Periprandial Changes in Metabolite and Metabolic Hormone Concentrations in High-Genetic-Merit Dairy Heifers and Their Relationship to Energy Balance in Early Lactation

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ABSTRACT

Sixteen high-genetic-merit Holstein-Friesian heifers were offered a complete diet of grass silage, maize silage, and concentrates ad libitum through mo 1 to 3 postpartum. Open-circuit calorimetry and a 6-d digestibility balance were performed on each heifer at the end of each month, and energy balance (EB) was calculated. After each digestibility balance, heifers were blood sampled hourly from 1 h before to 9 h after feeding. Prefeed plasma and 3-h composites of postfeed plasmas were analyzed for selected metabolites and hormones. Levels of nonesterified fatty acids (NEFA) decreased, whereas those of β -hydroxybutyrate (BHBA) increased after feeding in each month. Urea levels increased after feeding, whereas glucose levels decreased in each month. Insulin increased after feeding but with reducing significance as lactation progressed. Insulin was always lower before feeding, and mean insulin increased from mo 1 to mo 3. Levels of insulin-like growth factor-1 (IGF-I) increased across mo 1 to 3, but were unaffected by feeding except in mo 2, whereas leptin levels varied significantly on each sampling occasion, but showed no increase between mo 1 and mo 3. Multiple regression of all data showed no significant correlation between EB and either BHBA or NEFA levels. However, EB was negatively correlated with leptin levels ($r = -0.632$), which were themselves positively associated with IGF-I ($r = 0.498$) and glucose ($r = 0.565$). A relationship was established between overall mean EB and prefeed leptin, insulin, and urea ($R^2 = 0.63$) in the first 3 mo of lactation. A potentially useful relationship was also established between EB and prefeed concentrations of leptin, IGF-I, urea, and glucose ($R^2 = 0.80$) only in mo 1 of lactation.

Key words: energy balance, metabolic hormone, lactation, metabolite

INTRODUCTION

Energy balance (EB) is a key concern in modern dairy herd management and represents the difference, on a daily basis, between the energy consumed by a cow and the energy expended in milk output and all supporting functions (locomotion, eating, digestion, and metabolism). Energy balance is strongly positive until a few weeks before calving but decreases with reducing feed intake in very late pregnancy. After calving, EB becomes negative (NEB) as the energy cost of milk production is factored in. The level of milk output from modern, high-genetic-merit (HGM) dairy cows means that EB is invariably negative and sometimes strongly so, well into lactation. Doepel et al. (2002) and Ferris et al. (2002b) identified the most negative EB (the EB nadir) within the first 2 wk postpartum in HGM cows, but NEB persisted for several months.

The significance of NEB for dairy farmers lies in the putative link between EB and cow fertility, which declined by almost 1% annually between 1975 and 1998 in the United Kingdom (Royal et al., 2000), threatening the economic viability and sustainability of many herds (Esslemont et al., 2001). Beam and Butler (1999) proposed that the depth or duration of the NEB, or the timing of the EB nadir affect the interval to resumption of reproductive activity in individual cows, through effects on follicular development (Lucy et al., 1992a,b; Beam and Butler, 1997), but supporting evidence for this has not always been forthcoming (Spicer et al., 1990; Snijders et al., 2001; Reist et al., 2003b). A reliable marker of EB might help identify the earliest realistic opportunity for insemination, reducing the costs of wasted semen and enhancing cow welfare.

Changes in BW and BCS have long been used by farmers as an intuitive indicator of EB but are insuffi-

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ciently sensitive to pinpoint the EB nadir in a postpartum cow. Blood concentrations of NEFA and BHBA (a product of liver NEFA metabolism) have been used to suggest or confirm nutritional adequacy or inadequacy in farm animals since the 1960s (Ingvarsen et al., 2003) but the value of single and multiple indicators, such as those in the Compton metabolic profile (Payne et al., 1970), has been questioned (Adams et al., 1978; Rowlands, 1980). Blood levels of 3 functionally related metabolic hormones (insulin, IGF-I, and leptin) also change with nutritional status and one or more of these may have potential as markers of EB (Wathes et al., 2007a). There has been much interest in using leptin alone to indicate the EB nadir and predict the time of the first postpartum ovulation. Kadokawa et al. (2000) found a link between the interval from parturition to the leptin nadir and the time to first postpartum ovulation, with a delay in leptin recovery postpartum increasing time to first ovulation, but Liefers et al. (2003) could not identify a clear leptin nadir in a study of over 300 primiparous Holstein cows up to 80 d postpartum.

The current study set out to investigate if a reliable prediction of EB can be made in early lactation in dairy cows using concentrations of a small number of nutritionally dependent metabolites and hormones. Energy metabolism was assessed over 48 h in each of 16 first-lactation Holstein-Friesian cows at the end of mo 1 to 3 of lactation and used with milk yield (MY) and food intake data to calculate EB. Cows were then blood sampled from 1 h before to 9 h after feeding for analysis of NEFA, BHBA, urea, glucose, insulin, IGF-I, and leptin, and correlations were then sought between the determined EB and concentrations of these metabolites and hormones.

MATERIALS AND METHODS

Animal Management and Measurements

Sixteen HGM autumn-calving heifers, with a PTA₂₀₀₅ (PTA using 2005 as base year) for fat plus protein of 21.7 ± 7.47 kg (mean $\pm$ SD), were used. Artificial insemination commenced at 14 mo (>315 kg), and heifers calved between September and January at 24.5 ± 1.31 mo and were then moved into individual cubicles. Feed intake and MY were recorded daily and BW and BCS weekly (scale of 1 to 5). Milk fat, protein, and lactose were determined every 2 wk on 3-d milk composites using a Milkoscan 605 (Foss Electric, Hillerød, Denmark). The study was approved by the AFBI (Hillsborough) Ethical Review Committee.

Diets and Diet Measurements

After calving, animals had ad libitum access to a complete diet of grass silage/maize (*Zea mays* L. cv.

Loft) silage (60:40 DM basis) and concentrates (barley, wheat, molassed sugar beet pulp, citrus pulp, maize gluten, soybean meal, and rapeseed meal supplemented with minerals and vitamins). Concentrates were introduced at 4.0 kg/d and increased to 9.5 kg/d by d 20 of lactation. A Calan gate feeding system (American Calan, Northwood, NH) linked to Griffith-Elder weighing platforms (Griffith Elder, Bury St. Edmunds, UK) recorded intake at every meal. The amount of feed offered was adjusted to ensure approximately 10% orts. In mo 1 to 3 postpartum, feed was given once daily about 1030 h. In mo 7 only, cows grazed a grass pasture (except on the day of blood sampling, when harvested grass was fed). The silage was sampled daily and DM content was determined by drying overnight at 85°C. Dried samples were bulked weekly, milled, and analyzed for ADF, NDF, and ash (Cushnahan and Gordon, 1995). Fresh silage was analyzed weekly for volatiles-corrected oven DM, pH, nitrogen, VFA, gross energy, alcohols, and ammonia (Porter and Murray, 2001). Concentrate samples were bulked weekly and analyzed for DM, nitrogen, ash, ADF, NDF, and gross energy (Cushnahan and Gordon, 1995).

Energy Metabolism and Diet Digestibility Studies

At the end of mo 1, 2, and 3 postpartum, each animal was adapted to a tie-stall for 4 d and then transferred to an open-circuit respiration chamber for 3 d. Chamber operation was described by Gordon et al. (1995) with calibration according to Yan et al. (2000). Gas exchange and heat production data were collected over the last 48 h. After removal from the chamber, a 6-d digestibility balance (Mayne and Gordon, 1984) was performed on each cow.

Blood Sampling and Analysis

After each digestibility balance, a sterile jugular catheter was inserted (between 0800 and 0900 h). Patency was maintained by injecting 3 mL of a 50 IU/mL solution of sodium heparin (CP Pharmaceuticals, Wrexham, UK) in sterile physiological saline (Baxter, Northants, UK). Blood was sampled into 20-mL polyethylene syringes 0.5 to 1 h before feeding (approximately 1000 h) and then hourly from 1 h to 9 h after feeding. Samples were immediately divided between plain glass tubes (for analysis of hormones), tubes containing fluoride oxalate (for analysis of glucose), and heparinized tubes (for analysis of metabolites). Serum and plasma were recovered by centrifugation and kept at -20°C until analyzed. Prefeed sera and plasma samples and three 3-h postfeed composites, comprising equal volumes of serum or plasma (taken at 1 to 3, 4

to 6, and 7 to 9 h), were analyzed for each animal. The postfeeding composites were prepared from once-thawed sera and plasmas.

Metabolite and Hormone Analyses

Metabolites were assayed as described by Keady et al. (1998) using auto-analyzer kits for NEFA (WaKo Chemicals, Neuss, Germany), urea, and BHBA (Randox, Antrim, UK). Hormones were determined by RIA. Insulin was assayed in all hourly samples, IGF-I in the prefeed sample and postfeed composites in mo 1, 2, 3, and 7, and leptin in the prefeed sample and postfeed composites in mo 1, 2, and 3 only (due to limited availability of radiolabeled leptin).

Insulin was determined using guinea pig anti-bovine insulin (Sigma, Poole, UK) at a final dilution of 1:80,000 bovine pancreatic insulin (Sigma) as assay standard (6.25 to 0.049 ng/mL) and ^{125}I -3-iodotyrosyl recombinant human insulin (Amersham, Bucks., UK) as label. Second antibody was SacCel donkey anti-guinea pig IgG (IDS, Washington, UK). Before IGF-I assay, binding proteins were removed by overnight extraction with ethanol:2 M HCl (7:1). An International Reference Standard recombinant human IGF-I (NIBSC, Potters Bar, UK) was used to construct the standard curve (3.0 to 0.0156 ng/tube). Primary antibody (UB3-189) was rabbit anti-human IGF-I (a gift from A. F. Parlow of the US National Hormone and Pituitary Program, Torrance, CA) used at a final dilution of 1:18,000, and ^{125}I -IGF-I (Amersham, UK) was used as label. This assay used goat anti-rabbit IgG (Sigma, UK) and polyethylene glycol to precipitate bound label. For the leptin assay, a standard curve was prepared using purified recombinant bovine leptin (Diagnostic Systems Laboratories, Oxford, UK), with a concentration range of 50 to 0.049 ng/mL. Pooled, previously characterized bovine serum was included at regular intervals in each assay for quality assurance purposes. The primary antibody (GP-OL3) was raised in guinea pigs against recombinant ovine leptin (**reOL**) generously donated by A. Gertler (Hebrew University of Jerusalem, Israel) and used at 1:160,000 final dilution. The second antibody was donkey anti-guinea pig IgG (IDS). ^{125}I -Recombinant ovine leptin was prepared by iodination of this same reOL with ^{125}I -sodium iodide (Amersham) using iodogen-coated tubes (Perbio Science, Northumberland, UK). Iodinated leptin was separated from unreacted iodine by gel filtration on G-75 Sephadex (GE Healthcare, Bucks, UK).

Statistical Analysis

Repeated-measures ANOVA using Genstat v.6 (Lawes Agricultural Trust, 2002) was used to allow for

Table 1. Mean, minimum, and maximum values for metabolite and hormone concentrations and animal parameters across all time periods and sample times

Parameter	Mean	Minimum	Maximum	SD
Milk yield (kg/d)	28.4	23.7	35.5	2.76
Glucose (mmol/L)	3.24	2.36	3.93	0.311
BHBA (mmol/L)	0.6	0.3	0.9	0.19
NEFA (mEq/L)	0.12	0.04	0.87	0.112
Urea (mmol/L)	5.69	3.10	8.00	1.078
EB (MJ/d)	-12.5	-60.5	+30.8	22.96
ME intake retained (%)	-0.08	-0.37	0.12	0.132
Insulin (ng/mL)	0.49	0.07	1.51	0.235
Leptin (ng/mL)	3.92	0.24	6.63	1.091
IGF-I (ng/mL)	187	130	275	28.3
BW (kg)	506	429	601	42.9
BCS (0 to 5 scale)	2.33	1.75	3.00	0.273
Feed intake (kg/d)	35.6	23.1	53.0	6.68

the successive time periods postcalving. To predict EB across the first 3 mo of lactation, forward stepwise regressions were run to generate multiple regression equations from mean concentration data for each metabolite and hormone and BCS, milk yield, feed intake, and BW data. To establish if sampling pre- or postprandially affected the predictability of EB, the regressions were carried out first using the prefeed data alone, then using postfeed data alone, and finally, using all of the data collected across the first 3 mo of lactation. All data were next reanalyzed by stepwise regression using forward selection of independent variables to establish relationships between all parameters, using probability levels of 5%. Regressions were calculated using total 3-mo data and then separately for data from each individual month.

RESULTS

Diet Characterization and Animal Performance Data

The grass silage had a DM content of 348 g/kg of fresh weight, an ME content of 12.5 MJ/kg DM, and a CP content of 158 g/kg of DM with a pH of 3.98. The maize silage had a DM content of 243 g/kg of fresh weight and a CP content of 81 g/kg of DM with a pH of 3.86. The grass and maize silages contained 292 and 311 g of ADF/kg of DM and 533 and 587 g of NDF/kg of DM, respectively, and ash contents were 89 and 57 g/kg of DM. The concentrates contained 218, 95, and 194 g/kg of DM of CP, ADF, and NDF, respectively, and had a gross energy of 17.8 MJ/kg DM. Mean and range values for EB, percentage ME retained, BW, BCS, MY, and feed intake are presented in Table 1.

All 16 heifers were bred again after calving and all were confirmed pregnant during the study. However, 2 were not pregnant when blood-sampled at the end of mo 7. In one of these, the first observed heat was in wk

Table 2. Periprandial changes in metabolite concentrations in calved heifers (n = 16)

Hormone/month postcalving	Prefeed	Time postfeeding (h)			SED	Level of significance
		1 to 3	4 to 6	6 to 9		
BHBA (mmol/L)						
1	0.50 ^a	0.68 ^b	0.70 ^b	0.66 ^b	0.031	***
2	0.43 ^a	0.63 ^b	0.69 ^b	0.63 ^b	0.047	***
3	0.41 ^a	0.76 ^b	0.79 ^b	0.70 ^b	0.047	***
7	0.44 ^a	0.49 ^{ab}	0.52 ^b	0.60 ^c	0.035	**
NEFA (mEq/L)						
1	0.37 ^b	0.13 ^a	0.09 ^a	0.09 ^a	0.034	***
2	0.16 ^b	0.08 ^a	0.08 ^a	0.08 ^a	0.016	***
3	0.19 ^b	0.09 ^a	0.08 ^a	0.08 ^a	0.029	***
7	0.17	0.13	0.16	0.15	0.020	NS
Urea (mmol/L)						
1	5.31 ^a	5.84 ^b	6.01 ^b	5.75 ^b	0.180	***
2	5.65 ^{ab}	6.07 ^{bc}	5.87 ^{ab}	5.58 ^a	0.218	**
3	5.58 ^a	6.07 ^c	6.00 ^{bc}	5.71 ^{ab}	0.161	***
7	4.36 ^{ab}	4.23 ^a	4.61 ^{bc}	4.77 ^c	0.133	***
Glucose (mmol/L)						
1	3.31 ^{bc}	3.10 ^a	3.19 ^{ab}	3.38 ^c	0.089	**
2	3.27 ^c	3.05 ^a	3.13 ^b	3.27 ^c	0.010	*
3	3.42 ^b	3.14 ^a	3.27 ^{ab}	3.46 ^b	0.080	***
7	3.24	3.23	3.37	3.36	0.110	NS

^{a-c}Identical superscripts within rows indicate nonsignificant ($P > 0.05$) differences between means.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

16 but this cow was still not pregnant at the end of mo 7. In the other, the first observed heat was in wk 22. This cow became pregnant but lost her fetus before the end of mo 7.

Plasma Metabolite Concentrations

Table 2 shows the mean concentrations of each metabolite at each time point. Concentrations of NEFA decreased ($P < 0.001$) after feeding in mo 1, 2, and 3 but did not differ between the 3 postprandial composites in any of these months and were unaffected by feeding in mo 7. Concentrations of BHBA varied significantly ($P < 0.001$) across the 10-h sampling period in each of mo 1, 2, and 3 and also in mo 7 ($P < 0.01$). The BHBA levels were consistently lower before feeding than in any postprandial composite but this was significant in mo 1 to 3 only and there were no significant differences in mean BHBA between any of the postprandial composites in any month. In mo 7, BHBA concentrations increased steadily across the sampling period.

Urea concentrations increased after feeding in mo 1, 3, and 7 ($P < 0.001$) and also in mo 2 ($P < 0.01$), but postprandial levels were not significantly different from each other in mo 1. Postprandial urea concentrations had returned to preprandial levels by the end of sampling in mo 2 and 3 only. Glucose concentrations decreased after feeding in each of mo 1 to 3 (mo 1, $P < 0.01$; mo 2, $P < 0.05$; mo 3, $P < 0.001$) but not in mo 7. In mo 1 to 3, glucose concentrations had increased again to near-prefeed levels by 9 h after feed allocation.

Serum Hormone Concentrations

Insulin levels increased significantly after feeding in each of mo 1 ($P < 0.001$), mo 2 ($P < 0.01$), and mo 3 ($P < 0.05$) but not in mo 7, and there was no significant difference between the hourly postprandial concentrations (Figure 1) or the 3-h postprandial composites (Table 3) in any of these months. In each of mo 1, 2, and 3, the greatest insulin concentration occurred between 4 and 5 h after feed was replenished. Insulin concentrations differed significantly ($P < 0.05$) in samples taken at 2, 3, and 4 h after feeding in mo 1 and 3 (Figure 1). Serum leptin concentrations (Table 3) were initially

Figure 1. Periprandial changes in insulin concentrations at the end of mo 1, 2, 3, and 7 postcalving (n = 16). PF = prefeed; bars = SEM.

Table 3. Periprandial changes in hormone concentrations in calved heifers (n = 16)

Hormone/month postcalving	Prefeed	Time postfeeding (h)			SED	Level of significance
		1 to 3	4 to 6	6 to 9		
Insulin (ng/mL)						
1	0.24 ^a	0.47 ^b	0.50 ^b	0.51 ^b	0.056	***
2	0.30 ^a	0.55 ^b	0.60 ^b	0.58 ^b	0.085	**
3	0.33 ^a	0.67 ^b	0.73 ^b	0.67 ^b	0.137	*
7	0.23	0.27	0.30	0.27	0.031	NS
Leptin (ng/mL)						
1	3.86 ^a	3.77 ^a	3.58 ^a	4.21 ^b	0.159	***
2	3.79 ^{ab}	4.18 ^{bc}	3.91 ^{ab}	3.72 ^a	0.221	*
3	4.00 ^a	3.99 ^a	4.38 ^{bc}	4.13 ^{ab}	0.170	**
IGF-I (ng/mL)						
1	169	174	170	170	5.4	NS
2	189 ^{ab}	181 ^a	185 ^{ab}	192 ^{bc}	4.4	*
3	202	195	195	206	9.6	NS
7	195	195	196	192	6.9	NS

^{a-c}Identical superscripts within rows indicate nonsignificant ($P > 0.05$) differences between means.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

unaffected by feeding in mo 1, increasing only by 6 to 9 h after feeding ($P < 0.001$), whereas in mo 2, leptin levels increased immediately after feeding ($P < 0.05$) before quickly returning to prefeed levels. In mo 3, leptin concentrations increased some 4 to 6 h postprandially ($P < 0.01$) before returning to prefeed values. There was no significant difference between monthly mean leptin concentrations. Concentrations of IGF-I were unaffected by feeding in any month except mo 2 (Table 3), in which levels at 6 to 9 h after feeding were significantly greater than those immediately after feeding ($P < 0.05$). Overall mean IGF-I (pre- and postprandial) increased steadily but not significantly across the first 3 mo of lactation.

Regression Analysis

The correlation coefficients (r) for relationships between concentrations of selected metabolites and hormones and MY, EB, BW, BCS, and feed intake are presented in Table 4. The initial data analysis used individual animal means for all of the metabolites and hormones from mo 1 to 3 of lactation. Mean EB was not significantly correlated with mean plasma BHBA or NEFA concentrations but was negatively correlated with mean serum leptin ($r = -0.632$; $P < 0.01$). Body condition score was positively correlated with BW ($r = 0.607$; $P < 0.05$) and feed intake ($r = 0.573$; $P < 0.05$) and also with blood plasma concentrations of BHBA ($r = 0.494$; $P < 0.10$) and NEFA ($r = 0.678$; $P < 0.01$). Levels of NEFA were also correlated with BW ($r = 0.500$; $P < 0.01$), whereas BHBA levels were correlated with both urea ($r = 0.500$; $P < 0.05$) and NEFA levels ($r = 0.545$; $P < 0.05$). Leptin concentrations were positively correlated with both plasma glucose ($r = 0.565$; $P < 0.05$)

and serum IGF-I concentrations ($r = 0.498$; $P < 0.05$), and plasma glucose concentrations were positively correlated with serum IGF-I concentrations ($r = 0.541$; $P < 0.05$).

Separate regression analyses using data for each of the monthly sampling days gave the following best-fit relationships: mo 1 $EB = -109.6 - 18.2 \text{ leptin (ng/mL)} + 36.5 \text{ glucose (mM)} - 0.466 \text{ IGF-I (ng/mL)} + 19.67 \text{ urea (mM)}$ ($R^2 = 0.80$; $P < 0.001$) and mo 3 $EB = -42.4 - 76.0 \text{ NEFA (mEq/L)} + 10.49 \text{ urea (mM)}$ ($R^2 = 0.51$; $P < 0.05$). There was no significant relationship between EB and any of the analytes in mo 2.

DISCUSSION

The mean EB nadir value of -60.5 MJ/d in the current work was close to the -63.9 MJ/d estimated by Sutter and Beever (2000) for HGM Holstein-Friesian cows.

As anticipated, blood concentrations of all of the selected metabolites and hormones (except IGF-I) were significantly affected by feeding in each of the first 3 mo postpartum, with postprandial increases in BHBA, urea, insulin, and leptin and decreases in NEFA and glucose. In most instances where metabolite levels increased, they remained elevated throughout the 9-h postprandial sampling period. Urea, BHBA, and insulin showed the most immediate and proportionately greatest increases. In contrast, NEFA and glucose concentrations decreased sharply after feeding, whereas glucose levels recovered to preprandial values by the end of each sampling period. The contrast between pre- and postprandial metabolite concentrations highlights a longstanding concern about reliance on a single blood sample of variable and uncertain timing to provide information on an animal's metabolic status.

Table 4. Correlations of mo 1 to 3 postpartum means of metabolite and hormone concentrations with cow characteristics milk yield (MY), energy balance (EB), percentage ME intake retained, BW, BCS, and feed intake¹

Item	MY	Glucose	BHBA	NEFA	Urea	EB	ME intake retained (%)	Insulin	Leptin	IGF-I	BW	BCS	Intake
MY (kg/d)	1												
Glucose (mmol/L)	0.128	1											
BHBA (mmol/L)	0.172	0.213	1										
NEFA (mEq/L)	0.177	0.438	0.545	1									
Urea (mmol/L)	0.248	-0.097	0.500	0.210	1								
EB (MJ/d)	0.126	-0.225	0.135	-0.216	0.125	1							
ME intake retained (%)	0.119	-0.188	0.170	-0.170	0.078	0.991	1						
Insulin (ng/mL)	-0.334	0.295	-0.039	-0.190	-0.071	-0.241	-0.215	1					
Leptin (ng/mL)	0.020	0.565	-0.022	0.248	0.266	-0.632	-0.647	0.137	1				
IGF-I (ng/mL)	0.100	0.541	-0.061	0.035	0.325	-0.253	-0.289	0.397	0.498	1			
BW (kg)	0.358	0.463	0.215	0.500	0.103	-0.133	-0.097	-0.137	0.250	0.269	1		
BCS	0.301	0.378	0.494	0.678	0.273	-0.176	-0.092	-0.031	0.236	-0.025	0.607	1	
Feed intake (kg/d)	0.190	0.211	0.437	0.156	-0.009	0.104	0.186	0.293	-0.150	-0.185	0.397	0.573	1

¹Correlations in bold type are significant ($P < 0.05$).

Elevated blood BHBA levels are commonly used to confirm nutritional inadequacy in farm animals, and because BHBA is itself a product of NEFA metabolism by ruminant liver (Zammit, 1999), unusually high BHBA values (>1.5 mM) are taken to indicate low energy status and associated fat mobilization in cattle and sheep. But BHBA is also a product of butyrate metabolism by rumen epithelium and liver, which convert 75 and 60%, respectively, of butyrate passing through them to BHBA (Pennington, 1952). Rumen butyrate is increased in silage-fed ruminants offered barley-based supplements (Wylie et al., 1984), so high blood BHBA levels may not exclusively reflect mobilization of adipose reserves. β -Hydroxybutyrate is also a substrate for de novo fatty acid synthesis in the lactating bovine mammary gland (Vernon, 2005) so that elevated BHBA levels can make a positive contribution to lactation. In general, undernutrition is associated with a decrease in EB, suggesting an inverse correlation between EB and BHBA concentrations. In the current study, a similar postprandial BHBA increase was seen in each of mo 1 to 3 postpartum despite a very different EB in each. Conversely, Ferris et al. (2002b) saw no BHBA change in the first 52 d postpartum in HGM cows despite large EB changes and similarly concluded that BHBA levels are more closely related to diet type than to EB in early lactation. The sharp postprandial decrease in NEFA was greatest (65%) in mo 1. Because cows had no access to feed from the start of milking (~0500 h) until 1030 h, and because Ferris et al. (2002a) noted that food intake was lowest between 2400 h and 0900 h in cows, then cows in the current study may have eaten little in the 10 or 11 h before feed renewal, promoting the very high prefeeding NEFA concentrations.

Plasma urea levels rose immediately after feeding in mo 1, 2, and 3 postpartum when the diet was a mixture of silage and concentrates. In mo 7, when fresh grass was given, an increase in plasma urea also occurred but was delayed until more than 3 h after feeding. In ruminants, high blood urea levels can follow an increase in protein intake, a change in protein quality, or a less synchronous supply of nitrogen and energy that limits the amount of ammonia assimilated into ruminal microbial protein (Sinclair et al., 1995). The small increase in plasma urea in the current study suggests that dietary protein was efficiently utilized despite the high protein level in the diet. The significant decrease in plasma glucose immediately after feeding in each month may have been linked to the near-doubling of insulin soon after feeding. In early lactation, insulin resistance in muscle (Bauman and Vernon, 1993) and adipose tissue (Etherton and Bauman, 1998) means that more glucose is partitioned to the mammary gland for lactose and milk fat synthesis, and the rapid fall in glucose in the current study may simply reflect a high glucose use for milk production in these HGM cows.

The incremental increase in insulin concentrations between mo 1 and mo 3 in the current study was similarly seen in postpartum cows by Ingvarsen and Andersen (2000) and in a shorter study by Spicer et al. (2002). Insulinogenic diets have been advocated in early lactation to encourage an early resumption of ovulation and help slow or reverse the decline in fertility in HGM cows with high levels of Holstein genetics (Gong, 2002). In general, in ruminants and nonruminants, a high plane of nutrition sustains insulin secretion and, through insulin, the activity of the hepatic growth hormone receptor (GHR) such that GH-induced IGF-I levels in blood are maximized (Breier et al., 1988). In dairy

cows, however, expression of liver-specific GHR1A (the higher-affinity GHR isoform) is depressed at calving and recovers to precalving values only after 10 or more days (Kim et al., 2004). Thus, IGF-I levels decline by as much as 70% in the last few weeks prepartum (Block et al., 2001) but begin to rise again in well-fed cows from about 2 to 3 wk postpartum (Spicer et al., 1990; Doepel et al., 2002). Levels of IGF-I in the current study increased linearly across mo 1, 2, and 3 in line with increasing feed intake and insulin, but there was no further increase in IGF-I in mo 7 when cows were at grass and insulin levels were at their lowest. Taken collectively, studies by Beam and Butler (1999), Gutierrez et al. (1999), and Lucy et al. (1999) point to the insulin-GHR interaction that promotes IGF-I levels as a strong influence on ovarian follicular development. Disruption of this interaction by or during the NEB of early lactation (Fenwick et al., 2008) impedes resumption of ovulation and extends the calving interval. However, Gong et al. (2002) reported that high-insulin diets do not affect circulating IGF-I levels and argued that it is insulin's direct effect on the differentiation and maturation of dominant follicles that increases the chance of ovulation in response to an LH surge.

Leptin concentrations varied significantly across the sampling day in each of mo 1 to 3 but with no consistent pattern, and mean leptin levels did not differ between mo 1 and 3. Leptin secretion in cattle is pulsatile (Ingvartsen and Boisclair, 2001; Wylie et al., 2001) but this was not considered to be of any practical significance in the current work in which postprandial leptin was assayed in 3-h composite samples. The earlier and proportionately greater increase in IGF-I than in leptin across mo 1, 2, and 3 paralleled the results of Meikle et al. (2004) in cattle, and those of Wylie et al. (2002a) in progressively refed fasted sheep, and is consistent with the prioritization of nutrient use for the restoration of muscle mass before fat mass as nutrition increases again above maintenance in previously underfed animals.

Of the metabolic hormones, only leptin was strongly correlated with mean EB through the first 3 mo of lactation ($r = -0.632$; $P < 0.01$). Reist et al. (2002) found only a weak association between EB and leptin ($r = -0.027$; $P = 0.437$) in wk 1 to 10 postpartum in HGM cows, whereas in a subsequent study (Reist et al., 2003b), they found no relationship between leptin and reproductive performance. Reist et al. (2003a) proposed that leptin is important in regulating feed intake, body fat stores, and energy homeostasis in transitional cows. An apparent lack of consistency across studies in relationships between leptin, energy status, and fertility may reflect the complexity of the multifactorial regulation of leptin in cows in early lactation (reviewed by Chilliard et al.,

2005), but there is an absolute consensus that the management of adipose tissue reserves across the reproductive cycle is hugely important in meeting the reproductive priorities in dairy cows (Friggens, 2003).

Leptin and IGF-I concentrations were positively correlated ($r = 0.498$; $P < 0.05$) in the current study as in that by Reist et al. (2003a). Leptin and insulin were poorly correlated ($r = 0.137$), but IGF-I levels tended to be positively related to those of insulin ($r = 0.397$). Houseknecht et al. (2000) noted a correlation between leptin mRNA and IGF-I mRNA in cows and a positive association of each with insulin suggesting a functional triumvirate between these 3 metabolic hormones. Leptin concentrations have been correlated, to varying degrees, with body fat mass and BCS in sheep (Delavaud et al., 2000; Wylie, 2004), heifers (Leon et al., 2004), and dairy cows in mid and late lactation (Ehrhardt et al., 2000; Wylie et al., 2002b) making leptin a potentially useful marker of EB in dairy cows as suggested by Block et al. (2001). Further, IGF-I concentrations have been positively associated with muscle growth in several farm species (Oddy and Owens, 1996; Lobley, 1998) and with backfat thickness in 8-wk-old pigs (Suzuki et al., 2004), but not in beef bulls (Davis and Simmen, 2000), where the correlation was negative.

Given the inadequacy of single metabolites as predictors of EB, forward stepwise regressions were initially conducted using all preprandial and postprandial metabolite and hormone concentrations. The best-fit equation ($R^2 = 0.89$; $P < 0.001$; Table 5) used both preprandial and postprandial metabolite and hormone concentrations, but sampling and analysis on such a scale is unrealistic. The next-best-fit equation used only pre-fed samples ($R^2 = 0.87$; $P < 0.01$; Table 5) but required accurate estimates of feed intake, making it also of no practical use. After omitting parameters that are not easily measured, the best-fit equation involved only pre-fed values for leptin, urea, and insulin ($R^2 = 0.63$; $P < 0.01$; Table 5). However, this equation links mean EB in mo 1 to 3 of lactation to analyte levels determined throughout mo 1 to 3 and is, by definition, of no use as an anticipatory predictor of EB. When the analytical data and determined EB were regressed within each month, a potentially useful prediction for EB was obtained in mo 1 only [Mo 1 EB = $-109.6 - 18.2$ leptin (ng/mL) + 36.5 glucose (mM) - 0.466 IGF-I (ng/mL) + 19.67 urea (mM); $R^2 = 0.80$; $P < 0.001$]. Month 3 EB was also significantly fitted to metabolite and hormone data but the relationship was not as strong [Mo 3 EB = $-42.4 - 76.0$ NEFA (mEq/L) + 10.49 urea (mM); $R^2 = 0.51$; $P < 0.05$]. There was no significant relationship between EB and any of the analytes in mo 2.

The strength of the regression equation for EB in mo 1 suggests that using multiple regression equations to

Table 5. Predictions produced by forward stepwise regression for the estimation of energy balance (EB) and serum leptin concentrations from metabolite and hormone concentrations (all 3-mo postpartum means)

Prediction	R ²	Significance
All samples		
EB (MJ/d) = 18 – 7.90 leptin (ng/mL)	0.36	**
Leptin (ng/mL) = –8.98 – 0.05 EB (MJ/d) + 5.07 glucose (mmol/L) + 1.33 urea (mmol/L) – 5.28 BHBA (mmol/L) – 0.03 IGF-I (ng/mL) – 1.24 BCS	0.89	***
Postprandial samples only		
EB = 18 – 7.87 leptin (ng/mL)	0.35	**
Leptin (ng/mL) = –6.82 – 0.045 EB (MJ/d) + 2.28 glucose (mmol/L) + 0.494 urea (mmol/L)	0.66	***
Preprandial samples only		
EB = 0.1 – 8.96 leptin (ng/mL) + 6.84 urea (mmol/L) – 53.2 insulin (ng/mL)	0.63	**
Leptin (ng/mL) = –2.54 – 11.87 EB (MJ/d) + 0.50 urea (mmol/L) – 6.15 insulin (ng/mL) + 2.15 glucose (mmol/L) – 0.074 feed intake (kg/d)	0.87	***

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

link metabolite and hormone concentrations to EB may be a useful way forward for development of transitional cow management strategies that seek to get higher-yielding cows back into calf as early as possible. The absence of a significant relationship between EB and analytes in mo 2 may reflect more dynamic changes in EB in mo 2 of lactation, in particular because this period encompasses peak milk yield in most cows and, in some, the point of EB inflexion, so that EB is falling for some cows in mo 2 but rising for others. This contrast has consequences for analytes making it difficult to develop a single relationship between analyte levels and EB in mo 2. This difficulty might be reduced if more data were obtained by concentrating more future research effort on this key period.

Wathes et al. (2007a) collated data for the same metabolites and hormones as in the current study over 500 individual lactations in primiparous and multiparous cows to test relationships with cow fertility characteristics. Blood samples were taken from each cow (about 2 h after morning feeding) in the last 2 wk before calving and then in wk 2 to 3, 4 to 5, and 7 to 8 postpartum. A longer calving-to-conception interval was associated with greater prepartum leptin and lower prepartum NEFA and urea levels in multiparous cows. In a separate paper, Wathes et al. (2007b) compared interrelationships between the same metabolites and hormones and MY and BCS and stressed the importance of inherent differences in the control of nutrient partitioning between primiparous and multiparous cows that are consistent with primiparous cows diverting a greater proportion of their nutrient supply to tissue growth.

From the current work in primiparous cows, the most appropriate and useful equation for deriving EB used only leptin, urea, and insulin concentrations before feeding, whereas the best overall equation, for mo 1 only, used leptin, glucose, IGF-I, and urea concentrations. With current laboratory-based technology, measurement of these substances is expensive and out-

weighs any potential benefit. However, insulin, urea, and leptin are either already determinable or potentially determinable by immunobiosensors. Furthermore, all 3 are present and determinable in milk. Given the rapid pace of developments in nanotechnology and specifically in immunobiosensors, real-time milk hormone and metabolite assays may not be far away and could enable more accurate prediction of EB by noninvasive sampling.

CONCLUSIONS

The present study failed to establish significant correlations between NEFA or BHBA concentrations and EB, reinforcing skepticism about the reliability of these particular metabolites as sole indicators of nutritional status in early lactating dairy cows. Furthermore, although most of the hormones and metabolites measured in the present study exhibited significant periprandial variation, regression analysis of the data suggested that the most useful and opportune time to blood-sample cows in early lactation for estimation of EB is before morning feeding. This is an encouraging observation because it eliminates potential problems arising from greater postprandial variation in hormone levels, especially due to differences in feed intake between animals and because sampling can be more easily done at morning milking. The predictive equations generated by the current study may be improved by data from further similar studies and, with continuing progress in nanotechnology and the drive toward development of rapid, automated biosensors for human metabolic monitoring, there is a likelihood of being able to more accurately monitor the metabolic status of dairy cows in a timely way. What will then be needed is further reliable animal data on which to develop necessarily robust prediction equations.

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