Relationship Between Milk Progesterone Profiles and Genetic Merit for Milk Production, Milking Frequency, and Feeding Regimen in Dairy Cattle

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ABSTRACT

Milk progesterone profiles were determined from samples obtained twice weekly for 100 d postpartum in 100 Holstein primiparous cows at a Dutch experimental farm. Three treatments were applied in a $2 \times 2 \times 2$ factorial arrangement with high-low genetic merit for overall production, high-low caloric density diet, and 2–3 times milking/day as factors. Milk progesterone profiles were characterized by start of first ovarian cyclical activity (commencement of luteal activity, C-LA), length and peak milk progesterone concentration of first ovarian cycle, and number of ovarian cycles in first 100 d postpartum, as well as classified into normal, delayed, prolonged, and interrupted ovarian cyclical activity. Cows with a greater milk production had lower peak progesterone concentrations, especially if the high milk production was caused by milking 3 times a day. A more negative energy and protein balance was associated with later C-LA and less ovarian cycles within 100 d postpartum. Relationships between protein balance and C-LA differed between cows with a high genetic merit and a low genetic merit. Cows with a high genetic merit for production showed delayed C-LA with more negative protein balances, whereas this association was not observed among cows with a low genetic merit. Cows in negative energy balance had greater risk for prolonged ovarian cycles when there was no delay in C-LA than when C-LA was delayed.

Key words: milk yield, high production, fertility, progesterone

INTRODUCTION

Breeding for increased production in dairy cattle has negative side effects on fertility traits (Pryce et al., 1997; Rauw et al., 1998; Roxstrom et al., 2001; Royal

et al., 2002a). Specifically, these negative side effects include abnormal ovarian cyclicity (Opsomer et al., 1998), poor estrus expression, and failure to establish successful pregnancy after AI or embryonic loss (Sheldon et al., 2006). In the Netherlands, success rate at first AI decreased from 55.5 to 45.5% over a 10-yr period, during which 305-d milk production increased by 1,186 kg (Jorritsma and Jorritsma, 2000). Lucy (2001) reported an increase in the number of AI per conception from 1.75 to more than 3 over a 20-yr period for US Holstein. A steady decline in pregnancy rate of about 1% per year, resulting in pregnancy rates at first service of 40%, has been observed in the United Kingdom (Royal et al., 2000b). As a result, calving intervals are typically longer than is economically optimal, at about 1 yr in primiparous cows and somewhat shorter in older cows (Huirne et al., 2002). Poor fertility results in high replacement rates (Beaudeau et al., 2000) and, consequently, may have implications for animal welfare and sustainability of animal production.

Fertility of the dairy cow is determined by the interaction of management decisions and the biology of the cow. This may be one of the reasons why the relationship between milk production level and fertility is subtle and varies from herd to herd, both phenotypically (Windig et al., 2005) and genetically (Windig et al., 2006). Delayed ovulation has been attributed to negative energy balance (Darwash et al., 1997; De Vries and Veerkamp, 2000; Royal et al., 2002a; Jorritsma et al., 2005), and, in general, the decline in fertility is associated with increased direction of energy reserves toward milk production. It is unclear if the effect of negative energy or protein balance is the same in cows with a high and low genetic merit for milk production.

The quality of the ovarian cycle is an important aspect of female fertility, and it can be monitored by progesterone concentrations in blood or milk. Postpartum rises in progesterone levels indicate commencement of luteal activity (C-LA), and cows with a high milk production tend to have long intervals to C-LA (Darwash et al., 1997; Royal et al., 2002a; Mann et al., 2005). Cows with a high breeding value for milk production

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Table 1. Compositions of the high caloric density ration and the low caloric rations fed to the cows¹

Item	High caloric density ration	Low caloric density ration
Partial mixed ration (PMR) components	Corn silage (49%) Grass silage (30%) Soybeans, extracted/customized meals (21%)	Grass silage (86%) Concentrates (14%)
PMR supply	Ad libitum	Ad libitum
PMR DM, g/kg of product	377	451
PMR NE _L , MJ/kg of DM	6.5	5.9
PMR DVE, ² g/kg of DM	77.5	65.0
PMR OEB, ³ g/kg of DM	7.4	7.3
Concentrate supply, kg/d	8	3
Concentrate DM, g/kg of product	877	881
Concentrate NE _L , MJ/kg of DM	7.5	7.4
Concentrate DVE, g/kg of DM	119	112
Concentrate OEB, g/kg of DM	27	47

¹The high caloric density ration was balanced and attuned to expected mean milk production; the low caloric density ration was adapted to reduce energy content with minimal effects on other aspects of the ration.

²DVE = true protein digested in the small intestine.

³OEB = degraded protein balance.

experience a delay in ovulation that is independent of phenotypic milk production. These high genetic merit cows may be better adapted to mobilize body energy reserves to ensure nutrient availability for milk production, which may have negative effects on fertility (Gutierrez et al., 2006). Progesterone profiles can be constructed for individual dairy cows, and ovarian cycles can be classified as normal or various forms of abnormal (Darwash et al., 1997; Opsomer et al., 1998; Mann et al., 2005; Petersson et al., 2005). Moreover, various aspects of the cycle such as peak progesterone concentrations and C-LA can be quantified.

The objective of this study was to investigate the relationship between fertility and milk production levels as determined by genotype, environment, and genotype × environment interactions, by monitoring the ovarian cycle of the cow. A second objective was to assess the role of energy and protein balances in the relationship between milk production level and ovarian cyclicity.

MATERIALS AND METHODS

Experimental Animals and Management

Holstein-Friesian primiparous cows with high or low genetic merit for milk, fat, and protein production were kept under 4 different conditions expected to have a marked influence on milk production level, energy, and protein balances. The experimental design enables the assessment of the different roles of genotype, environment, and genotype × environment interactions in relationships between ovarian cyclicity, as determined by milk progesterone profiles, and milk production level, energy, or protein balances.

Experiments were performed at the research farm Nij Bosma Zathe in Goutum, the Netherlands. Heifers were purchased from 61 different farms throughout the Netherlands and before purchase were screened for disease and subclinical infections. Farms from which the heifers were obtained had low risks for harboring paratuberculosis, leptospirosis, or infectious bovine rhinotracheitis, as evidenced by results from specific disease eradication and prevention programs conducted by the Dutch Animal Health Service. Heifers that showed signs of disease, including skin lesions and leg or claw disorders, were not purchased and not included in the study. A total of 100 heifers were used in this study. The experimental animals in this study calved from May 2003 until August 2004.

To create differences in milk yield, cows were selected to have either a relatively high overall genetic merit (**Hgen**) or a relatively low overall genetic merit (**Lgen**). Selection was made on the basis of the Dutch production index (Inet; <http://www.nrs.nl/index-eng.htm> - E9):

$$\text{Inet} = (-0.06 \times \text{EBV milk yield}) + (0.7 \times \text{EBV fat yield}) + (4.2 \times \text{EBV protein yield}).$$

The Hgen cows had an average Inet value of 171, ranging from 112 to 241, and Lgen cows had an average Inet value of -24, ranging from -117 to 34, whereas at the time of the experiment, the average Inet of the Dutch population was 64, with a standard deviation of 99. Differences in Inet were associated with differences in EBV for milk production and fat and protein percentages. The mean milk EBV were 771 (from -6 to 1,704) and -68 (from -729 to 561) for Hgen and Lgen cows, respectively. The EBV for fat percentage was on aver-

Table 2. Number of cows in experimental groups

Item	Low energy		High energy	
	Low merit	High merit	Low merit	High merit
Milking (2×)	11	14	11	14
Frequency (3×)	11	14	11	14

age 0.07 for Hgen (ranging from −0.66 to 0.57) and −0.04 for Lgen (from −0.41 to −0.40). For protein percentage, the mean EBV were 0.08 (from −0.19 to 0.34) and −0.01 (from −0.16 to 0.20) for Hgen and Lgen cows, respectively. The mean reliability of the EBV of the sires of the heifers was 95%. The heifers were sired by 53 different bulls, and the size of half-sib groups was limited to 6 individuals or less. Heifers were 100% Holstein-Friesian or of a mixed breed with 82.5% Holstein-Friesian and 12.5% Dutch Friesian ($n = 8$) bloodlines.

Further differences in milk production were created by splitting the cows in 2 groups with different milking intensity, milking 2 times (2× milk) or 3 times (3× milk) per day. The 2× milk cows were milked at 0600 and 1700 h, the 3× milk cows at 0600, 1400, and 2200. Further differences in milk production were created by splitting the cows in 2 groups receiving different feed rations, a high-quality, high caloric density ration (**Hfeed**) and a low-quality, low caloric density ration (**Lfeed**; Table 1). Water and partial mixed ration were available ad libitum for both groups, and daily roughage intake was recorded per individual. The feeding regimens are described in detail elsewhere (Beerda et al., 2007).

Experimental Design

The treatments were applied in a $2 \times 2 \times 2$ factorial design (Table 2). The primiparous cows were housed indoors in 4 adjacent sections of a cubicle house. The 4 sections each represented 1 of the 4 combinations of milking frequency and ration. Each of the 4 sections consisted of 16 cubicles and 16 feeding troughs and housed both Hgen and Lgen primiparous cows. The unbalanced number of animals between Hgen and Lgen groups (Table 2) was caused by difficulties in finding Lgen heifers on farms with a demonstrable good health status. Cows that were not part of the experiment were added to each group to reach a level of 16 cows per group. Treatments of the cows for disorders were restricted to a minimum as to prevent the masking of variation in health status by management. For ethical reasons, obvious cases of disease were treated. Two cows suffered a light form of endometritis and received antibiotics (ceftiofur hydrochloride) once.

Measurements

During the first 100 DIM, measurements were done to assess milk composition and milk progesterone levels. On an individual level, milk production was registered every milking. Fat and protein content in milk were measured in composite milk samplings from 4 successive milkings per week. Milk samples for progesterone measurements were collected twice a week, with at least 2 d between samplings, in pony vials (Perkin Elmer 6000292, Waltham, MA) containing 2.5 mg of thimerosal (Sigma T-5125, St. Louis, MO), and stored for a maximum of 2 wk at 4°C until analysis by enzyme immunoassay. Progesterone levels in unextracted mixed milk samples were assayed following Van de Wiel and Koops (1986) with minor modifications. Microtiter plates (Costar 3590, Corning Inc., Corning, NY) were coated overnight at 4°C with a 1:10,000 dilution antiserum (rabbit anti-progesterone) in coating buffer (15.02 mM Na_2CO_3 + 35.27 mM NaHCO_3 pH 9.6; 100 μL /well). Eight wells were left empty to serve as background controls. Next, plates were emptied and coated for 50 min at room temperature with 200 μL /well 1% BSA in PBS-T (9.17 mM NaH_2PO_4 /33.79 mM Na_2HPO_4 /145.45 mM NaCl/0.02% thimerosal, pH 7.2). Before adding the diluted milk samples, plates were washed 5 times with 0.05% Tween 80. Milk samples, as well as a high and a low milk progesterone standard (100 μL /well; 1:200 diluted in assay buffer: PBS-T containing 0.1% BSA), were tested in duplicate, and progesterone standard solutions (0, 12.5, 25, 50, 100, and 200 pg/mL in assay buffer containing 0.5% progesterone-free milk; 100 μL /well) were tested in quadruplicate. Fifty microliters of enzyme-labeled progesterone (horseradish peroxidase conjugated progesterone, 10 ng/ μL) was added to each well. The plate was incubated for 2 h at room temperature in the dark. The plates were washed 5 times with 0.05% Tween 80 before 150 μL of substrate was put into each well and incubated for 40 min at room temperature in the dark. Substrate was prepared fresh by adding 1.6 mL of peroxidase buffer (0.48% H_2O_2 in 1 M sodium acetate/27.16 mM citric acid, pH 5.6) and 200 μL of 3,3',5,5'-tetramethyl benzidine (**TMB**) solution [6 mg/mL of TMB (Fluka-87748, Fluka Chemie AG, Buchs, Switzerland) in dimethyl sulfoxide] to 14.4 mL of milli-Q water. After adding 50 μL of 4 M H_2SO_4 , the optical density was read at 450 nm using a Victor multilabel reader. Intraassay variations based on 12 replicates were 10% (7 ng/mL), 3% (11 ng/mL), 3% (22 ng/mL), and 2% (35 ng/mL). Interassay variations were 20% (2 ng/mL) and 16% (12 ng/mL).

The following variables were calculated from the milk recordings and feed intake measurements:

- Milk production was expressed as ECM:

$$ECM = (0.337 + 0.116 \text{ fat}\% + 0.06 \text{ prot}\%) \text{milk}$$

where *milk* is in kilograms per day and *fat%* and *prot%* in percentages.

- Energy balance (EB) was expressed in megajoules per day as:

$$EB = (DMI \times NE_L) - 0.0069[(42.4BW^{0.75} + 442ECM)(0.00165ECM + 0.97525) + 660]$$

where DMI = DMI per day; $NE_L = NE_L$ in total ration; ECM = ECM defined above; and 660 = the correction for growth in primiparous cows.

- Protein balance (PB) in grams per day was calculated as:

$$PB = PI - 54 + 0.1BW + 13.96 \text{ milk} \times \text{prot}\% + [0.000195 \times (10 \times \text{milk} \times \text{prot}\%)^2]$$

where PI = intake of true protein digested in the small intestine and the remainder requirement true protein digested in the small intestine with milk being milk yield in kilograms per day and *prot%* being milk protein as a percentage of milk. These formulas are based on the Dutch energy and protein systems (Van Es, 1978; Tamminga et al., 1994). All 3 variables were calculated per day and then summed over the first 100 d postpartum to get the total production and balances over the experimental period.

Progesterone Profile Analysis

The progesterone profiles were evaluated by 4 quantitative measurements and classified according to Mann et al. (2005). The quantitative measurements were number of days until C-LA, length of first ovarian cycle in days, peak progesterone concentration of first cycle in nanograms per milliliter and number of cycles until 100 d postpartum. Because C-LA was different for cows with prolonged cycles (see results), analyses for C-LA were also done with cows with prolonged cycles excluded, in which a prolonged cycle was defined as a cycle >24 d. A cycle was defined as a period with progesterone >5 ng/mL in at least 2 consecutive samples. Number of ovarian cycles was determined by counting all completed cycles in the first 100 d postpartum. Incomplete cycles were counted as half if at least 3 samples >5 ng/mL occurred before 100 d postpartum. If no cycles were observed in the first 100 d postpartum, the start of the first cycle was set at 106 d. This value was determined by regressing the number of cycles on the start of the first cycle for animals with cyclicity. The

correlation was 0.932, and 0 cycles was reached at 105.98 d. The length and peak progesterone concentration were also determined for the second cycle, but these were strongly correlated to the first cycle, and no qualitative differences were found with the first cycle, so the results will not be presented here. Uterine infections or retained placenta influencing length of progesterone cycles (Royal et al. 2002a, Petersson et al. 2006) did not occur during the experiment, so no analyses for these conditions were needed.

Profiles were also classified according to Mann et al. (2005) into 4 types of ovarian cyclicity:

1. Normal cyclicity: progesterone >5 ng/mL in 2 consecutive samples before 65 d postpartum, without later cessation (see 3) or prolonged luteal activity (see 4).
2. Delayed onset of luteal activity: progesterone <5 ng/mL until >65 d postpartum.
3. Cessation of luteal activity: progesterone <5 ng/mL for >2 wk after at least 2 samples >5 ng/mL.
4. Prolonged luteal activity: progesterone >5 ng/mL for >24 d.

Statistical Analysis

There were 4 continuous outcome variables: C-LA, length and peak progesterone concentration of first cycle, and number of cycles. There were 3 class variables: milking frequency, genetics, and feed regimen. Relationships between the 2 types of variables were analyzed with a 3 factor ANOVA with 2-way interactions included. The relationship of milk yield, energy balance, and protein balance with the class variables was analyzed in the same way. A more elaborate model, in which month of calving, age at calving, and sire of the cow were included as fixed effects, was also analyzed. However, results were very similar, and no difference in significance of effects was detected. Therefore, only results of the simpler model will be presented.

Relationships between 2 continuous variables, such as C-LA and energy balance, were evaluated by simple regression analyses. Interactions of the regressions with the class variables were evaluated by comparing slopes. For example, the slope of C-LA on energy balance was calculated separately for Hgen and Lgen cows to test whether the relationship between energy balance and C-LA differed between Hgen and Lgen cows. Relationships between class variables (e.g., type of ovarian cyclicity) and feed regimen were assessed with χ^2 analyses. All statistical tests were performed with Statistix 8.1.

RESULTS

Associations with Genetic Merit, Milking Frequency, and Feed Ration

The Hgen cows produced significantly more milk than Lgen cows, Hfeed cows more than Lfeed cows, and 3× milk cows more than 2× milk cows (Table 3). Energy and protein balance were both lower for Hgen, Lfeed, and 3× milk cows. These differences were significant ($P < 0.05$) except for the effect of genetics on energy balance ($P = 0.0502$). Two-way interactions were significant for milk yield by genetics and feed, for milk yield by genetics and milking frequencies, and for milk yield by feed and milking frequencies. The interaction between genetics and feed was also found to be significant both for protein balance and energy balance (Beerda et al., 2007). Commencement of luteal activity and number and length of ovarian cycles were not significantly different between categories of genetic merit, milking frequency, feed rations, and their interactions (Table 3), although Hgen cows tended to have later C-LA ($P = 0.10$; $P = 0.069$ for cows with prolonged cycles excluded). The association of milking frequency with peak concentration of progesterone was significant ($P < 0.001$), with 3× milk cows having lower peak progesterone concentrations.

Associations Between Ovarian Activity and Milk Yield Level, Energy Balance, and Protein Balance

Milk yield did not significantly influence progesterone traits (Table 4). Energy balance influenced the number of ovarian cycles ($r = 0.24$) and peak concentration of progesterone ($r = 0.26$). Cows with a more negative energy balance had fewer cycles with lower peak concentrations. Protein balance significantly influenced number of cycles ($r = 0.21$) and C-LA ($r = -0.19$). Cows with a more negative protein balance had fewer cycles and later C-LA (Table 4). When prolonged cycles were excluded, there was a significant association between C-LA and both energy and protein balance (Table 4, Figure 1). Relationships between peak progesterone concentrations and milk yield, energy, and protein balance differed with feed ration (Table 5). Whereas peak progesterone concentrations decreased for Lfeed cows with a greater milk yield or a more negative energy or protein balance, it did not for Hfeed cows.

Relationships Between Treatments and Ovarian Cycle Type

There was no significant difference in percentage of abnormal cycles between cows with different feed ration, genetic merit, and milking frequency (Figure 2,

$\chi^2 = 3.53$, $P = 0.17$). The different types of ovarian cycles (normal, interrupted, prolonged, delayed) did not differ with respect to milk production, energy balance, and protein balance.

Relationships Between Ovarian Cycle Type and Progesterone Profile Parameters

Prolonged cycles had earlier C-LA than normal cycles (75% of prolonged vs. 40% of normal cycles started before 35 d postpartum; $\chi^2 = 4.76$; $P = 0.029$). For all types of ovarian cycles, excluding the prolonged cycles, the regression of C-LA on protein balance was negative (i.e., negative protein balances seemed to delay C-LA when ovarian cycles were not prolonged). For prolonged ovarian cycles, the regression of C-LA on protein balance was not significantly different from 0 ($P = 0.9$), whereas for all other cycles, the regression was -0.24 and significantly different from 0 (Table 4).

DISCUSSION

The influence of genetics, management, and their interaction on ovarian cycles was studied in primiparous cows with high or low genetic merit for overall production that were milked 3 or 2 times a day and fed a high or low caloric density ration. Increased milking frequency reduced peak progesterone concentrations, and there were indications that C-LA was delayed in Hgen cows. Negative energy and protein balances were associated with reduced progesterone peak progesterone concentrations and delayed C-LA, respectively, and both were associated with relatively few ovarian cycles during the first 100 DIM. Some relationships differed between Lfeed and Hfeed cows. In Lfeed cows, low peak progesterone concentrations were associated with relatively high yield and poor energy and protein balances. In Hfeed cows, low peak progesterone concentrations were not associated with high yield and poor protein balances and only slightly with poor energy balance (Table 5). Milk production levels in the different groups differed significantly, but whereas several aspects of ovarian cycles varied over groups, milk yield had no effect. Energy and protein balances explained more variation in ovarian cycles.

Negative effects of milk production on parameters of the ovarian cycle have been reported both phenotypically (Royal et al., 2000a; Wiltbank et al., 2006) and genetically (Veerkamp et al., 2000; Royal et al. 2002a). The present results provide some evidence for the latter, because Hgen cows under conditions of poor protein balance tended to have delayed C-LA compared with Lgen cows when prolonged cycles were excluded from analyses. The present study was performed on primipa-

Table 3. Average 100-d production and progesterone profile traits (SEM in parentheses)¹

Item	Genetic merit			Feed			Milking frequency (Milkfreq)			Genetics × feed			Genetics × Milkfreq			Feed × Milkfreq		
	High (SEM)	Low (SEM)	<i>P</i>	High (SEM)	Low (SEM)	<i>P</i>	3× (SEM)	2× (SEM)	<i>P</i>	HH LH	HL LL	<i>P</i>	High 3× Low 3×	High 2× Low 2×	<i>P</i>	High 3× Low 3×	High 2× Low 2×	<i>P</i>
Milk yield (HL)	25.5 (4.3)	23.9 (4.0)	**	28.9 (4.4)	20.7 (4.2)	***	26.3 (4.3)	23.3 (4.2)	***	30.6 26.8	20.5 21.0	**	26.5 26.2	24.6 21.6	**	31.4 21.4	26.5 20.1	*
Energy balance (MJ)	-1,422 (123)	-1,061 (186)	†	-893 (165)	-1,634 (119)	**	-1,656 (124)	-870 (160)	***	-1,218 -478	-1,625 -1,644	*	-1,733 -1,559	-1,111 -564		-1,403 -1,910	-382 -1,358	
Protein balance (kg)	-4.4 (1.8)	4.6 (2.4)	***	3.2 (2.5)	-4.1 (1.5)	***	-5.2 (1.7)	4.4 (2.3)	***	-4.2 12.7	-4.6 -3.4	**	-7.3 -2.7	-1.6 11.9	†	-3.4 -7.1	9.8 -1.1	
C-LA (d)	52.6 (4.1)	42.6 (3.9)	†	48.1 (4.2)	48.3 (4.1)		51.6 (4.2)	44.8 (4.1)		52.4 42.6	52.8 42.6		58.0 43.5	47.2 41.8		53.2 50.0	43.0 46.6	
C-LA of cycles <25d	55.7 (4.5)	44.3 (4.2)	†	49.9 (4.6)	51.3 (4.4)		53.6 (4.5)	47.3 (4.5)		55.4 43.6	55.9 45.1		60.1 45.1	47.2 43.6		53.2 52.6	44.7 49.9	
Peak concentration (ng/mL)	19.7 (1.1)	21.8 (1.2)		19.9 (1.1)	21.3 (1.2)		17.6 (1.0)	23.6 (1.2)	***	18.9 21.2	20.6 22.3		17.0 18.3	22.4 25.2		17.0 18.1	22.6 24.6	
Cycle length (d)	22.9 (1.6)	23.1 (1.8)		21.5 (1.1)	24.4 (2.1)		21.6 (1.3)	24.4 (2.0)		21.7 21.2	24.1 24.9		21.3 21.9	24.4 24.4		19.1 23.8	23.8 25.0	
Cycles (n)	2.1 (0.2)	2.4 (0.2)		2.3 (0.2)	2.2 (0.2)		2.2 (0.2)	2.3 (0.2)		2.1 2.5	2.0 2.3		2.0 2.4	2.2 2.5		2.2 2.1	2.4 2.2	

¹*P* = significance as determined in a 3-way ANOVA; C-LA = commencement of luteal activity in days after parturition; C-LA of cycles <25 d = C-LA of animals with a first ovarian cycle longer than 24 d excluded; peak concentration = highest progesterone concentration in first ovarian cycle after parturition; cycle length = duration of first ovarian cycle; cycles (n) = number of ovarian cycles in first 100 d after parturition; HH = high genetic merit and high caloric density feed; HL = high genetic merit and low caloric density feed; LH = low genetic merit and high caloric density feed; LL = low genetic merit and low caloric density feed.

†*P* < 0.10; **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

Table 4. Correlation coefficients (r) between milk progesterone traits and production-related traits¹

Item	Milk yield (kg)		Energy balance (MJ)		Protein balance (g)	
	r	P	r	P	r	P
C-LA	0.12		-0.20	†	-0.19	*
C-LA of cycles <25d	0.13		-0.24	*	-0.24	**
Peak concentration	-0.14		0.26	*	0.17	
Cycle length	-0.10		-0.01		-0.02	
Cycles (n)	-0.08		0.24	*	0.21	*

¹Milk yield, energy balance, and protein balance refer to first 100 d postpartum. C-LA = commencement of luteal activity in days after parturition; C-LA of cycles <25 d = C-LA of animals with a first ovarian cycle longer than 24 d excluded; peak concentration = highest progesterone concentration in first ovarian cycle after parturition; cycle length = duration of first ovarian cycle; cycles (n) = number of ovarian cycles in first 100 d after parturition.

†P < 0.10; *P < 0.05; **P < 0.01.

rous cows that exhibit less variation in energy balance and milk yield than in later parities, so stronger differences between Hgen and Lgen cows may exist in later parities. More than Lgen cows, Hgen cows may direct resources to milk production and postpone luteal activity when protein balances are relatively poor. The effects of milk production on ovarian cycles may be indirect, because milk production level per se and management factors that increased milk production

Figure 1. Regression of first luteal activity [commencement of luteal activity (C-LA) in days] on protein balance for Hgen cows and Lgen cows. Cows with prolonged first cycles (>24 d) indicated separately. Regression lines are based on cows with short first cycles (<25 d) only. Hgen = cows of high genetic merit; Lgen = cows of low genetic merit.

Figure 2. Frequency of regular and abnormal ovarian cycles observed for different treatments of primiparous cows. Delayed = commencement of luteal activity >65 d postpartum; cessation = cycles interrupted >2 wk; prolonged = cycle of >24 d; Hgen = cows of high genetic merit; Lgen = cows of low genetic merit; Hfeed = cows fed high caloric density rations; Lfeed: cows fed low caloric density rations; 2× = cows milked twice a day; 3× = cows milked three times a day.

significantly (i.e., milking 3 times a day and feeding a high caloric density ration) were not linked to suboptimal ovarian cycles (that is, either interrupted, prolonged, or delayed cycles). Feeding low caloric density rations was associated with poor energy and protein balances, and in turn, these were associated with unfavorable progesterone profile parameters (few cycles per 100 DIM, low peak progesterone concentrations, and delayed C-LA). In situations of relatively low protein and energy supply, when energy and protein balances are unfavorable anyway, high yield may further compromise the balances and may affect the reproductive system negatively. Milk production levels did not seem to affect the ovarian cycles of the dairy cows directly but rather the energy and protein balances. Gutierrez et al. (2006) suggested that high genetic merit cows are better adapted to mobilize body energy reserves to ensure nutrient availability for milk production, which may have detrimental effects on fertility. Gong et al. (2002) confirmed that high genetic merit cows had later C-LA but showed that a dietary-induced increase in circulating insulin concentrations could alleviate the problem.

The relationship between energy balance and fertility in the dairy cow has been extensively studied, and an unfavorable relationship between negative energy balance and fertility has been confirmed (De Vries and Veerkamp, 2000; Veerkamp, 2002). These results are

Table 5. Slopes of regression lines for milk progesterone traits on production-related traits¹

Item	Milk yield			Energy balance			Protein balance		
	High	Low	<i>P</i>	High	Low	<i>P</i>	High	Low	<i>P</i>
C-LA									
Genetic merit	0.88	−0.26		−0.90	0.18		−0.66	−0.02	†
Feed	1.53	1.53		−0.85	−0.84		−0.41	−0.74	
Milking frequency	0.89	0.39		−0.68	−0.58		−0.40	−0.56	
C-LA of cycles <25 d									
Genetic merit	1.16	−0.42		−1.16	−0.20	†	−0.87	−0.01	*
Feed	1.28	1.53		−0.75	−0.36		−0.35	−0.58	
Milking frequency	0.45	0.56		−0.56	−0.46		−0.53	−0.24	
Peak concentration									
Genetic merit	−0.14	−0.27		0.18	0.18		0.03	0.13	
Feed	0.00	−1.18	*	0.12	0.47	*	0.01	0.40	**
Milking frequency	−0.03	−0.16		0.14	0.08		0.00	0.04	
Cycle length									
Genetic merit	−0.18	−0.27		0.03	−0.06		0.07	−0.06	
Feed	0.03	−0.27		0.13	−0.14		0.05	−0.03	
Milking frequency	−0.27	0.02		0.00	−0.12		−0.01	−0.03	
Cycles (n)									
Genetic merit	−0.028	−0.016		0.039	0.017		0.025	0.007	
Feed	−0.051	−0.084		0.029	0.028		0.001	0.003	
Milking frequency	−0.003	−0.035		0.023	0.034		0.002	0.002	

¹Slopes are compared between groups with different genetic merit, feed ration, and milking frequency, in which columns marked high or low refer, respectively, to high or low genetic merit for overall production, high or low caloric density in feed ration, and 3× and 2× milking per day. C-LA = commencement of luteal activity in days after parturition; *P* = significance of differences in slopes; C-LA of cycles <25 d = C-LA of animals with a first ovarian cycle longer than 24 d excluded; cycle length = duration of first ovarian cycle; cycles (n) = number of ovarian cycles in first 100 d after parturition.

†*P* < 0.10; **P* < 0.05; ***P* < 0.01.

supported by large population studies in which BCS was used as a measure of energy balance (Pryce et al., 2001; Veerkamp et al., 2001; Dechow et al., 2002, 2004; Dal Zotto et al., 2005). Energy balance as measured in the current study was based on inputs and outputs only but ignores other sources of variation in energy balance such as body composition (Veerkamp and Emmans, 1995; Veerkamp, 2002). Body condition score reflects the net result of inputs and outputs and thus is an interesting alternative for calculations of energy balance based solely on inputs and outputs (Coffey et al., 2003; Banos et al., 2004). A strongly negative genetic correlation between C-LA and BCS has been found (Royal et al., 2002b). Unfortunately, BCS were not available at the time of analysis, but future work will involve the relationship between BCS, energy balance, and fertility.

This study did not discover a direct significant relationship between genetic merit and length of cycles, but this could be due to small sample sizes. Royal et al. (2002a) found a strong negative genetic correlation between both milk yield and protein content and length of progesterone cycle. The Hgen cows with a negative protein balance generally started luteal activity later, but if they did start early, they more often had prolonged ovarian cycles (Figure 1). In other words, poor protein balance delayed C-LA when prolonged cycles

were excluded from analyses, but this was not true for prolonged cycles. The latter seems a negative consequence of not postponing luteal activity when protein resources are limited. Opsomer et al. (2000) suggested puerperal problems as the cause of prolonged cycles (rather than metabolic influences), whereas McCoy et al. (2006) linked prolonged luteal phases to retained fetal membranes. Petersson et al. (2006) found a pronounced effect of endometritis on prolonged luteal phases. Clinical health problems were recorded during the present study, but prolonged luteal activity could not be attributed to parturition-related complications. In part, the reported effects in literature of impaired health on prolonged luteal activity may be the consequence of limited availability of resources such as protein, because unhealthy animals are likely to reduce their feed intake. Results of this study suggest that Hgen cows either prolong or delay cycles under poor protein balance.

The observation that a reverse relationship existed in Hgen cows between interval to C-LA and protein balance, but not energy balance, is unexpected. The Hgen cows had significantly lower BCS scores than Lgen cows in the absence of differences in calculated negative energy balance (Beerda et al., 2007). Assuming that these results reflect the biological reality more than inaccuracies in measuring methods, the Hgen

cows may have mobilized more tissue that included a relatively high proportion of protein than Lgen cows. One unit of fat from body tissue contains more energy than 1 unit of protein from body tissue, and use of protein for energy supply could lead to increased loss of body tissue and a decreased BCS in Hgen cows. High levels of protein intake (Elrod and Butler, 1993) as well as low levels of protein intake (Gustafsson and Carlsson, 1993) can impair fertility, and cows with delayed C-LA may have low milk protein content (Gravert et al., 1986). Thus, protein intake and metabolism may influence fertility via different mechanisms such as ammonia detoxification and amino acid availability. The interpretation of the reverse relationship in Hgen cows between interval to C-LA and protein balance remains speculative but may indicate that Hgen cows mobilized body reserves to the extent that relatively large amounts of protein tissue were used as an energy supply, which could compromise the availability of amino acids necessary for the onset of luteal activity.

A negative correlation between milk production level and peak concentration of progesterone (in the first ovarian cycle) was found for cows that received low caloric density rations, and it could be interpreted as a dilution effect. However, within Hfeed cows, there was no relationship between milk production and peak progesterone concentration (Table 5). Three times milking decreased progesterone peak levels more than a simple dilution effect could explain. Progesterone peak levels were 35% lower in cows milked 3 times, whereas milk production was only 14% higher. Differences in DMI could have played a role, because high feed intake stimulates liver blood flow and, in this way, progesterone metabolism (Lucy, 2001). This should have induced lower levels of progesterone in Hfeed cows than in Lfeed cows, because the former had a DMI that was about 4 kg/d higher (Beerda et al., 2007). The more likely interpretation is that high yield contributes to poor energy and protein balances and through this route reduces, in some situations, progesterone peak values. Similarly, cows with strong negative energy balance early postpartum had decreased serum progesterone levels during their third ovarian cycle (Villa-Godoy et al., 1988; Reksen et al., 2002), and diets that increase energy intake promote ovarian activity after calving (Formigoni et al., 1996). Milk releases are associated with increases in oxytocin, brief releases of prolactin, and cortisol responses (Gorewit et al., 1992), and these hormonal responses could affect luteal activity. For example, prolactin can be luteotrophic and increase progesterone secretion by means of potentiating steroidogenic effects of luteinizing hormone. At the same time, it can inhibit enzymatic deactivation of progesterone (Freeman et al., 2000).

In this paper, differences in cycles were analyzed by several continuous variables, as it has been done before (Darwash et al., 1999; Royal et al., 2002a). Others have classified ovarian cycles, for example, as normal, prolonged, or delayed (Opsomer et al., 1998; Royal et al., 2000a, 2002a; Mann et al., 2005; Petersson et al., 2005, 2007). Classes of ovarian cycles have the advantage that they can be used as a diagnostic tool indicating whether or not a cycle is abnormal. Continuous variables have the statistical advantage that stronger parametric test statistics are possible (e.g., ANOVA instead of χ^2 tests). Moreover, classes of ovarian cycles have been defined differently by different authors. For example, some authors classify cycles with progesterone concentrations <3 ng/mL until after 45 d postpartum as delayed (Lamming and Darwash, 1998; Taylor et al., 2003; McCoy et al., 2006), whereas others define delayed cycles as <5 ng/mL until after 65 d postpartum (Mann et al., 2005). The use of different threshold values (i.e., 3 or 5 ng/mL) may cause some variation between studies but will minimally affect the findings regarding study-specific research questions. By using continuous variables, discussions on classifications are bypassed, but classification has its own merits. In the present study, for example, cows with a poor protein balance and early C-LA seem to be at risk for prolonged cycles, and analysis of different classes of cyclicity is a useful addition to the continuous variables describing various aspects of cyclicity.

CONCLUSIONS

Increased milking frequency was associated with reduced peak progesterone concentrations. A poor protein balance was also associated with reduced peak progesterone in Lfeed cows. In Hfeed cows, however, a poor protein balance was not associated with reduced peak progesterone. Delayed C-LA was associated with a more negative protein balance in Hgen cows but not in Lgen cows. Cows in negative energy balance had a high risk for prolonged ovarian cycles when there was no delay in C-LA. Significant indications were found that under conditions of negative energy balance, Hgen cows had a relatively strong tendency to direct resources to milk production and postpone luteal activity, the latter being a route to escape abnormal (i.e., prolonged) ovarian cycles.

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