Milking Frequency, Estradiol Cypionate, and Somatotropin Influence Lactation and Reproduction in Dairy Cows¹

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ABSTRACT

Our objectives were to determine lactational and reproductive outcomes in response to increased milking frequency (MF), injection of estradiol cypionate (ECP), and treatment with bovine somatotropin (bST). Lactating dairy cows ($n = 144$) were blocked by lactation number (1 vs. 2+) and assigned randomly to a $2 \times 2 \times 2$ factorial experiment consisting of 8 treatment combinations: 1) MF consisting of 4× daily milking (4×) for the first 30 d in milk (DIM) vs. 2× daily milking (2×), with all cows milked 2× after 30 DIM; 2) 10 mg of ECP given postpartum at 8 ± 3 DIM versus controls that received ECP diluent (oil); and 3) biweekly bovine somatotropin (bST), starting sometime after 60 DIM, versus no bST. Ovulation before the first artificial insemination was synchronized by using Heatsynch (GnRH injection 7 d before PGF_{2α} followed in 24 h by ECP), and cows were artificially inseminated after detected estrus or at 48 h after ECP, whichever came first. Pregnancy was assessed by transrectal ultrasonography 28 to 30 d after artificial insemination. Daily yield and weekly components of milk were measured during the first 90 DIM. Intervals to first and second postpartum ovulation were unaffected by treatment, but cows were in estrus earlier after 2× (24 ± 4 d) than 4× (41 ± 4 d) daily MF, and sooner after ECP (25 ± 3 d) than after oil (39 ± 4 d) treatment. Pregnancy rates among 4× cows increased for ECP versus oil (52.8 vs. 27.8%) more than for cows with 2× MF treated with ECP versus oil (50.0 vs. 39.4%). Increased MF increased daily milk yields and energy-corrected milk yields during the first 30 DIM. Although milk yields were increased acutely by ECP during the 10 d after its injection, subsequent milk yields were decreased for ECP-treated cows previously milked 4× daily.

Treatment with bST increased overall daily milk yields most in cows previously milked 2× daily and treated with oil and those milked 4× daily and treated with ECP. We concluded that early postpartum ECP injection increased pregnancy rates, but generally had detrimental effects on milk yields after 30 DIM for ECP-treated cows previously milked 4× daily, unless those cows also were treated with bST.

Key words: estradiol cypionate, bovine somatotropin, milking frequency, pregnancy rate

INTRODUCTION

The dairy industry must produce milk efficiently to survive economically. Milk yields can be increased by greater daily milking frequency (MF; Bar-Peled et al., 1995), administration of bST (McBride et al., 1988), or both (Speicher et al., 1994). The optimal number of daily milkings is between 3× and 4×, whereas no biological advantages are likely with milking more than 4× daily (Stelwagen, 2001). When cows had the freedom to choose their own MF by robotic milking processes, they chose an average of 3.9 times per day (Ipema et al., 1987).

To decrease fixed costs and justify the use of idle milking parlors, studies have examined the consequences of increased MF only during early lactation (Bar-Peled et al., 1995). Milk yields were increased by more frequent milkings during early lactation, which persisted even after switching to less frequent milking during later lactation (Bar-Peled et al., 1995). Further, bST is commonly used to increase milk yields (McBride et al., 1988).

An inverse relationship between milk yield and conception rates was detected by analyses of DHIA herd records (Lucy, 2001). Genetic emphasis is placed on selection for milk that is correlated negatively with some reproductive traits (Lucy, 2001). Conception rates (Stevenson et al., 1983) and expression of estrus (Lopez et al., 2004) are compromised by increasing milk yields. The greater the milk yield, the more apparent the compromise on reproductive performance (Harrison et al., 1990).

When administered to postpartum cows, estrogen affects ovarian activity and uterine health. Various

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hormone therapies are common practice in many dairy herds, and are part of fresh cow management. Researchers and veterinarians anecdotally suggest that postpartum prophylactic treatment with estradiol cypionate (ECP) may improve reproductive performance and uterine health. When multiparous dairy cows received ECP (10 mg) at 7 d postpartum, a delay in first postpartum ovulation and subsequent estrus was detected because of reduced circulating FSH (Haughian et al., 2002). Benefits of delayed first postpartum ovulation may include reduced services per cow, reduced interval from calving to conception, and improved conception rates.

Use of bST before AI increased conception rates in cycling cows inseminated at 1 fixed time (timed AI, TAI; Moreira et al., 2001) or inseminated after detected estrus (Santos et al., 2004). Results of embryo studies indicated that bST may be increasing pregnancy rates in lactating dairy cows via enhancing egg maturation, increasing fertilization rates, accelerating early embryonic development, and affecting factors within pregnant cows that enhance embryo development (Thatcher et al., 2002).

All 3 of these managerial tools (bST, ECP, and MF) affect dairy production in different ways and have been researched individually. Our objective was to determine the effects and potential interactions of bST, postpartum ECP injection, and MF (2× vs. 4×) on reproductive and lactational outcomes. We hypothesized that short-term (first 30 DIM) increased MF, forced delayed onset of the estrous cycle in response to ECP injection, and administration of bST in lactating dairy cows may increase reproductive outcomes and overall milk yields.

MATERIALS AND METHODS

Herd Management

The experiment was conducted at the Kansas State University Dairy Teaching and Research Center with cows that had calved between September 2000 and October 2001. Lactating Holstein cows ($n = 144$) were housed in covered free stalls bedded with sand, and twice or thrice (summer) daily were fed a TMR that met or exceeded National Research Council (1989) requirements for lactating cows. The TMR consisted of 30% chopped alfalfa hay, 19% wet corn gluten meal, 15% corn silage, 9.3% whole cottonseed, 4.4% solvent-extracted soybean meal, 3.3% expeller soybean meal, 13% corn grain, 1.3% menhaden fish meal, 1% sugar cane wet molasses, and 3.7% mineral-vitamin premix. Cows had ad libitum access to fresh water. During the summer, pens were covered with shade cloth and water was applied by sprinklers 6×/h for 1 min.

Figure 1. Experimental protocol used in lactating Holstein cows between September 2000 and October 2001. Cows were milked either 2× or 4× daily during the first 30 DIM and then milked 2× thereafter. Cows also received either 10 mg of estradiol cypionate (ECP) in oil or cottonseed oil between d 2 and 15 (8 ± 2.6 d). In addition, cows received either bST (500 mg of Posilac, Monsanto Co., St. Louis, MO) every 14 d starting at approximately 60 DIM, or did not receive bST (no bST). Ovulation was synchronized before AI by using the Heatsynch protocol starting 56 to 75 DIM. Blood was collected (B) before each injection (GnRH, $\text{PGF}_{2\alpha}$, and ECP) during ovulation synchronization to determine cycling status before the onset of the Heatsynch protocol. In the first 82 of 144 cows studied, blood also was collected twice weekly during the first 60 DIM to determine ovulatory patterns. During the first 90 DIM, milk yields were recorded daily, and milk was collected once weekly to determine milk components (fat, protein, lactose, SNF, SCC, and MUN). TAI = timed AI.

Experimental Design

Clusters ($n = 22$) of cows were formed as calving occurred. Within a cluster, cows were assigned to treatments. Variation associated with clusters included seasonal and other climatic differences. The experimental design consisted of a $2 \times 2 \times 2$ factorial arrangement of 8 treatment combinations (Figure 1). The 3 main effects were MF (2× vs. 4× daily), injection of either oil or 10 mg of ECP (Pharmacia and Upjohn, Kalamazoo, MI), and either no or 500 mg of bST (Posilac; Monsanto, St. Louis, MO). Gestating heifers were paired based on their individual predicted transmitting ability for milk, and dry cows were assigned based on the previous 305-2×-ME. Pairs were allocated randomly for milking either 2× or 4× daily. Cows in the 4× treatment were milked 4× daily during the first 30 DIM (0400, 1000, 1600, and 2000 h) and subsequently were milked 2× daily (0500 and 1700 h) until the end of lactation. Remaining cows were milked 2× daily throughout lactation (2× treatment). Within each MF group, alternate cows within lactation block (1 vs. 2+) received an injection of ECP or a cottonseed oil placebo. Injection of ECP or oil was given at 8 ± 2.6 DIM (range = 2 to 15). Within each of the preceding 4 treatments,

beginning at 67 ± 0.4 DIM (range = 57 to 75), alternate cows, within lactation block, received bST every 14 d or received no bST. A total of 18 cows were assigned to each of the 8 treatment combinations, with 10 to 11 first-lactation and 7 to 8 multiple-lactation cows in each treatment combination. Because of differences in when bST was initiated, a somewhat unequal distribution of cows receiving and not receiving bST occurred relative to first AI. Further, similar inequalities occurred as cows approached 90 DIM, when all cows not previously receiving bST were subsequently treated with bST every 2 wk during the remainder of lactation. Body condition scores and BW were assessed weekly for each cow during the first 9 wk of lactation until AI occurred. The BCS was based on a 5-point scale (1 = thin and 5 = fat).

Lactational Traits. Lactational responses to the 3 main effects (MF, ECP, and bST) were assessed in various ways. Individual milk yields were recorded daily during the first 90 DIM. Energy-corrected milk yields were calculated for the first 90 DIM [$ECM = (0.3246 \times \text{milk}) + (0.1286 \times \text{milk} \times \text{fat } \%) + (0.0704 \times \text{milk} \times \text{protein } \%)$]. Milk components (fat, protein, lactose, MUN, and SCC) were measured weekly in composite samples (a.m. and p.m.) per cow by the Heart of America DHIA (Manhattan, KS) for samples collected during the first 90 DIM. Daily yields of fat and protein were calculated.

Reproductive Traits. Blood was collected via coccygeal venipuncture twice weekly from the first 82 of the 144 cows studied and from all cows before each of 3 hormonal injections used to synchronize ovulation before the first AI. Blood was stored at 5°C for 24 h until serum was harvested following centrifugation. Serum was stored at -20°C until assayed by radioimmunoassay to determine concentrations of progesterone (P4; Skaggs et al., 1986). Inter- and intraassay coefficients of variation for 12 assays were 14.8 and 12.1%, respectively. Day of first and second postpartum ovulation was estimated from serum P4 concentrations according to defined criteria (Stevenson and Call, 1983).

Estrus, ovulation, or both were synchronized beginning at 56 to 75 DIM by using the Heatsynch protocol (Figure 1). Relative to TAI (d 0), a GnRH injection (100 µg; Merial Ltd., Iselin, NJ) was administered (i.m.) on d -10, PGF_{2α} (25 mg; Lutalyse, Pharmacia Animal Health, Kalamazoo, MI) was administered on d -3, and ECP (1 mg; ECP, Pharmacia Animal Health) was administered on d -2. Chalk applied over the tail head and pin bones of each cow aided detection of estrus. Cows detected in estrus after PGF_{2α} were AI according to the a.m.-p.m. rule. When no estrus was detected, cows received TAI 48 h after ECP. All AI were per-

formed by the same technician. Transrectal ultrasonography was used to determine pregnancy between 28 and 30 d after AI and later confirmed by uterine palpation by rectum (40 and 53 d after AI). Pregnancy rates (number of cows pregnant divided by number treated) were calculated for each treatment combination.

Cyclicity of each cow was determined by concentrations of serum P4 collected on d -10, -3, and -2 relative to TAI. When P4 was >1 ng/mL (high) in any of these 3 samples, or if estrus was detected (including cows that had tail chalk rubbed off), the cow was classified as cycling. If P4 was <1 ng/mL (on all 3 d), the cow was anestrus at AI.

Statistical Analyses

All data were analyzed by using ANOVA [GLM for categorical variables (GENMOD), continuous variables (GLM), or for repeated measures (MIXED models procedure); SAS Institute, Inc., Cary, NC]. Various reproductive traits were assessed in the reduced group of 82 cows from which blood samples were collected during the pre-AI period and in all 144 cows. Traits assessed before bST included days to first and second ovulation, number of estrous cycles before AI, and days to first behavioral estrus after calving and after ECP treatment, and were analyzed by using procedure GLM with MF, ECP, MF × ECP, lactation block (1 vs. 2+), interactions of the 2 main effects with lactation block, and cluster, as well as BCS as a covariate. Reproductive traits assessed after bST treatment included pregnancy rates and cycling rates based on concentrations of P4 during the 10 d before AI, which were analyzed by using procedure GENMOD with MF, ECP, bST, and all 2-way interactions, as well as lactation block and season of calving (n = 2).

Lactational traits assessed included ECM yields, unadjusted milk yields, milk components, and yields of milk protein and milk fat during the pre-bST period (first 60 DIM and changes in milk yield during the 10 d after ECP injection), and post-bST period (61 to 90 DIM). Changes in BCS and BW during the first 9 wk of lactation were analyzed. In the case of daily changes in milk yield during the 10 d after ECP and weekly changes in BCS and BW, data were normalized relative to the day of ECP administration (percentage basis) or to the first postpartum measure of BCS or BW because of subtle differences among cows milked 4× daily and treated with ECP or oil. Percentage changes were then analyzed by using a mixed models procedure (SAS Institute, Inc.) consisting of MF, ECP, MF × ECP, cow within (MF × ECP), week or day, and all 2- and 3-way interactions with week or day. Initial models

Table 1. Intervals to ovulation, ovulation frequency, and number of estrous cycles before first AI in lactating dairy cows

Item	Treatment ¹				P		
	Milked 2×		Milked 4×		MF	ECP	MF × ECP
	Oil	ECP	Oil	ECP			
Cows ovulating within 7 d after estradiol cypionate, %	15 (20) ²	29 (21)	30 (23)	39 (18)	0.15	0.22	0.59
Days to first ovulation after estradiol cypionate	18 ± 3 (20)	17 ± 3 (21)	18 ± 3 (23)	18 ± 3 (18)	0.82	0.71	0.75
Days to first postpartum ovulation ³	26 ± 3 (20)	25 ± 3 (21)	24 ± 3 (23)	26 ± 3 (18)	0.99	0.94	0.55
Days to second postpartum ovulation ³	44 ± 3 (14)	44 ± 3 (16)	48 ± 3 (20)	45 ± 3 (14)	0.50	0.60	0.70
Interval between first and second ovulation, ⁴ d	20 ± 2 (14)	22 ± 2 (16)	24 ± 2 (20)	22 ± 2 (14)	0.44	0.86	0.40
Number of estrous cycles before AI							
1	100 (20)	100 (21)	100 (23)	100 (18)	0.28	—	—
2	70 (20)	76 (21)	87 (23)	78 (18)	—	0.87	0.39
3	45 (20)	33 (21)	22 (23)	39 (18)	0.40	0.86	0.15

¹Only the first 82 of the 144 cows had blood sampled to obtain these data. Daily milking frequency (MF) was either 2× or 4× during the first 30 DIM and then 2× thereafter. Cows received 10 mg of estradiol cypionate (ECP) or oil between d 2 and 15 postpartum (8 ± 3 d).

²Number of cows.

³Based on an increase in serum progesterone (P4) ≥ 1 ng/mL (above baseline) followed by a normal luteal-phase pattern of P4.

⁴Interaction ($P = 0.05$) of MF × lactation number (2×-first = 24 ± 2; 2×-2+ = 19 ± 2; 4×-first = 21 ± 2; 4×-2+ = 25 ± 2) and tendency ($P = 0.06$) for interaction of ECP × lactation number (oil + first = 24 ± 2; oil + 2+ = 20 ± 2; ECP + first = 20 ± 2; ECP + 2+ = 24 ± 2).

excluded lactation number as a significant source of variation.

The pre-bST model (milk traits assessed during the first 60 DIM) included MF, ECP, MF × ECP, lactation number (1 vs. 2+), and all 2-way interactions with lactation number. The post-bST model included bST in the preceding model with appropriate 2- and 3-way interactions. Full-lactation yields of milk, milk components, and SCS were analyzed by procedure GLM, including MF, ECP, and the MF × ECP interaction in the model. No other independent variables were considered in the model because full-lactation traits were mature-equivalent, 305-d standardized lactation records, which already accounted for age and month of calving and for lactation length.

RESULTS

Reproductive Traits

Occurrence of Ovulation and Estrus. Neither proportions of cows ovulating in response to ECP nor time to ovulation after ECP injection were different from those of cows ovulating spontaneously in response to oil (Table 1). When the timing of the ECP or oil injection was categorized into 3 periods (2 to 6 DIM, $n = 22$; 7 to 9 DIM, $n = 35$; or 10 to 15 DIM, $n = 27$), the percentage of cows that ovulated within 7 d of ECP treatment increased ($P < 0.05$) linearly: 0, 26.5, and 51.9%, respectively (data not shown). Days to first postpartum ovulation ranged from 11 to 61 d, but were

not affected by MF or ECP injection (Table 1). Days to second ovulation did not differ among treatments.

Intervals between first and second ovulations were affected by an interaction ($P = 0.05$) between MF and lactation number (1 vs. 2+). When cows were milked 2× daily, days between ovulations were greater for first-lactation cows (24 ± 2 d) than for multiple-lactation cows (19 ± 2 d). In contrast, cows milked 4× daily and in their first lactation had fewer days between ovulations (21 ± 2 d) than did multiple-lactation cows (25 ± 2 d). A similar tendency ($P = 0.06$) for an interaction was detected between ECP treatment and lactation number. First-lactation cows receiving oil had greater intervals between ovulations (24 ± 2 d) than did multiple-lactation cows (20 ± 2 d). The opposite trend was detected in first-lactation cows receiving ECP (20 ± 2 d) compared with multiple-lactation cows (24 ± 2 d).

Number of estrous cycles per cow before first AI was determined in 82 cows. All cows tested had at least 1 cycle before AI (Table 1). The percentage of cows that had 2 or more cycles did not differ among treatments, with 78% having 2 cycles and 34% having 3 or more cycles.

Estrual Traits. Estrual traits and incidence of cyclicity, assessed in all 144 cows, are presented in Table 2. A greater ($P < 0.01$) percentage of cows given ECP (average = 8 ± 3 d postpartum) were detected in estrus (28%) within 7 d of injection compared with cows receiving oil (5%). When the timing of the ECP or oil injection was categorized into 3 periods (2 to 6 DIM,

Table 2. Estrual response to increased milking frequency (MF) and estradiol cypionate (ECP) injection, occurrence and percentage of cows in estrus, and cyclicity before first AI

Item	Treatment ¹				<i>P</i>		
	Milked 2×		Milked 4×		MF	ECP	MF × ECP
	Oil	ECP	Oil	ECP			
Cows in estrus within 7 d after ECP injection, %	11 (35) ²	42 (36)	0 (36)	14 (37)	<0.01	<0.01	0.17
Days from ECP to first estrus after calving	22 ± 3 (17)	9 ± 4 (23)	39 ± 4 (20)	26 ± 3 (21)	<0.01	0.01	0.99
Days to first behavioral estrus	30 ± 4 (17)	17 ± 3 (23)	47 ± 4 (20)	34 ± 3 (21)	<0.01	<0.01	0.92
Cows in estrus once before AI, %	49 (35)	64 (36)	56 (36)	57 (37)	0.99	0.32	0.42
Cows in estrus twice before AI, %	14 (35)	28 (36)	3 (36)	16 (37)	0.05	0.02	0.99
Cows cycling before AI, ³ %	100 (33)	97 (36)	97 (36)	78 (36)	0.01	0.01	0.13
Cows in estrus before AI, ⁴ %	67 (33)	50 (36)	42 (36)	44 (36)	0.53	0.68	0.15
Hours to estrus after prebreeding ECP injection	27 ± 5 (22)	31 ± 6 (17)	23 ± 6 (15)	28 ± 6 (16)	0.33	0.31	0.86

¹Daily MF was either 2× or 4× during the first 30 DIM and then 2× thereafter. Cows received 10 mg of ECP or cottonseed oil between 2 and 15 postpartum (8 ± 2.6 d).

²Number of cows.

³Based on elevated concentrations of progesterone during the 10 d before AI. Missing blood samples in 3 cows.

⁴Detected in estrus (visual standing) during the 3 d between PGF_{2α} and timed AI.

n = 42; 7 to 9 DIM, *n* = 58; or 10 to 15 DIM, *n* = 44), the proportion of cows in estrus within 7 d of treatment increased ($P < 0.05$) with increasing DIM at the ECP injection: 9.5, 13.8, and 27.3%, respectively. In addition, cows treated with ECP displayed estrus 12 ± 3 d sooner ($P < 0.001$) than cows receiving oil.

Milking frequency influenced the proportion of cows in estrus by 7 d after ECP injection. Cows milked 2× daily (27%) were more likely ($P < 0.01$) to be in estrus than cows milked 4× daily (7%). For cows treated with ECP that showed estrus after ECP, those milked 2× daily were in estrus 24 ± 3 d earlier ($P < 0.01$) than those milked 4× daily.

Both main effects altered days to first postpartum behavioral estrus similarly to alterations in days to first estrus after ECP injection (Table 2). Days to first behavioral estrus in ECP-treated cows (25 ± 3) were fewer ($P < 0.01$) than those in cows treated with oil (39 ± 3). Increasing MF from 2× to 4× daily increased ($P < 0.01$) the interval to first behavioral estrus by 18 ± 3 d.

Similar proportions of cows in each treatment combination were detected in estrus once before insemination (Table 2). More cows ($P < 0.05$) injected with ECP were in estrus twice before AI (22%) than were those after oil injection (10%). In addition, cows milked 2× daily were twice ($P = 0.05$) as likely to have 2 estrous cycles before AI than cows milked 4× daily (21.1 vs. 9.6%).

Incidence of cyclicity in all 144 cows was based on the presence of elevated concentrations of blood P4 sometime during the 10-d period of the Heatsynch protocol. Increased MF ($P < 0.01$) and ECP ($P < 0.01$)

decreased the percentage of cows classified as cycling before first AI. Only 78% of the cows were cycling before AI when milked 4× daily and injected with ECP, compared with a range of 97 to 100% of cows cycling in the other 3 treatment combinations (Table 2).

The proportion of cows in which estrus was detected during a 3-d period between injection of PGF_{2α} and subsequent TAI was determined. Expression of estrus in cows milked 4× daily was not affected by ECP treatment. The interval to estrus after PGF_{2α} ranged from 18 to 39 h, but did not differ among treatments (Table 2).

Pregnancy Rates. The average pregnancy rate was 42.6% in the 141 cows inseminated. Three cows were not included because one was culled before AI, one was inseminated at estrus before the Heatsynch protocol, and the third was inseminated late off schedule. Injection of ECP improved ($P < 0.01$) pregnancy rates, compared with treatment with oil (51.4 vs. 33.3%), but differences were more prominent in cows milked 4× daily than in cows milked 2× daily (Table 3). Cycling cows (*n* = 131) had greater ($P < 0.05$) pregnancy rates than 10 noncycling cows (43.5 vs. 30%; respectively). Cows treated with bST before TAI had similar pregnancy rates as those not treated with bST (45.3 vs. 40.9%, respectively). Pregnancy rates did not differ between cows in estrus before AI and those that received the TAI (45.7 vs. 39.4%, respectively).

Body Condition

Body condition scores were assessed weekly during the first 9 wk of lactation. Initial BCS ranged from

Table 3. Pregnancy rates in lactating dairy cows in response to milking frequency (MF), estradiol cypionate (ECP) injection, and bST¹

bST	2× milked		4× milked		bST	<i>P</i>							
	Oil	ECP	Oil	ECP		MF	ECP	bST	MF × ECP	MF × bST	ECP × bST	MF × ECP × bST	
	(%)												
–	36.8	47.4	28.0	52.0	40.9								
+	42.9	52.9	27.3	54.5	45.3	0.58	<0.05	0.64	0.32	0.63	0.64	0.89	
Σ	39.4	50.0	27.8	52.8	—								
	(No. of cows) ²												
–	19	19	25	25	88								
+	14	17	11	11	53								
Σ	33	36	36	36	141								

¹Cows were milked either 2× daily or 4× daily for the first 30 DIM and then milked 4× thereafter. Cows received 10 mg of ECP or cottonseed oil between d 2 and 15 postpartum, and either received bST or did not, beginning the ninth week of lactation. Cows were treated with GnRH, 7 d later with PGF_{2α}, 24 h later with 1 mg of ECP, and fixed-time inseminated (TAI) 48 h after ECP when estrus did not occur earlier after PGF_{2α}. Three of the original 144 cows were not included because one was culled before AI, one was inseminated early, and one was inseminated well after the scheduled TAI.

²Numbers of cows were not evenly distributed because some cows were inseminated before initiation of biweekly bST treatment.

2.0 to 4.5 and were 2.8 ± 0.04 during the first week postpartum. The average percentage change in BCS declined after parturition in all treatment combinations (Figure 2). Cows milked 4× daily lost more ($P < 0.001$) body condition ($86.6 \pm 4.8\%$ of first BCS) than those milked 2× daily ($90.7 \pm 5.8\%$ of first BCS), with the 4× cows treated with oil tending ($P = 0.06$) to have the greatest loss ($85.7 \pm 7\%$ of first BCS) in BCS (MF × ECP interaction).

Figure 2. Percentage changes in BCS relative to that assessed during the first week postpartum until approximately 9 wk of lactation. Cows were milked either 2× or 4× daily during the first 30 DIM, then 2× thereafter and injected with 10 mg of ECP or cottonseed oil between d 2 and 15 postpartum (8 ± 3 d). Body condition scores ($\pm$ SE) during the first week postpartum for each treatment combination were: 2× Oil = 2.7 ± 0.08 ($n = 35$); 2× ECP = 2.7 ± 0.08 ($n = 35$); 4× Oil = 2.9 ± 0.08 ($n = 35$); and 4× ECP = 2.7 ± 0.07 ($n = 37$).

BW

Percentage change in BW followed similar trends as changes in BCS. Nearly all cows first lost BW and then began to regain lost BW during the fifth or sixth week after calving (Figure 3). Initial BW ranged from 429 to 866 kg and was 606 ± 9 kg during the first week postpartum. Cows milked 4× daily lost more ($P < 0.001$) BW ($98.7 \pm 3.7\%$ of first postpartum BW) than those milked 2× daily ($96.4 \pm 3.2\%$ of first postpartum BW).

Figure 3. Percentage changes in BW relative to that assessed during the first week postpartum until about 9 wk of lactation. Cows were milked either 2× or 4× daily during the first 30 DIM, then 2× thereafter and injected with 10 mg of ECP or cottonseed oil between d 2 and 15 postpartum (8 ± 3 d). Body weights ($\pm$ SE) during the first week postpartum for each treatment combination were: 2× Oil = 603 ± 18 ($n = 35$); 2× ECP = 605 ± 18 ($n = 35$); 4× Oil = 612 ± 18 ($n = 35$); and 4× ECP = 604 ± 17 ($n = 37$).

Figure 4. Percentage changes in daily milk yields were analyzed for the first 10 d after estradiol cypionate (ECP) injection. Cows were either milked 2× or 4× daily during the first 30 DIM, then 2× thereafter and injected with 10 mg of ECP or cottonseed oil between d 2 and 15 postpartum (8 ± 3 d). Milk weights ($\pm$ SE) on d 0 (day of ECP or oil injection) for each treatment combination were: 2× Oil = 26.9 ± 1.5 kg ($n = 36$); 2× ECP = 27.5 ± 1.8 kg ($n = 35$); 4× Oil = 29.2 ± 1.4 kg ($n = 36$); and 4× ECP = 32.3 ± 1.9 kg ($n = 40$).

Lactational Traits

Acute Effects of ECP. Initial effects of ECP injection on milk yields relative to the first 10 d after injection were analyzed after being normalized on a percentage basis. Average milk per day ranged from 7 to 81 kg at the time of ECP injection (Figure 4). Milk yields were increased ($P < 0.01$) consistently by ECP ($112.8 \pm 1.7\%$) compared with oil ($106.3 \pm 1.7\%$). In addition, daily milk yields were increased ($P < 0.001$) by increased MF ($116.2 \pm 1.7\%$ vs. $103 \pm 1.7\%$, respectively) for cows milked 4× versus 2×. No interaction was detected between MF and ECP.

Yield and Composition of Milk During the First 30 DIM. During the first 30 DIM, when half of the cows were milked 4× daily, greater MF increased ($P \leq 0.05$) daily yields of milk, ECM, and MUN (Table 4). An interaction ($P = 0.05$) of MF × ECP indicated that ECP attenuated the increased effect of MF on daily milk yield. Although the percentages of milk fat (4.1 ± 0.08 vs. 3.94 ± 0.08), lactose (4.83 ± 0.04 vs. 4.82 ± 0.04), SNF (8.76 ± 0.07 vs. 8.68 ± 0.07), and SCC (586 ± 112 vs. 333 ± 112) did not differ among cows milked 4× versus 2× daily, respectively, the protein percentage (3.1×0.03 vs. 2.96×0.03) and protein yield tended ($P = 0.06$) to increase in cows milked 4× daily.

As expected, compared with first-lactation cows, older cows had greater ($P < 0.01$) uncorrected milk yields, as well as more ECM; had greater ($P < 0.01$) percentages and yields of fat and protein, and percentages of SNF; and had increased ($P < 0.01$) concentrations of MUN. Somatic cell counts and percentages of

lactose in milk did not differ between first- and multiple-lactation cows. No interactions were detected between lactation number and treatment.

Yield and Composition of Milk During 31 to 60 DIM. Milking frequency was reduced from 4× to 2× daily at 30 DIM. The resulting milk yields and compositions during the subsequent 30 d are summarized in Table 5. With the exception of an effect on MUN, MF had no carryover effects on yield and the composition of milk. Concentrations of MUN, however, remained elevated ($P < 0.05$) in cows previously milked 4× daily. For daily milk yields, a tendency ($P = 0.08$) for a MF × ECP interaction accounted for the lower milk yield in ECP-treated cows previously milked 4× daily, whereas the milk yield was greater for their oil-treated contemporaries milked 4× daily. Older cows produced more milk, more ECM, and greater concentrations of MUN, whereas lactation number had no effect on the percentages of fat, protein, lactose, and SNF, or on SCC. No interactions were detected between lactation number and treatment.

Yield and Composition of Milk from 61 to 90 DIM after Initiation of bST. After 60 DIM, bST treatment was initiated (Table 6). A suppression in milk yield (ECP × MF interaction; $P < 0.05$) was a direct effect of ECP because cows milked 2× versus 4× daily and treated with oil had similar daily milk yields (39.2 vs. 39.6 ± 1 kg, respectively), whereas yields in cows treated with ECP were suppressed (39.8 vs. 36.0 ± 1 kg, respectively). Treatment with bST increased ($P < 0.05$) daily milk yields by 1.8 ± 0.7 kg and decreased concentrations of MUN by 0.6 ± 0.2 mg/dL.

Fairly strong evidence for an interaction ($P = 0.06$) among the main effects (MF, ECP, and bST) for daily milk yield is summarized in Table 6. Previous suppression of daily milk yields by ECP during the first 60 DIM was restored by bST administration, particularly in those cows previously milked 4× and treated with ECP.

Full Lactation Yields and Composition of Milk. After 90 DIM, all cows not previously treated with bST were treated every 2 wk with bST. Mature-equivalent, 305-d standardized lactation records for milk, ECM, fat, protein, and SCS are summarized in Table 7. Neither MF nor ECP had a significant effect on any of the lactation traits.

DISCUSSION

Reproductive Traits

Neither MF nor ECP injection altered the days to first ovulation (based on the onset of the first postpartum increase in P4 concentration in serum, followed by a normal or abbreviated luteal phase) in 82 of 144

Table 4. Lactation traits during the first 30 DIM, during which milking frequency (MF) was 2× or 4× daily and cows were treated during early lactation with cottonseed oil or estradiol cypionate (ECP)¹

Trait	MF	Treatment		MF average	P		
		Oil	ECP		MF	ECP	MF × ECP
No. of cows	2×	36	36	72			
	4×	36	37	73			
		(Mean ± SE)					
Milk, ² kg	2×	29.0 ± 1.1	31.3 ± 1.1	30.1 ± 0.8	<0.01	0.86	0.05
	4×	34.5 ± 1.1	32.6 ± 1.1	33.6 ± 0.8			
ECM, ² kg	2×	31.1 ± 1.2	33.0 ± 1.2	32.1 ± 0.8	0.03	0.91	0.13
	4×	35.4 ± 1.2	33.8 ± 1.2	34.6 ± 0.8			
Fat, ² kg	2×	1.2 ± 0.05	1.3 ± 0.05	1.2 ± 0.04	0.14	0.79	0.24
	4×	1.3 ± 0.05	1.3 ± 0.05	1.3 ± 0.04			
Protein, ² kg	2×	0.9 ± 0.03	0.9 ± 0.03	0.9 ± 0.02	0.06	0.63	0.21
	4×	1.0 ± 0.03	1.0 ± 0.03	1.0 ± 0.02			
MUN, ² mg/dL	2×	11.9 ± 0.3	12.1 ± 0.3	12.0 ± 0.2	<0.01	0.32	0.12
	4×	13.5 ± 0.3	12.6 ± 0.3	13.1 ± 0.2			

¹Daily MF was either 2× or 4× during the first 30 DIM and then 2× thereafter. Cows received 10 mg of ECP or cottonseed oil between d 2 and 15 postpartum (8 ± 3 d).

²Effect of lactation (1 vs. 2+; $P \leq 0.001$). No lactation × treatment interactions.

cows studied (Table 1). In contrast, administration of the same dose (10 mg) of ECP on d 7 postpartum delayed time to first ovulation to 56 d (particularly in multiparous cows; Haughian et al., 2002). Previous studies have demonstrated that increased milk production, which is correlated with increased MF (Bar-Peled et al., 1995), results in negative energy balance and prolongs the days to first postpartum ovulation in dairy cattle (Butler and Smith, 1989).

The ECP injection used in the present study decreased the percentage of cows cycling before AI (particularly those milked 4× daily; Table 2), confirming

recent observations (Haughian et al., 2002). In addition, the postpartum ECP injection decreased the percentage of cows in estrus twice before AI (Table 2).

Postpartum ECP injection and milking 2× daily decreased the time to first postpartum behavioral estrus and increased the percentage of cows in estrus within 7 d of injection. When ECP injection was administered later (10 to 15 d postpartum), more cows showed estrus and ovulated within 7 d of treatment. Expression of estrus did not lead to ovulation when cows were treated before d 10; thus, ECP treatment only triggered sexual behavior. For ovulation to occur, a surge

Table 5. Lactational traits (31 to 60 DIM) during the 30 d after milking frequency (MF) was reduced from 4× to 2× daily in one-half of the cows and when cows were treated during early lactation with either oil or estradiol cypionate (ECP)¹

Trait	MF	Treatment		MF average	P		
		Oil	ECP		MF	ECP	MF × ECP
No. of cows	2×	36	36	72			
	4×	36	37	73			
		(Mean ± SE)					
Milk, ² kg	2×	38.4 ± 1.2	39.5 ± 1.2	39.0 ± 0.8	0.34	0.44	0.08
	4×	39.3 ± 1.2	36.4 ± 1.1	37.9 ± 0.8			
ECM, ² kg	2×	37.9 ± 1.1	37.8 ± 1.1	37.9 ± 0.8	0.31	0.36	0.42
	4×	37.7 ± 1.1	35.8 ± 1.1	36.8 ± 0.8			
Fat, ² kg	2×	1.4 ± 0.04	1.3 ± 0.04	1.3 ± 0.03	0.47	0.33	0.94
	4×	1.3 ± 0.04	1.3 ± 0.04	1.3 ± 0.03			
Protein, ² kg	2×	1.1 ± 0.03	1.1 ± 0.03	1.1 ± 0.02	0.12	0.47	0.22
	4×	1.1 ± 0.03	1.0 ± 0.03	1.1 ± 0.02			
MUN, ² mg/dL	2×	14.2 ± 0.3	14.1 ± 0.3	14.2 ± 0.2	0.03	0.52	0.66
	4×	15.1 ± 0.3	14.7 ± 0.3	14.9 ± 0.2			

¹Daily MF was either 2× or 4× during the first 30 DIM and then 2× thereafter. Cows received 10 mg of ECP or oil between d 2 and 15 postpartum (8 ± 3 d).

²Effect of lactation (1 vs. 2+; $P \leq 0.001$). No lactation × treatment interactions.

Table 6. Lactation traits (61 to 90 DIM) after recombinant bST treatment was initiated in cows whose milking frequency (MF) was reduced from 4× to 2× in one-half of the cows at 30 DIM, for cows treated during early lactation with either cottonseed oil or estradiol cypionate (ECP)¹

Trait	bST	2× milked				4× milked				P					
		Oil		ECP		Oil		ECP		MF	ECP	bST	MF × ECP	MF × bST	ECP × bST
		16	20	12	24	16	20	14	23						
No. of cows	-	16	20	12	24	16	20	14	23			58			
	+											87			
(Mean ± SE)															
Milk, ² kg	-	38.8 ± 1.5	39.8 ± 1.7	39.7 ± 1.5	39.7 ± 1.3	39.7 ± 1.5	39.7 ± 1.6	32.7 ± 1.6	32.7 ± 1.3	0.11	0.14	0.05	<0.05	0.16	0.06
	+	39.6 ± 1.4	39.7 ± 1.3	39.7 ± 1.4	39.7 ± 1.3	39.5 ± 1.4	39.3 ± 1.3	39.3 ± 1.3	39.5 ± 0.6						
ECM, ² kg	-	38.3 ± 1.5	38.4 ± 1.8	38.4 ± 1.5	38.4 ± 1.3	39.2 ± 1.5	33.7 ± 1.7	33.7 ± 1.7	37.4 ± 0.8	0.39	0.12	0.17	0.20	0.36	0.18
	+	39.2 ± 1.4	38.5 ± 1.3	38.5 ± 1.4	38.5 ± 1.3	39.2 ± 1.4	38.7 ± 1.3	38.7 ± 1.3	38.9 ± 0.7						
Fat, ² kg	-	1.4 ± 0.07	1.4 ± 0.07	1.4 ± 0.07	1.4 ± 0.07	1.4 ± 0.07	1.3 ± 0.04	1.3 ± 0.04	1.3 ± 0.04	0.96	0.14	0.42	0.72	0.55	0.32
	+	1.4 ± 0.06	1.3 ± 0.05	1.3 ± 0.06	1.3 ± 0.06	1.4 ± 0.06	1.4 ± 0.06	1.4 ± 0.06	1.4 ± 0.03						
Protein, ² kg	-	1.1 ± 0.04	1.1 ± 0.05	1.1 ± 0.05	1.1 ± 0.05	1.2 ± 0.04	1.0 ± 0.05	1.0 ± 0.05	1.1 ± 0.02	0.15	0.19	0.08	0.04	0.51	0.35
	+	1.1 ± 0.04	1.2 ± 0.04	1.2 ± 0.04	1.2 ± 0.04	1.2 ± 0.04	1.1 ± 0.04	1.1 ± 0.04	1.2 ± 0.02						
MUN, ² mg/dL	-	14.9 ± 0.5	15.0 ± 0.5	15.0 ± 0.5	15.0 ± 0.5	15.9 ± 0.5	15.1 ± 0.5	15.1 ± 0.5	15.2 ± 0.2	0.35	0.82	<0.05	0.82	0.43	0.27
	+	14.8 ± 0.4	14.3 ± 0.4	14.3 ± 0.4	14.3 ± 0.4	14.6 ± 0.4	14.6 ± 0.4	14.6 ± 0.4	14.6 ± 0.2						

¹Daily MF was either 2× or 4× during the first 30 DIM and then 2× thereafter. Cows received 10 mg of ECP or cottonseed oil between d 2 and 15 postpartum (8 ± 3 d).

²Effect of lactation (1 vs. 2+; $P \leq 0.01$). No lactation × treatment interactions.

in LH must be induced by the elevated blood concentrations of estrogen. Induction of the surge in LH is limited by various factors (Short et al., 1979), including suckling, DIM, and dose of estrogen. The gradual increase in the estrual and ovulatory activity observed in the present study is consistent with that report.

As an assessment of energy balance and nutrient status, changes in BW and BCS differed among treatments and between lactation groups. Cows having a BCS <3 are less likely to be detected in estrus and inseminated (Suriyasathaporn et al., 1998). High-producing Holstein cows may lose as much as 10% of their BW and be in negative energy balance for as long as 10 wk after calving (Harrison et al., 1990). Negative energy balance leads to lipolysis, increased plasma concentrations of NEFA, and increased concentrations of β -endorphin (Baile and Della-Fera, 1981). The β -endorphins inhibit secretion of GnRH from the hypothalamus and decrease the number of LH pulses released from the anterior pituitary. In conjunction with having an increased appetite, cows that are milked more frequently have greater DMI (Bar-Peled et al., 1995). On the basis of decreased BCS and BW, it is reasonable to suppose that cows milked 4× daily had a greater negative energy balance than did cows milked 2× daily (Figures 2 and 3). Cows that lost >1 unit of BCS did not show estrus and did not ovulate as soon as cows that lost <1 unit of BCS (Butler and Smith, 1989). Greater losses in BW and BCS, particularly in older cows, partly explain why expression of first estrus, even after ECP treatment, was delayed for cows milked 4× daily. In addition, fewer cows milked 4× daily were cycling before first AI (Table 2).

Postpartum ECP injection improved pregnancy rates (Table 3). Some have suggested anecdotally that ECP may induce myometrial contractions and increase sensitivity of uterine oxytocin and PGF_{2 α} receptors or facilitate uterine involution. But no beneficial effects were detected after a metaphylactic ECP (4 mg) treatment (24 h after calving) given to cows prone to developing uterine infections in response to retained fetal membranes (Risco and Hernandez, 2003). Estimates of postpartum uterine infections in dairy cattle generally are in the 17 to 37% range (Stevenson and Call, 1989). Treatments that specifically improve uterine health, however, as a prerequisite to improved fertility are not well documented (LeBlanc et al., 2002).

Increased MF is usually associated with greater milk yields (Bar-Peled et al., 1995). However, increased milk yields often reduce expression of estrus (Lopez et al., 2004) and reduce conception rates (Stevenson et al., 1983; Lucy 2001). In the current study, pregnancy rates were equally as good in cows milked 4× daily as those milked 2× daily as long as they were

Table 7. Mature-equivalent, 305-d standardized lactation records for cows in which milking frequency (MF) was 2× or 4× daily during the first 30 DIM and cows were treated during early lactation with cottonseed oil or estradiol cypionate (ECP)

Trait	MF	Treatment ¹		P		
		Oil	ECP	MF	ECP	MF × ECP
No. of cows	2×	34	36			
	4×	36	37			
		(Mean ± SE)				
Milk, kg	2×	13,527 ± 393	14,025 ± 381	0.91	0.99	0.20
	4×	14,062 ± 381	13,572 ± 376			
ECM, kg	2×	13,443 ± 393	14,013 ± 374	0.56	0.98	0.14
	4×	14,225 ± 374	13,671 ± 369			
Fat, kg	2×	472 ± 15	496 ± 15	0.28	0.88	0.14
	4×	510 ± 15	490 ± 15			
Protein, kg	2×	404 ± 11	417 ± 11	0.98	0.78	0.15
	4×	420 ± 11	401 ± 11			
SCS	2×	2.6 ± 0.2	3.0 ± 0.2	0.82	0.97	0.16
	4×	2.9 ± 0.2	2.6 ± 0.2			

¹Daily milking frequency (MF) was either 2× or 4× during the first 30 DIM and then 2× thereafter. Cows received 10 mg of estradiol cypionate (ECP) or cottonseed oil between d 2 and 15 postpartum (8 ± 3 d). Treatment with bST was initiated in all cows (not yet treated after 60 DIM) after 90 DIM.

treated with ECP (Table 3). Pregnancy rates achieved in our study after the Heatsynch protocol are consistent with results achieved in other studies (Pancarci et al., 2002).

Use of bST before AI increased conception rates in cycling cows inseminated at one fixed time (Moreira et al., 2001) or at estrus (Santos et al., 2004), in contrast to our results. Results of embryo studies previously indicated that bST may be increasing pregnancy rates in lactating dairy cows via enhancing egg maturation, increasing fertilization rates, accelerating early embryonic development, and affecting factors within pregnant cows that enhance embryo development (Thatcher et al., 2002).

Pre-bST Lactational Traits

Acute effects of MF and ECP were detected during the 10 d immediately after ECP, as well as during the first 30 DIM, before bST treatment was initiated. Greater MF increased milk yields (Bar-Peled et al., 1995) and yields of milk components (Allen et al., 1986). Studies have shown that mammary cells increase in number and metabolic activity with increased MF (Hillerton et al., 1990) and estrogen stimulation (Tucker, 2000), thus leading to increased milk yields (Stelwagen, 2001). Postpartum estrogen may increase milk yields because of mammary cell proliferation (Haughian et al., 2002), increased release of prolactin, and increased receptors for prolactin in mammary alveolar epithelial cells (Tucker, 2000). Findings of increased milk yields during the first 30 DIM in our study (Table 4) confirm earlier observa-

tions for 2× milked, first-lactation cows administered 10 mg of ECP on d 7 postpartum (Haughian et al., 2002).

Administration of estrogen may interfere with the milk-ejection reflex and decrease milk yield (Bruce and Ramirez, 1970). Another hypothesis is that increased milk production in response to greater MF led to an increased energy demand that was suppressed by ECP administration (Table 4). Because estrogen initially reduces postpartum DMI (Invartsen and Anderson, 2000), its use may exacerbate the deficiency in energy intermediates necessary for milk synthesis.

Post-bST Lactational Traits

Overall, daily milk yields were increased in cows treated with bST (Table 6), but not consistently in all treatment combinations. Injections of bST every 2 wk did not improve milk yields in the 2× ECP-treated or 4× oil-treated cows, but it was effective in the 2× oil-treated cows and was greatest in the 4× ECP-treated cows. Although MF increased yields before administration of bST (Table 4), MF had limited effects after bST administration. Increased MF during the entire lactation, plus concurrent administration of bST beginning at 75 DIM, additively increased FCM yields (Speicher et al., 1994).

None of the milk components was altered significantly by bST treatment, except for MUN (Table 6). Composition of milk is generally not altered by bST treatment unless nutritional needs are not met (McBride et al., 1988), so increased milk yield in conjunction with an inadequate supply of dietary energy

and protein often leads to a decrease in milk constituents.

Concentrations of MUN were decreased in response to bST (Table 6). Likewise, in one study (Molento et al., 2002) in which lactating cows were infused during 6 d with saline, bST, insulin + glucose, or bST + glucose + insulin, researchers reported that the protein percentage in milk and MUN were reduced after bST administration during the last 3 d of infusion, regardless of whether insulin was infused.

None of the interactions of MF $\times$ ECP were significant ($P \leq 0.20$) for full-lactation standardized yields (Table 7). Lack of significance is likely attributable to insufficient numbers of cows per treatment. In spite of this, cows milked 2 $\times$ daily and treated with ECP consistently had numerically greater yields of milk, milk fat, and protein, and higher SCS, whereas the reverse was true for cows milked 4 $\times$ daily and treated with oil.

In summary, postpartum ECP injection improved pregnancy rates, regardless of MF. Improved fertility, however, was not related to the timing of first postpartum ovulation before AI or the greater frequency of estrus. Acute increases in milk yield during the 10 d after ECP injection initially were detected in all cows regardless of MF. Greater MF increased daily milk yields and daily ECM during the first 30 DIM, but the increased milk yield was maintained during the next 60 DIM after 4 $\times$ milking was discontinued only in oil-treated cows. Treatment with bST increased overall daily milk yields, but especially those in cows previously milked 2 $\times$ daily and treated with oil, and in cows milked 4 $\times$ daily and treated with ECP. In conclusion, early postpartum ECP injection increased pregnancy rates in lactating cows, regardless of MF, but had later detrimental effects on milk yields for cows previously milked 4 $\times$ daily, unless they were also treated with bST.

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