Short communication: Milk fat depression induced by conjugated linoleic acid and a high-oil and low-fiber diet occurs equally across the day in Holstein cows

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ABSTRACT

Recently, a circadian rhythm of milk and milk component synthesis has been characterized that is partially dependent on the timing of feed intake. Our objective was to determine if inhibition of milk fat synthesis during diet-induced milk fat depression occurred to a higher degree during certain phases of the day. A retrospective analysis was conducted on 2 experiments that induced milk fat depression while milking cows 3 times per day at equal intervals. The response at each milking was analyzed using mixed model ANOVA with repeated measures. In experiment 1, nine multiparous Holstein cows were arranged in a 3 × 3 Latin square design, and treatments were control, 3-d intravenous infusion of 10 g/d of *trans*-10,*cis*-12 conjugated linoleic acid (CLA), and a low-forage and high-fat diet for 10 d. In experiment 2, ten multiparous ruminally cannulated cows were arranged in a replicated design and milk samples were collected during a control period or after 5 d of abomasal infusion of 10 g/d of CLA. The daily pattern of milk fat concentration and yield did not differ between treatments in either experiment. In experiment 1, an effect was found of treatment and milking time on milk fat concentration and yield. Similarly, in experiment 2, main effects were found of treatment and milking time on milk fat concentration and an effect of treatment, but no effect of milking time on milk fat yield. Milk fat percent was increased from 3.41 to 4.06% and 3.25 to 3.48% from the morning to the afternoon milking in experiments 1 and 2, respectively. Additionally, milk fatty acid profile, including *trans* intermediates, was changed over the day in experiment 1, but the magnitude of the changes were small and the pattern did not differ among treatments. A daily rhythm of milk fat concentration and yield was observed in cows

milking 3 times a day, but milk fat depression decreases milk fat yield equally over the day.

Key words: milk fat depression, circadian, diurnal, conjugated linoleic acid

Short Communication

Fat is the most variable component of milk and its synthesis is affected by many factors, with diet being a major factor. Diet-induced milk fat depression (MFD) is a specific reduction in milk fat synthesis caused by feeding diets with high levels of rapidly fermentable carbohydrates and unsaturated FA (reviewed by Bauman and Griinari, 2003; Harvatine et al., 2009). The reduction in milk fat synthesis is attributed to reduced mammary capacity for lipid synthesis caused by bioactive *trans* FA intermediates generated during altered rumen biohydrogenation of unsaturated FA by rumen microbes. Specifically, *trans*-10,*cis*-12 CLA is one of the bioactive isomers that has been extensively studied with its specific effects confirmed by intravenous and abomasal infusion experiments in dairy cows. Importantly, infusion of CLA replicates the phenotype and molecular events associated with MFD without other confounding factors such as changes in the amount and profile of absorbed VFA.

Most physiological processes follow a daily or circadian rhythm that is controlled by endogenous time-keeping mechanisms commonly referred to as biological clocks. Clocks enable the synchronization of behaviors and physiological processes with daily changes in the external environment. Clocks also permit the coordinated activities between organs and circadian rhythms have been well described for most physiological processes [reviewed by Dibner et al. (2010)]. In mammals, the dominant circadian pacemaker is located in the suprachiasmatic nucleus of the hypothalamus, which organizes the temporal activity of peripheral clocks located throughout different organs of the body by regulating a series of neural and hormonal signals. A daily rhythm of intake also creates a daily rhythm of rumen fermentation and nutrient absorption. For ex-

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ample, Loor et al. (2004) reported a daily pattern of ruminal *trans* FA isomers in rumen fluid of cows fed high-oil diets.

Dairy farmers commonly recognize that morning and evening milking differ in milk yield and composition. Gilbert et al. (1972) reported 0.65 kg higher milk yield at the morning milking, but 0.32 and 0.09 percentage unit higher milk fat and protein, respectively, at the evening milking in cows milked at 12-h intervals. More recently, Quist et al. (2008) conducted a survey of the milking-to-milking variation in milk yield and composition on 16 dairy farms. Milk yield and milk fat concentration showed a clear repeating daily pattern over the 5 d of observation in herds that milked 2 and 3 times per day. Morning milking was over 1 percentage unit lower in milk fat than the evening milking. We recently observed milk and milk component yield at each milking while milking every 6 h and feeding cows 1 time per day at 0800 h or in 4 equal feedings every 6 h and demonstrated that the daily rhythm of milk fat synthesis was dependent on the timing of nutrient intake (Rottman et al., 2014). Specifically, feeding cows 4 times per day in equal meals increased milk fat yield and milk fat concentration across the entire day and reduced the amplitude of the daily rhythm of milk fat synthesis. However, no daily rhythm of milk *trans* C18:1 FA occurred and only minor differences in concentrations of FA <16 carbons (de novo synthesized) and FA >16 carbons (preformed from plasma) were observed.

The daily rhythm of milk fat synthesis in the dairy cow may be due to the daily rumen dynamics including *trans* FA synthesis and passage to the intestine, an endogenously generated rhythm driving mammary metabolic capacity, or a within-day change in the responsiveness of the mammary gland to bioactive FA. To our knowledge, the daily pattern of milk fat during MFD has not been investigated. Therefore, the objective of the current study was to determine whether bioactive FA decreased milk fat synthesis equally across the day. To answer this question, the daily rhythm of milk synthesis from 2 previously conducted experiments that induced MFD by either feeding a low-fiber, high-oil diet or by intravenous or abomasal infusion of *trans*-10,*cis*-12 CLA was analyzed. Our hypothesis was that MFD occurs equally across the day during both diet- and CLA-induced MFD.

All animal procedures were approved by the Cornell University Institutional Animal Care and Use Committee. Briefly, 9 mid-lactation cows (193 ± 32 d postpartum; mean $\pm$ SD) were housed in tie-stalls with approximately a 20L:4D cycle and assigned randomly to a 3×3 Latin square design. Treatments were control (CON1), 3 d intravenous infusion of *trans*-10,*cis*-12 CLA (iCLA; 10 g/d in intralipid emulsion), and feed-

ing a low-forage, high-oil diet for 10 d (LF/HO; 3.0% soybean oil and 1.5% fish oil). Cows were milked every 8 h (0800, 1600, and 2400 h) and milk yield and composition were determined at each milking on the last day of each experimental period (described in Harvatine and Bauman, 2006). The LF/HO treatment decreased daily milk fat yield 38% and intravenous CLA infusion decreased daily milk fat yield 24% (Harvatine and Bauman, 2006). To investigate the daily rhythm of milk synthesis, data from a single day for each cow-period were analyzed using the PROC MIXED procedure in SAS with the repeated statement (version 9.3, SAS Institute Inc., Cary, NC). The model included the fixed effect of treatment, milking time, and the interaction of treatment and milking time and the random effect of cow and period. Subject was cow by period, and the Kenward-Rogers denominator degree of freedom adjustment and the ARH1 covariance structure were used. Cosine analysis was not conducted because of the lower frequency of data collection.

No treatment by milking time interaction was found for milk fat yield and concentration when MFD was induced by intravenous CLA infusion and feeding the LF/HO diet (Table 1). The decreases in milk fat yield and concentration were similar at all milkings (24 to 25% for iCLA and 38 to 42% for LF/HO; Table 1). A main effect was found of time on fat yield and concentration, with milk fat yield in control cows ranging from 392 to 445 g/milking (Table 1; $P < 0.05$). A treatment by milking time interaction was found for milk and milk protein yield, supporting a daily rhythm of milk synthesis.

Milk FA profile was analyzed as described by Rico and Harvatine (2013) to characterize *trans* FA isomers and de novo synthesized FA that are known to change during MFD. An effect was observed of treatment and time, but no treatment by time interaction was observed for the proportion of de novo synthesized and preformed FA and the 16-carbon desaturase ratio (Table 2). De novo synthesized FA were lowest at the 1600 h milking and highest at the 2400 h milking, although the difference between milkings was less than a 5% change. Similarly, there was an effect of treatment and time and no interaction of treatment and milking time on the proportion of *trans*-10 C18:1, an isomer of the alternate biohydrogenation pathway associated with diet-induced MFD. *Trans*-10 C18:1 was lowest at 2400 h, although the difference between milkings was between a 6.5 to 13% change. Lastly, a treatment by milking time interaction was found for *trans*-11 C18:1, which peaked at 1600 h. *Trans*-11 C18:1 was almost 11% different between milkings in the LF/HO treatment, whereas little difference occurred over the day in control and CLA infusion. The complete FA profile is reported in Supplemental

Table 1. Milk yield and milk component yield and composition by milking during CLA- and diet-induced milk fat depression in cows milked every 8 h in experiment 1

Item	Milking ¹			SE	P-value ²		
	0800 h	1600 h	2400 h		Trt	Time	Trt × time
Milk, kg					<0.01	0.02	0.03
CON1	11.5	11.0	11.7	0.56			
iCLA	11.7	10.4	11.6	0.56			
LF/HO	10.3	10.1	9.3	0.58			
Fat yield, g					<0.001	0.04	0.95
CON1	392	445	399	25			
iCLA	298	332	301	11			
LF/HO	243	268	233	11			
Fat, %					<0.001	<0.001	0.39
CON1	3.41	4.06	3.40	0.23			
iCLA	2.56	3.26	2.63	0.23			
LF/HO	2.39	2.66	2.58	0.25			
Protein yield, g					<0.01	0.07	0.02
CON1	346	343	369	15			
iCLA	345	319	367	15			
LF/HO	309	309	288	16			
Protein, %					0.73	<0.001	0.32
CON1	3.01	3.12	3.15	0.09			
iCLA	2.95	3.09	3.08	0.09			
LF/HO	2.96	3.08	3.17	0.95			

¹Least squares means at each milking of cows fed a high-forage and low-oil TMR once per day (CON1), control TMR and 3-d intravenous infusion of 10 g/d of *trans*-10,*cis*-12 CLA (iCLA), and cows fed a low-forage, high-oil diet for 10 d (LF/HO).

²Significance of model effect including the main effect of treatment (Trt), milking time (Time), and their interaction (Trt × time).

Table S1 (<http://dx.doi.org/10.3168/jds.2014-8614>). A treatment by time interaction was observed for C6:0, C14:1, C18:0, *trans*-9 18:1), but the magnitude of the differences was very small and the differences do not appear to be biologically relevant. Collectively, these data demonstrate a daily rhythm of milk fat synthesis and that the mammary gland is equally responsive to bioactive FA across the day.

Data from a second experiment were analyzed to validate that the mammary gland is equally responsive to CLA across the day. In experiment 2, ten multiparous ruminally cannulated cows were randomly arranged in a replicated design and managed similar to described above. Treatment period was 14 d and treatments were control (CON2) and 5 d abomasal infusion of *trans*-10,*cis*-12 CLA (aCLA; a priming dose of 7.5 g followed by 10 g/d infused in equal amounts 4 times per d). The time course of response to this dosing procedure was previously published and demonstrated complete induction of MFD within 3 d (Harvatine and Bauman, 2011). Milk samples were collected at each milking on the last day of each treatment period and data were analyzed as described above. Milk fat yield and concentration were decreased by CLA infusion, although the decrease was similar across the day (39 to 44% decreased in fat percent and 38 to 49% decrease in fat yield). However, milk fat concentration in both control and treatments varied 0.21 and 0.15 units between milkings (Table 3). A treatment by milking time interaction was observed for milk protein concentration and a tendency for a

treatment by milking time interaction for milk protein yield (Table 3; $P < 0.05$). Milk FA profile was not analyzed because of the limited differences observed with CLA treatment in the first experiment described above.

The role of *trans*-10,*cis*-12 CLA in MFD has been extensively studied and the mechanism is well established (reviewed by Harvatine et al., 2009), although the daily pattern has not been specifically investigated. In the current experiment, a rhythm of milk fat synthesis was observed over the day, although CLA- and diet-induced MFD failed to modify this daily pattern. Although the magnitude of the change in *trans*-10 C18:1 and *trans*-11 C18:1 over the day is small, peak concentration of *trans*-10 C18:1 and *trans*-11 C18:1 coincided with the lowest and highest milk fat concentration and yield in the LF/HO treatment, respectively. A larger amplitude may occur in the daily rhythm of intestinal absorption of *trans* isomers, but the amplitude of the rhythm is expected to be decreased by dilution in the plasma lipid pool. The present experiment recorded milk yield and composition 3 times per day, and the ability to observe changes in the rhythm is dependent on the frequency and timing of milking intervals. Higher resolution data may allow identification of a change in the rhythm that was below the level of detection in the current experiment. However, the change in amplitude or phase of the rhythm is expected to be quite small based on observations at 8-h intervals.

The lactating rat has a daily rhythm in mammary metabolism with approximately a 1-fold change in lactose

Table 2. Milk fatty acid profile by milking during CLA- and diet-induced milk fat depression in cows milked every 8 h in experiment 1

FA, % FA	Milking ¹				P value ²		
	0800 h	1600 h	2400 h	SE	Trt	Time	Trt × time
<i>trans</i> -10 C18:1					<0.001	<0.01	0.79
CON1	0.39	0.41	0.36	0.06			
iCLA	0.45	0.46	0.40	0.06			
LF/HO	8.91	8.72	8.33	1.60			
<i>trans</i> -11 C18:1					<0.001	<0.01	0.01
CON1	1.08	1.07	1.03	0.25			
iCLA	1.32	1.32	1.27	0.29			
LF/HO	6.12	6.87	6.62	1.71			
FA <16 carbons ³					<0.001	<0.001	0.52
CON1	28.98	27.91	29.26	1.05			
iCLA	27.34	26.24	27.41	1.01			
LF/HO	21.15	20.82	21.58	1.13			
16-carbon FA ⁴					<0.001	<0.001	0.46
CON1	34.28	33.72	35.35	0.84			
iCLA	32.53	31.47	33.41	0.80			
LF/HO	29.03	27.59	28.95	0.89			
FA >16 carbons ⁵					<0.001	<0.001	0.33
CON1	29.99	31.26	28.85	1.12			
iCLA	32.93	34.99	32.33	1.07			
LF/HO	39.29	40.97	39.40	1.20			
16 Carbon desaturase ratio ⁷					<0.001	0.02	0.55
CON1	0.057	0.061	0.057	0.008			
iCLA	0.060	0.063	0.059	0.009			
LF/HO	0.099	0.099	0.098	0.014			

¹Least squares means at each milking of cows fed a high-forage and low-oil TMR once per day (CON1), control TMR, and 3-d intravenous infusion of 10 g/d of *trans*-10,*cis*-12 CLA (iCLA), and cows fed a low-forage, high-oil diet for 10 d (LF/HO).

²Significance of model effect including the main effect of treatment (Trt), milking time (time), and their interaction (Trt × time).

³FA <16 carbons originate from de novo synthesis.

⁴16-carbon FA originate from both de novo synthesis and plasma.

⁵FA >16 carbons originate from plasma.

⁶Calculated as C16:1/(C16:0 + C16:1).

synthesis and approximately a 2-fold change in lipid synthesis activity occurring over the day (Carrick and Kuhn, 1978; Munday and Williamson, 1983). Maningat

et al. (2009) estimated that ~7% of the genes assayed in mRNA extracted from human milk fat displayed a daily pattern of expression. The circadian-regulated

Table 3. Milk yield and milk component yield and composition by milking during abomasal CLA infusion in cows milked every 8 h in experiment 2

Item	Milking ¹				P-value ²		
	0800 h	1600 h	2400 h	SE	Trt	Time	Trt × time
Milk yield, kg					0.37	0.61	0.14
CON2	9.8	9.0	9.3	1.3			
aCLA	8.4	8.8	8.3	1.4			
Fat yield, g					<0.01	0.64	0.36
CON2	323	317	324	41			
aCLA	166	198	167	45			
Fat, %					<0.001	0.03	0.86
CON2	3.25	3.48	3.46	0.16			
aCLA	1.81	2.14	1.96	0.19			
Protein yield, g					0.43	0.86	0.08
CON2	290	275	292	32			
aCLA	250	275	251	37			
Protein, %					0.14	<0.001	0.01
CON2	3.03	3.12	3.19	0.12			
aCLA	3.16	3.27	3.18	0.13			

¹Least squares means at each milking of cows fed a high forage and low oil TMR once per day (CON2) or 5-d abomasal infusion of 10 g/d of *trans*-10,*cis*-12 CLA (aCLA).

²Significance of model effect including the main effect of treatment (Trt), milking time (time), and their interaction (Trt × time).

genes include many enzymes and regulatory factors important to milk synthesis. For example, thyroid hormone responsive spot 14 expression in the liver follows a robust circadian rhythm with a ~10-fold change in expression over the day that is under control of both the light- and food-entrainable oscillator (Ishihara et al., 2007). Additionally, transcription of sterol response element binding protein 1 (**SREBP1**) was responsive to the time of feeding (Brewer et al., 2005; Matsumoto et al., 2010) and regulation of SREBP1 was responsive to a circadian clock ancillary gene (Le Martelot et al., 2009). We have previously shown that expression of thyroid hormone responsive spot 14 and SREBP1 are decreased in the mammary gland during CLA- and diet-induced MFD in the cow (Harvatine and Bauman, 2006). However, there does not appear to be a circadian-dependent interaction of bioactive FA and these regulatory mechanisms in the mammary gland based on the equal inhibition of milk fat synthesis across the day.

The CLA infusion was applied equally across the day, but the LF/HO diet was fed ad libitum, allowing the cow to select the timing of intake. Changing the timing of food intake entrains peripheral circadian rhythms in rodent models, and changing the timing of feeding, timing of feed availability, or composition of nutrient absorbed over the day may change the daily rhythm of milk and milk component synthesis in dairy cows. However, rumen fermentation and the large rumen volume would tend to reduce the magnitude of changes in absorbed nutrients associated with meals compared with nonruminants. However, we recently reported that feeding cows 4 times per day in equal meals increased daily milk fat yield and decreased the amplitude of the daily rhythm (Rottman et al., 2014). This provides intriguing questions for future experiments.

In conclusion, the current study demonstrates a daily rhythm of milk fat synthesis that is maintained during MFD, whether induced by CLA or by a diet resulting in alternate rumen biohydrogenation. We do not find support for within-day changes in the sensitivity or responsiveness of the mammary gland to bioactive FA or for a daily rhythm of *trans* isomer absorption as the driver of the daily rhythm of milk fat synthesis. Other possible mechanisms of the daily rhythm of milk and milk fat synthesis include a daily rhythm of substrate available for milk synthesis or an endogenous rhythm of mammary synthetic capacity driven by a biological clock.

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