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Diurnal variation in blood plasma metabolites and insulin of lactating dairy cows grazing pasture and supplemented with a high-lipid supplement either once or twice a day

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ABSTRACT

The experiment determined the diurnal variation of a blood plasma hormone and three metabolites (insulin, glucose, NEFA and triglyceride) and how these varied with different frequencies of supplementary feeding. Twenty-four cows in early lactation (DIM 81 ± 20) were divided randomly into three treatments; grazed pasture only and pasture supplemented with 2 kg/cow/day of ruminally protected oilseed supplement (metabolisable energy 19 MJ/kg DM) either once a day after morning milking or twice a day after morning and afternoon milking. On day 12 of the experiment, 12 cows (4 cows/treatment) were jugular catheterised and commencing at 1600 h, blood sampled at 2-hourly intervals for 24 h. This protocol was repeated for the remaining 12 cows on day 13. Concentrations of the selected blood plasma hormone and metabolites varied diurnally ($P < 0.001$). The difference between the average peak and nadir concentrations over the 24 hours were 54%, 66%, 224% and 63% for plasma insulin, glucose, NEFA and triglyceride respectively. Feeding the lipid supplement increased blood plasma NEFA ($P < 0.01$), and triglycerides ($P < 0.001$) but had no effect on insulin, or glucose concentrations. Frequency of supplementary feeding influenced ($P < 0.05$) the diurnal variation of NEFA but not, insulin, glucose, or triglyceride. Concentrations of NEFA, glucose, and triglyceride were variable between 0600 h and 1000 h and stabilised between 1600 h to 2000 h. Plasma insulin concentrations were low and stable between 0400 h to 0800 h. Consideration of diurnal variation and the stability of the blood plasma components measured led to the recommendation that the best time to blood sample for insulin is between 0400 – 0800 h and for glucose, NEFA, and triglyceride, between 1600 2000h.

Key Words: diurnal variation; plasma insulin; plasma glucose; plasma NEFA; plasma triglyceride.

INTRODUCTION

Concentrations of a range of blood metabolites, obtained from single blood samplings have been used as indicators of health and nutritional status in commercial dairy herds (Payne *et al.*, 1970; Payne *et al.*, 1974) and, in many dairy experiments, as either background descriptive data or as a means of interpreting treatment effects. Many authors (Coggins & Field 1976; Sutton *et al.* 1988; Kolver & Macmillian 1993; Eicher *et al.* 1999; Blum *et al.* 2000) have reported that common metabolites used in determining health and nutritional status of dairy cows vary diurnally, and with the frequency of feeding. These metabolites also vary between days (Kolver & Macmillian 1993) and with stage of lactation (Blum *et al.* 2000). Under controlled feeding situations, diurnal variation is reduced as the frequency of feeding increases (Sutton *et al.* 1988; Eicher *et al.* 1999) and these authors stressed that, in situations where only one blood sample was taken, standardisation, at least in respect of time of feeding, was necessary for reliable interpretation of blood parameters. To minimise the effects of within- and between-day variation on trial results, Kolver & Macmillian (1993) recommended that blood sampling should be done on specific animals at a standard time over consecutive days. In the grazing situation, the pattern of feed intake is predetermined by cow behavioural patterns, milking times and grazing management. In commercial dairying, and nutritional experiments, supplementary feeding at set times is common practice. This usually occurs once a day after the morning milking or twice a day after both the morning and afternoon milkings. With consideration to the published literature, such a practice may exacerbate the diurnal variation of

blood metabolites that occurs in a grazing situation. The objective of this experiment was to investigate the diurnal variation and effect of supplementary feeding frequency on insulin and various blood metabolites in grazing dairy cows.

MATERIALS AND METHODS

Twenty-four multiparous Friesian cows in early-mid lactation, 81 ± 20 days in milk, were randomly allocated to one of three treatments based on milk yield, milk composition, live weight and body condition score. Treatments were: pasture only (P), pasture plus 2 kg/cow/day lipid supplement (70% full-fat canola:30% full-fat soy ruminally protected meal, Rumentek Industries, Australia) fed once a day after morning milking at 0800 h (PS1), and pasture plus 2 kg/cow/day of the same lipid supplement fed twice a day; 1 kg/cow/day after morning milking at 0800 hr and 1 kg/cow/day at the end of the afternoon milking at 1600 hr (PS2). At each feed the cows were held in individual stalls until each cow had consumed all supplement. All cows were returned to pasture at the same time after supplementary feeding. The experiment consisted of a one-week preliminary period and a two-week treatment period. The preliminary period was used to allow cows to adjust to trial management conditions and to obtain covariate of production parameters to use in the statistical analyses. During the preliminary and experimental period the animals grazed together as one herd on high quality pasture. Following each milking, cows were offered a fresh allocation of pasture with an average daily allowance of 30 kg DM/cow/day.

Cows were milked twice daily at approximately 0800 and 1600 h. Twice-weekly milk yield was recorded using

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in-line milk meters (TruTest, Palmerston North, New Zealand). Individual milk samples were taken from consecutive PM/AM milkings and daily composites based on weight proportionality according to milk yields were prepared. A composite aliquot was analysed for fat, crude protein, true protein, casein, lactose, and total solids using an infrared milk analyser (FT120; Foss Electric, Hillerød, Denmark). Cows were weighed and assessed for body condition score (CS) before morning milking for two consecutive days at the end of the preliminary and experimental periods. The averages of the respective two-day assessments were used in the analyses.

On day 12 of the experimental period, 12 cows (four from each treatment) were jugular catheterised and blood sampled at 2-hourly intervals (commencing at 1600 h) for 24 hours. On day 13, the remaining cows were catheterised and blood sampled in a similar manner. Throughout the blood-sampling period, all cows grazed adjacent to the sampling facilities and were brought in at 2-hourly intervals throughout the 48-hour sampling period.

Blood samples were collected from the jugular catheter by syringe and deposited into a heparinised vacutainer placed on ice. Plasma was separated following centrifugation within 1h of collection and the supernatant split into two aliquots. One aliquot was sent to Alpha Scientific, Hamilton for analysis of non-esterified fatty

acids (NEFA), glucose, and triglycerides and the second aliquot was frozen and analysed later for insulin at the Dexcel laboratories, Hamilton. NEFA (calorimetric method), glucose (hexokinase method) and triglyceride (lipoprotein lipase/peroxide method) concentrations were determined on the Hitachi 717 (Boeringer-Mannheim) automatic auto analyser. The coefficient of variation for these assays were less than 5%. Insulin was determined by radioimmunoassay (Coat-A-Count, Diagnostic Products Corporation, Los Angeles).

Dairy cow production data was analysed using ANOVA with pre-trial uniformity data as a covariate. The blood plasma data was analysed using repeated measures ANOVA with Greenhouse-Geiser (1959) adjustment. All analyses were done using Genstat.

RESULTS

The chemical composition of pasture and the lipid supplement is presented in Table 1. The ruminally protected lipid supplement comprised a 70:30 canola:soy meal with high concentrations of total long-chain fatty acids (LCFA) and protein. It was estimated, assuming a daily pasture intake of 16 kg DM/cow and 560 g LCFA/cow, that cows on the PS1 and PS2 treatments would have been consuming approximately twice the amount of LCFA/cow/day as those on the P treatment. The composition of the LCFA differed considerably between

TABLE 1: Average composition and long-chain fatty acids (LCFA) of pasture and the 70:30 canola: soy ruminally protected meal fed during the period of monitoring diurnal variation of blood plasma metabolites in grazing dairy cows. DM = dry matter, NDF = neutral detergent fibre, DOM = digestible organic matter.

Feed	DM %	% of DM			
		Protein	NDF	DOM	LCFA
Pasture	16.3	19.5	23.6	80.2	3.5
Supplement	92.0	28.1	16.3	-	25.4
		Long chain fatty acids (% total LCFA)			
	C _{16:0}	C _{18:0}	C _{18:1}	C _{18:2}	C _{18:3}
Pasture	15	4	3	13	59
Supplement	8	3	46	31	10

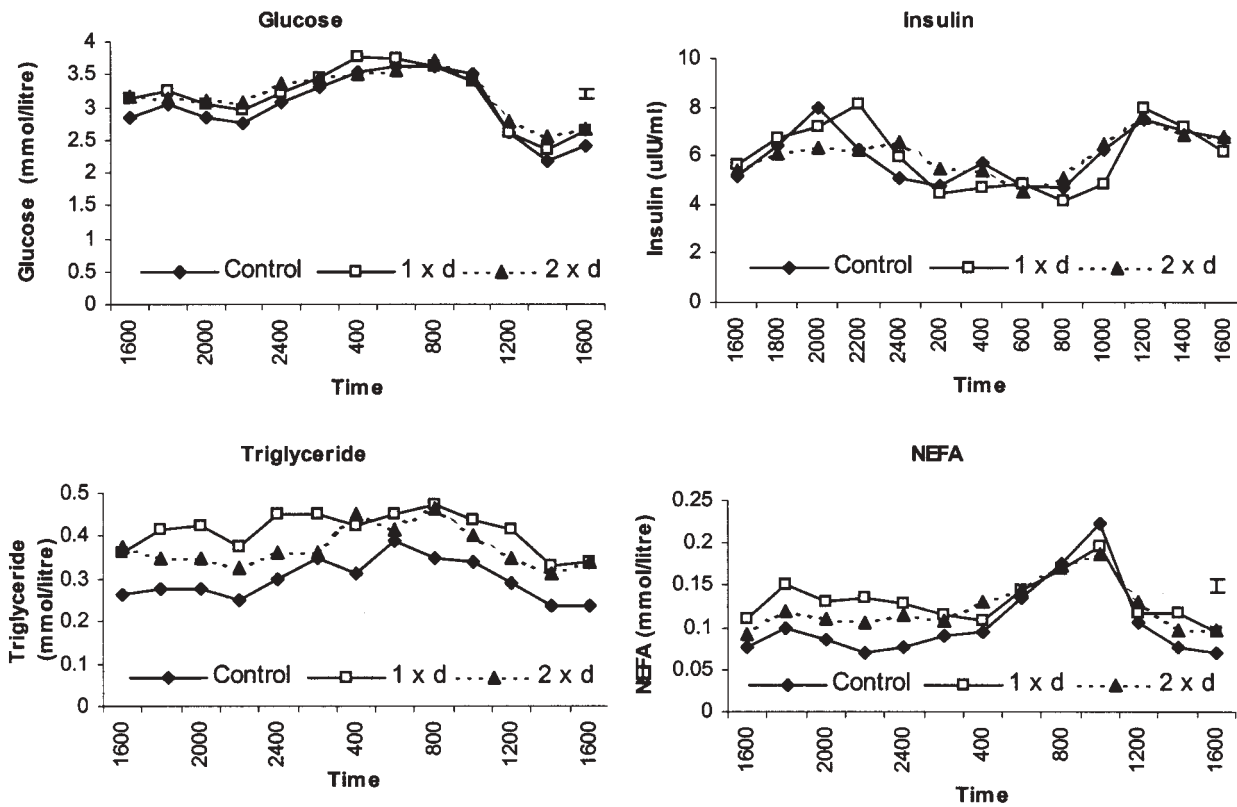
TABLE 2: Average (over a 14 day period) live weight, body condition score (CS), milk yield and composition recorded for cows 81 ± 20 days in milk grazing pasture only (P) or supplemented with 2 kg/cow/day of a lipid supplement fed either once (PS1) or twice (PS2) a day (n=24). The means presented have been adjusted by covariant analyses.

	Treatment			Supplement significance level	s.e.d.
	P	PS1	PS2		
Live weight (kg/cow)	447	458	460	***	3.4
CS	3.9	4.0	4.0	NS	0.7
Milk yield (kg/cow/day)	16.8	18.2	18.6	**	0.5
Fat %	4.1	4.4	4.5	*	0.03
Crude protein %	3.23	3.26	3.22	NS	0.06
Casein %	2.52	2.55	2.51	NS	0.05
Lactose %	5.03	4.93	4.90	NS	0.07
Fat yield (kg/cow/day)	0.69	0.78	0.82	**	0.02
Crude protein yield (kg/cow/day)	0.54	0.59	0.60	**	0.02
Milk solids yield(kg/cow/day)	1.21	1.38	1.42	***	0.05

TABLE 3: Statistical comparisons of blood plasma insulin and metabolites determined at 2-hourly intervals during the last 2 days of the 14 day experimental period. Blood samples were collected from cows on pasture only or fed a lipid supplement (2 kg/cow/day) either once or twice daily (n = 24).

	Feed	Day	Freq.	Time	Time x feed	Time x day	Time x feed x day
Glucose	NS	*	NS	***	NS	***	NS
Insulin	NS	NS	NS	***	NS	***	NS
NEFA	**	NS	NS	***	*	***	NS
Triglycerides	***	NS	NS	***	NS	**	NS

FIGURE 1: Effect of lipid supplementation (2 kg/cow/day) and frequency of supplementation (once: 1 x d, twice: 2 x d) on diurnal variation (2-hour sampling intervals) of the concentrations in blood plasma of insulin, glucose, NEFA, and triglycerides of lactating cows 81 ± 20 days in milk (n = 24).



pasture and the supplement, the fat in pasture was dominant in linolenic (C_{18:3}) acid and the lipid supplement dominant in oleic (C_{18:1}) and linoleic (C_{18:2}) fatty acids.

At the end of the two week experimental period cows on the PS1 and PS2 treatments were heavier and produced 9.5% more milk with an 8.5% higher concentration of milk fat, but similar concentration of crude protein and casein to the P treatment (Table 2). Associated with the response in milk yield and milk fat concentration seen with feeding the lipid supplement, there was an increase in the daily yield of milk fat (16%), crude protein (10.2%) and milksolids (15.7%). There was no effect of feeding frequency (once or twice a day) on these parameters.

The average concentrations of the metabolites investigated over the two-day period were 0.76 ± 0.06 µIU/ml, 3.12 ± 0.16, 11 ± 0.04 and 0.35 ± 0.04 mmole/litre, for insulin, glucose NEFA and triglyceride respectively. Plasma insulin, glucose, NEFA and triglyceride exhibited diurnal variation (Table 3, Figure 1). The difference between the highest and lowest concentrations that occurred over the 24-h sampling period were: 54%, 66%, 224% and 63% for insulin, glucose, NEFA and triglyceride respectively. Not only did the extent of the variation differ, the time that peaks and troughs in values occurred also differed (Figure 1). Peak values for glucose occurred between 0400 h and 0800 h, peak triglyceride values occurred between 0400 h and 0800 h, and peak NEFA occurred at 1000 h. Plasma insulin peaked twice over the 24-hour period, at 1200 h and between 2000 h to 2400 h. Feeding frequency of the

lipid supplement had no effect on the diurnal variation in the plasma concentrations of insulin, glucose, and triglyceride (Table 3). For NEFA, there was a small supplement x time interaction with the magnitude of the diurnal variation being less for PS2 than PS1 and P (Table 3 & Figure 1). Feeding the lipid supplement did not affect the average plasma concentrations of insulin or glucose, but elevated the concentrations of NEFA and triglyceride (Figure 1). The effect of the lipid supplement on the concentration of blood plasma insulin and selected blood plasma metabolites was similar irrespective of feeding frequency (Figure 1).

DISCUSSION

Milk production responses to feeding lipid supplements reported in the literature are inconsistent. Ashes *et al.* (1992) fed an equivalent amount of a similar ruminally protected lipid supplement as used in this study to cows fully fed on a total mixed ration (TMR) diet and found no increase in milk yield or milk protein concentration, but increased milk fat concentration by 13.6%. Handy & Kennelly (1983) also reported that there was no effect on milk yield or milk fat concentration when a proportion of a TMR diet was replaced with canola oil, crushed canola seed or a ruminally protected canola seed. Murphy *et al.* (1995), however, reported that supplementing cows grazing pasture with crushed full-fat rapeseed mixed with a cereal-based supplement, increased milk yield but depressed milk fat concentration. These reports indicate that, in the present experiment, the

cows were getting insufficient energy from pasture alone and the supplementation of a high-energy supplement (38 MJ ME/cow/day) resulted in an increase in energy intake and, thus, an increase in milk yield. The lack of depression in milk fat concentration was possibly due to the effectiveness of ruminal protection of the lipid supplement. High ruminal protection would have resulted in minimal biohydrogenation of the dietary unsaturated fatty acids that lead to the formation of *trans*-10, *cis*-12 conjugated linoleic acid, a component shown to cause milk fat depression (Baumgard *et al.*, 2001). Also, due to the effectiveness of the ruminal protection, greater portions of the supplemented fatty acids were available for transfer into milk fat (Doreau *et al.*, 1999).

The blood plasma metabolite data must then be considered with the knowledge that the cows were obtaining insufficient energy from pasture alone and supplementation with the canola:soy meal was minimising this energy deficit, thus, possibly influencing blood plasma: insulin, glucose and NEFA concentrations. The availability of increased dietary lipid through supplementation possibly influenced blood plasma triglyceride (Blum *et al.*, 2000), and NEFA concentrations (Tesfa *et al.*, 1992).

A distinct diurnal pattern for insulin and each metabolite was observed and supplementary feeding did not influence this. The only other reports of variation in blood metabolites from cows grazing pasture are those of Kolver & Macmillan (1993) who sampled blood at 0630 h, 1030 h, 1430 h, and 0630 h the following day and Fisher *et al.* (1975) who sampled on only two occasions each day: 0400 h and 1100 h. Both found that glucose and NEFA were higher at 0400 h - 0630 h than at 1030 h - 1100 h. The data presented in Figure 1 shows a similar trend for glucose but not for NEFA. Instead, in the present study, NEFA concentrations rose to peak at 1200 h in a trend opposite to that shown by Fisher *et al.* (1975). Blum *et al.* (2000) presented a very similar diurnal pattern to our study for NEFA stall-fed lactating cows. The only difference was Blum *et al.* (2000) measured the sharp peak in NEFA concentration earlier in the day, at 0700 h. In that study the cows were fed twice daily, at 0700 h and at 1500 h in equal amounts, but feed was available to the cows throughout the 24 hours that blood samples were collected. The peak in NEFA concentrations presented in Figure 1 occurs later than reported from either grazing or TMR feeding studies. However, in most of the studies reviewed, the blood sampling regimens adopted to determine diurnal variation were either at irregular intervals or, a proportion of the 24-hour period was missed. The only regular continuous sampling (at one-hour intervals) was undertaken by Sutton *et al.* (1988). Under very similar conditions to the study of Blum *et al.* (2000), Sutton *et al.* (1988) reported peak NEFA concentrations occurring earlier, at 0630 h. In the situation of a blood metabolite with a diurnal pattern similar to NEFA, then single, irregular, or infrequent sampling regimens may misrepresent what is actually occurring. Additionally, if a blood sample was collected between 0400 to 1200 h and analysed for NEFA, the results may not truly represent the average daily value. The

concentration would then depend on the time the sample was taken in relation to the time that the peak concentration occurred. This may, however, vary depending on herd management (Cisse *et al.*, 1991) and frequency of feeding (Sutton *et al.*, 1988; Eicher *et al.*, 1999; Blum *et al.*, 2000).

The diurnal pattern for glucose as presented in Figure 1, was similar to those published by Coggins & Field (1976), Sutton *et al.* (1988) and Blum *et al.* (2000) but the actual time of peak and nadir concentrations varied. In the present study, glucose concentrations were high between 0400 to 1000 h and were lowest over a short period at around 1400 h. In previous reports (Coggins & Field, 1976; Sutton *et al.*, 1988; and Blum *et al.*, 2000) low glucose concentrations occurred at 1000 and 1600 h, and high glucose concentrations occurred at 0900, 2300 and 0800 h. Sutton *et al.* (1988) reported a marked reduction of diurnal variation of glucose concentrations when feeding times were increase from twice to six times daily.

Blum *et al.* (2000) reported similar diurnal variation for plasma insulin and triglyceride concentrations as presented in Figure 1. This was especially so for triglyceride, but the time of peak insulin concentrations differed. Compared with the pattern of diurnal variation for insulin measured in the present study, each of the two insulin peaks presented by Blum *et al.* (2000) are offset by four hours. The period when insulin concentrations were low, however, occurred at a similar time (0200 – 0800 h) for both studies.

The comparisons made between the results in the present paper and those reported in previous studies have shown that considerable diurnal variation in the concentration of blood plasma insulin and metabolites exists irrespective of whether cows were grazed on pasture or fed a TMR-type ration indoors. Two of the metabolites, glucose and NEFA, and insulin are commonly used to determine nutritional disorders and health status of lactating dairy cows (Blum *et al.* 2000). Because of the recent inclusion of triglycerides and free fatty acids into TMR, (Blum *et al.* 2000) suggested that blood plasma triglyceride concentrations may also indicate energy status. Coggins & Field (1976) and Sutton *et al.* (1988) both concluded that diurnal variation of blood metabolites was almost entirely due to level, frequency and type of feeding. Similarly, Eicher *et al.* (1999) reported minimal diurnal variation in concentration of blood metabolites in cows fed continuously by an automated feeding system when compared to cows fed the same daily allowance, but fed only twice a day. Thus, when considering the best time to blood sample, time and frequency of feeding must be considered. Cisse *et al.* (1991) report that under stall-feeding conditions, NEFA and glucose concentrations stabilised 2, and 3 hours respectively after the commencement of the morning feed. Eicher *et al.* (1999) concluded that blood samples collected in a non-standardised manner provided uninterpretable results, and recommended that more interpretable results would be obtained by taking two blood samples, one in the morning and one in the afternoon. The information reported in the present study indicates that plasma glucose, NEFA, and

triglyceride values and treatment effects were consistent from 1600 to 2200 h. However, plasma insulin concentrations were low and stable from 0200 to 0800 h. These observations indicate that the best time to blood sample for glucose, NEFA and triglyceride analysis will be late afternoon, and for insulin analysis, early morning.

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