatty acids decreased. The insulin peak was attenuated during the first month of lactation, whereas postpartum growth hormone spiked on average 1 h after the first meal in cows during their first lactation. This peak occurred earlier than during the second lactation. Most of the differences in diurnal trends between lactations were observed in the postpartum period (and in some cases even in early lactation). Glucose and insulin were higher during the first lactation throughout the day, and the differences increased 9 h after feeding. Conversely, nonesterified fatty acids and β-hydroxybutyrate showed the opposite trend, and their plasma concentrations at 9 and 12 h after feeding differed between lactations. These results confirmed the differences observed between the first 2 lactations in prefeeding metabolic marker concentrations. Furthermore, plasma concentrations of investigated analytes showed high variability during the day, and thus we advise caution when interpreting metabolic biomarker data in dairy cows, especially during the periods close to calving.

Key words: metabolic profile, parity, circadian rhythm, diurnal pattern

INTRODUCTION

Lactating dairy cows have massive energy requirements that are required to support milk production, reproduction, and growth at a time when they also need energy to maintain their health status and welfare. The onset of lactation represents the most critical phase for nutrient regulation as an impressive number of physiological adaptations occur aimed at supporting mammary activity (Baumgard et al., 2017). During this period, lactating mammals mobilize body reserves to prioritize milk synthesis, in particular if the animals are highly selected and highly producing, such as Holstein cows (Coffey et al., 2004). In fact, the hierarchy of nutrient partitioning becomes completely reorganized, and dairy cows’ metabolism is finely coordinated to support the increased metabolic demands of milk synthesis.

Glucose plays a pivotal role in this scenario because it is the main source of energy for nervous tissue and the only precursor of lactose (Kronfeld, 1982). To produce 1 kg of milk, 72 g of glucose is required (Kronfeld, 1982), and the mammary gland is responsible for about 50 to 85% of whole-body glucose consumption (Rigout et al., 2002; Lemosquet et al., 2009). After calving, glucose requirements dramatically increase by up to 2.5-fold compared with those of the dry period (Bell and Bauman, 1997; Drackley et al., 2001) and even more if an immune response occurs during this period (Kvidera
markers and hormones in the same dairy cows during
12 h after the first meal) of the main plasma metabolic
study was to characterize postprandial variations (until
and second calving. Thus, the aim of this observational
turn, can lead to alterations in plasma concentrations
many hormones (Bruckmaier and Blum, 1998) that, in
milking and mammary uptake influence the release of
of several metabolites.
rumen pH and VFA production vary
throughout the day (van Lingen et al., 2017; Salfer et
al., 2013) and changes that occur in the somatotropic axis
(Lucy et al., 2001).
To further complicate this situation, heifers that give
birth for the first time have a different metabolic and
dermic status than that of mature cows. In fact,
despite lower milk yield, growth requirements add
up to those of lactation (NASEM, 2021). Moreover,
compared with multiparous cows, they have different
feeding behavior, eating less DM during the transition
period and taking more time to eat. Thus, because of
the slower rate of consuming food, they suffer more
from competition and can be replaced at the feeder
by multiparous cows (Neave et al., 2017). Conversely,
previous studies carried out later during lactation
showed the inverse trend with multiparous (Dado and
Allen, 1994; Maekawa et al., 2002). In addition, lying
and activity times differ with parity number (Stone et
al., 2017). Taken together, these behavioral changes can
influence metabolic responses throughout the day.
The analysis of plasma metabolites is commonly used
to monitor the metabolic status of dairy cows (Bertoni
and Trevisi, 2013; Calamari et al., 2016; Premi et al.,
2021). Samples are usually collected once a day before
the morning feeding. However, many metabolites have
large variations during the day, and sampling at dif-
hers might lead to a different interpretation of results (Fröhli and Blum, 1988; Blum et al., 2000;
Piccioli-Cappelli et al., 2022). Diurnal patterns of
plasma metabolites may better explain the metabolic
condition of animals. Daily variations can be related to
feeding frequency, behavior, and rumen fermentation
pattern. In fact, rumen pH and VFA production vary
throughout the day (van Lingen et al., 2017; Safer et
al., 2018) in relation to feeding and eating frequency
(Sutton et al., 1986). Also, feed intake shows a diurnal
rhythm, with the frequency and distribution of meals
mostly related to the time of feed delivery and milking,
and more frequent meals during the day and less during
the overnight period (DeVries et al., 2005). Moreover,
milking and mammary uptake influence the release of
many hormones (Bruckmaier and Blum, 1998) that, in
turn, can lead to alterations in plasma concentrations
of several metabolites.
We hypothesized that hormonal and metabolic diur-
nal variations would differ between cows at their first
and second calving. Thus, the aim of this observational
study was to characterize postprandial variations (until
12 h after the first meal) of the main plasma metabolic
markers and hormones in the same dairy cows during
their first and second lactations from late gestation
through the first 4 mo of lactation.

**MATERIALS AND METHODS**

**Animal Management and Experimental Design**

The research was carried out at the Università Cattolica del Sacro Cuore research dairy barn (Experiment
Station, San Bonico, Piacenza, Italy) in accordance with Italian laws on animal experimentation (DL n.
116, 27/01/1992) and ethics. This study used the same experimental design and animals as described by Cat-
taneo et al. (2023). Briefly, 8 Holstein dairy cows were
housed in individual tiestalls under controlled environ-
mental conditions (room temperature of 20°C, relative
humidity of 65%, 14 h of light) from −55 to 120 d rela-
tive to calving (DRC). The same group of animals was
monitored throughout their first and second calvings.
From −55 until −7 DRC, animals received hay-based
feed combined with corn silage (10 kg) and concentrate
(1.5 kg). Seven days before the expected day of calving,
1 kg of lactation concentrate was added to the diet, and
just after calving, alfalfa-dehydrated hay was fixed to
3 kg and grass hay was gradually reduced to 2.0 kg/d.
Moreover, after calving, corn silage was incremented
at a rate of 2 kg/wk (to a maximum of 20 kg/d), and
concentrate was increased by 0.5 kg/d until it satis-
fied the requirement of 1 kg of concentrate for every
3 kg of produced milk. Daily amounts of forage were
individually fed twice a day (0730 and 1930 h), and
the daily amount of concentrate (delivered in pelleted
form) was fed in 8 equal meals at 3-h intervals during
the day, using an automatic feeder; 2 of the 8 meals
were programmed to deliver the concentrate 30 min
before the forage meals (Figure 1). Thus, the diet was
fed in 2 identical modules repeated every 12 h, and the
2 main meals were given at 0730 and 1930 h. Represen-
tative samples were collected from each feed at every
batch change; after DM determination, samples were
analyzed for CP, crude fiber, NDF, ether extract, ash,
and starch contents (AOAC International, 2012). Full
details of feeding and diet composition can be found in
Cattaneo et al. (2023). Average DMI and composition

---

**Figure 1.** Schematic representation of the daily schedule of meal delivery times and blood samples collected during the trial.
of the diet consumed by cows are reported based on physiological phase in Table 1.

### Blood Sample Collection and Analysis

At −21, −7, 7, 21, 35, 49, 63, 90, and 120 DRC immediately before the delivery of morning feed (0 h), and after 1, 2, 3, 4.5, 6, 9, and 12 h, blood samples were harvested from the jugular vein (Figure 1). For the metabolic profile assessment, samples were collected into 10-mL heparinized vacuum tubes (Vacutainer, Becton Dickinson) and placed on ice until centrifugation. Within 1 h of collection, a small amount of blood was used for the determination of packed cell volume (Centrifugette 4203, ALC International Srl), and the remainder was centrifuged at 3,500 ×g for 16 min at 4°C (Calamari et al., 2016). Aliquots of the resulting plasma were frozen at −20°C until further analysis (Cattaneo et al., 2023). Briefly, blood metabolites were analyzed at 37°C using a clinical analyzer (ILAB 600, Werfen). Commercial kits were used to determine plasma concentrations of glucose, urea, and creatinine (Werfen), BHB (Randox Laboratories Ltd.), and nonesterified fatty acids (NEFA; Wako Chemicals GmbH).

Plasma concentration of bST was quantified by a heterologous double-antibody RIA using materials and procedures obtained from the National Hormone and Peptide Program and the National Institute of Diabetes and Digestive and Kidney Diseases (Torrance, CA). Highly purified bST (reagent AFP-11182B/AFP-9884C) was used for standards (useful concentration range: 0–100 ng/mL) and for iodination (based on the iodogen method of Salacinski et al., 1981). Monkey anti-bovine bST serum (AFP-B55Bb) was used as the primary antibody (1:500,000 final tube dilution). Precipitation of the antigen–antibody complexes was obtained using a goat anti-human γ-globulin (Jackson ImmunoResearch Labs) as a second antibody (2.0% final dilution) together with normal human serum (0.2%) and diluted polyethylene glycol (PEG 6000, 3.0% final tube dilution). Spike, recovery, and linearity testing yielded results within the 85 to 120% range of expected concentrations. Inter- and intraassay coefficients of variation (CV) were 4.0 and 6.2%, respectively. Insulin concentrations in plasma were assayed using a double-antibody RIA kit for human insulin (DSL 1600; Diagnostic Systems Laboratories Inc.) that used a polyclonal antibody with high cross-reactivity to bovine insulin. For bovine plasma samples, the kit was validated by performing linearity testing in which observed, compared with expected results were in the range of 85 to 115%. The intra- and interassay CV were 7.5 and 9.5%, respectively.
The sample size was calculated to achieve a power >0.80 with an $\alpha = 0.05$ using the G*Power package (Faul et al., 2007). The effect size was calculated using the variance of blood parameters (mainly glucose and insulin) as observed in our previous studies. Statistical analysis was performed using SAS software (release 9.4, SAS Institute Inc.). Data were subjected to ANOVA using repeated-measures mixed models (GLIMMIX procedure of SAS). Sampling days were grouped into physiological phases, considering −21 and −7 DRC as dry period (DP), 7 and 21 DRC as postpartum period (PP), 35 and 49 DRC as early lactation (EL), and 63, 90, and 120 DRC as mid lactation (ML). The model included the fixed effects of lactation (L; first and second), physiological phase (P; dry period, onset of lactation, early and mid lactation), hour of the day (H; 0, 1, 2, 3, 4.5, 6, 9, 12 h), and the interactions L × H, P × H, and L × P × H. The random effect of cow was included in the model. Hour of the day was specified as a repeated measure with compound symmetry covariance structure, and the subject was defined as cow × physiological phase (Tao et al., 2015). Distributions of residuals were visually assessed. The pairwise comparisons were performed using the least significant difference test with Tukey adjustment. Statistical significance was declared at $P \leq 0.05$, and differences among means with $0.05 < P \leq 0.10$ were considered in the context of tendencies.

**RESULTS**

**Blood Biomarkers**

Least squares means of plasma biomarker concentrations are shown in Table 2. Hematocrit was constant throughout the day (H; $P = 0.57$) without relevant differences in daily patterns among lactations and physiological phases. Glucose concentration peaked 1 h after the morning feeding, decreased for 3 h thereafter, and increased again ($3.89$ vs $3.73$ vs $3.82 \pm 0.08$ mmol/L, respectively at 1, 3, and 6 h; H, $P < 0.01$; Figure 2A). Plasma glucose diurnal patterns varied with phases between lactations (L × P × H; $P < 0.01$), whereas trends did not differ between lactations in DP and ML. In the PP period, glucose showed the lowest values, particularly during the second lactation. In EL, glycemia did not differ during the first hours of the day but second-lactation cows had more marked declines in glucose concentrations between 2 and 9 h.

Urea concentrations increased from $5.02$ to $5.29 \pm 0.19$ mmol/L at baseline to 3 h after the main meal, after which they decreased, reaching their lowest concentration at 12 h ($4.82 \pm 0.19$ mmol/L; H, $P < 0.01$; Figure 2B). No significant interaction effects were achieved, with urea having the same diurnal pattern among lactations and physiological phases, maintaining during the day the differences observed between the lactations at the baseline (time zero) with gradually increasing concentrations from DP to the ML period, as reported in the companion paper (Cattaneo et al., 2023).

The concentration of NEFA was at its daily highest concentration at the moment of the morning feeding ($0.26 \pm 0.02$ mmol/L; H, $P < 0.01$; Figure 2C) and then stabilized around $0.16$ mmol/L and increased again 9 h later ($0.20$ mmol/L). Cows in their second lactation tended to have a slower decrease after the main meal and an earlier increase in the evening (L × H; $P = 0.10$). The latter was more evident during PP when there was a plunge in both groups after the main feeding (though slower in second lactation) and a large increase at the end of the 12-h period (L × P ×

### Table 2: Least square means of plasma metabolites and hormones from 0 to 12 h from the morning feeding in different physiological phases (dry period, postpartum, early lactation, and mid lactation) in 8 Holstein dairy cows during their first and second lactations

<table>
<thead>
<tr>
<th>Item</th>
<th>Lactation</th>
<th>P-value$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>First</td>
<td>Second</td>
</tr>
<tr>
<td>Packed cell volume, L/L</td>
<td>0.30</td>
<td>0.29</td>
</tr>
<tr>
<td>Glucose, mmol/L</td>
<td>3.91</td>
<td>3.72</td>
</tr>
<tr>
<td>Urea, mmol/L</td>
<td>4.80</td>
<td>5.38</td>
</tr>
<tr>
<td>NEFA, mmol/L</td>
<td>0.17</td>
<td>0.21</td>
</tr>
<tr>
<td>BHB, mmol/L</td>
<td>0.51</td>
<td>0.62</td>
</tr>
<tr>
<td>Creatinine, μmol/L</td>
<td>94.6</td>
<td>94.2</td>
</tr>
<tr>
<td>Insulin, mU/mL</td>
<td>10.2</td>
<td>8.9</td>
</tr>
<tr>
<td>bST, ng/mL</td>
<td>2.32</td>
<td>2.16</td>
</tr>
</tbody>
</table>

$^1$Each physiological phase included 2 or 3 sampling days. Means present summarized diurnal data by parity within cow and across physiological phases for the first 120 DIM.

$^2P$-values of the main effects: lactation (L), physiological phase (P), hour of the day (H), and their interactions.

$^3$NEFA = nonesterified fatty acids.
H; \( P < 0.01 \)); diurnal patterns of NEFA were similar in the other periods. Plasma BHB tended to peak at 3 h (0.61 ± 0.06 mmol/L; H, \( P = 0.06 \); Figure 2D), and a significant L × P × H interaction was observed (\( P < 0.01 \)). Overall, values were similar and stable during the day throughout the phases considered, except during PP. In fact, in this period, BHB concentrations were higher compared with other phases, especially during the second lactation, when they were significantly higher than during the first lactation (\( P < 0.01 \)). Furthermore, primiparous cows showed a marked decline in BHB concentrations at 9 and 12 h, whereas values were stable during second lactation. Creatinine did not show any difference with regard to parity and hour of the day.

**Blood Hormones**

During the day, circulating insulin was lowest before the meal, peaked 1 h later (7.26 and 11.07 ± 0.58 mU/mL, respectively; H, \( P < 0.01 \); Figure 3A) and increased again at 12 h (10.04 ± 0.60 mU/mL). Regardless of parity, the periods under consideration showed different diurnal patterns (P × H; \( P = 0.04 \)). Circulating insulin was lower in PP and EL, and the postprandial peak was reduced during these phases compared with that during DP and ML. Moreover, there was a tendency toward a triple interaction effect (\( P = 0.06 \); Figure 3A) as a result of the specific pattern of the 2 phases mentioned before. That is, while both lactations in DP and ML showed the usual pattern in terms of the postprandial peak,
in PP and EL the aforementioned peak was greatly reduced, and the 2 lactations showed a divergent trend starting from 6 h with an increase of plasma insulin in first-lactation cows and a decrease in second-lactation cows. No differences were observed throughout the day in blood bST. Compared with second-lactation cows, cows in their first lactation had an anticipated peak after a meal and a second peak at 9 h (L × H; \( P = 0.08 \); Figure 3B), which was particularly noticeable postpartum. Although overall bST concentrations were relatively stable during the day, a peak at 1 h during PP and another peak at 9 h in PP and EL were found (P × H; \( P = 0.04 \)).

**DISCUSSION**

Large differences exist in dairy cattle productivity, BW, DMI, and metabolic and endocrine profiles between their first and second lactations (Meikle et al., 2004; Wathes et al., 2007; Cattaneo et al., 2023). However, these comparisons were carried out only once daily, and samples for metabolic profile assessment and hormone determination are usually collected before the morning feeding. Much effort has been directed toward disentangling the diurnal patterns of blood metabolites and hormones in response to different diets or feeding patterns (Ametaj et al., 2009; Rottman et al., 2015; Salfer et al., 2018) but, to our knowledge, this study is the first to investigate the differences in these patterns in cows during their first and second lactations. Moreover, we followed the same cows raised under equal conditions (environment, diet, and management) in the 2 lactations considered, thus limiting as far as possible the confounding effects of genetics and environment. Cows were in good health overall, as supported by the absence of severe periparturient diseases and the low levels of SCC and haptoglobin (Cattaneo et al., 2023), except for the first week of lactation when a certain degree of inflammation is necessary to support adaptation to the transition period (Bionaz et al., 2007; Bradford et al., 2015; Trevisi and Minuti, 2018). The main target of the present research was to investigate postprandial variations in a condition where cows were fed according to a feeding system that included meals of forage and concentrates distributed at identical intervals every 12 h, with no competition among animals for feeding or bedding space. Therefore, samples were not harvested during the overnight period, somewhat limiting the visualization of the complete rhythms over a day. Particularly, bST has a pulsatile secretion, and we were not able to identify the peaks in its secretion.

In the companion paper, we highlighted how different DMI (18.5 kg/d overall during the first lactation, and 21.2 kg/d during the second) and milk yields (33.4 kg/d in the first 4 mo of the first lactation, and 42.1 kg/d in the second) drive variations between the 2 lactations, resulting in a more marked negative energy balance during second lactation (Cattaneo et al., 2023). In the present work, we investigated the postprandial trends of the main metabolism biomarkers and hormones that have shown diurnal changes. Glucose is the key molecule in energy metabolism and nutrient partitioning (De
Koster and Opsomer, 2013). Plasma glycaemia showed similar patterns during the day in the different phases under consideration but, consistent with baseline concentrations, was constantly higher during the dry period and lower during the postpartum period compared with early and mid lactation, reflecting the overall energy balance. An increase immediately after the first meal was observed only during the dry period. During lactation, glucose concentration decreased, likely as a result of mammary uptake and insulin activity, which redirects glucose toward peripheral tissues. The likely higher intensity of rumen fermentation, supported by the concentrate meals delivered with automatic feeders, and the greater availability of substrates for gluconeogenesis likely caused the slight increase observed in the evening, similar to the results observed by Rottman et al. (2015) with different feeding strategies. Moreover, glucose had a similar pattern during first and second lactations, but concentrations were markedly different, particularly during the first stage of lactation, a result consistent with the difference observed in terms of the energy balance (Cattaneo et al., 2023). The relevant decrease in glycaemia observed during the central hours of the day, particularly at the onset of second lactation and despite the applied feeding strategy, suggests the need for feeding strategies that allow for a more constant supply of gluconeogenetic substrates after the main meal (Bertoni et al., 2004).

During the dry and mid-lactation periods, blood urea concentrations increased during the first hours of the day. Urea concentration usually peaks and subsequently declines during the first few hours after a meal (Gustafsson and Palmquist, 1993; Rottman et al., 2015) due to hepatic conversion of greater ammonia flux resulting from microbial dietary protein degradation (Spek et al., 2013). However, the variations after the meal were dampened in the first weeks of lactation, likely due to a higher rate of gluconeogenesis from AA due to the negative energy balance. The diurnal pattern of urea was not different between parities, but there was a higher concentration during second lactation due to the higher DM and protein intake (and likely an increased ammonia flux from the rumen, despite not being measured). The latter occurred although the same protein sources were used, thus with the same rumen degradability and the likely higher rumen passage rate during the second lactation. Particularly, during the first stage of lactation, this difference between lactations was more evident as a result of higher AA catabolism related to more pronounced negative energy balance during second lactation.

The effect of the negative energy balance and fat mobilization can also be observed in terms of NEFA and BHB trends. At times other than the postpartum period, NEFA concentrations decreased immediately after the morning feeding and remained relatively stable during the day, as previously observed (Bertoni et al., 1994). Nonesterified fatty acids usually increase overnight (Niu et al., 2017; Couperus et al., 2021; Seely et al., 2021) because of low intake paired with the constant demand for milk synthesis (Rottman et al., 2015), which results in an increase in lipid mobilization. Without overnight data, we could not observe this course in our study. Nevertheless, a decline in plasma NEFA during the first hours of the day was observed and could be justified by insulin and bST changes. Particularly during the postpartum period, the bST variations were larger with a likely reduction in lipolysis and a higher peripheral NEFA utilization facilitated by the hormone (Burton et al., 1994). Nevertheless, the limited postprandial variation in insulin concentration postpartum was not consistent with the concurrent decrease in NEFA, suggesting a modest effect of insulin on lipolysis in this phase, in agreement with the reduced adipose tissue sensitivity to insulin at the turn of the calving event (Vernon and Sasaki, 1991). Particularly during the transition period, the upregulated basal and catecholamine-stimulated lipolytic activity might explain these variations (De Koster et al., 2016; Contreras et al., 2017). During the postpartum phase, samples taken at 9 and 12 h after the morning feeding showed a relevant increase in NEFA concentration, similar to those observed at night during the second lactation (0 h), consistent with the findings of other authors (Fröhli and Blum, 1988; Blum et al., 2000). This fact could be indicative of a different feeding pattern exacerbated by a more severe negative energy balance, likely with lower DMI in these cows late in the afternoon, or a different endocrine framework. Moreover, this was the only different pattern observed between lactations; in the other phases, NEFA had a similar pattern and concentration between the 2 lactations. For BHB, we observed a moderate postprandial increase. Other authors (Piccioli-Cappelli et al., 2014; Couperus et al., 2021) reported an increase in BHB after meals due to an increase in conversion of butyrate into BHB in the rumen epithelium (Borregaard et al., 1990). Furthermore, patterns of BHB after a meal were similar among parity and stage of lactation, except for a higher overall concentration postpartum during the second lactation. A possible explanation could be related to a higher basal BHB concentration maintained by liver ketogenesis (Nielsen et al., 2003) and driven by the negative energy balance. Moreover, the concentrations of NEFA and BHB seemed negatively correlated with those of glucose, and consequently insulin, and positively correlated with those of bST.

All trends and differences among phases reported for metabolic markers were consistent with those observed
by Plaizier et al. (2005) in multiparous Holstein cows. Hormonal and intake patterns, also mediated by vagal reflexes (Weekes and Godden, 1981), might help explain the metabolic variations. The postprandial peak in insulin concentration was present in all phases, although that postpartum (and to a lesser extent in early lactation) was minimal. The latter is the most complex phase in which insulin secretion is reduced in addition to the tissue sensitivity to its action (De Koster and Opsomer, 2013). During the first 2 mo of lactation, an opposite trend in circulating insulin in the afternoon was observed between the 2 lactations. This process occurred during a phase of negative energy balance, particularly during second lactation (Cattaneo et al., 2023), as supported by the low glucose concentrations. Glucose itself was the only other variable showing differences until early lactation.

Conversely, bST was much higher in the postpartum phase compared with the other parameters considered, with a peak immediately after the main meal. These results are consistent with the findings of Bradford and Allen (2008). They suggested that, in cows in negative energy balance (as cows physiologically are in the immediate postpartum period), the postprandial bST surge can be explained by an adaptation mechanism involving increased premeal ghrelin secretion and greater bST response to ghrelin. Another possible explanation might be related to lower insulin concentration (Steyn and Ngo, 2017), with the variations in the 2 hormones often linked by a negative relationship. A 1-h shift in postprandial bST peak between the 2 lactations and a decline during the first lactation at 12 h occurred, concurrent with the insulin increase. Although the bST surge in this phase was previously reported (Bradford and Allen, 2008), the difference in the peak between the 2 lactations might have been influenced by the pulsatile secretion of this hormone or the different glycemia.

Particularly in multiparous cows, the first 2 wk of lactation were confirmed to be crucial to manage the negative energy balance, and feeding strategies have a key role in mitigating this deficit. In the present study, the feeding system used (autofeeders and equal intervals between concentrate and forage distributions) ensured that meals were well distributed through the day, likely enabling a more constant flux of nutrients compared with TMR feeding. Additionally, the gradual increase in dietary concentrates (and energy and protein contents) could improve DMI and energy intake in a critical phase when DMI and gut capacity are limiting factors (Drackley, 1999). In our opinion, cows fed TMR may have more pronounced variations in the metabolic markers investigated, either because of meal frequency or drastic diet change (Eicher et al., 1999; Esposito et al., 2014). Developing alternative feeding systems, either in terms of gradual release of gluconeogenic substrates or feeding frequency after the main meal, might help cows better support the onset of lactation and improve rumen fermentation profile and feed efficiency.

**CONCLUSIONS**

Milk yield and energy balance differ greatly between first and second lactation, resulting in altered metabolic responses. The analysis of plasma markers and hormones during the day not only confirmed the differences observed at the prefeeding state but also provided information about nutrient utilization in different stages of lactation. Our results confirmed that the blood markers of energy (glucose, NEFA, and BHB) and protein (urea) metabolism undergo relevant changes during the lactation cycle. During the second lactation, the more pronounced negative energy balance, which reflects the greater difficulty in guaranteeing an appropriate flux of glucogenic substrates during the day (in particular some hours after the main meal), resulted in exacerbated responses. Regardless of parity, diurnal variations in plasma metabolic markers and hormones investigated were relevant, with large differences throughout the lactation cycle. Therefore, when analyzing metabolic responses in blood, consideration of these changes and sampling time is essential.

**ACKNOWLEDGMENTS**

This study was funded by the Romeo e Enrica Invernizzi Foundation (Milan, Italy) and supported by the Doctoral School on the Agro-Food System (Agri-system) of the Università Cattolica del Sacro Cuore (Italy). The authors are grateful to Emeritus Professor Giuseppe Bertoni (Università Cattolica del Sacro Cuore, Piacenza, Italy) for the advice and assistance during the planning and realization of the experiment. The authors have not stated any conflicts of interest.

**REFERENCES**


**ORCIDs**

L. Cattaneo https://orcid.org/0000-0001-6027-7536
F. Piccioni-Cappelli https://orcid.org/0000-0003-1277-7821
A. Minuti https://orcid.org/0000-0002-0617-0571
E. Trevisi https://orcid.org/0000-0003-1644-1911