EFFECT OF FEED DELIVERY TIME AND CONCENTRATE LEVEL ON RHYTHMS OF PLASMA METABOLITE, ENZYME, AND HORMONE LEVELS, GLUCOSE TOLERANCE AND MILK PRODUCTION IN DAIRY COWS

BY

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A Thesis
Submitted to the Faculty of Graduate Studies
In Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

Department of Animal Science
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ABBREVIATIONS

Diets

Experiment 1

HC	High concentrate diet (38.54:61.46 forage to concentrate ratio)
LC	Low concentrate diet (50.62: 49.38 forage to concentrate ratio)

Terms

ADF	acid detergent insoluble fibre
ADIP	acid detergent insoluble protein
ALP	alkaline phosphatase
AST	aspartate aminotransferase
AUC	area under the curve
CP	crude protein
DIM	days in milk
DM	dry matter
DMI	dry matter intake
dL	decilitre
F:C	forage to concentrate ratio
FT	feeding time
GGT	gamma glutamyl transferase
GLDH	glutamate dehydrogenase
I/G ratio	insulin to glucose ratio
IU	international unit
i.v. GTT	intravenous glucose tolerance test
Kg	kilogram
mL	millilitre
mg	milligram
NDF	neutral detergent fibre
NEFA	non-esterified fatty acids
NFC	non-fibre carbohydrate
pg	picogram
ppm	parts per million
PSPS	Penn State Particle Separator
TMR	total mixed ration
µIU	micro international unit
VFA	volatile fatty acid

ABSTRACT

Furedi, Carole J. M. Sc. The University of Manitoba, May 2007. Effect of feed delivery time and concentrate level on rhythms of plasma metabolite, enzyme, and hormone levels, glucose tolerance and milk production in dairy cows. Advisors: A. D. Kennedy & J. C. Plaizier.

The objective of the first study was to determine the effects of evening feeding (2100h) versus morning feeding (0900h) on plasma hormone, enzyme and metabolite levels and glucose tolerance in high-producing dairy cows in two separate experiments. Shifting the delivery of fresh TMR to 2100h from 0900h shifted and increased the variability of diurnal rhythms in plasma glucose, insulin, cholesterol, liver enzymes but not melatonin levels. Glucose tolerance tests performed at 3, 10, 15 and 22 hours post-feeding showed that peak glucose and insulin response varied with hours post-feeding. This is the first study to demonstrate the effect of hours post-feeding on glucose tolerance in dairy cows. The objective of the second study was to examine the production response to evening feeding. The effect of evening feeding on patterns of plasma insulin and glucose levels at 2 and 14 hours post-feeding were similar to those observed during the first study. An unexpected low milk fat content (1.7%) may have masked any possible beneficial effects of evening feeding on production.

1.0 INTRODUCTION

High producing dairy cows in early lactation are often in negative energy balance because the demands for nutrients by the mammary gland far surpass the intake of nutrients. Energy reserves are mobilized in order to maintain milk production during this time and excessive fat mobilization can lead to health problems such as ketosis. Attempts to minimize the gap between energy intake and energy requirements typically involve feeding diets that have a low forage:concentrate ratio in order to maximize daily energy intake (Zimmerman et al., 1991). However, feeding high energy diets has been associated with health (sub-acute ruminal acidosis) (Kennelly et al., 1999) and production (low milk fat) (Bauman and Griinari, 2003) problems in dairy cows.

The use of bovine somatotropin (bST) in the U.S. has allowed dairy producers to increase milk production and improve the milk:feed ratio by making dairy cows insulin insensitive (Bauman and Vernon, 1993). However, bST treatment does not replace proper management of dairy cows and is not effective while the animal is in negative energy balance (Bauman and Vernon, 1993). Furthermore, Health Canada has not approved the use of bST in Canadian dairy herds and so other methods of improving production while maintaining cow health are needed.

Low milk fat production in cows fed high concentrate diets is caused by incomplete biohydrogenation of unsaturated fatty acids by rumen microbes that inhibit milk fat synthesis (Bauman and Griinari, 2003). When milk fat synthesis is inhibited, the energy balance of the cow increases and, as a result, insulin level rises to begin storing the excess energy. Studies with humans have shown a period of insulin insensitivity (poor glucose tolerance) during the night, where the glucose and insulin responses to a

meal consumed in the evening are greater than the responses to the same meal in the morning (Van Cauter et al., 1992). Similar patterns in glucose tolerance have been suggested for dairy cows since plasma glucose and insulin responses to meals show comparable daily variation as seen in humans (Plaizier et al., 2005; Lefcourt et al., 1999). If a period of insulin insensitivity does occur in dairy cows then offering feed at a time that would synchronize the availability of nutrients with this period of insensitivity may allow nutrients such as glucose and volatile fatty acids to be available for milk production and milk fat synthesis.

The objectives of Experiment 1 (Study 1) were to investigate the effects of a 12h shift in feed delivery on diurnal variations in plasma insulin, glucose, cholesterol, liver enzymes, melatonin and glucose tolerance. The objectives of Experiment 2 (Study 1) were to investigate the effects of evening feeding on daily plasma glucose and insulin levels, and glucose tolerance at four different times within a 24h period. The objectives of Study 2 were to investigate the production responses to a change in feed delivery time with a larger sample size than Study 1.

2.0 LITERATURE REVIEW

2.1 Carbohydrate Metabolism in Ruminants

2.1.1 Glucose & VFAs

Carbohydrate metabolism in ruminants differs from monogastrics in that most dietary carbohydrates are converted to volatile fatty acids (VFAs) in the gastro-intestinal tract (GIT) (Brockman and Laarveld, 1986). As a result, very little glucose is absorbed from the GIT of ruminants unless corn starch is fed (Bassett, 1974b). The VFAs are mainly (in order of most absorbed) acetate, propionate and butyrate (Brockman and Laarveld, 1986). The proportion of VFAs absorbed from the rumen can be altered by altering the diet composition. For example, a diet with a forage:concentrate ratio of 50:50 had an acetate:propionate ratio of 3.14; whereas, a diet with a forage:concentrate ratio of 25:75 had an acetate:propionate ratio of 2 ($P < 0.05$) (Kennelly et al., 1999). Acetate is the main precursor for short and medium chain fatty acid synthesis; propionate is a major precursor for glucose synthesis; butyrate is used as fuel by the rumen epithelium and circulates in the blood as β-hydroxybutyrate (βHB) where it can be incorporated into milk fat (Hanson and Ballard, 1967; Brockman and Laarveld, 1986).

Glucose is a very important source of energy for ruminants since it is needed for nervous & muscle tissue activity, fat synthesis, foetus growth and lactation. A dairy cow producing 40kg of milk requires on average 3045g of glucose per day to maintain lactation (Hart, 1983). Since very little glucose comes from the diet, the majority of the glucose requirement is fulfilled by gluconeogenesis. Gluconeogenesis is the synthesis of glucose from precursors which include carbohydrates, amino acids (mostly Asp, Asn, Ser, Glu, Gln, Ala, Tyr, Cys, His and Arg), and volatile fatty acids (Bergman, 1973). In

monogastrics, gluconeogenesis is highest during a fast when glucose must be synthesized in the liver to maintain euglycemia (Bucolo et al., 1974). In ruminants, gluconeogenesis is highest immediately after feeding when glucose precursors are most readily available (Bergman, 1973). After feeding, propionate and amino acids are absorbed from the GIT and 90% of propionate is removed from the blood by the liver before it reaches general circulation (Bergman, 1973). The liver produces around 85% of the required glucose and the kidneys produce most of the remaining 10-15% (Bergman, 1973). Propionate is less responsive to the gluconeogenesis inhibiting effects of insulin than other precursors in ruminants, allowing lactate and amino acids to be available for other synthetic processes (Brockman, 1990).

Dairy cows experience a period of negative energy balance during the first three months of lactation because the peak in milk production occurs before the peak in daily feed intake (Hart, 1983). It is estimated during this time that 33% of milk fat is produced from the mobilization of fat reserves (lipolysis) (Hart, 1983). During this time insulin level is low and glucagon level is high allowing for increased rates of lipolysis and gluconeogenesis (Herbein et al., 1985).

When energy supply exceeds requirements, lipogenesis occurs with the incorporation of acetate into long chain fatty acids and glucose is used for the glyceride backbone of triglycerides and as a source of reducing agents (NADPH) (Hanson and Ballard, 1967; Brockman and Laarveld, 1986). Acetate is incorporated into adipose tissue 10 times more in ruminants than in rats (0.52 vs 0.05 $\mu moles \cdot g^{-1} \cdot 3hr^{-1}$) (Hanson and Ballard, 1967). The rate of acetate incorporation increases with the presence of

insulin and glucose since glucose is required to provide NADPH (Hanson and Ballard, 1967).

2.1.1.1 Glucose Transport

A group of 14 facilitative transporters (GLUTs) transport glucose down its concentration gradient into target cells (Dühlmeier et al., 2007; Scheepers et al., 2004; Watson and Pessin, 2001). Most GLUTs are tissue specific and have varying affinities for glucose; GLUT1 to GLUT4 are the most important in glucose homeostasis (Scheepers et al., 2004). GLUT1 and GLUT3, for example, are important in glucose transport across the blood-brain barrier and into neurons (Watson and Pessin, 2001). GLUT1 is ubiquitously expressed and is responsible for the fundamental supply of glucose to cells; GLUT1 is also the principal glucose transporter in mammary tissue (Scheepers et al., 2004; Zhao et al., 1996). GLUT2 is involved in glucose absorption from the GIT, and in glucose-sensing by β-cells and hepatocytes (Tappy et al., 2000; Watson and Pessin, 2001). GLUT4 is the sole insulin-dependent transporter and is distributed mostly in muscle, adipose tissue and the liver (Del Prato, 2003; Watson and Pessin, 2001). Another set of transporters (SGLTs) couple glucose transport with the electrochemical gradient of sodium and are distributed along the luminal brush-border of the intestinal tract; therefore, SGLTs transport glucose into the enterocytes from the apical surface and GLUT2 transports glucose out of the enterocytes from the basal surface (Scheepers et al., 2004; Rhoads et al., 1998).

The majority of GLUT4 proteins are in intracellular vesicles (unlike the other glucose transporters which are located primarily in the plasma membrane) with a small

percentage found in the plasma membrane in the basal state (Watson and Pessin, 2001; la Fleur, 2003). Insulin binding to the insulin receptor increases GLUT4 translocation to the plasma membrane and increases glucose entry into cells 10- to 20-fold (Scheepers et al., 2004; Watson and Pessin, 2001). Exercise will also increase GLUT4 translocation to the plasma membrane with the use of a distinct signalling pathway that is independent of insulin (Goodyear and Kahn, 1998; King et al., 1993). GLUT1 is also expressed in muscle and adipose tissue and transport by GLUT1 is dependent only on glucose concentration outside the cell (Dühlmeier et al., 2007; Scheepers et al., 2004; Garvey et al., 1991).

Studies indicate that there is a difference in the distribution of GLUT1 and GLUT4 between ruminant and monogastic skeletal muscle and between oxidative (red) and glycolytic (white) skeletal muscles (Dühlmeier et al., 2005; Dühlmeier et al., 2007). GLUT4 is distributed mostly in oxidative muscles (red muscle) in most mammals, and GLUT1 is distributed equally between the two muscle types in monogastrics but is mostly distributed in glycolytic (white) muscles of ruminants (Dühlmeier et al., 2005; Dühlmeier et al., 2007). The insulin-dependent translocation of GLUT4 to the plasma membrane is 10% lower in cattle than in swine myocytes ($P < 0.05$) (Dühlmeier et al., 2005).

2.1.2 Insulin

Insulin, the hormone required for the facilitated transport of glucose across cell membranes by GLUT4 proteins, is secreted by the β-cells of the islets of Langerhans in the pancreas in response to glucose in the blood (Marks et al., 1992). Insulin reduces

blood glucose level by stimulating lipogenesis in adipose tissue, glycogenesis in muscle and the liver and inhibiting gluconeogenesis in the liver, glycogenolysis in liver and muscle tissue and lipolysis in adipose tissue (Morgan, 1992). Insulin binds to insulin receptors on the plasma membrane of target cells and, through a series of post-receptor signalling pathways, triggers the translocation of GLUT4 proteins to the plasma membrane of striated muscle and adipose tissue cells and thereby increases the rate of glucose entry into those cells (Watson and Pessin, 2001). Skeletal muscle accounts for nearly 90% of insulin-stimulated glucose disposal *in vivo* and therefore is the most important site of insulin action (King et al., 1992; Garvey et al., 1991).

The pancreas receives inputs from both autonomic divisions via the pancreatic nerve which is composed of fibres from the vagus and splanchnic nerves (Holst, 1992). Parasympathetic input from the vagus nerve promotes insulin release related to blood glucose level detected by glucosensing neurons in the brain (Rohner-Jeanrenaud et al., 1983). Rising blood glucose level in the brain causes an increased firing rate of glucose-responsive neurons and a decreased firing rate in glucose-sensitive neurons (Scheepers et al., 2004). It is thought that GLUT2 or another isoform of the glucose transporters is involved in neuronal glucosensing (Scheepers et al., 2004). Sympathetic input from the splanchnic nerve inhibits insulin release when glucose level is low and also attenuates the effects of the vagus inputs to prevent hypoglycaemia (Rohner-Jeanrenaud et al., 1983). GLUT2 is also responsible for glucosensing in the β-cells and insulin release increases with increasing portal blood glucose level (Scheepers et al., 2004). Increasing propionate levels in the portal blood of ruminants is also a potent insulin secretagogue (Lomax et al., 1979). Insulin level is lower during early lactation in dairy cows than the rest of the

lactation cycle and this is related to the energy balance of the animal (Bauman and Griinari, 2003).

Epinephrine infusion causes an increase in glucose level through increased gluconeogenesis and glycogenolysis in humans without a concomitant increase in insulin level (Porte, 1967). During stressful situations (indicated by high cortisol level), splanchnic input inhibits insulin secretion regardless of blood glucose level (Rohner-Jeanrenaud et al., 1983). This was tested in sheep by using cold stress. Sheep exposed to a warm environment (18-22 °C) for 4-19d had four times higher insulin level ($P < 0.01$) 10min after a glucose infusion than did sheep exposed to a cold environment (0 ± 0.5 °C), even though the blood glucose level was similar for both groups (Sasaki et al., 1982).

Insulin secretion in response to a meal is considered biphasic with an immediate (within 2 to 3 min), short lasting (5 to 9 min) and small peak followed by a slower (within 20 min), higher and more sustained (15 to 25 min) peak (Rohner-Jeanrenaud et al., 1983). The first phase of insulin secretion is termed the "cephalic phase" and is under parasympathetic control (Rohner-Jeanrenaud et al., 1983; Grill et al., 1984). A biphasic release of insulin is also present in sheep with the first peak within 15min and the second peak from 1h to 3h of feeding (Godden and Weekes, 1981). The cephalic phase of insulin release in sheep was not diminished in one sheep by sham feeding, which involves the installation of an oesophageal fistula that permits the animal to ingest feed but does not allow the feed to enter the digestive tract (Bassett, 1974a). In support of the findings of Basset (1974a), vagotomy (cutting of the hepatic, abomasal, pyloric and duodenal branches of the vagus nerve) was proven to inhibit the cephalic phase of insulin secretion in sheep (Herath et al., 1999). Only 2 to 3% of β-cell insulin content is released

during the cephalic phase; whereas, approximately 20% is released during the second phase (Del Prato, 2003). However, when the cephalic phase of insulin secretion is inhibited or reduced, glucose level rises progressively and results in increased insulin secretion during the second phase in order to return glucose to basal level (Del Prato, 2003).

2.1.2.1 Incretins and Insulin

Insulin release and the GIT are linked by the entero-insular axis composed of a neuronal component (cephalic phase of insulin secretion) as well as a hormonal component (incretins) that regulate pancreatic hormone secretion (Morgan, 1992). Incretins include glucagon-like-peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP); insulinotropic hormones are released by enterocytes of the intestine in response to the active absorption of carbohydrates, amino acids, and long chain fatty acids (Morgan, 1992; Vilsboll et al., 2002). Insulin secretion following an oral glucose load is enhanced compared to an equivalent i.v. glucose load (Berthoud, 1984). Plasma concentrations of GIP increase by 2h post-feeding in sheep and this is thought to be mainly from the absorption of long chain fatty acids in the small intestine (McCarthy et al., 1992). Incretin secretion can be modified by diet as GIP level increases in proportion to the size of an oral glucose load along with insulin level in healthy humans (Hampton et al., 1986). Plasma glucose level accounts for only 19% of the early insulin secretion response following a meal in the rat; whereas, neural signals contribute to 46%, and incretins are responsible for 22% of the insulin secretion response to a meal (Berthoud, 1984).

2.1.3 Glucagon and Insulin:Glucagon Ratio

Glucagon, a hormone produced by the α-cells of the islets of Langerhans in the pancreas, increases blood glucose level by promoting gluconeogenesis and glycogenolysis in the liver and lypolysis in adipose tissue (Marks et al., 1992). Glucagon also has a paracrine role as it acts directly on the neighbouring β-cells to stimulate insulin secretion in order to moderate the ensuing hyperglycemia (Marks et al., 1992). Insulin also has a paracrine role as it inhibits glucagon secretion from the α-cells to inhibit any further increase in hyperglycaemia (Marks et al., 1992).

Electrical stimulation of the vagus nerve in pigs causes a euglycemic promoting response of the pancreatic hormones that is glucose dependent; glucagon secretion is inhibited at glucose level above 144 mg/dL and insulin secretion is inhibited at blood glucose level below 81 mg/dL (Holst et al., 1981).

The opposing relationship between insulin and glucagon allows the islets of Langerhans to control glucose level as well as direct the deposition or mobilization of energy stores (Unger, 1971). An increase in the insulin:glucagon ratio (*i.e.*, insulin secretion surpasses glucagon secretion) is considered anabolic and will promote energy and protein storage and a reduction in blood glucose level; whereas, a decrease in the insulin:glucagon ratio (*i.e.*, insulin secretion lags behind glucagon secretion) is considered catabolic and will promote the mobilization of energy and protein stores (Unger, 1971). After a meal, the insulin:glucagon ratio rises if the blood glucose level is within or above the euglycaemic range; however, if there is hypoglycaemia, the normal response is gone and the ratio will decrease to ensure that the ingested nutrients will be converted to glucose (Unger, 1971). This pattern is also seen in dairy cows during early

lactation: glucose level is low and the insulin:glucagon ratio is around 0.9 (Herbein et al., 1985). The ratio increases as the lactation cycle progresses but does not surpass 1.0 until approximately 150 days in milk (DIM), well after the peak in milk production which occurs from 60 to 90 DIM (Herbein et al., 1985). Therefore the low ratio in early lactation would favour gluconeogenesis and the higher ratio in late lactation would favour lipogenesis.

2.1.4 Milk Fat and Lactose

Acetate, βHB and glucose are very important during lactation since acetate and βHB are incorporated into milk fat and glucose is used to produce lactose, the limiting factor of milk volume since it is the major osmoregulator in the mammary gland (Lemosquet et al., 2004). Fifty to 85% of glucose entering the mammary gland is converted to lactose (Annison et al., 1974). Glucose entry into secretory cells of the mammary gland is via GLUT1 and is independent of insulin (Zhao et al., 1996; Watson and Pessin, 2001).

Milk fat is derived from *de novo* synthesis or circulating fatty acids that originate from either dietary sources or the mobilization of body reserves (McGuire et al., 1995). Short chain and medium chain fatty acids ($C_4 - C_{14}$) in milk are synthesized *de novo* from acetate and βHB, long chain fatty acids (C_{18} +) are derived from circulating fatty acids, and palmitic acid (C_{16}) is derived from both *de novo* and circulating fatty acids (McGuire et al., 1995; Griinari et al., 1997). Approximately 50% of milk fat is derived from *de novo* synthesis in ruminants (Bauman and Griinari, 2003). Glucose is needed for milk fat

synthesis as reducing agents and also forming the glycerol backbone of the triglycerides in milk fat (McGuire et al., 1995).

2.1.4.1 Milk Fat Depression

According to the glucogenic theory by McClymont and Vallance (1962) the milk fat depression (MFD) seen in ruminants with low forage/high concentrate diets is caused by an increase in circulating glucose and therefore an increase in insulin which preferentially shifts nutrients toward deposition in body reserves and inhibits the mobilization of adipose tissue for milk synthesis in ruminants. However, recent studies have shown that increased insulin level may not be the cause of MFD but rather an effect of increased nutrient availability as a result of decreased milk fat synthesis (Bauman and Griinari, 2003).

There is often large individual variation in the development of MFD in cows (Evans et al., 1975; Gaynor et al., 1995). Gaynor et al. (1995) investigated MFD caused by feeding a diet high in concentrate (80%) compared to a diet low in concentrate (40%). Cows were classified as responders or non-responders based on whether or not milk fat was depressed by more than one percentage unit when fed the 80% diet. Blood metabolites and milk fat composition were also compared. Blood glucose and insulin levels increased uniformly with the 80% diet but the responders (-1.37% units) had a greater depression in milk than non-responders (-0.53% units): 37% and 14%, respectively (Gaynor et al., 1995).

MFD is typically associated with a shift in the fatty acid composition of milk fat; the concentrations of short chain fatty acids decrease while the concentrations of long

chain fatty acids increase suggesting that *de novo* synthesis of fatty acids is reduced (Annison et al., 1974; Gaynor et al., 1995). However, at the same time, the concentrations of unsaturated fatty acids increase while concentrations of saturated fatty acids decrease, suggesting that insulin is inhibiting the mobilization of energy reserves (Gaynor et al., 1995). Insulin level was similar in both responders and non-responders and, in fact, the level of non-esterified fatty acids (NEFA), a by product of lipolysis, is reduced in both groups of cows (Gaynor et al., 1995). Therefore, this indicates that both groups had lower rates of lipolysis when fed the 80% concentrate diets (Gaynor et al., 1995).

An abomasal infusion of glucose has the opposite effect to high concentrate diets on the fatty acid composition of milk fat in ruminants: long chain fatty acids decrease whereas short chain fatty acids increase (Lemosquet et al., 1997). Hyperinsulinemic-euglycemic clamps (where insulin and glucose are infused into the blood at rates that will increase insulin level while maintaining euglycemia) also cause a reduction in long chain fatty acids compared to short chain fatty acids (Corl et al., 2006). These data indicate that lipolysis is reduced and the magnitude of the reduction in milk fat will depend on the importance of body reserves as a source of fatty acids for milk fat synthesis (Bauman and Griinari, 2003).

Cows in early lactation depend highly on body reserves for milk fat synthesis since their daily intake does not meet the demands of milk production, as such, these cows tend to experience a much greater reduction in milk fat (20% to 30%) with high level of plasma insulin associated with high concentrate feeding (Bauman and Griinari, 2003). Cows in mid or late lactation are able to consume enough feed to meet the

demands of milk production and as such only 4% to 8% of milk fat originates from body reserves, therefore, these cows tend to experience a smaller (6%) reduction in milk fat with high concentrate feeding (Bauman and Griinari, 2003). This agrees with Gaynor et al., (1995) who observed a 14% decrease in non-responders and 37% decrease in responders during peak lactation (109 ± 9 DIM) with similar insulin level.

The bacteria in the rumen of ruminants saturate unsaturated fatty acids in a process called biohydrogenation and diets that are high in concentrate typically have higher level of unsaturated fatty acids (Gaynor et al., 1994). Incomplete biohydrogenation results in the formation of *trans*-$C_{18:1}$ fatty acids and diets containing higher level of concentrate are typically associated with increased production of *trans*-$C_{18:1}$ fatty acids in the rumen (Gaynor et al., 1994). The infusion of *trans*-$C_{18:1}$ fatty acids into the abomasums of dairy cows in early lactation reduced milk fat content by 25% (Gaynor et al., 1994). Responders, in Gaynor et al. (1995), had 38% more *trans*-$C_{18:1}$ fatty acids in the milk than non-responders. Cows fed either a diet with high fibre (HF) or low fibre (LF) and added unsaturated fatty acids (UFA) or saturated fatty acids (SFA) had milk fat of 3.58% (HF + SFA), 3.36% (HF + UFA), 3.33% (LF + SFA) and 2.49 % (LF + UFA); the LF + UFA treatment caused a 30% reduction in milk fat percentage compared to the HF + SFA treatment (Griinari et al., 1998). The shift in milk fatty acids composition with these treatments mimics the shift seen in MFD diets (Bauman and Griinari, 2003). Piperova et al. (2000) compared the activity and mRNA level of Acetyl-CoA carboxylase, the rate-limiting enzyme in *de novo* fatty acid synthesis, in the mammary gland of cows fed either a MFD diet (25% forage:70% concentrate) with 5% added soybean oil or a control diet (60% forage:40% concentrate). Milk fat percent and

yield was reduced by 43% and the amount of *trans*-$C_{18:1}$ fatty acids in the milk was increased in cows fed the MFD diet. Also, Acetyl-CoA carboxylase activity and mRNA were both reduced by 62% with the MFD diet (Piperova et al., 2000). This has lead investigators to propose the "biohydrogenation theory" in which a change in rumen conditions (*i.e.* reduced pH induced by increased concentrate:forage ratio of feed) causes the incomplete biohydrogenation of UFA and results in specific *trans*-$C_{18:1}$ fatty acid isomers which inhibit milk fat synthesis in the mammary gland (Bauman and Griinari, 2003).

2.1.5 Growth Hormone and Milk Yield

Exogenous growth hormone or somatotropin (ST) given as a daily injection or as a constant infusion has milk yield promoting effects that include increased peak yield as well as increased persistency over the lactation cycle in dairy cattle (Bauman and Vernon, 1993). However, ST has no effect on milk yield if the animal is in negative energy balance such as in early lactation (Bauman and Vernon, 1993; Bauman, 1999). Chronic ST treatment has anti-insulin-like effects and causes a repartitioning of nutrients toward milk production and away from energy storage by decreasing peripheral tissue sensitivity to insulin and stimulating lipolysis (Zhao et al., 1996; Bauman, 1999).

Peripheral tissue sensitivity to insulin is reduced by the inhibition of GLUT4 gene expression in muscle and adipose tissue (Zhao et al., 1996). Treatment of lactating Holsteins with ST caused a 44% decrease in skeletal muscle GLUT4 mRNA and reduced adipose tissue GLUT4 mRNA to undetectable levels (Zhao et al., 1996), thereby rendering adipose tissue insensitive to insulin (Hart et al., 1985). The insulin-

independent GLUT1 found in mammary glands is not affected by ST treatment and thus the reduction in GLUT4 in peripheral tissues allows more glucose to be available for lactose synthesis (Zhao et al., 1996).

2.1.6 Glucose Tolerance

Glucose tolerance (GT) is a measure of the ability of the body to clear glucose from the blood and has been studied in several species including humans (Van Cauter et al., 1989), rats (Kalsbeek and Strubbe, 1998), horses (Hoffman et al., 2003), pigs (Pere et al., 2000) and cattle (Mir et al., 1998). Glucose tolerance is of particular interest in humans as it is impaired with Type II diabetes mellitus and the number of people with Type II diabetes is growing each year (World Health Organization, 1999). In 2000, 17.7 million people in the United States (171 million world-wide) were living with Type II diabetes; in 2030 the World Health Organization estimates that number will nearly double to 30.3 million (366 million world-wide) (Wild et al., 2004). Type II diabetes is defined as a metabolic disorder characterized by high blood glucose level caused by β-cell (insulin) insensitivity to glucose and/or insulin-dependent tissue insensitivity to insulin (World Health Organization, 1999).

Type II diabetes is considered a bihormonal disorder: a reduction in insulin secretion or action as well as an insensitivity of the α-cells (glucagon) to insulin (Unger, 1971). As discussed earlier, insulin and glucagon have an opposing relationship in order to regulate plasma glucose concentrations. A rise in plasma glucose level will stimulate the secretion of insulin from the β-cells (in order to lower plasma glucose) which, in turn, inhibits the secretion of glucagon from the α-cells (in order to minimize a further increase

in plasma glucose). This typical relationship between insulin, glucagon and glucose is lost in individuals with impaired GT (Ahren and Larsson, 2001). Ahren and Larsson (2001) found that insulin and glucose levels are higher in individuals with impaired GT, but glucagon level is the same as in individuals with normal GT. Usually, high glucose and insulin levels would cause a reduction in glucagon as in the case of individuals with normal GT. The high glucagon level in people with impaired GT indicates that the suppression of glucagon secretion by insulin is reduced (Ahren and Larsson, 2001).

The cephalic phase of insulin release is also lost or impaired in individuals with impaired GT or Type II diabetes (Del Prato, 2003). The loss of the cephalic phase causes postprandial plasma glucose level to rise higher than in individuals with normal cephalic phase release and, in turn, causes an increased release of insulin in the second phase in order to return glucose to basal level (Del Prato, 2003).

Individuals with impaired GT or Type II diabetes also experience a reduction in the effectiveness of incretins to promote insulin secretion in response to a meal (Nauck et al., 1986). Insulin level was 1.6-fold higher in response to an oral glucose load ($50g \cdot 400mL^{-1}$) in control individuals compared to Type II diabetics even though the plasma GIP profiles were similar in both groups (Nauck et al., 1986). The response to GLP-1, however, seems to remain intact because the infusion of GLP-1 increased the insulin response during a hyperglycaemic clamp in Type II diabetics, but GIP infusion had no effect on insulin level (Vilsboll et al., 2002).

The Zucker rat is an animal model of insulin resistance (glucose intolerance) and work involving obese (fa/fa) and lean (fa/?) litter mates has shown that individuals with impaired GT (fa/fa) have reduced GLUT4 gene expression in adipose tissue compared to

those with normal GT (fa/?) but both groups have similar expression in skeletal muscle (Goodyear and Kahn, 1998). This was also found in humans with impaired GT or Type II diabetes when compared to normal controls (Garvey et al., 1992). Therefore, insulin resistance seems to be caused by impaired intracellular signalling, and not a deficiency in glucose transporters, in skeletal muscle which is the most important site of insulin-dependent glucose disposal (Goodyear and Kahn, 1998; King et al., 1992, Garvey et al., 1992). Exercise will increase GLUT4 translocation to the plasma membrane, independent of insulin, in individuals with impaired GT or Type II diabetes suggesting that the impaired intracellular signalling is isolated to the insulin-dependent pathways (King et al., 1993).

Glucose tolerance testing in ruminants has revealed a decrease in GT during early lactation in dairy cows (Chelikani et al., 2003; Holtenius et al., 2003), and goats (Debras et al., 1989) but not in beef cows (Sano et al., 1991). This is likely related to the negative energy balance of the high-producing lactating animal shortly after parturition when energy demands exceed nutrient intake (Bell and Bauman, 1997). Early lactation is also associated with increased ST levels, lower insulin and glucose levels (Reist et al., 2003) and increased glucagon and NEFA levels (Hayirli et al., 2001). As mentioned earlier, chronic elevation in ST has anti-insulin effects and this causes a down-regulation of the insulin-dependent glucose transporter (GLUT4) in muscle and adipose tissue. These adaptations allow ruminants to spare glucose for lactose synthesis (the primary determinant of milk volume).

2.1.6.1 Glucose Tolerance Tests

Glucose tolerance, tested by giving an i.v. glucose bolus preceded and followed by frequent blood sampling is called the frequent sampling intravenous glucose tolerance test (i.v. GTT) and is most commonly used in research settings (Lemosquet and Faverdin, 2001). Clinical tests in humans usually involve an oral glucose bolus with one to two blood samples collected before and after the glucose bolus (Kritz-Silverstein et al., 1989). Oral glucose administration stimulates incretin release which enhances insulin secretion (Kritz-Silverstein et al., 1989). A duodenal glucose bolus or a ruminal VFA bolus has sometimes been used in ruminants but most commonly the glucose bolus is administered into the jugular vein (Lemosquet and Faverdin, 2001).

During an oral GT test, delayed and elevated insulin level coupled with normal or elevated glucose level indicate insulin insensitivity in humans (Bergman et al., 1985). There is a closed-loop feedback relationship between β-cells and glucose-dependent tissues: hyperglycemia causes increased insulin secretion which in turn inhibits hepatic glucose production and increases peripheral glucose uptake to reduce glucose level (Bergman et al., 1985). The oral GT test, therefore, does not allow researchers to determine whether the altered glucose and insulin responses to a glucose load are a result of insulin-dependent tissue insensitivity to insulin or initial β-cell insensitivity to glucose or incretins (Bergman et al., 1985).

The hyperinsulinemic-euglycemic clamp is another approach to testing GT but is a more complex procedure as it involves infusing insulin and measuring the amount of glucose that must be infused to maintain euglycemia (Bergman et al., 1985). These tests are called open-loop because they remove the feedback relationship between the β-cells

and insulin-dependent tissues and allow researchers to determine insulin-dependent glucose use (Bergman et al., 1985). They are most often used in ruminants while examining the effects of insulin on milk production, because they allow researchers to determine the consequences of hyperinsulinemia without the confounding effects of hypoglycaemia on milk yield (Debras et al., 1989).

2.2 Circadian Rhythms

An intrinsic biological clock exists in all organisms tested to date and acts to synchronize physiological and/or behavioural activities to the 24h cycle of day and night (Devlin, 2002). The most clearly understood circadian (*circa* (about); *dies* (day)) clock in mammals is found in the suprachiasmatic nuclei (SCN), a group of cells in the hypothalamus, which receive input of daylength from the retina (Moore and Leak 2001). External cues (*zeitgebers* = time givers) such as light and temperature, entrain the circadian clock and the ensuing rhythms so that they are synchronized precisely with the changing day/night cycle of the seasons (Daan and Aschoff, 2001; Devlin, 2002).

A rhythm is defined as a cycle of metabolites, hormones, or behaviour that varies in intensity and occurs with a set period (duration) (Sehgal, 2004). The rhythm is considered circadian if it has a period of 24h under natural conditions, resists entrainment to periods that are dramatically different from 24h and continues "free-running" with a period of roughly 24h in the absence of its *zeitgeber* (Moore, 1978). Therefore, in this thesis, a rhythm will be termed circadian if the above criteria have been demonstrated. If a rhythm has not yet been proven to be circadian, it will be considered to be diurnal;

having an acrophase (peak) and nadir (trough) within a 24h period but is not known to exist without its *zeitgeber*.

The retinohypothalamic tract connects the retina to the ventral SCN and provides the SCN with photoperiod information (Moore and Leak, 2001; Zucker 2001). During darkness, when SCN activity is low, high level of norepinephrine is released in the pineal gland to stimulate the production of the hormone melatonin (Moore and Leak, 2001; Takahashi and Zatz, 1982). During daylight, exposure of the retina to light increases SCN activity which inhibits norepinephrine release in the pineal gland and melatonin production is not stimulated (Takahashi and Zatz, 1982). Under constant light (LL) or constant dark (DD), circadian rhythms in the activities of the rat (e.g., drinking, wheel running, sleep) free-run with a period of 24.3 to 24.9h (Boulos et al., 1980; Armstrong et al., 1986). Ablation of the SCN or the retinohypothalamic tract abolishes these circadian rhythms in rats (Moore, 1978).

Clock-genes found in the SCN have also been demonstrated in several other parts of the body including the liver and are referred to as peripheral oscillators (Cailotto et al., 2005; Giebultowicz, 2004). The SCN is responsible for synchronizing the peripheral oscillators that control tissue-specific functions (Giebultowicz, 2004). Therefore, more than one endogenous clock exists in mammals; one is the master oscillator (the SCN or light-entrainable oscillator - LEO), and the others are slave oscillators (Alvarez, 2004). Some slave oscillators (e.g., liver) are said to be food-entrainable oscillators (FEO) since they can be uncoupled from the LEO by restricted feeding (Alvarez, 2004; Mistlberger, 1994).

2.2.1 Melatonin and Light Entrainable Rhythms

Melatonin is the hormone that links the LEO of the SCN to various organs or systems that have circadian rhythms (Zucker ,2001). Melatonin has the most well-known and robust circadian rhythm. The melatonin circadian rhythm is closely associated with the *zeitgeber* of the circadian clock (*i.e.*, darkness) and melatonin rhythms are thought to reflect SCN rhythms (Van Cauter and Buxton, 2001).

Peak melatonin level as well as total melatonin secreted during the dark phase can vary greatly among individual humans (Van Cauter and Buxton, 2001) but is reproducible within an individual human (Morgan et al., 1998; Deacon and Arendt, 1994). Genetic variability in pineal weight has been found to be the only cause for the individual differences in night-time melatonin production in sheep (Coon et al., 1999). Although total melatonin secreted and peak melatonin level varies, the presence and the length of a melatonin surge are important for the synchronization of other rhythms to the external photoperiod (Devlin, 2002).

Photoperiod and melatonin has been studied in dairy cows and it was found that milk production is maximized with a 16L:8D to 18L:6D photoperiod (Dahl et al., 2000). Although light of 5 and 10 lux has no effect on night-time melatonin level, light of 50 lux caused 50 to 70% suppression of night-time melatonin level in the first 2 to 3h of darkness in dairy heifers (Muthuramalingam et al., 2006; Lawson and Kennedy, 2001). A light intensity of 400 lux was required in order to suppress night-time melatonin level for the entire 8h night in dairy heifers (Lawson and Kennedy, 2001).

Pinealectomy (PNx) does not alter free-running behaviours (e.g., wheel running) in rats under DD (Armstrong et al., 1986); however, melatonin injection will shift free-

running behaviours so that the active period follows the melatonin injection (Armstrong et al., 1986). SCN-lesions (SCNx) in addition to PNx abolish rhythms altogether in rats under DD and melatonin no longer had an effect (Armstrong et al., 1986). Therefore, the endogenous oscillator of the SCN is required to sustain rhythms and melatonin from the pineal gland is required to set rhythms to the precise LD cycle.

2.2.2 Food Related Rhythms

2.2.2.1 Food Entrainable Circadian Rhythms

Rhythms generated by the FEO can persist indefinitely with no access to food (Mistlberger, 1994). Under either 24h LD (12L:12D) or LL and *ad libitum* feed access, the feeding and sleeping behaviours of rats will have a period of roughly 24h with the minimum and maximum activity timed to the current or prior LD cycle (Boulos et al., 1980). However, under 24h LD or LL and restricted feeding, the same behaviours will maintain a 24h period but will become timed to the feeding schedule with an increase in activity prior to the availability of food (Boulos et al., 1980). The increase in activity (e.g., wheel running) preceding food availability is termed anticipatory activity (AA) and is a robust circadian rhythm in rats that does not entrain well to periods of less than 19h or greater than 29h and persists during food deprivation for 5d (Boulos et al., 1980; Stephan, 2001).

When under LL or DD and restrictive feeding, rats can have two simultaneous rhythms in AA; one that is linked to feeding time and another that free-runs in LL or DD, suggesting that the FEO is coupled but separate from the LEO (Mistlberger, 1994). Timed caloric restriction causes a phase advance in several circadian rhythms in rats

(Challet et al., 1997). To differentiate between meal timing and caloric input, Challet et al. (1997) fed rats either 50% of previous *ad libitum* feed intake (feed restricted) or 100% of previous *ad libitum* feed intake (sham restricted) at 2h after lights on (1000h) and monitored several circadian rhythms for several months. They found that the feed restricted rats had phase advances in the rhythms of melatonin (-2hr), body temperature (-7hr) and activity patterns (-6hr); whereas, the sham restricted did not exhibit any shifts in the measured circadian rhythms. The difference in phase shifts suggests that timed caloric restriction may affect the synchronization of the two oscillators regulating the circadian system in rats (Challet et al., 1997).

Recently, Kalsbeek et al. (2004) have demonstrated that the liver is connected to the SCN through both the sympathetic and parasympathetic nervous systems. They suggest that the daily rhythms of plasma glucose seen in rats and humans (discussed below) are caused by daily fluctuations in hepatic glucose production (Kalsbeek et al., 2004). In another study, Cailotto et al. (2005) determined that the sympathetic connection to the liver is needed to generate the 24h rhythm in plasma glucose concentrations but is not needed for the rhythmic expression of clock-gene mRNA in the liver. Other products of the liver have been tested including cholesterol, and liver enzymes (e.g., aspartate aminotransferase – AST, alkaline phosphatase – ALP) which support a rhythm in liver function (Gabris and Duran, 1988; Piccioni et al., 2003).

2.2.2.1.1 Cholesterol

Piccioni et al. (2003) were able to demonstrate that cholesterol has a clearly defined circadian rhythm controlled by the FEO in goats which persisted even during

food deprivation. Under LL and food deprivation, cholesterol level dropped but still maintained a faint rhythm with a period of 24.5 ± 0.2h (Piccione et al., 2003). Under LL coupled with a shift (-6hr) in feeding time the rhythm became erratic (Piccione et al., 2003).

A study of the 24-h rhythm of hormones and blood metabolites of dairy cows (Simmental x Red Holstein, Schweizerisches Brauvich, and Schweizerisches Brauvich x Brown Swiss) fed different amounts of energy (83.2 MJ NE_L/d, 119.9 MJ NE_L/d, or 128.3 MJ NE_L/d) during peak lactation did not demonstrate a 24h rhythm in cholesterol level (Blum et al., 1985).

2.2.2.1.2 Glucose

A circadian rhythm in glucose level is found in rats with a reproducible peak, just before the onset of the dark period, regardless of feeding regime (la Fleur et al., 1999). The circadian rhythm in rats occurs with *ad libitum* access to feed, fasting and scheduled feeding but disappears with SCNx (la Fleur et al., 1999). The authors suggested that the daily variation in glucose level in rats was caused by an increase in sympathetic activity to the liver from the SCN to promote gluconeogenesis (la Fleur et al., 1999). This was recently confirmed by Cailotto et al. (2005), who found that the removal of the sympathetic connection to the liver from the SCN caused a loss of the circadian rhythm in glucose level in rats.

Glucose also has a marked circadian rhythm in humans with peak level occurring between 0015h and 0545h despite fasting while receiving a constant rate of glucose infusion that was started either at 1100h or 2300h (Van Cauter et al., 1989). Since

gluconeogenesis has been shown to be suppressed during constant glucose infusion (Wolfe et al., 1980) other factors must also be responsible for the glucose circadian rhythm in humans. The night-time peak in glucose level in humans may be due to a reduced glucose requirement by peripheral tissues, reduced insulin secretion, insulin insensitivity or a combination of these factors (Van Cauter et al., 1989).

In cattle fed twice daily (Sutton et al., 1988; Blum et al., 1985; Andersson, 1982), or once daily (Ross and Kitts, 1973), blood glucose level dropped at 2h after feeding and rose 8 to 10h after feeding. A similar drop in blood glucose level shortly after feeding is found in sheep (2h post-feeding) and goats (30min post-feeding) fed once or twice daily (de Jong, 1981; Bassett, 1974b). The rise in plasma glucose in response to the mid-afternoon feeding (for cows fed twice daily) occurred during the night and was typically 8 to 13% higher than the rise in plasma glucose in response to the early morning feeding even though twice as much feed was consumed in the morning (Andersson, 1982; Blum et al., 1985). Similarly, humans who consume meals twice daily will have a glucose area under the curve (AUC) after an evening meal that is two-fold greater than after the morning meal (Van Cauter et al., 1992). Glucose responses to meals appear to be linked to cortisol level in the blood, because cortisol level decreases throughout the day (light phase), reaches a minimum value at 0200h and rises to a peak at 0600h; therefore, the higher glucose AUC coincides with a lower cortisol level (Van Cauter et al., 1992).

Higher blood glucose level at night in cattle fed twice daily coincided with a period of decreasing insulin concentration (Blum et al., 1985). A similar effect of time of day has been found in humans where a meal consumed at 2000h caused 22% higher peak

blood glucose and 15% lower peak plasma insulin than an identical meal consumed at 0800h (Van Cauter et al., 1992). Kalsbeek and Strubbe (1998) were also able to demonstrate a diurnal rhythm of food induced responses of glucose and insulin in rats. Postprandial glucose and insulin levels rose regardless of meal time but the rise was delayed for meals offered at 6h and 10h after lights on (reduced glucose tolerance in light phase), when the rats were fed six meals daily (Kalsbeek and Strubbe, 1998).

2.2.2.1.3 Insulin

Only 50% of rats that are fed *ad libitum* or at scheduled meal times will show a rhythm in insulin level, and those rhythms disappear with SCNx (la Fleur et al., 1999). In the same experiment, no detectable rhythm in insulin level was found in fasted rats. Conversely, Bizot-Espiard et al. (1998) reported that insulin rhythms in fasted and hyperglycaemic rats are similar with two peaks; one at the beginning of the light phase and the other at the beginning of the dark phase. The rhythms in insulin were unrelated to the variation in blood glucose which suggests that there is an endogenous rhythm of insulin secretion in rats regardless of glucose level (Bizot-Espiard et al., 1998). However, the rhythm found by Bizot-Espiard et al. (1998) may have been caused by residual absorption from the GIT since food was withdrawn at the start of the sampling period; whereas, food was withdrawn 12h prior to the start of sampling for la Fleur et al. (1999). This suggests that the dominant influence in insulin level may be feeding.

However, insulin level did not rise as a consequence of the larger glucose response to an evening meal and this seems to be partly a result of reduced insulin secretion (Van Cauter et al., 1992). The level of C-peptide, a by-product of insulin

synthesis that is released into the blood at the same rate as insulin, increased equally in response to each meal and therefore insulin secretion did not increase along with postprandial glucose level (Van Cauter et al., 1992). This suggests that, as well as differences in insulin sensitivity (discussed below), there may be differences in β-cell sensitivity to glucose throughout the day (Van Cauter et al., 1992).

Isolated rat pancreatic islets exhibit a strong daily rhythm in glucose-stimulated insulin secretion with peak insulin secretion at 2400h and lowest secretion at 0300h to 0800h when exposed to 8.3 mM of glucose (Picinato et al., 2002a). Pancreatic islets isolated from PNx rats exhibit a phase advance (-3h) of insulin secretion when exposed to 8.3 mM of glucose (Picinato et al., 2002a). Melatonin treatment inhibits insulin secretion in isolated rat pancreatic islets (Picinato et al., 2002b). Untreated islets had increased insulin secretion in response to glucose level of 16.7 mM but not 5.6 mM, and treated islets did not increase insulin secretion regardless of glucose level (Picinato et al., 2002b). Glucose uptake by the islets did not appear to be altered by melatonin treatment because there was no difference in glucose oxidation between the treated and untreated islets. However, protein kinase A (an enzyme activated by glucose metabolism and involved in glucose-induced insulin secretion) level were lower ($P < 0.05$) in the treated islets compared to the untreated islets (Picinato et al., 2002b).

A diurnal rhythm in insulin level is also seen in Holsteins fed once daily with peak level occurring in the light phase (4 to 6h after feeding) (Lefcourt et al., 1999). Cattle fed twice daily had an ultradian (period < 24h) insulin rhythm with a peak 2 to 4h post-feeding (Blum et al., 1985).

2.2.2.1.4 Glucose Tolerance

As mentioned earlier, glucose tolerance (GT) is a measure of the ability of the body to clear glucose from the blood. Delayed and elevated insulin level coupled with normal or elevated glucose level, during a GT test, indicate poor glucose tolerance in humans. A diurnal rhythm in glucose tolerance seen in rats (higher insulin response to an i.v. glucose load at 1600h than at 0800h) is removed with PNx (response equal at both times and similar to the 0800h response in intact rats) causing glucose level to rise higher than in intact rats and stay elevated longer ($P < 0.05$) (Lima et al., 1998). Peak insulin level can be compared to peak glucose level in response to a glucose load by determining the insulin/glucose ratio (I/G ratio) (Lima et al., 1998). Intact rats tended to have a higher I/G ratio at 1600h than at 0800h whereas PNx rats had a reduced I/G ratio at both times ($P < 0.05$).

Pinealectomy caused a 4-fold reduction in adipose tissue GLUT4 expression compared to intact rats but skeletal muscle GLUT4 remained unchanged (Lima et al., 1998; Zanquetta et al., 2003). Melatonin treatment reversed the reduction in GLUT4 expression in PNx rats (Zanquetta et al., 2003). Therefore, the glucose intolerance seen in PNx rats seems to be caused by decreased insulin sensitivity of adipose tissue (Lima et al., 1998).

A diurnal rhythm was found in SGLT1 expression in rat and rhesus monkey intestinal mucosa (Rhoads et al., 1998). Rat mucosa had 3.7-fold higher SGLT1 expression at 1600h and 2200h than 1000h and 0400h, whereas rhesus monkey mucosa had 5-fold higher SGLT expression at 0900h compared to 2000h; the diurnal rhythm is shifted by approximately 12h in the diurnal primate compared to the nocturnal rodent

(Rhoads et al., 1998). These results indicate that glucose absorption is highest during times of activity and feeding but does not necessarily indicate that this is a true circadian rhythm as constant conditions (LL or DD) or feed deprivation conditions were not examined.

2.2.2.2 Food Dependent Diurnal Rhythms

2.2.2.2.1 Urea

Urea has a diurnal rhythm that is dependent upon feeding in ruminants. Dairy cows have a peak urea level that corresponds with feeding time (Blum et al., 1985; Lefcourt et al., 1999). Piccione et al. (2003) demonstrated that, although cholesterol maintained a rhythm in a 12L:12D cycle with food deprivation, urea no longer had a rhythm after 3d of food deprivation in the goat. In fact, under various experimental conditions, the acrophase of the urea rhythm occurred consistently 9 to 12h after feeding time regardless of the LD cycle but disappeared completely with food deprivation (Piccione et al., 2003).

2.2.2.2.2 NEFA

Non-esterified fatty acids (NEFA), end-products of lipolysis, also have a food dependent rhythm in ruminants. The NEFA level rises with food deprivation and NEFA will have a rhythm with a period that is related to feeding time in cows that are fed once or twice daily (Blum et al., 1985). The NEFA level drops at feeding and the decrease is dependent upon the energy value of the food; a lower energy diet will cause a smaller

drop in NEFA than a diet higher in energy because the animal on a low energy diet will still need to rely on lipolysis for energy (Blum et al., 1985).

2.2.2.2.3 Liver Enzymes

A diurnal rhythm for AST (aspartate aminotransferase) was found in rats that shifted with a shift in feeding time (Sitren and Stevenson, 1978). Rats fed in the morning (MF, 0700h to 1100h) had peak AST level at 0600h (end of dark period and before feeding) whereas, rats fed in the afternoon (AF, 1400h to 1800h) had peak AST levels at 1700h (end of light period and after feeding). Even with the earlier peak, MF rats had minimum AST level at 8h post-feeding just as in AF rats (Sitren and Stevenson, 1978). The same pattern was seen in lactic dehydrogenase (LDH) level with MF rats having a peak before feeding and AF rats having a peak after feeding. However, alanine aminotransferase (ALT) level did not follow the same pattern as the other two liver enzymes. Feeding time coincided with peak ALT level in MF rats but with minimum level in AF rats (Sitren and Stevenson, 1978). Although AST and LDH levels appear to be linked to feeding, ALT level may be more closely linked to the LEO. However, measurements were not made during food deprivation or constant light or dark and therefore no conclusions can be made regarding whether these are true circadian rhythms or food dependent fluctuations of plasma level.

2.3 Time of Feed Delivery

The eating pattern of range cattle is crepuscular with peak grazing occurring at dawn and at dusk and peaks in eating behaviours shift along with seasonal changes in day

length (Stricklin and Kautz-Scanavy, 1984). A minor peak in eating is seen at mid-day and also in the middle of the night, when day length shortens in northern latitudes (Stricklin and Kautz-Scanavy, 1984). The most potent stimulus for eating in cattle is the provision of fresh feed and any change in the pattern of feed delivery will change the pattern of lying and feeding (Stricklin and Kautz-Scanavy, 1984; DeVries et al., 2005). Time of feed delivery has a greater effect than time of day on the daily feeding pattern of cattle housed indoors (DeVries and von Keyserlingk, 2005). Adaptation to a new feeding regime can take up to 14d but typically occurs within 3 to 4d in mammals (Mistlberger, 1994).

Phillips and Rind (2001) found that Holstein cows fed several times daily (0600, 1000, 1400, and 1900) spent 10% more time eating in the late afternoon (1800h) than in the morning (0800h). Also, when fed on alternate days they spent 10 to 30% more time eating in the afternoon (1800h) than in the morning (0800h) even on mornings when feed was delivered (Phillips and Rind, 2001). Regardless of feeding time, cattle consumed feed at a faster rate in the first 2h after feed delivery (0800h or 1800h) and then consumed the remainder of their daily intake throughout the day (Moshtaghi Nia et al., 1995).

2.3.1 Production Responses and Preferred Time of Feeding

Studies with beef cattle have shown an improvement in average daily gain (ADG) and feed:gain ratio when feed was delivered in the evening (Knutsen et al., 1995; Kennedy et al., 2004; Schwartzkopf-Genswein et al., 2004). One theory was that evening feeding is beneficial to beef cattle because it reduces cold stress; providing fresh feed in

the evening allows the heat increment of feeding to coincide with the lower nightly temperatures (Kennedy et al., 2004). In support of this theory, Schwartzkopf-Genswein et al. (2004) found that the greatest improvement of ADG occurred during the winter backgrounding stage which coincided with the lowest nightly temperatures.

A decrease in milk production is seen in dairy cattle under heat stress and investigators have theorized that night feeding should be beneficial as it shifts the heat increment of feeding to the cooler night hours rather than the warmer day hours (Ominski et al., 2002; Aharoni et al., 2005). However, evening feeding did not result in an increase in production when compared to day-fed controls during short-term, moderate heat stress (Ominski et al., 2002) or long-term, high heat stress (Aharoni et al., 2005).

No work has been done, according to the author's knowledge, on the effects of evening feeding on dairy cattle production in the thermoneutral zone.

2.3.2 Metabolic Responses

As mentioned earlier, the metabolic response to a meal consumed in the evening is different than to a meal consumed during the day. Diurnal changes in insulin sensitivity cause a reduction in glucose tolerance in humans at night characterized by elevated glucose level even during constant glucose infusion (Van Cauter et al., 1989). The higher level of glucose at night is not accompanied by an equal rise in insulin level (Van Cauter et al., 1992). The insulin secretion rate in response to a meal consumed at night is the same as the secretion rate in response to the same meal during the day. A diurnal variation in the set point of β-cell responsiveness to glucose may account for the lack of an increased insulin secretion rate response in proportion to the increased glucose

(Van Cauter et al., 1992). The insulin clearance rate is also higher at night than the day (1.81 L/min vs 1.22 L/min; $P < 0.01$), which may account for lower insulin level at night (Van Cauter et al., 1992).

2.4 Summary

Diurnal rhythms are found in every species studied to date, from single-cell organisms to complex organisms and plants (Devlin, 2002). Although there may be differences in the timing of rhythms between species (*i.e.*, nocturnal vs. diurnal) there are often striking similarities between species in the triggers and influences of diurnal rhythms (Rhoads et al., 1998). Therefore, it is not unlikely that patterns in glucose and insulin found in rats and humans could also be found in ruminants even though differences in carbohydrate metabolism exist.

Since cattle also have a night time peak in glucose level without a concomitant rise in insulin level (Andersson, 1982; Blum et al., 1985), it is possible that cattle also have a period of insulin insensitivity at night. If so, provision of fresh feed in the evening might synchronize nutrient availability (*i.e.*, glucose, acetate, and propionate) with this period of insulin insensitivity and allow more of those nutrients to be available for milk production.

2.5 Hypotheses

Study 1 Evening feeding will result in post-feeding patterns in glucose and insulin that are characteristic of insulin insensitivity such as elevated insulin and glucose soon after feeding. Plasma glucose and insulin responses to an i.v. glucose load will be elevated at night because of insulin insensitivity.

Study 2 Delivering feed in the evening will increase productivity in dairy cows as demonstrated by improved body conditioning and higher milk fat.

2.6 Objectives

Study 1. Investigate diurnal variations in plasma insulin, glucose, cholesterol, liver enzymes and melatonin with respect to evening feeding and diet concentrate level. Also, investigate the diurnal changes in glucose tolerance with respect to evening feeding and diet concentrate level.

Study 2. Investigate the production responses to evening feeding in a larger sample size than Study 1.

3.0 STUDY 1: Effect of Feed Delivery Time and Concentrate Level on Daily Rhythms of Plasma Metabolite, Enzyme, and Hormone Levels and Glucose Tolerance in Dairy Cows

3.1 ABSTRACT

The first experiment was a 4x4 replicated Latin Square with four 21d periods (14d adaptation, 7d sampling), and primiparous (n = 4) and multiparous (n = 4) Holsteins received either a high concentrate (38.5:61.5 F:C) or low concentrate (50.6:49.4 F:C) TMR at 0900h or 2100h. Blood samples were taken every 2h for two non-consecutive days during the sampling week each period and analysed for insulin, glucose, cholesterol, GGT, GLDH, AST, ALP and melatonin. An i.v. GTT was performed at 1200h in all cows during periods 2, 3, and 4. The second experiment was a cross-over design with two 6wk periods (3wk adaptation, 3wk sampling) and primiparous (n = 4) and multiparous (n = 4) Holsteins received fresh TMR at either 0900h or 2100h. The same sampling procedure as the first experiment was followed during week 5 of each period but blood was only analysed for insulin and glucose. Four i.v. GTTs were performed during week 5 of each period over two consecutive days at 1200h, 1900h, 0000h and 0700h. Each test corresponded to 3, 10, 15, or 22h post-feeding for the 0900h-fed or 2100h-fed cows. Shifting the delivery of fresh TMR to 2100h from 0900h, in the first experiment, shifted the rhythm and increased the variability of plasma level of insulin, glucose, cholesterol, GLDH, AST, ALP and tended to shift the rhythm of GGT. The rhythm of plasma melatonin was not affected by changes in feed delivery time. In the second experiment, peak glucose and insulin response varied with hours post-feeding. This is the first study to demonstrate the effect of hours post-feeding on GT in dairy

cows. Milk fat production might be optimal at 10h post-feeding because glucose sparing is taking place for milk production when glucose is most abundant, and this effect is enhanced with evening feeding. Therefore, evening feeding may provide a natural management way to control glucose sparing that is available to Canadian farmers.

(**Key words**: diurnal rhythms, carbohydrate metabolism, glucose tolerance)

Abbreviation key: **VFA** = volatile fatty acids; **i.v. GTT** = intravenous glucose tolerance test; **HC** = high concentrate diet (38.5:61.5 forage:concentrate ratio); **LC** = low concentrate diet (50.6:49.4 forage:concentrate ratio); **TMR** = Total Mixed Ration; **I/G ratio** = insulin/glucose ratio (uIU/mg•100); **GGT** = gamma-glutamyl transpeptidase; **AST** = aspartate aminotransferase; **ALP** = alkaline phosphatase; **GLDH** = glutamate dehydrogenase.

3.2 INTRODUCTION

Carbohydrate metabolism in dairy cows involves the conversion in the rumen of dietary carbohydrates to volatile fatty acids (VFAs), acetate, propionate and butyrate (Brockman and Laardveld, 1986). These VFAs are then absorbed and used by the animal for a source of energy (Brockman and Laardveld, 1986). Dairy cows rely on gluconeogenesis for the majority of their glucose needs which, for high producing cows (40kg/d), may exceed 3000g a day during lactation (Hart, 1983). The highest rates of gluconeogenesis in dairy cows are in the hours following feeding when glucose precursors are abundant; therefore, glucose level typically increase post-feeding

(Bergman, 1973, Blum et al., 1985; Sutton et al., 1988). Insulin secretion also increases post-feeding in ruminants as a result of rising glucose level as well as the entero-insular axis which is composed of sympathetic inputs to the pancreas (cephalic phase) and hormonal signals (incretins) from the gastro-intestinal tract in the form of GIP and GLP-1 (Godden and Weekes, 1981; McCarthy et al., 1992).

In cattle fed twice daily, the rise in plasma glucose in response to the mid-afternoon feeding occurred during the night and was typically 8-13% higher than the rise in plasma glucose in response to the early morning feeding, even though twice as much feed was consumed in the morning (Andersson et al., 1982; Blum et al., 1985). Similarly, humans who consume meals twice daily will have a glucose response after the evening meal that is two-fold greater than the morning meal (Van Cauter et al., 1992). In humans and cattle, the higher plasma glucose level in response to the meal later in the day does not coincide with higher plasma insulin level suggesting a diurnal variation in glucose tolerance (Van Cauter et al., 1992; Blum et al., 1985). Glucose tolerance, a measure of the ability of the body to metabolize glucose, is often tested using oral or i.v. glucose tolerance tests (GTT) (Van Cauter et al., 1989; Mir et al., 1998).

The most potent stimulus for eating in cattle housed indoors is the provision of fresh feed (De Vries and Von Keyserlingk, 2005; De Vries et al., 2005; Stricklin and Kautz-Scanavy, 1984). Research has shown that cattle will spend 10% to 30% more time eating in the evening (1800h) than the morning (0800h) when fresh feed was delivered daily or on alternate days at 0800h (Phillips and Rind, 2001). Evening feed delivery has been shown to improve average daily gain (ADG) and feed:gain ratio during winter in beef cattle (Knutsen et al., 1995; Kennedy et al., 2004; Schwartzkopf-Genswein et al.,

2004). Evening feeding did not translate into an increase in production when compared to day-fed controls during short-term, moderate heat stress (Ominski et al., 2002) or long-term, high heat stress (Aharoni et al., 2005). Robinson et al. (1997) observed higher milk fat and milk energy in four cows receiving a protein supplement at 0030h vs. controls receiving the supplement at 0830h. No studies have been completed, according to the author's knowledge, on the effects of evening feeding on dairy cattle in the thermoneutral zone.

In light of the evidence of circadian rhythms in plasma hormone, metabolite and enzyme levels, these studies were undertaken in order to determine if the rhythms found in cattle are related to feeding or the prevailing light-dark cycle. I hypothesize that shifting the delivery of fresh TMR to the evening (2100h) instead of the morning (0900h) will cause post-feeding patterns in insulin and glucose that are characteristic of insulin insensitivity such as elevated insulin and glucose soon after feeding. Plasma glucose and insulin in response to an i.v. glucose load will be elevated at night because of insulin insensitivity.

3.3 MATERIALS AND METHODS

3.3.1 Experiment 1

3.3.1.1 Animals and Housing

Eight Holstein dairy cows (4 primiparous, 4 multiparous) were chosen from the Glenlea Dairy Barn herd based on days in milk (82 ± 22 DIM), parity and milk yield (37 kg/d) at the start of the experiment and were moved to the adjacent Glenlea Metabolism Unit 3d prior to the start of the experiment. The cows were housed individually in tie-

stalls and had free access to feed and water in individual feeders and waterers. Cows were cared for in accordance with the Canadian Council on Animal Care (CCAC, 1993) guidelines. Cows were milked twice daily starting at 0400h and 1600h. Cows were allowed 2h of exercise outdoors every second day except during sampling weeks. Lights came on at 0345h and then off at 2230h.

3.3.1.2 Experimental Procedure

Cows were fed a ration of one of two diets *ad libitum* (adjusted daily for 5% orts) at 0900 (0900h-fed) or 2100 (2100h-fed). The diets were High Concentrate (HC - 38.5:61.5 forage:concentrate ratio) and Low Concentrate (LC – 50.6:49.4 forage:concentrate ratio) (Tables 1 and 2). The experimental design was a replicated 4x4 Latin Square with four 21d periods (14d adaptation, 7d sampling). There was a 2x2 factorial arrangement of diet and time of feeding. Cows were randomly assigned to one of four treatments in Period 1 and were randomly rotated through the other three treatments during Periods 2 to 4 so that each cow received each treatment once. The treatments were HC + 0900h-fed, HC + 2100h-fed, LC + 0900h-fed, and LC + 2100h-fed. During each period there were 2 cows on each treatment. Cows were weighed at the start of the experiment and at the end of each period at 0830h.

The sampling week consisted of 24h blood sampling on Tuesday and Thursday (all periods) and an i.v. glucose tolerance test (i.v. GTT) on Friday (Periods 2 to 4). Each cow was fitted with a long term catheter (Mila International, Florence, KY) and a catheter extension (Mila International, Florence, KY) in the jugular vein on the Monday of each sampling week (alternate sides were used at each period). All catheters were flushed

with 10mL of sterile heparinized 0.9% saline (5000 IU/100mL) solution to minimize clotting after installation and after each blood sample collection. Catheters were replaced as needed due to blockage or accidental removal.

The 24h blood samples were taken at 2h intervals starting at 0900h and ending at 0700h the following day. The first 10mL of blood drawn were discarded and 40mL of blood were then collected at each sample time. Three 10mL aliquots of blood were transferred to plasma vacutainers (sodium heparin, 143 USP units, Fisher Scientific, Fairlawn, NJ) and one 10mL aliquot was transferred to a serum vacutainer (no anti-coagulant, Fisher Scientific, Fairlawn, NJ). Blood was spun at 1900 x *g* for 15min and subsequently the plasma was harvested and stored (-20°C) for later analysis. The serum vacutainers were allowed to clot at room temperature for 30 to 60min and then spun. All samples taken during darkness period were done using red lighting (<5 lux).

The i.v. GTT was performed at 1200h (noon), which was 3h and 15h post-feeding for the 0900h-fed and 2100h-fed cows, respectively. After two 10mL blood samples were taken prior to the glucose injection (-5 and -2 min), each cow was injected with 150mg/Kg body weight of sterile 50% dextrose solution (Midwest Veterinary Distribution Cooperative, Winnipeg, MB) over 1 min using pre-filled sterile 60mL syringes. The catheter was then flushed with 20mL sterile 0.9% saline solution to ensure that all the dextrose solution had passed into the jugular vein. Blood samples (10mL) were taken at 5, 10, 15, 20, 25, 30, 35, 45, 60, 90, 120, 150 and 180 min post-injection. All blood samples were analysed for glucose, however, only the samples up to and including 120min post-injection were analysed for insulin due to budget constraints. Therefore, data presented are only up to and including 120min post-injection for both

Table 1. Ingredient and nutrient composition of the experimental diets in Experiment 1. Standard deviations are within brackets.

	Diet[1]	
Diet ingredients, % of DM	HC	LC
Alfalfa silage	15.87	20.96
Corn silage	22.67	29.66
Energy supplement	49.89	37.15
Protein supplement	11.57	12.23
Forage:concentrate (F:C) ratio	38.5:61.5	50.6:49.4

	Forage		TMR	
Nutrient Composition[2]	Alfalfa Silage	Corn Silage	HC	LC
Dry matter, %	34.8 (1.9)	44.0 (1.1)	63.53 (0.79)	55.94 (0.92)
CP, % DM	18.2 (2.1)	7.42 (0.6)	18.08 (0.3)	17.25 (0.58)
ADIP, % DM	1.3 (0.24)	0.55 (0.13)	1.5 (0.22)	1.55 (0.13)
NFC[3], % DM	21.0 (5.6)	37.6 (3.5)	39.67 (1.34)	35.9 (2.5)
NDF, % DM	47.1 (7.0)	47.4 (4.2)	28.6 (0.75)	33.77 (2.6)
ADF, % DM	37.8 (4.4)	26.1 (1.6)	15.08 (0.25)	19.4 (0.78)
Ether extract, % DM	2.9 (0.16)	2.3 (0.35)	5.85 (0.17)	5.29 (0.21)
Ash, % DM	10.7 (1.1)	5.2 (0.54)	7.75 (0.37)	7.79 (0.37)
Ca, % DM	1.36 (0.33)	0.27 (0.04)	1.09 (0.1)	1.1 (0.17)
P, % DM	0.29 (0.06)	0.17 (0.02)	0.58 (0.1)	0.51 (0.1)
K, % DM	2.87 (0.39)	1.19 (0.07)	1.22 (0.04)	1.03 (0.03)
Mg, % DM	0.38 (0.06)	0.28 (0.02)	0.32 (0.01)	0.32 (0.02)
Na, % DM	0.04 (0.01)	< 0.01	0.39 (0.03)	0.37 (0.03)

[1] HC = high carbohydrate, LC = low carbohydrate
[2] n = 4 for each forage
[3] Nonfibre carbohydrates = 100 – (NDF% + CP% + EE% + Ash%)

Table 2. Ingredient composition of energy supplement and protein supplement (%) in Experiments 1 and 2.

Ingredient	Energy Supplement	Protein Supplement
Rolled barley	54.0	—
Luprosil salt (calcium propionate)	0.2	—
Protein pellet[1]	1.8	—
Dairy supplement[2]	40.0	—
Tallow (feed grade rendered fat)	4.0	
Dried distillers grain	—	42.0
Fish meal	—	7.0
Canola meal	—	22.8
Soybean meal	—	20.0
Beet molasses	—	3.2
Niacin (Vit. B3)	—	0.3
Sodium bicarbonate	—	5.0

[1]Protein pellets contain 46.1% soybean meal, 2.6% wheat shorts, 40.0% canola meal, 5.0% oat hulls, 0.3% pellet binder, 1.0% cane molasses, and 5.0% corn gluten meal.
[2]Dairy supplement contains 0.13% vitamin ADE premix (Vit A, 16800 IU/kg; Vit D, 2215 IU/kg; Vit E, 75 IU/kg, DM basis), 0.13% trace mineral premix, 2.6% soybean meal, 0.06% selenium, 39.1% wheat shorts, 5.0% distillers grain, 17.5% canola meal, 15.0% ground wheat, 1.7% dicalcium phosphate, 1.6% salt, 2.0% dynamite, 0.3% pellet binder, 1.0% cane molasses, 3.7% calcium carbonate, and 10.0% corn gluten meal.

glucose and insulin. Aliquots of each blood sample were placed in two 3mL antiglycolytic tubes (Fisher Scientific, Fairlawn, NJ) to minimize glycolysis and tubes were immediately put on ice until they could be centrifuged at 1900 x *g* for 15min. The plasma was then aliquoted into 2mL cryovials (VWR International, Edmonton, AB) and frozen (-20°C) until later analysis.

3.3.1.3 Analysis

Plasma was analyzed for glucose (Stat Profile Critical Care Express, Nova Biomedical, Waltham, MA), insulin (Coat-a-Count RIA, Diagnostic Products Corporation, Los Angeles, CA) and melatonin using the Buhlmann kit (direct RIA, ALPCO Diagnostics, Salem, NH). The insulin intra-assay coefficient of variation was 9.0% from six different runs and the inter-assay coefficient of variation was 6.2%. Samples with melatonin level exceeding the standard curve were diluted up to 1:7 using the supplied sample dilution buffer. Dilutions of plasma containing various concentrations of melatonin were examined for parallelism with a curve generated from the standard solutions (Appendix 1). The melatonin intra-assay coefficient of variation for the high standard from two different runs was 16.9%, for the medium standard from six different runs was 12.6%, and for the low standard from three different runs was 11.3%. The melatonin inter-assay coefficient of variation was 12.7%, 9.6% and 8.7% for the high, medium and low standards respectively. Serum was analysed for cholesterol, gamma-glutamyl transpeptidase (GGT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and glutamate dehydrogenase (GLDH) at the University of Guelph (Guelph, ON) using a Hitachi 911 with Roche/Hitachi reagents.

3.3.1.4 Statistics

Area under the curve was calculated for glucose and insulin during the i.v. GTT after correcting for basal level (average of -5 and -2 min samples) using the trapezoidal rule (Lemosquet et al., 1997). Data were tested for normality and then log transformed and analyzed using the PROC MIXED procedure with repeated measures in SAS (SAS, 1999). Tables and figures display the non-transformed means and standard errors and all *P*-values are calculated using the transformed data. The model for the 24h data included feeding time, diet, parity, and hours post-feeding and the repeated measure was hour post-feeding. The model for the i.v. GTT data included feeding time, diet, parity and minute from glucose injection and the repeated measure was minute from glucose injection. The effects of feeding time, diet, parity and their interactions were considered fixed. The effects of period, cow within parity, sampling day within period, and the interaction of diet, feeding time and parity with cow within parity and day within period were considered random. Covariance structures for hourly measurements were tested and the covariance structure that resulted in the lowest value for the fit statistic was chosen (Plaizier et al., 2005). Tukey's mean comparison test was used to compare mean values when the main or interaction effect was significant (Steel et al., 1997).

3.3.2 Experiment 2

3.3.2.1 Animals and Housing

Four primiparous and four multiparous cows (84 ± 29 DIM, 35 Kg/d) were housed as outlined for Experiment 1. All cows were received a 50.2:49.8

(forage:concentrate) ration (Tables 2 and 3), formulated for the level of production *ad libitum* allowing for 5 to 10% orts at either 0900h or 2100h.

3.3.2.2 Experimental Procedure

The experiment was set up with a cross over design with two 6wk periods (3wk adaptation, 3wk sampling). Cows were randomly assigned to the delivery of fresh feed at either 0900 (0900h-fed) or 2100 (2100h-fed). In Week 5 blood sampling took place over 24h (Tuesday) and an i.v. GTT was conducted four times per cow over Wednesday and Thursday.

Jugular catheters were installed on the Sunday of Week 4 and maintained as in Experiment 1. Blood samples were drawn and processed as in Experiment 1. Cows were weighed twice daily (0800h and 2000h) at the end of Week 4 each period.

The four i.v. GTTs performed each period at 1200h (noon) on Wednesday and 0000h (midnight, using red lights <5 lux), 0700h and 1900h on Thursday corresponded to 3h, 10h, 15h or 22h after feed delivery for either the 0900h-fed or 2100h-fed cows. The data from one cow was not obtained during the final i.v. GTT due to a lost catheter, therefore, in all, 63 tests were completed. The sampling procedure for the i.v. GTT was similar to Experiment 1 except that samples following the glucose infusion were at 5, 10, 20, 30, 40, 50, 60, 90, 120min. Each cow was infused with 300mg/Kg body weight (average of morning and evening weight) of sterile 50% dextrose solution over 2min using pre-filled sterile 60mL syringes.

Table 3. Ingredient and nutrient composition of the experimental diets in Experiment 2.

Ingredient	% dry matter
Alfalfa silage	25.1
Barley silage	25.1
Energy supplement	39.2
Protein supplement	10.6
F:C ratio	50 : 49

	Forage		Concentrate		
Item	Alfalfa silage	Barley silage	Energy supplement	Protein supplement	TMR
DM, %	57.5	26.5	87.9	88.8	52.4
CP, % DM	18.5	9.9	17.0	34.2	17.3
ADIP, % DM	1.4	1.4	0.29	6.5	2.5
NFC[1], % DM	20.1	16.7	49.3	9.1	29
NDF, % DM	48.5	58.8	19.3	35.2	39.2
ADF, % DM	38.8	39.4	10.1	18.4	27.1
Ash, % DM	10.3	11.4	6.5	13.0	9.8
Ca, % DM	1.19	0.46	0.90	3.71	1.09
P, % DM	0.36	0.39	0.62	1.17	0.57
K, % DM	2.83	2.24	0.84	1.0	1.88
Mg, % DM	0.40	0.34	0.30	0.32	0.36
Na, % DM	0.09	0.07	0.27	1.48	0.32
Zn, ppm	31.7	37	116	70	71.7
Mn, ppm	39.8	41	113	53	74.5
Cu, ppm	11.5	9	31	9	15.2
Fe, ppm	311	522	198	408	322

[1]Nonfiber carbohydrates = 100 – (NDF% + CP% + EE% + Ash%).

3.3.2.3 Analysis

Blood was analyzed for glucose and insulin as indicated for Experiment 1. The insulin intra-assay coefficient of variation was 12.4% for four different runs and the inter-assay coefficient of variation was 8.2%.

3.3.2.4 Statistics

Area under the curve was calculated for glucose and insulin during the i.v. GTT as in Experiment 1. Data were tested for normality and then log transformed and analyzed using the PROC MIXED procedure with repeated measures in SAS (SAS, 1999). Tables and figures display the non-transformed means and standard errors and all *P*-values are calculated using the transformed data. The model for the 24h data included feeding time, parity, and hours post-feeding and the repeated measure was hour post-feeding. The model for the i.v. GTT data included feeding time, parity and minute from injection and the repeated measure was minute from glucose injection. The effects of feeding time, parity and their interactions were considered fixed. The effects of period, cow within parity, and the interaction of feeding time and period with cow within parity were considered random. Covariance structures for hourly measurements were tested and the covariance structure that resulted in the lowest value for the fit statistic was chosen (Plaizier et al., 2005). Tukey's mean comparison test was used to compare mean values when the main or interaction effect was significant (Steel et al., 1997).

3.4 RESULTS

3.4.1 Experiment 1

3.4.1.1 Glucose and Insulin

Cows fed the HC diet had higher ($P < 0.001$) plasma glucose and insulin levels than cows fed the LC diet (Table 4). Diet X FT tended ($P < 0.1$) to affect plasma glucose level where HC feeding caused a greater rise (4.7 mg/dL) in plasma glucose in 0900h-fed cows than in 2100h-fed cows (3.0 mg/dL). Although the effect of parity on plasma glucose was not significant, the rise (4.7 mg/dL) in plasma glucose with HC feeding compared to LC feeding in primiparous cows (82.5 mg/dL vs 77.8 mg/dL, respectively) tended ($P < 0.10$) to be greater than the rise (3.0 mg/dL) in multiparous cows (78.3 mg/dL vs 75.3 mg/dL, respectively) (Table 5).

The interaction of FT and hours post-feeding was significant for both plasma glucose and insulin (Table 4, Figure 1). Plasma glucose level of 2100h-fed cows dropped to a low at 2h post-feeding ($P < 0.05$), increased by 6h post-feeding and remained elevated above pre-feeding level until 14h post-feeding ($P < 0.05$) whereas plasma glucose level of 0900h-fed cows did not vary significantly throughout the 24h period. Plasma insulin level rose ($P < 0.001$) by 2h post-feeding in 2100h-fed cows but not until 4h post-feeding ($P < 0.001$) in 0900h-fed cows. Plasma insulin level remained elevated until 8h post-feeding in 2100h-fed cows ($P < 0.001$). In contrast, plasma insulin level remained elevated for much longer (16h, $P < 0.001$; 20h, $P < 0.05$) in 0900h-fed cows. Plasma insulin level began to drop by 10h post-feeding in 2100h-fed cows even though plasma glucose was still elevated from pre-feeding level until 14h post-feeding (Figure

Table 4. Effect of diet, feed delivery time and hours post-feeding on daily plasma hormone, metabolite and enzyme levels in dairy cows of Experiment 1.

	Diet[1] (D)		Feedtime (FT)			*P*-value[2]				
	HC	LC	0900h	2100h	SEM	FT[3]	D	H[4]	FT x H	FT x D
Glucose, mg/dL	80.4	76.6	78.5	78.5	1.0	NS	***	***	***	†
Insulin, uIU/mL	17.5	11.9	14.5	14.8	2.5	NS	***	***	***	NS
Cholesterol, IU/mL	6.5	6.7	6.6	6.7	0.3	NS	NS	***	**	NS
GGT, IU/mL	28.5	29.1	28.4	29.2	1.9	NS	NS	***	†	NS
GLDH, IU/mL	30.8	32.0	32.1	30.8	4.0	NS	NS	***	***	NS
AST, IU/mL	95.4	92.5	96.9	91.0	6.3	NS	NS	†	***	NS
ALP, IU/mL	50.2	48.6	50.6	48.2	3.9	NS	NS	*	*	NS
Melatonin, pg/mL	23.0	31.6	28.9	25.6	5.9	NS	†	***	NS	NS
i.v. GTT results										
Glucose AUC, mg/min	1815	1994	1963	1846	78	NS	†	-	-	NS
Glucose peak, mg/dL	83.4	89.5	84.1	88.7	3.5	NS	†	-	-	NS
Insulin AUC, uIU/min	3549	2452	2944	3058	568	NS	*	-	-	NS
Insulin peak, uIU/mL	135.4	96.5	115.5	116.4	23.3	NS	*	-	-	NS
I/G ratio[5], (uIU/mg)*100	1.7	1.1	1.4	1.4	0.3	NS	*	-	-	NS

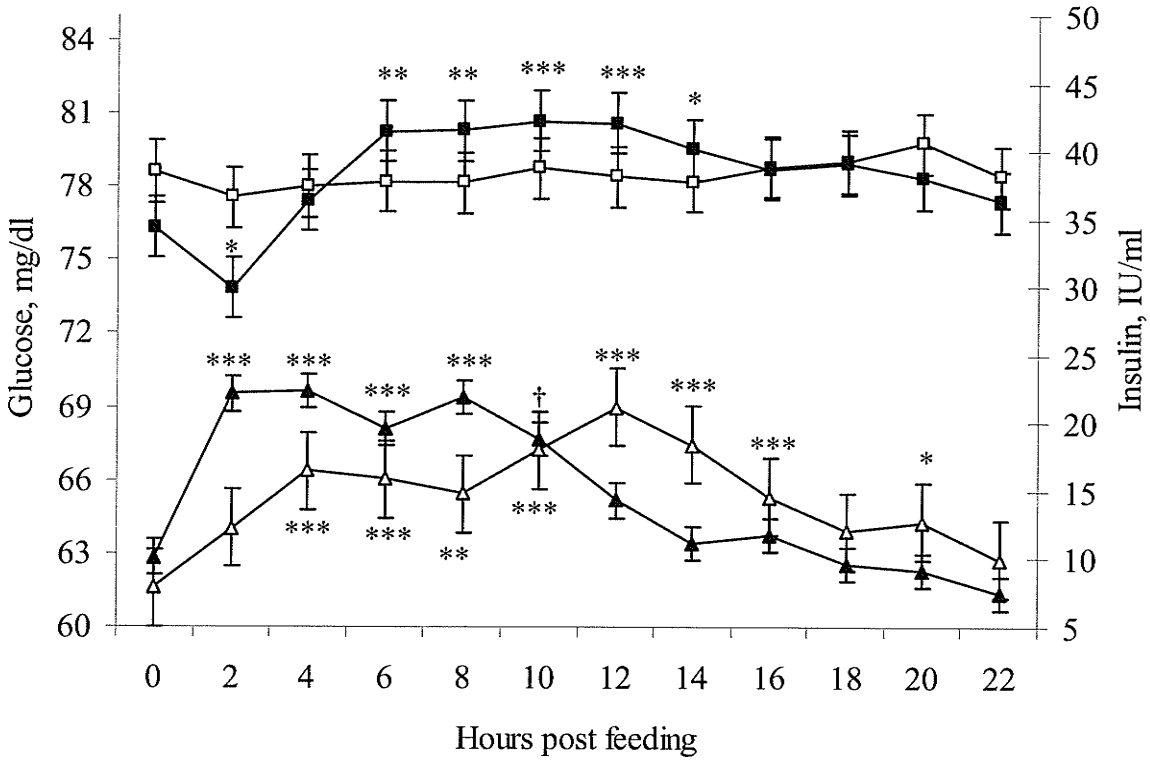
[1] Diet: HC = high concentrate (61% of dry matter), LC = low concentrate (51% of dry matter)
[2] NS = $P > 0.10$, † = $P < 0.10$, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$
[3] Feedtime
[4] Hours post-feeding
[5] (peak – basal insulin)/(peak – basal glucose)

Table 5. Effect of parity on daily plasma metabolite, hormone and enzyme levels in dairy cows of Experiment 1.

	Parity			*P*-value[2]						
	Primi-parous	Multi-parous	SEM	Parity (P)	P x H[4]	P x D[1]	P x FT[3]	P x D x H	P x FT x H	P x FT x D
Glucose, mg/dL	80.2	76.8	1.5	NS	NS	†	NS	NS	NS	NS
Insulin, uIU/mL	16.0	13.4	3.4	NS	NS	NS	NS	NS	NS	NS
Cholesterol, IU/mL	7.3	5.9	0.4	*	*	NS	NS	NS	NS	NS
GGT, IU/mL	25.6	32.0	2.6	†	NS	NS	NS	NS	NS	NS
GLDH, IU/mL	38.8	24.0	5.2	*	NS	NS	NS	NS	NS	NS
AST, IU/mL	94.4	93.4	7.6	NS	†	NS	NS	NS	†	NS
ALP, IU/mL	57.0	41.8	5.1	*	*	NS	NS	NS	*	NS
Melatonin, pg/mL	26.9	27.6	7.5	NS	†	NS	NS	NS	NS	NS
i.v. GTT results										
Glucose AUC, mg/min	2080	1729	82	*	-	NS	NS	-	-	NS
Glucose peak, mg/dL	91.5	81.4	4.3	NS	-	NS	NS	-	-	NS
Insulin AUC, uIU/min	2600	3401	715	NS	-	NS	NS	-	-	NS
Insulin peak, uIU/mL	105.2	126.8	30.7	NS	-	NS	†	-	-	†
I/G ratio[5], (uIU/mg)*100	1.2	1.6	0.4	NS	-	NS	†	-	-	†

[1] Diet: HC = high concentrate (61% of dry matter), LC = low concentrate (51% of dry matter)
[2] NS = $P > 0.10$, † = $P < 0.10$, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$
[3] Feedtime
[4] Hours post-feeding
[5] (peak – basal insulin)/(peak – basal glucose)

Figure 1. Plasma glucose of cows fed at 0900h (□) or 2100h (■) and plasma insulin levels of cows fed at 0900h (Δ) or 2100h (▲) in Experiment 1. Means within a treatment differ from 0 h post-feeding († = $P < 0.1$, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$). Means within a sampling time for either glucose or insulin did not differ between treatments.



1). There was no interaction of parity and FT or parity with hours post-feeding on plasma glucose or insulin levels (Table 5).

3.4.1.2 Cholesterol

There was no effect of diet on plasma cholesterol level (Table 4). Hour post-feeding and FT x H significantly affected plasma cholesterol level (Table 4, Figure 2). Plasma cholesterol level was reduced at 2 and 4h post-feeding in 2100h-fed cows ($P < 0.05$) and returned to pre-feeding level by 8h post-feeding while no significant changes over time in plasma cholesterol level were found in 0900h-fed cows.

Primiparous cows had higher ($P < 0.05$) plasma cholesterol level than multiparous cows (Table 5). Although parity X H was significant, the reason for this interaction was not apparent (Figure 3).

3.4.1.3 Liver Enzymes

The effects of parity, FT, diet and hour post-feeding on liver enzymes are shown in Tables 4 and 5. Diet did not affect the liver enzymes but the interaction of FT and hours post feeding was significant for GLDH ($P < 0.001$), AST ($P < 0.001$) and ALP ($P < 0.05$) and tended to be significant for GGT ($P < 0.1$) (Table 4, Figure 4). With the exception of GGT (Figure 4a), the difference between the 0900h-fed and 2100h-fed cows appeared in the first 12h post-feeding where 2100h-fed cows had lower enzyme levels (Figure 4b to d). In general, enzyme levels dropped immediately post-feeding in 2100h-fed cows and increased in 0900h-fed cows, except ALP level which dropped in both groups immediately post-feeding (Figure 4). At 12h post-feeding both AST and ALP levels were significantly reduced compared to 0h post-feeding but this was only

significant for the 2100h-fed cows. A drop in AST and ALP at 12h post-feeding was significant in primiparous 2100h-fed cows (Table 5, Figures 5 and 6), where the 3 way interaction tended to be (AST) and was (ALP) significant.

3.4.1.4 Melatonin

Cows fed the HC diet tended to have lower average melatonin level then cows fed the LC diet ($P < 0.1$, Table 4). Feed delivery time did not have an effect on average melatonin level and did not interact with hours post-feeding (Table 4). Melatonin level began to rise by 2100h (1.5h before to lights off), peaked between 2300h and 0300h and returned to daytime level by 0700h (1.5h after lights on) in all treatment groups (Figure 7). Melatonin level varied significantly between individual cows ($P < 0.001$) where the rise in melatonin during the dark phase was 10x greater in some cows than others. Also, melatonin level tended to vary between sampling days ($P < 0.1$) for an individual cow. The primiparous cows tended (Table 5, $P < 0.1$) to have peak melatonin level earlier (0100h) than the multiparous cows (0300h), however peak level did not differ between parities.

Figure 2. Interaction ($P < 0.001$) of feeding time (0900h-fed - □, 2100h-fed - ■) and hours post-feeding on plasma cholesterol level of dairy cows in Experiment 1. Means within a treatment differ from 0 h post-feeding († = $P < 0.1$, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$). Means within a sampling time did not differ between treatments.

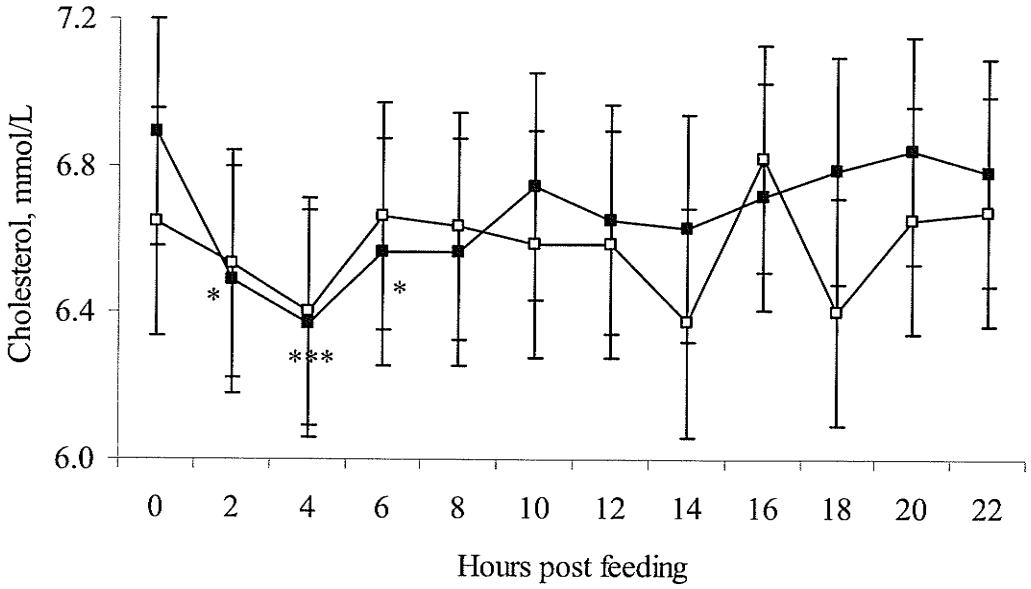


Figure 3. Interaction ($P < 0.05$) of parity and hours post-feeding on plasma cholesterol level of dairy cows in Experiment 1. Means within a parity differ from 0 h post-feeding († = $P < 0.1$, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$). Means within a sampling time did not differ between parities.

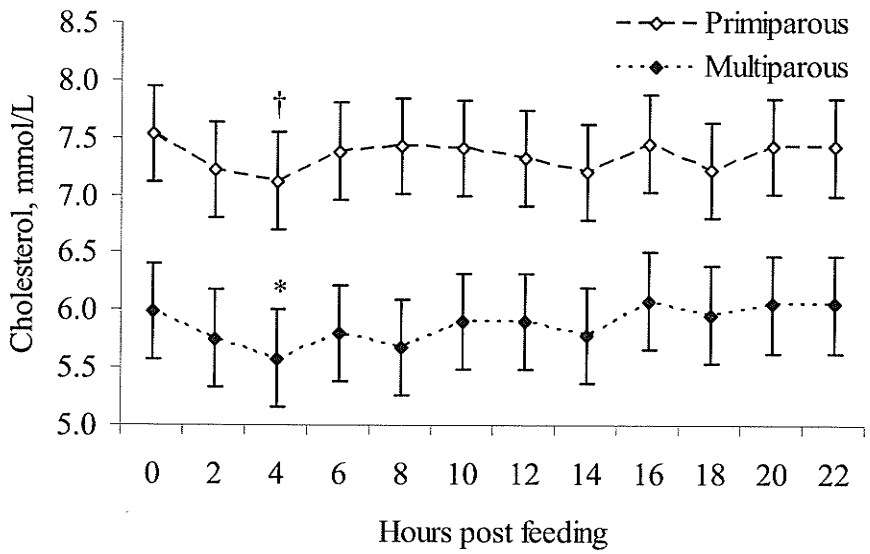


Figure 4. Interaction of feeding time (0900h-fed - □, 2100h-fed - ■) and hours post-feeding on plasma a) gamma glutamyl transferase (GGT) (interaction $P < 0.1$), b) glutamate dehydrogenase (GLDH) (interaction $P < 0.001$), c) aspartate aminotransferase (AST) (interaction $P < 0.001$) and d) alkaline phosphatase (ALP) (interaction $P < 0.05$) levels of dairy cows in Experiment 1. Means within a treatment differ from 0 h post-feeding († = $P < 0.1$, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$). Means within a figure within a sampling time did not differ between treatments.

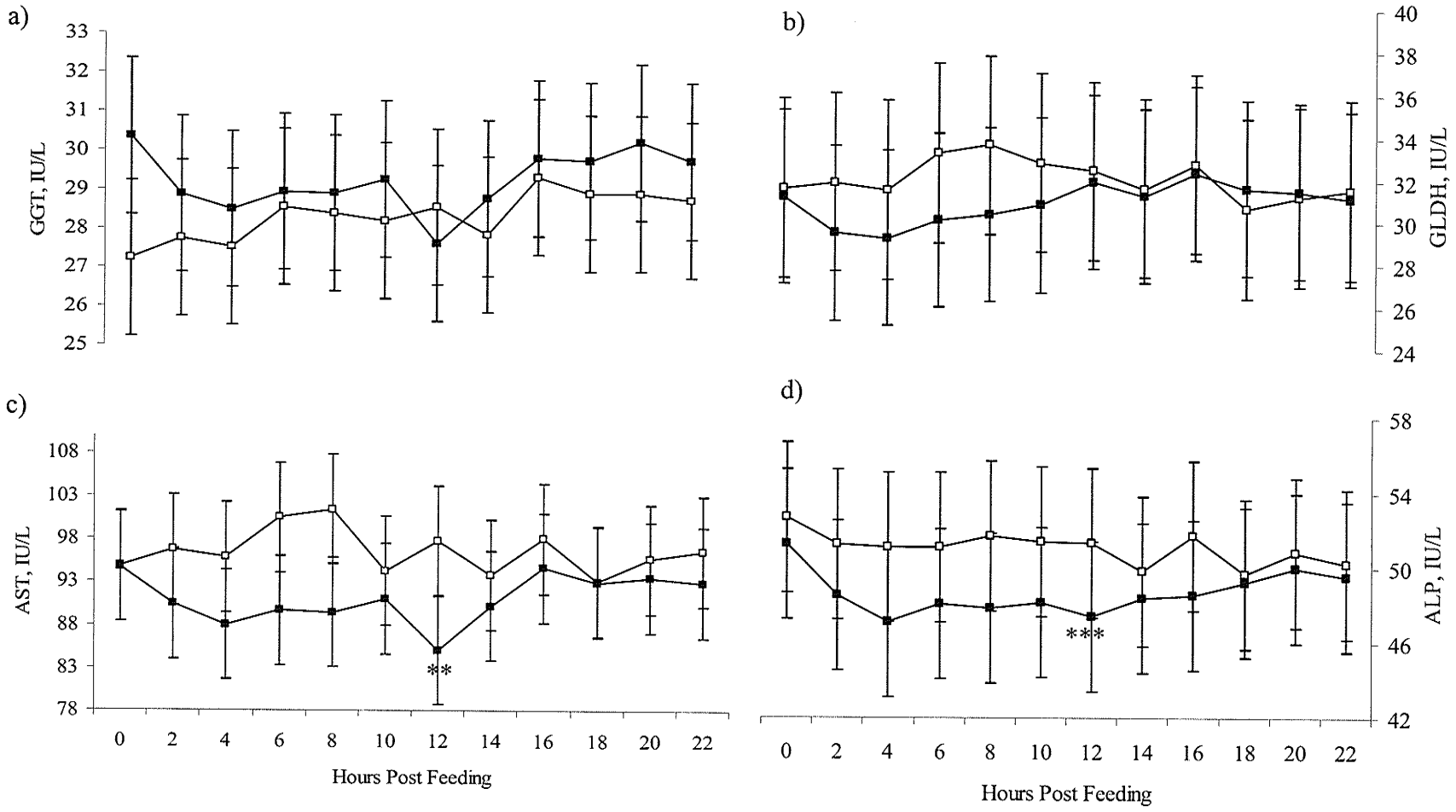
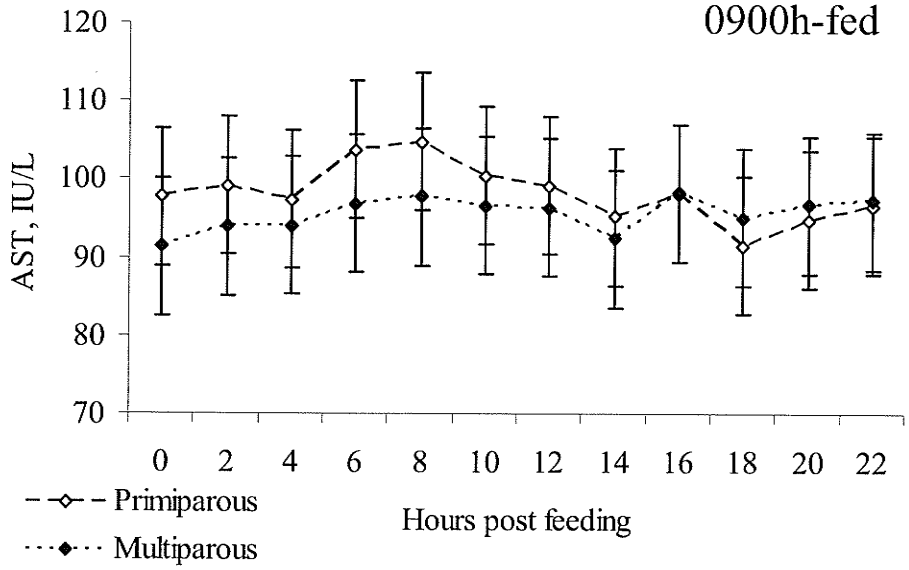


Figure 5. Interaction (P < 0.1) of parity, feeding time and hours post-feeding on daily plasma AST level of dairy cows in Experiment 1. Means within a parity differ from 0 h post-feeding († = $P < 0.1$, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$). Means within a figure within a sampling time did not differ between treatments or parities.



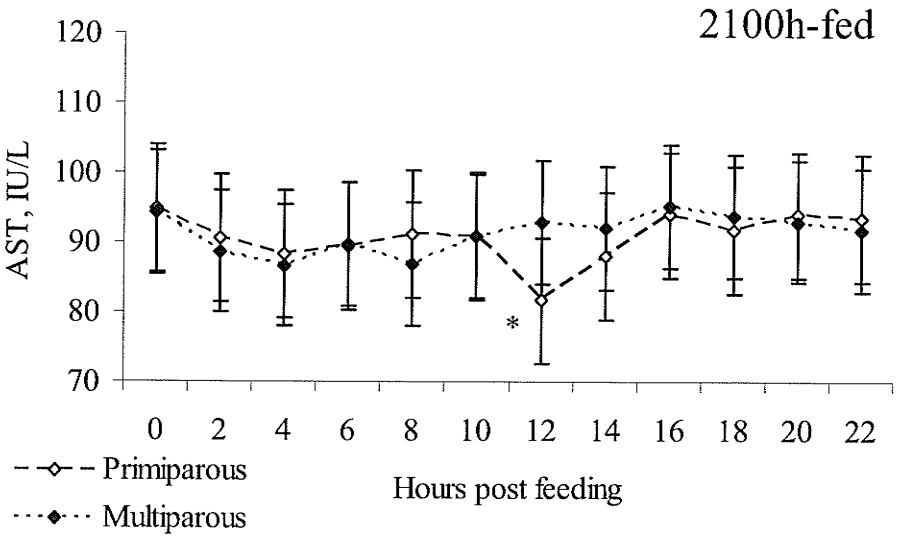
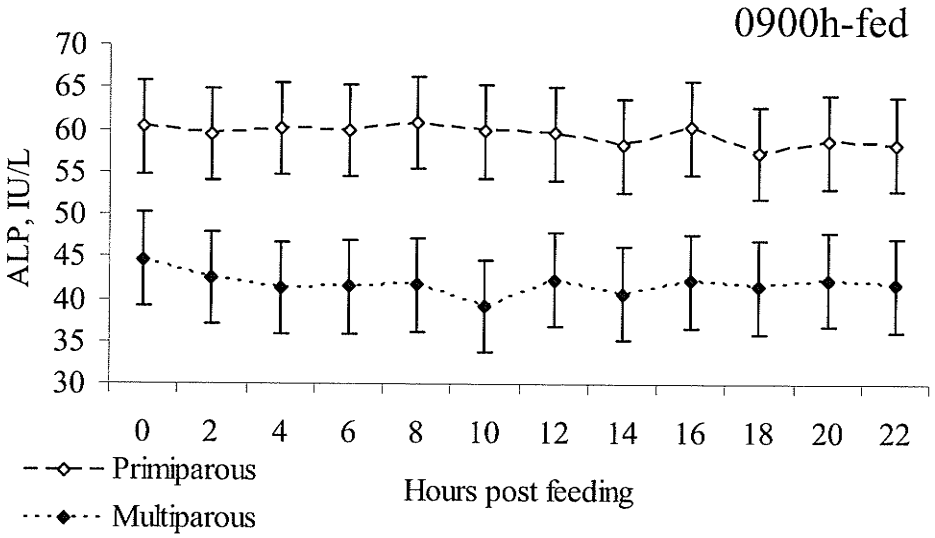


Figure 6. Interaction ($P < 0.05$) of parity, feeding time (0900h-fed - □, 2100h-fed, ■) and hours post-feeding on daily plasma ALP level of dairy cows in Experiment 1. Means within a parity differ from 0 h post-feeding († = $P < 0.1$, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$). Means within a sampling time did not differ between treatments or parities.



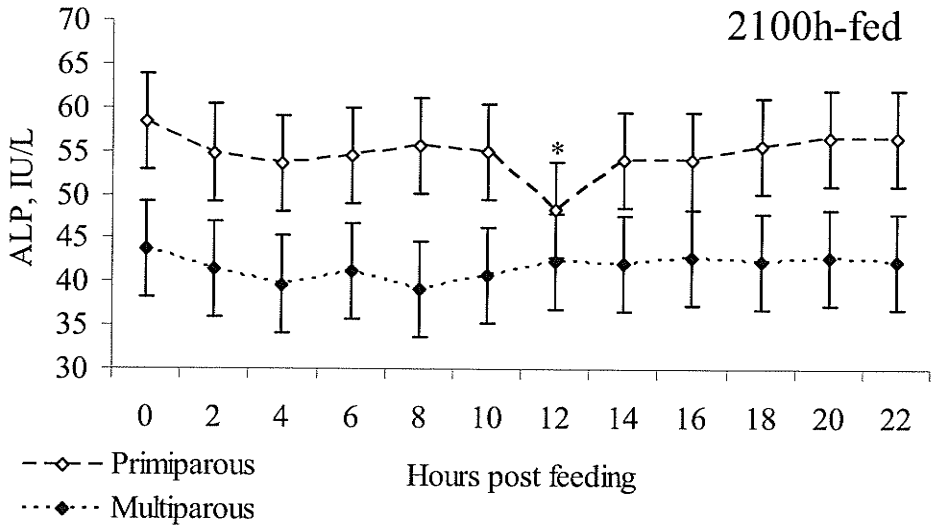
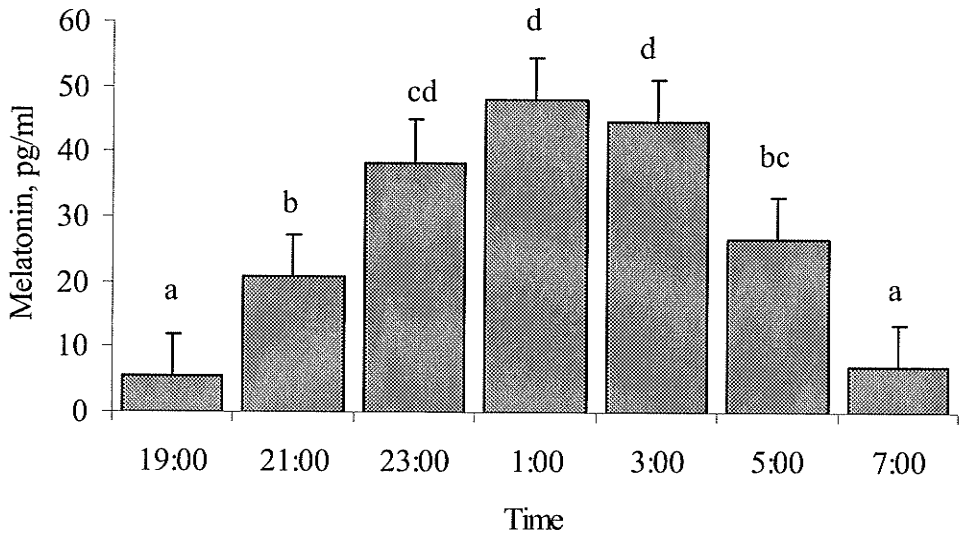


Figure 7. Mean plasma melatonin level of dairy cows in Experiment 1 across feedtimes, diets and parities from 1900h to 0700h (dark phase from 2230h to 0345). Means with different letters differed by $P < 0.05$.



3.4.1.5 i.v. GTT

Plasma glucose level peaked (2-fold increase) within 5min and plasma insulin peaked (8-fold increase) within 10min of glucose i.v. injection in both the 0900h-fed and 2100h-fed cows. Plasma glucose and insulin returned to pre-injection level by 60min of the glucose injection (Figure 8). Feed delivery time had no effect on any i.v. GTT parameter (Table 4). Cows fed the HC diet tended to have a 10% smaller ($P < 0.10$) glucose AUC and a 7% lower ($P < 0.10$) plasma glucose peak than cows fed the LC diet. Also, the insulin AUC was 45% higher ($P < 0.05$) and the insulin peak was 40% higher ($P < 0.05$) in cows fed the HC diet compared to the LC diet.

The ratio of peak insulin level to peak glucose level in plasma during the i.v. GTT at noon was calculated for the 0900h-fed and 2100h-fed cows as outlined in Lima et al. (1998). Briefly, the difference between the peak insulin concentrations (58% of cows had peak level at 10min sample) and the basal insulin concentration (average of -5min & -2min samples) was divided by the difference between the peak glucose concentrations (5min sample) and the basal glucose concentration (average of -5min and -2min samples). This ratio (I/G) allows the comparison of the magnitude of the insulin response to an equivalent glucose load between different treatments. As a result of having a higher insulin peak and lower glucose peak, the cows fed the HC diet had a 55% higher ($P < 0.05$) I/G ratio than the cows fed the LC diet (Table 4). There was no effect of FT on the I/G ratio (Table 4).

Primiparous cows had a significantly higher ($P < 0.05$) glucose AUC than multiparous cows during the i.v. GTT at 1200h. Although the insulin AUC was

numerically lower in primiparous cows there was no significant difference in the insulin AUC due to a large SEM (Table 5).

Parity did not influence peak plasma glucose or insulin levels during the i.v. GTT but there was a 3 way interaction of parity, FT and diet for peak plasma insulin level (Table 5). An increase in peak insulin level in multiparous cows and decreased peak insulin level in primiparous cows with 2100h-feeding was only found with the LC diet (Figure 9). The same pattern was seen for the I/G ratio (not shown).

Figure 8. Plasma glucose and insulin levels in response to an i.v. glucose load in dairy cows fed either at 0900 (0900h-fed - □) or 2100 (2100h-fed - ■) in Experiment 1. Means within a figure within a sampling time did not differ between treatments.

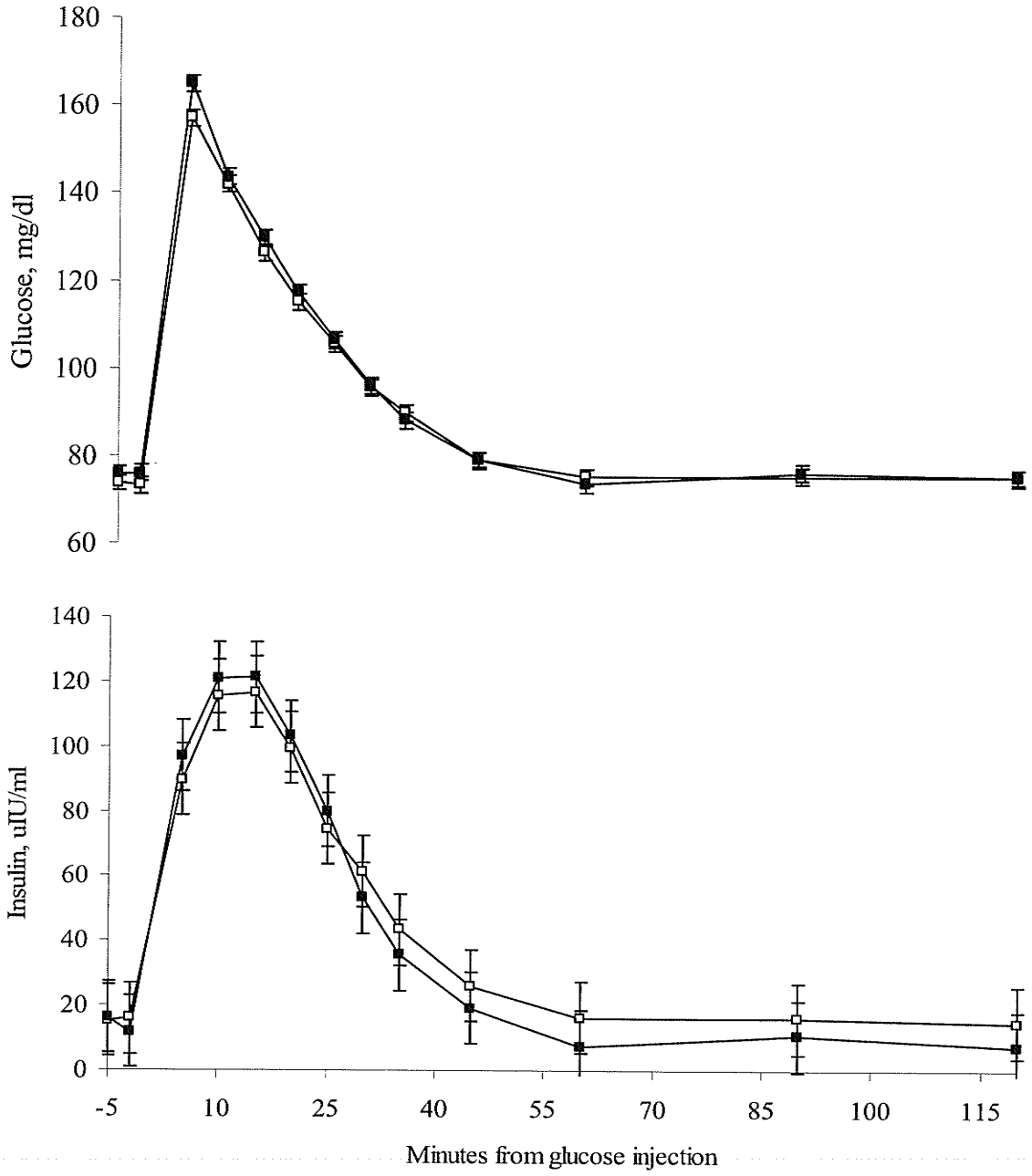
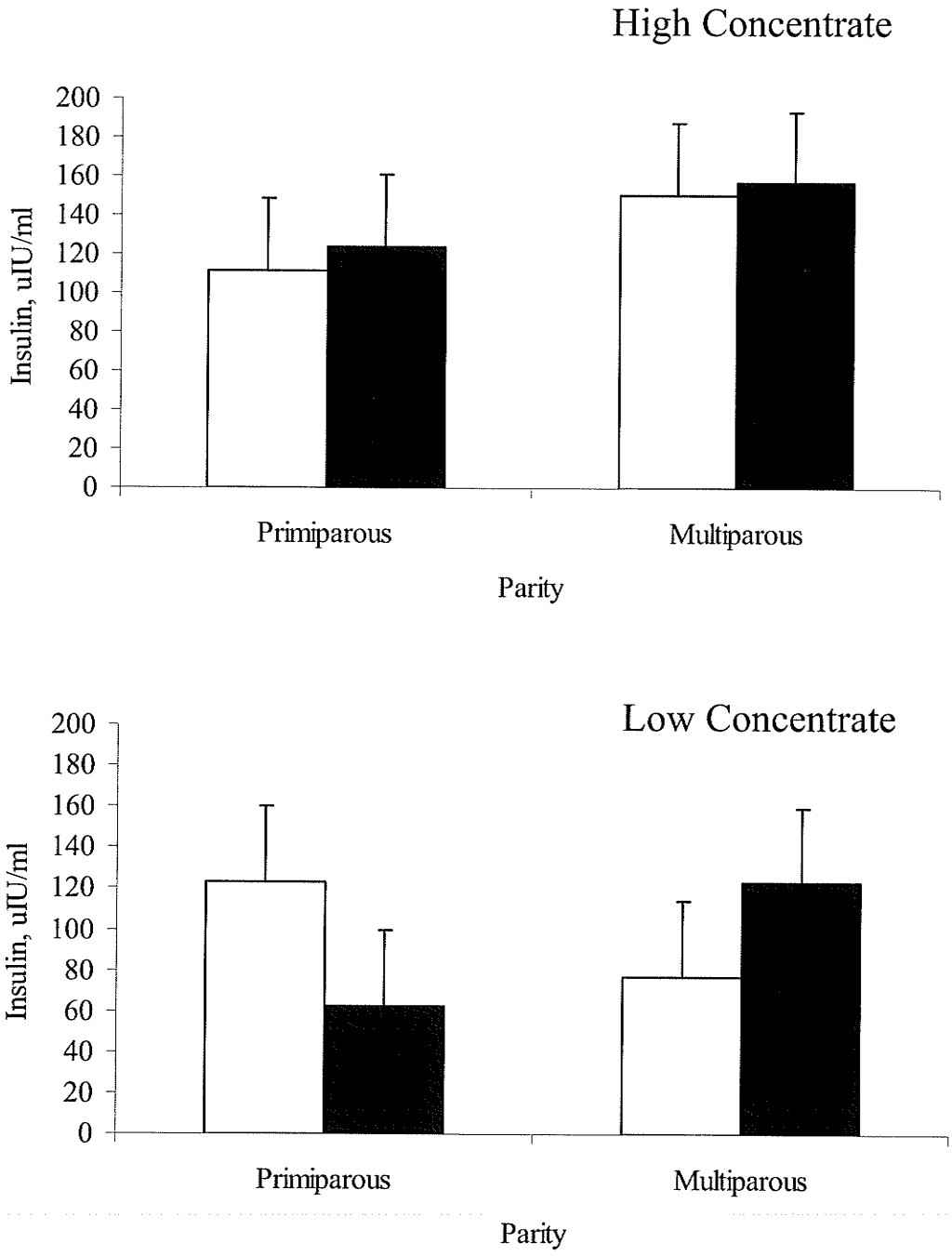


Figure 9. Interaction ($P < 0.1$) of parity, diet and time of feeding (0900h-fed - □, 2100h-fed - ■) on peak plasma insulin level during i.v. GTT in dairy cows of Experiment 1. Means did not differ between treatments, diets or parities.



3.4.2 Experiment 2

3.4.2.1 Glucose and Insulin

The interaction of FT and hours post-feeding was significant for plasma glucose ($P < 0.01$) and insulin ($P < 0.01$) levels (Table 6). Plasma glucose level was reduced at 2h post-feeding in 2100h-fed cows ($P < 0.001$) but not the 0900h-fed cows (Figure 10). Plasma insulin level were significantly higher than pre-feeding level from 2h to 10h post-feeding in the 2100h-fed cows but not in the 0900h-fed cows. The 2100h-fed cows had a 4-fold increase ($P < 0.01$) in plasma insulin level by 6h post-feeding, whereas a 2-fold increase by 6h post-feeding in the 0900h-fed cows was not significant (Figure 10).

Parity interacted with hours post-feeding on plasma glucose level but had no effect on plasma insulin level (Table 6). Glucose level dropped at 2h post-feeding in both primiparous and multiparous 2100h-fed cows ($P < 0.001$) but not 0900h-fed cows of either parity (Figure 11).

3.4.2.2 i.v. GTT

Feed delivery time had no effect on glucose AUC, glucose peak, insulin peak or I/G ratio but tended ($P < 0.1$) to influence insulin AUC (Table 6) in that the insulin AUC of the 2100h-fed cows tended ($P < 0.1$) to be greater than for 0900h-fed cows. The AUC of glucose and insulin during the i.v. GTTs was affected by hours post-feeding ($P < 0.05$ and $P < 0.001$, respectively) (Table 6). The insulin AUC at 10h post-feeding was larger ($P < 0.001$) than at 3h, 15h and 22h post-feeding but glucose AUC at 10h post-feeding did not differ from 3h, 15h or 22h post-feeding ($P > 0.1$) (Figure 12). The largest glucose AUC corresponded with the smallest insulin AUC at 22h post-feeding.

Table 6. Effect of feed delivery time, parity and hours post-feeding on plasma insulin and glucose levels throughout a 24h period and in response to i.v. GTT in dairy cows of Experiment 2.

	Feeding Time			Parity (P)			*P*-value[1]						
	0900h	2100h	SEM	Primi-parous	Multi-parous	SEM	P	FT[2]	P x FT	H[3]	FT x H	P x H	P x FT x H
Glucose, mg/dL	75.7	74.9	1.3	75.7	74.9	1.4	NS	NS	NS	***	**	**	**
Insulin, uIU/mL	10.6	10.9	1.8	12.1	9.5	2.0	NS	NS	NS	***	**	NS	NS
i.v. GTT results													
Glucose AUC, mg/min	4990	5051	190	4913	5127	231	NS	NS	NS	*	NS	NS	NS
Glucose peak, mg/dL	199.0	200.1	7.0	200.6	198.4	8.8	NS	NS	NS	**	**	NS	NS
Insulin AUC, uIU/min	3567	4609	525	4569	3607	625	NS	†	NS	***	NS	NS	NS
Insulin peak, uIU/mL	102.7	121.5	11.1	125.7	98.5	14.3	NS	NS	NS	***	NS	NS	NS
I/G ratio[4], (uIU/mg)*100	0.53	0.62	0.05	0.63	0.52	0.06	NS	NS	NS	***	†	NS	NS

[1]NS = $P > 0.10$, † = $P < 0.10$, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$
[2] Feedtime
[3] Hours post-feeding
[4] (peak – basal insulin)/(peak – basal glucose)

Figure 10. Plasma glucose level of cows fed at 0900h (□) or 2100h (■) and plasma insulin level of cows fed at 0900h (Δ) or 2100h (▲) in Experiment 2. Means within a treatment differ from 0 h post-feeding († = $P < 0.1$, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$). Means within a sampling time differ from each other (a = $P < 0.01$).

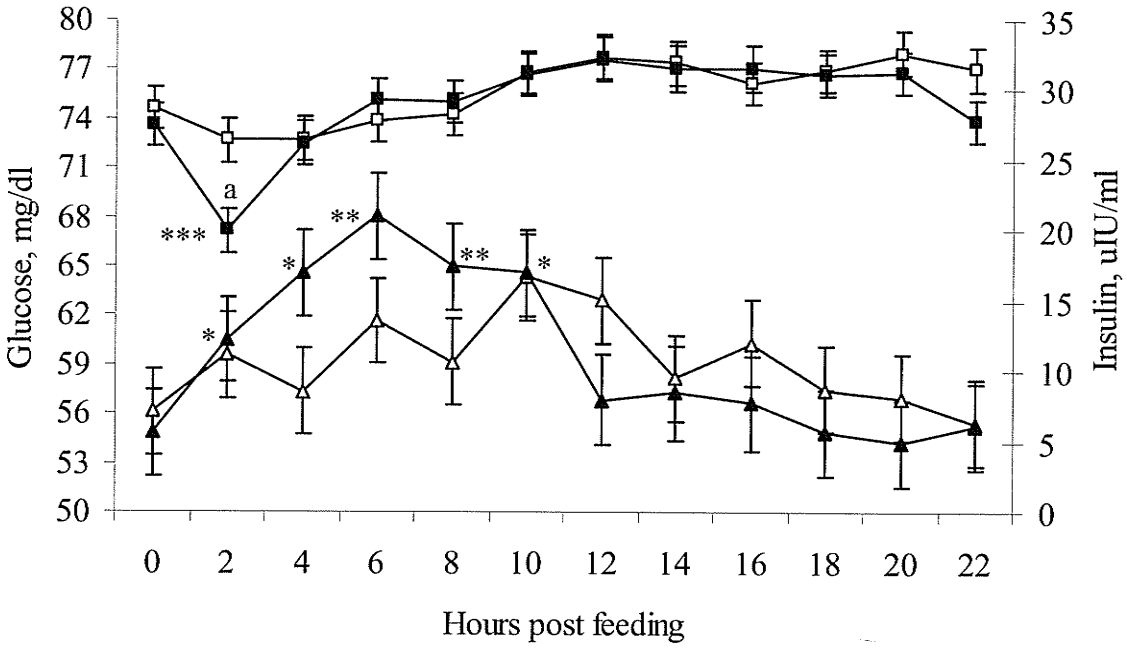


Figure 11. Interaction ($P < 0.01$) of parity, feeding time and hours post-feeding on plasma glucose level of dairy cows in Experiment 2. Means within a parity differ from 0 h post-feeding († = $P < 0.1$, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$). Means within a figure within a sampling time do not differ between parities.

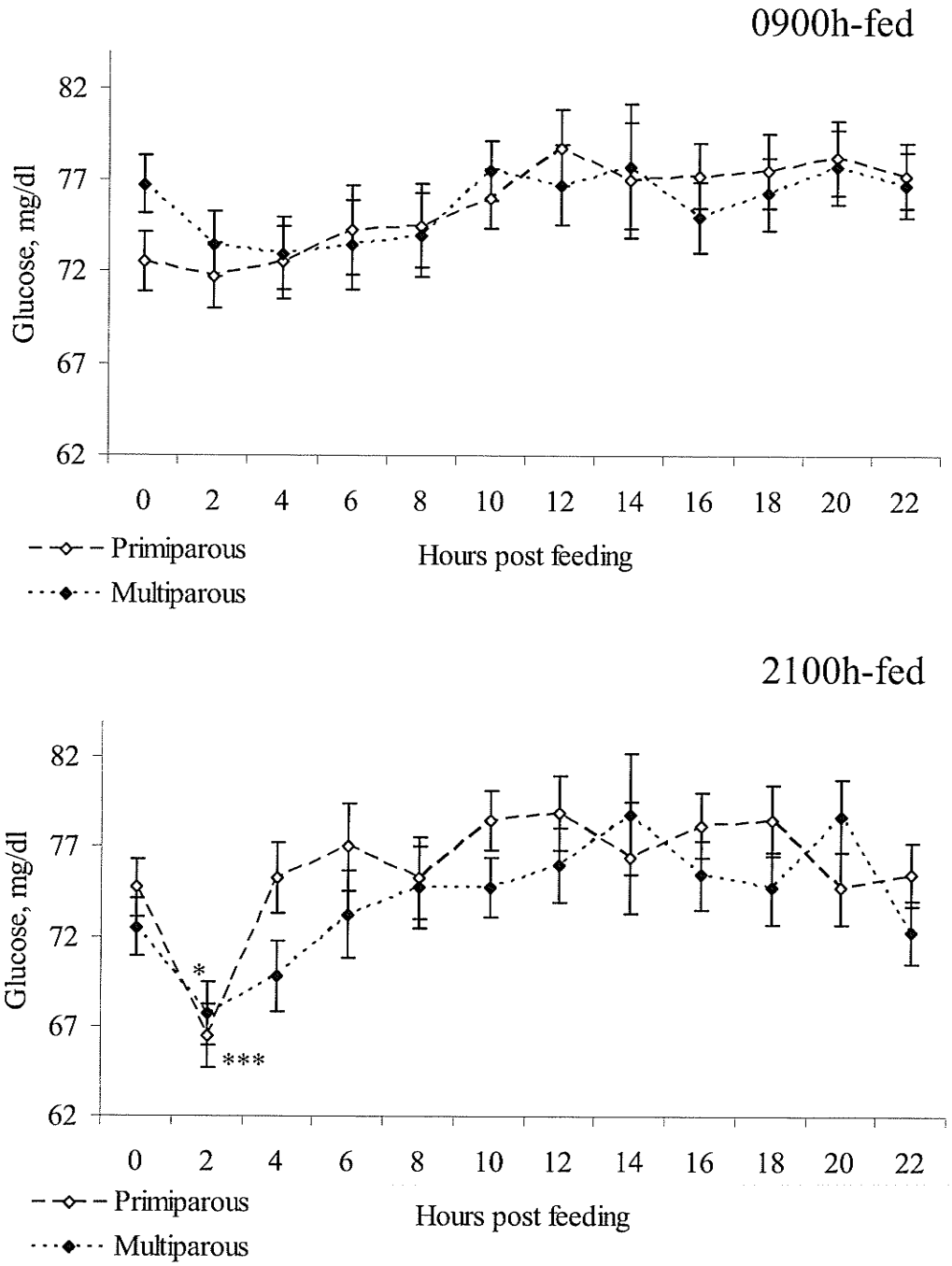
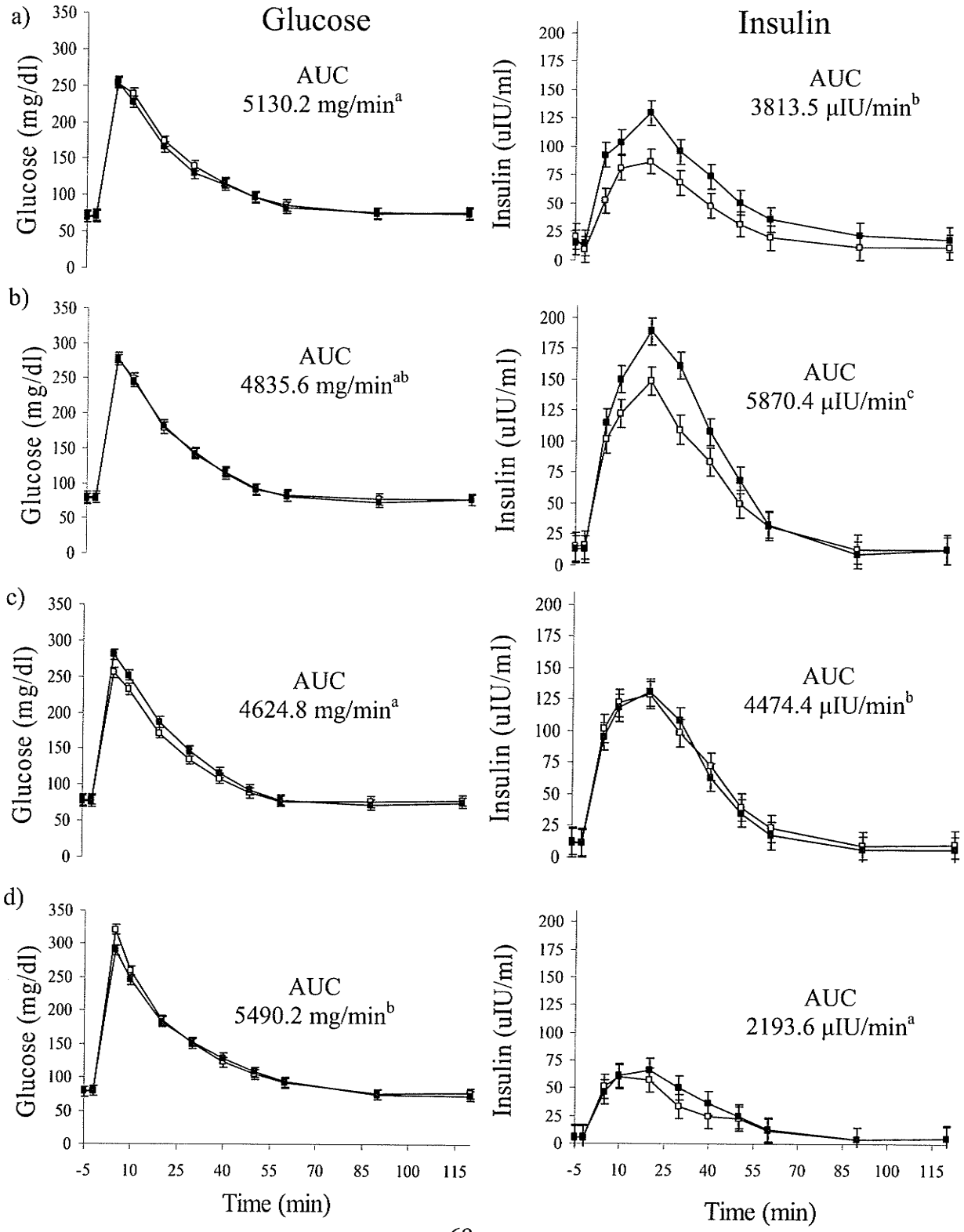


Figure 12. Plasma glucose and insulin response to an i.v. glucose load in cows fed either at 0900 (□) or 2100 (■) during i.v. GTTs performed at a) 3h, b) 10h, c) 15h, and d) 22h post-feeding in Experiment 2. The AUC values shown are for the respective hour post-feeding across feeding times. Letters indicate significant differences ($P < 0.05$) between AUC values of glucose or insulin.

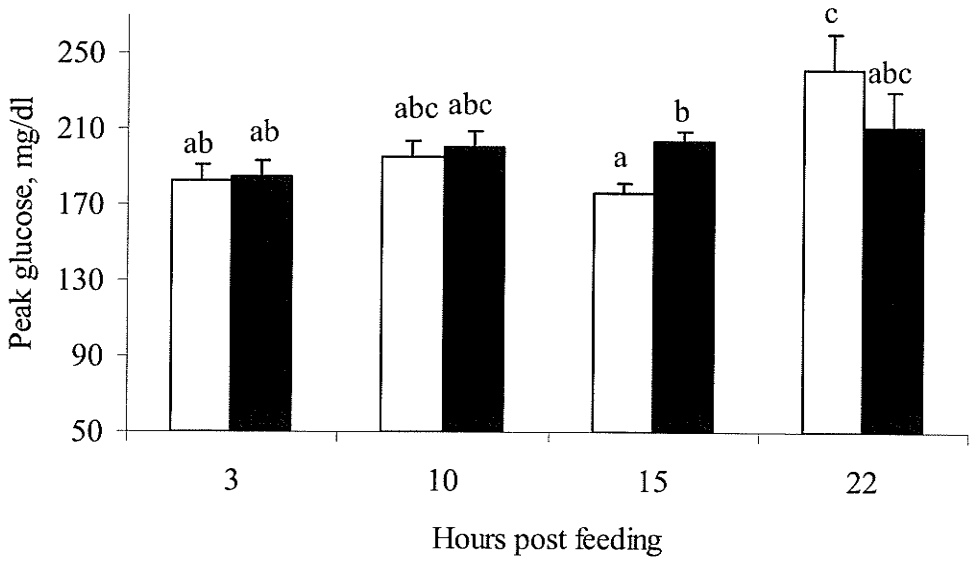


Peak plasma glucose level during the i.v. GTTs was affected by FT and hours post-feeding ($P < 0.05$, Table 6) with 2100h-fed cows having 15% higher glucose level during the i.v. GTT at 15h post-feeding than the 0900h-fed cows (Figure 13a) Peak plasma glucose was 37% and 33% higher at 22h post-feeding than at 15h and 3h, respectively, post-feeding in the 0900h-fed cows but peak plasma glucose did not vary with hours post-feeding in the 2100h-cows. Hours post-feeding also affected peak plasma insulin level during the i.v. GTT (Table 6). Peak plasma insulin level was highest and lowest when the i.v. GTT was performed at 10h and 22h post-feeding, respectively (Figure 13b).

The I/G ratio was affected by hours post-feeding ($P < 0.001$) in that the ratio at 10h post- feeding was 3-fold higher than at 22-h post feeding ($P < 0.05$) and 30% higher ($P < 0.05$) than at 3h post-feeding (Figure 14). The interaction of FT and hours post-feeding tended ($P < 0.1$, Table 6) to influence the I/G ratio, where the 2100h-fed cows tended to have higher I/G ratios at 3h and 10h post-feeding (0.65 μIU/mg•100 vs 0.90 μIU/mg•100, respectively) than the 0900h-fed cows (0.44 μIU/mg•100 vs 0.72 μIU/mg•100, respectively).

Figure 13. a) Effect of feeding time (0900h-fed - □, 2100h-fed, ■) and hours post-feeding on peak plasma glucose level and b) effect of hours post-feeding on peak plasma insulin level during i.v. GTTs performed on dairy cows in Experiment 2. Means within a figure with different letters differ by $P < 0.05$.

a)



b)

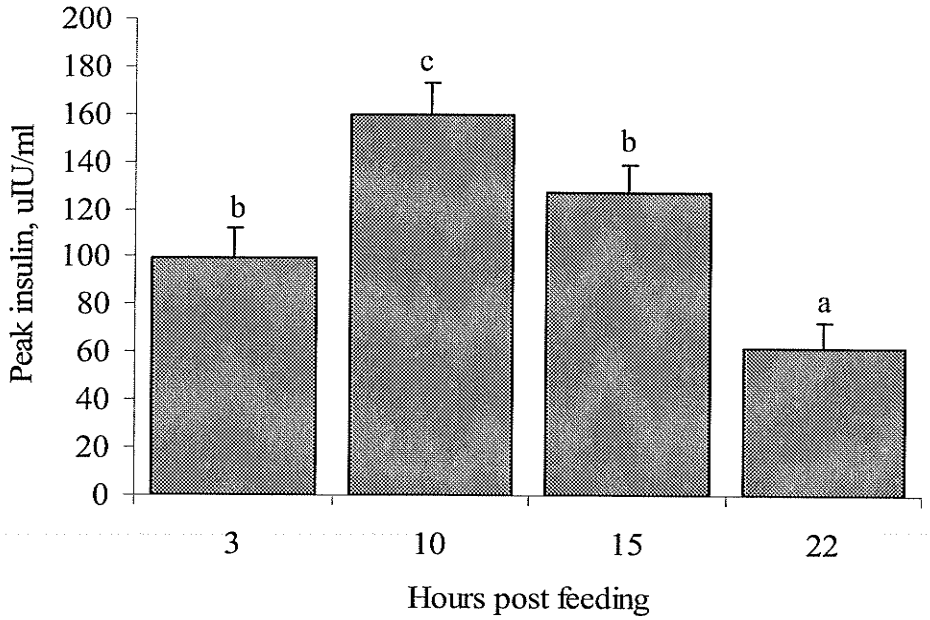
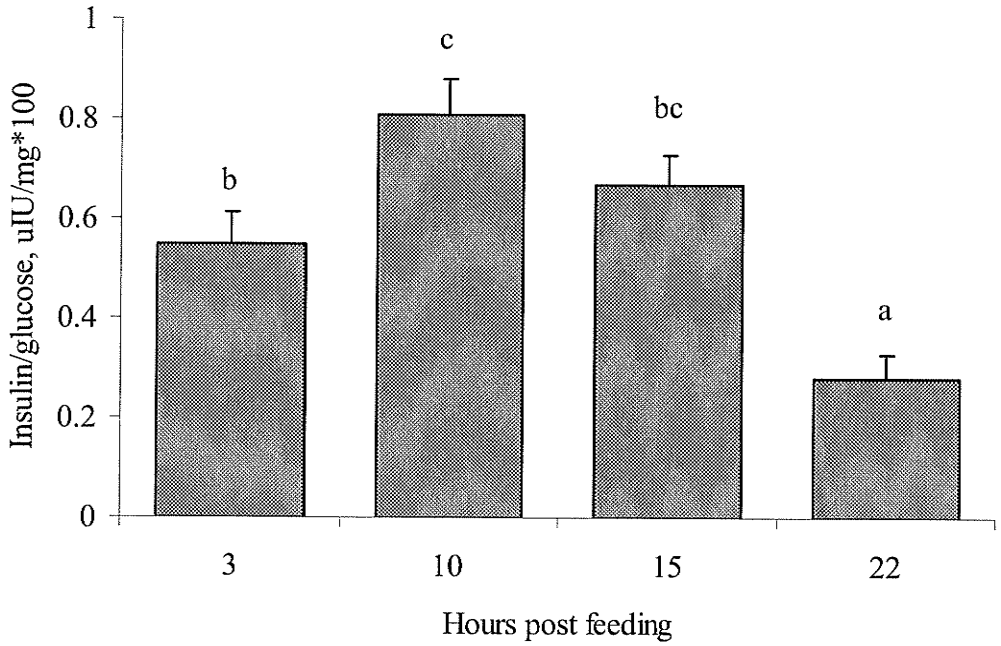


Figure 14. Effect of hours post-feeding on the insulin/glucose ratio of i.v. GTTs performed on dairy cows in Experiment 2. Means with different letters differ by $P < 0.05$.



3.5 DISCUSSION

3.5.1 Glucose and Insulin

Plasma glucose level had a similar pattern 24h post-feeding in both Experiments 1 and 2 (Figures 1 and 10) in that the level dropped significantly at 2h post-feeding in the 2100h-fed cows but not the 0900h-fed cows. The results for the 2100h-fed cows agree with findings by several other researchers where plasma glucose level fell shortly (2 to 3h) after feed delivery in ruminants fed twice daily (Sutton et al., 1988; Blum et al., 1985; Andersson, 1982; de Jong, 1981; Bassett, 1974b; Ross and Kitts, 1973). One study with a group of sheep fed once daily at 0800h also demonstrated a post-feeding drop in glucose level (Bassett, 1974b). The drop in glucose in 2100h-fed cows was likely related to insulin (see below).

After the early drop in plasma glucose, plasma glucose rose significantly above pre-feeding level in the 2100h-fed cows but not in the 0900h-fed (only in Experiment 1) which agrees with other studies that have found that the plasma glucose rise after a meal consumed in the evening is larger than a meal consumed in the morning in ruminants (Andersson, 1982; Blum, 1985) and humans (Van Cauter et al., 1992). The lack of a similar finding in Experiment 2 may have been a result of the difference in TMR; the diets in Experiment 1 contained corn and barley silage whereas the diet of Experiment 2 contained alfalfa and barley silage. Studies have shown that diets that include corn will allow more glucose to pass through the rumen and be absorbed in the small intestine (Bassett, 1974b) and this may have contributed to a) the higher daily plasma glucose level and b) the more dramatic increase in post-feeding glucose level seen in Experiment 1.

Post-feeding plasma insulin level began to rise at 2h post-feeding in the 2100h-fed cows in both Experiments compared to 4h post-feeding in Experiment 1 and no significant rise in Experiment 2 in the 0900h-fed cows (Figures 1 and 10). The higher plasma insulin level and lower plasma glucose level at 2h post-feeding in the 2100h-fed cows corresponded with higher feed intake in 2100h-fed cows during the first 3-h post-feeding in both Experiments (Nikkhah et al., 2005; Nikkhah et al., 2006). Studies support both cephalic phase (Basset, 1974a; Herath et al., 1999) and incretin-mediated (McCarthy et al., 1992) insulin release in ruminants. The higher feed intake in the first 3h post-feeding in the 2100h-fed cows may have caused a heightened cephalic insulin response and/or an accelerated incretin-mediated insulin release that resulted in a depression of glucose level. The control cows in this study differed from the literature possibly because the diet fed in this study was not as readily digestible as those from previous work. This is difficult to conclude, however, because the nutrient composition of diets in earlier studies was not reported.

The plasma insulin peak at 12h post-feeding in 0900h-fed cows coincided with a second significant eating period from 12h to 15h post-feeding in the 0900h-fed cows (Nikkhah et al., 2005). This second eating period also coincided with feed delivery for the 2100h-fed cows, which suggests that fresh TMR delivery to the 2100h-fed cows may have stimulated eating in the 0900h-fed cows.

The HC diet in Experiment 1 increased the average daily plasma glucose and insulin level in the dairy cows of both parities in agreement with earlier findings for diets higher in concentrates (de Jong, 1981). The greater increase in plasma glucose in primiparous cows with HC feeding suggests that primiparous cows are more insulin

insensitive, in agreement with previous work by Subiyatno et al (1996). Similar conclusions can be drawn with the Diet X FT interaction where 0900h-fed cows had a greater increase in plasma glucose with HC feeding than the 2100h-fed cows, indicating that 0900-h fed cows were more insulin insensitive than the 2100h-fed cows. However, these conclusions do not agree with the i.v. GTT data in Experiment 2 (see below).

3.5.2 Cholesterol and Liver Enzymes

There was no significant variation in post-feeding plasma cholesterol level in 0900h-fed cows but a drop in plasma cholesterol was observed in the 2100h-fed cows from 2h to 6h post-feeding (Figure 2). This drop was also observed in both primiparous and multiparous cows at 4h post-feeding (Figure 3). There have been no previous reports, to the best of the author's knowledge, of a 2h post-feeding drop in plasma cholesterol level in dairy cows. Bitman et al. (1990) observed that plasma cholesterol exhibited an ultradian rhythm in Holsteins with a period of 2.6h. High frequency rhythms can be obscured or create non-existing diurnal rhythms by infrequent sampling (Bitman et al., 1990). Bitman et al. (1990) suggest that sampling frequency should be three to five times the frequency of the rhythm; therefore, in the case of cholesterol samples should be taken every 30 to 50min. Thus, the apparent rhythm in cholesterol level of 2100h-fed cows may be an artefact of infrequent sampling and not a true rhythm. Average plasma cholesterol was significantly higher ($P < 0.05$) in primiparous cows than multiparous cows (Table 5) which has also never been reported previously, to the best of the author's knowledge.

The liver enzymes measured in Experiment 1 (GGT, GLDH, AST and ALP) are often used as markers for liver or muscle damage (Stojevic et al., 2005) and all measurements were in the normal range (R. Hodges, D.V.M., personal communication). Very little research has been done on the daily variation of these enzymes in healthy cattle. A recent study of multiparous Holstein cows reported mean plasma AST level of 45.8 ± 7.4 IU/L and GGT level of 15.2 ± 3.9 IU/L at peak lactation (Stojevic et al., 2005) which is 50% lower than the levels found in multiparous cows of Experiment 1 (93.4 ± 7.6 IU/L and 32.0 ± 2.6 IU/L respectively). It is unlikely that diet played a role in the difference between our study and that of Stojevic et al. (2005) because diet had no effect on enzyme levels in Experiment 1; however, this can not be concluded because Stojevic et al. (2005) did not report the diet composition. Stojevic et al. (2005) also found that AST and GGT levels were highest (57.8 ± 16.5 IU/L and 19.0 ± 4.2 IU/L, respectively) in early lactation (< 45DIM) and then decreased as lactation progressed to their lowest levels during the dry period. Thus, the difference in enzyme levels between Experiment 1 and those reported in Stojevic et al. (2005) may be due to differences in energy requirements. However, this can not be compared as the average milk yield of the cows was not reported by Stojevic et al. (2005). Energy requirement differences might also explain the difference in enzyme levels seen between the primiparous and multiparous cows of Experiment 1 (Table 5) with milk yields of 34 kg/d and 40 kg/d respectively (Nikkhah et al., 2005).

The 24h pattern in enzyme levels in Experiment 1 also suggests that dietary or energy requirement differences are most pronounced in the first 12h post-feeding (Figure

4). Thus, future studies investigating GGT, GLDH, AST and ALP could likely be restricted to only 12h of sampling following feed delivery.

3.5.3 Melatonin

Melatonin level in tissues of the GIT and in plasma increases in response to feeding in pigs (Bubenik et al., 1996; Bubenik et al., 2000). Melatonin concentrations have been found in the gastrointestinal tract (GIT) epithelium from the stomach to the colon of several animals including pigs and cattle (including the rumen) that are significantly (400 times) higher than pineal gland concentrations (Bubenik et al., 1999, Bubenik et al., 2000). The absorptive surface of the rumen in ruminants will increase through the development of longer papilla with diets that have a higher forage:concentrate (F:C) ratio (Hofmann, 1988). It is possible that the high F:C ratio of the LC diet caused proliferation of the rumen epithelium which resulted in higher plasma melatonin level in cows on the LC diet. No studies to date have investigated the effects of diet on melatonin level in cattle.

Melatonin level began to rise 1.5h prior to lights off which suggests that cows "anticipated" darkness (Figure 7). Critser et al. (1988) found the same anticipation of darkness in two groups of sheep exposed to two different photoperiods; melatonin level began to rise 1.5 to 2h prior to lights off. The variability in melatonin level observed among cows of Experiment 1 has been previously observed (Lawson, 2000) and is likely due to a genetic difference in pineal gland weight as has been found in sheep (Coon et al., 1999). Melatonin level for individual cows between sampling days tended to vary ($P < 0.1$) and this might be due to postural differences between corresponding samples on each

day. Deacon and Arendt (1994) found that plasma melatonin level dropped by 35% when humans changed position from standing to supine and was reversed when returned to standing 30min later. This is purpotively because blood volume drops when a human stands up increasing the concentration of blood constituents, thus changing from standing to supine increases plasma volume and dilutes melatonin level (Deacon and Arendt, 1994). Therefore, the position of an individual cow may have differed between corresponding sampling times between sampling days and this may have caused the tendency for variation within a cow. In the future, it may be beneficial to note the position of the cows during and around sampling to account for positions effect on melatonin.

The trend for an earlier peak in plasma melatonin level in primiparous cows than multiparous cows has not been previously observed but may also be related to the extra-pineal sources of melatonin.

3.5.4 i.v. GTT

The i.v. GTT in Experiment 1 corresponded to 3h post-feeding for the 0900h-fed cows and 15h post-feeding for the 2100h-fed cows where plasma glucose and insulin levels increased approximately 2-fold and 8-fold (respectively) in both 0900h-fed and 2100h-fed cows (Figure 8). During the i.v. GTT performed at equivalent times in Experiment 2, plasma glucose increased 3-fold for both the 0900h-fed and 2100-fed cows but plasma insulin increased and 4-fold and 12-fold, respectively (Figure 12a for 0900h-fed and Figure 12c for 2100h-fed). Greater elevations in plasma glucose and insulin in Experiment 2 may have been related to the injection of twice the glucose dose in

Experiment 2. A study investigating the insulin responses to various glucose loads (0, 75, 150 and 300 mg/Kg live weight) in Friesian heifers of high or low genetic merit for milk fat, found a significant glucose dose x genetic merit interaction where a larger difference in insulin responses was observed with only the higher doses of glucose (Xing et al., 1993). Heifers of high genetic merit had plasma insulin responses that were three times those of low genetic merit heifers (Xing et al., 1993). Thus the dose of 150 mg/Kg of body weight used in Experiment 1 may have been too low to elicit treatment effects.

The insulin AUC of the i.v. GTT performed at 10h post-feeding was greater than at any other hour post-feeding but this did not correspond with a low glucose AUC. The largest glucose AUC was observed during the i.v. GTT performed at 22h post-feeding which corresponded with the smallest insulin AUC. Taken together these data suggest that dairy cows are most insulin insensitive (glucose intolerant) at 10h post-feeding and have a poor insulin response to glucose at 22h post-feeding. Differences in the insulin response to a glucose load was also found to be dependent on the number of hours from feed delivery in sheep (Sasaki et al., 1984) and veal calves (Hugi et al., 1997). Sheep were offered orchard-grass hay for a period of 4h at 1200h and i.v. GTTs (288mg/Kg body weight) were performed at 30min, 2.5h, 11h, 16h and 22h post-feeding (Sasaki et al., 1984). The largest insulin AUC were observed during the GTTs performed during feed availability (30min and 2.5h post-feeding) (Sasaki et al., 1984). A larger insulin response to an i.v. glucose load (330mg/Kg^{75}•min for 5min) was also observed at 3h post-feeding compared to 15h post-feeding in veal calves fed milk replacer at 0730h (Hugi et al., 1997). This is the first study to demonstrate the effect of hours post-feeding on glucose tolerance in dairy cows.

The highest I/G ratio was observed at 10h post-feeding (0.8 uIU•mg^{-1}•100mL) and the smallest at 22h post-feeding (0.2 uIU•mg^{-1}•100mL). This agrees with findings in veal calves that show 14x higher ($P < 0.05$) I/G ratio at 3h post-feeding than at 15h post-feeding (Hugi et al., 1997). A high I/G ratio indicates reduced peripheral tissue sensitivity to insulin in dairy cows (Hayirli et al., 2001). The I/G ratio can be compared to the glucose clearance rate during an i.v. GTT, where reduced glucose clearance would indicate insulin resistance (Holtenius et al., 2003). Holtenius et al. (2003) observed a difference in glucose clearance rates during i.v. GTT conducted 3wk postpartum in Swedish Red and White cows fed low, medium or high energy diets (75%, 110% or 178%, respectively, of energy required for maintenance and pregnancy) during the dry period. The glucose clearance rate in cows receiving the high energy diet was 20% lower ($P < 0.005$) than cows receiving the low energy diet; therefore, cows receiving the high energy diet were more insulin resistant than those receiving the low energy diet (Holtenius et al., 2003).

In Experiment 1, the cows receiving the HC diet had a higher ($P < 0.05$) I/G ratio than those receiving the LC diet; therefore, the cows receiving the HC diet were more insulin resistant, which agrees with the results of Holtenius et al. (2003). The rise in plasma glucose with HC feeding was greater in 0900h-fed cows and primiparous cows, suggesting that 0900h-fed and primiparous cows are more insulin resistant. However, the i.v. GTT data in Experiment 2, where 2100h-fed cows have a tendency for higher I/G ratios than 0900h-fed cows at 3h and 10h post-feeding, suggests that 2100h-fed cows were more insulin resistant. Lower glucose clearance rates were observed in sheep when a GTT was performed at 11h and 22h post-feeding compared to 2.5h post-feeding;

therefore, sheep are more insulin resistant at 11h and 22h post-feeding than at 2.5h post-feeding (Sasaki et al., 1984). It seems that the presence of more glucose and glucose precursors in the plasma (i.e. with higher energy diets, or 10h to 15h post-feeding) may be the underlying cause of insulin resistance in ruminants. More pronounced insulin resistance at 3h and 10h post-feeding may have been the cause for the improved milk fat yield in the 2100h-fed cows compared to the 0900h-fed cows in Experiments 1 ($P < 0.1$) and 2 ($P < 0.05$) (Nikkhah et al., 2005 and 2006). Milk fat production might be optimal at 10h post-feeding when glucose sparing is maximized by insulin resistance. Evening feeding may be a management technique available to Canadian farmers to control glucose sparing and milk fat production.

Hugi et al. (1997) concluded that insulin resistance in veal calves is a postprandial condition and it is important to note that the differences in insulin resistance observed in the current experiments were linked to hours post-feeding and not time of day. On the other hand, Sasaki et al. (1984) observed a reduction in the magnitude of insulin AUCs when feeding time was at 0800h or 1600h compared to 1200h. Although not statistically significant ($P < 0.18$), the 2100h-fed cows in the current experiments had numerically higher insulin responses when early post-feeding coincided with night (2400h, Figure 12a) and early morning (0700h, Figure 12b), suggesting that time of day may affect post-feeding insulin responses to a glucose load in ruminants.

That multiparous cows tended to have greater peak insulin and a greater I/G ratio than primiparous cows when fed at either 0900h or 2100h during the i.v. GTT in Experiment 1 indicated greater insulin resistance in multiparous cows; however, similar patterns were not observed in Experiment 2. Subiyatno et al. (1996) observed the

opposite pattern between primiparous and multiparous cows during an i.v. GTT performed at 1000h in cows fed twice daily (times not specified). The primiparous cows tended to have a higher I/G ratio than multiparous cows in response to a dose of 300mg/kg body weight of glucose indicating reduced insulin sensitivity in primiparous cows (Subiyatno et al., 1996). The larger increase in plasma glucose with HC feeding in primiparous cows compared to multiparous cows in Experiment 1 agrees with Subiyatno et al. (1996) where primiparous cows seem to have greater insulin resistance. Obviously more research is required on the effect of parity on glucose tolerance in ruminants in order to draw conclusions.

The hyperglycemia induced by the i.v. GTTs in Experiment 2 (255 to 300 mg/dl) is greater than the physiological renal threshold (100 to 150 mg/dL, Blum et al., 1999) in cattle and so much of the glucose may have been lost in the urine as suggested by Hugi et al. (1997). As a result, glucose clearance from the blood may have only been in part dependent on peripheral tissue uptake of circulating glucose (Hugi et al., 1997). However, Grunberg et al. (2006) demonstrated that a dose of 400mg/Kg body weight of glucose administered i.v. in lactating dairy cows resulted in <5% of the administered dose to be excreted in the urine in 12h. Grunberg et al. (2006) indicated that glucose loss in the urine is not a major means of glucose disposal during GTT in lactating dairy cows.

3.6 CONCLUSION

When feed delivery time was changed from 0900h to 2100h the post-feeding rhythms of plasma glucose, insulin, cholesterol, GLDH, AST, ALP levels were significantly altered and the rhythm of plasma GGT level tended to be different. This

suggests that the diurnal variation observed in these hormones, enzymes and metabolites were influenced by the FEO rather than the SCN. The melatonin rhythm was not affected by FT and this was expected since melatonin has an undisputed SCN entrained rhythm. However, special care should be taken in interpreting diurnal variations when samples are taken every 2h since rhythms with high frequencies can mask or create diurnal rhythms.

The energy balance of the animal, the amount of energy in the diet and the number of hours post-feeding seem to be determinants of insulin resistance in dairy cows. The 2100h-fed cows consumed feed at a higher rate in the first 3h post-feeding and this resulted in an exaggeration of the postprandial insulin resistant state that appears to occur in ruminants. Milk fat production might be optimal at 10h post-feeding because glucose sparing is taking place for milk production when glucose is most abundant, and this effect is enhanced with evening feeding. Therefore, evening feeding may provide a natural management way to control glucose sparing that is available to Canadian farmers.

4.0 STUDY 2: Effect of Feed Delivery Time on Dairy Cow Production and Post-Feeding Plasma Glucose and Insulin Levels

4.1 ABSTRACT

A 6wk feeding trial with two groups of 14 lactating Holstein cows was conducted using a randomized complete block design. Cows received fresh TMR at 0900h or 2100h for *ad libitum* feeding allowing between 5 and 10% orts. Subcutaneous fat (back & hip) measurements were made using ultrasound at the beginning and end of the trial. Overall, a 39% decrease in milk fat yield was observed throughout the trial and there was no effect of treatment on milk fat. The dryness of the TMR allowed the cows to sort the feed which likely caused the milk fat depression. The large milk fat depression may have made it metabolically impossible to observe any beneficial effects of evening feeding. Dry matter intake, milk yield, milk fat percentage, milk protein percentage, body weight, and BCS were not affected by time of feed delivery. By the end of the trial, subcutaneous fat level of 2100h-fed cows was two times that of 0900h-fed cows but the treatment effect failed to reach significance ($P = 0.17$). A significant effect might be found with a larger number of cows and a longer treatment duration since the variation in subcutaneous fat was large.

(**Key words**: glucose, insulin, evening feeding, subcutaneous fat, milk fat)

Abbreviations: FT = feeding time; **BCS** = body condition score; **DMI** = dry matter intake; **MFD** = milk fat depression.

4.2 INTRODUCTION

Carbohydrate metabolism in dairy cows involves the conversion in the rumen of dietary carbohydrates to volatile fatty acids (VFAs), acetate, propionate and butyrate, which are then absorbed and used by the animal for a source of energy (Brockman and Laardveld, 1986). Glucose absorption from the gastrointestinal tract is minimal and so dairy cows rely on gluconeogenesis for the majority of their glucose needs, which for high producing cows (40Kg/d), may exceed 3000g a day during lactation (Hart, 1983). As such, the highest rates of gluconeogenesis in dairy cows are in the hours following feeding when glucose precursors are abundant (Bergman, 1973).

Insulin secretion increases post-feeding in ruminants as a result of rising glucose level as well as the entero-insular axis which is composed of sympathetic inputs to the pancreas (cephalic phase) and hormonal signals (incretins) from the gastro-intestinal tract in the form of GIP and GLP-1 (Godden and Weekes, 1981; McCarthy et al., 1992).

In general, 50% of milk fat is derived from *de novo* synthesis and the other 50% from circulating fatty acids that originate from dietary sources or the mobilization of body reserves (McGuire et al., 1995). During early lactation, dairy cows rely more on body reserves for sources of circulating fatty acids since milk production demands exceed feed intake (Bauman and Griinari, 2003). Milk fat depression (MFD) occurs when cows are fed diets that have a low forage:concentrate ratio and the extent of MFD depends on the reliance on body reserves as a source of fatty acids (Bauman and Griinari, 2003). Recently, it has been shown that MFD is a result of the increased *trans*-$C_{18:1}$ fatty acid production in the rumen through the incomplete biohydrogenation of dietary unsaturated fatty acids by rumen bacteria (Gaynor et al., 1994). *Trans*-$C_{18:1}$ fatty acids inhibit milk

fat synthesis in the mammary gland and therefore decrease demands for circulating fatty acids; as a result, insulin level increases to inhibit the mobilization of body stores (Bauman and Griinari, 2003).

Research has shown that cattle will spend 10% to 30% more time eating in the evening (1800h) than the morning (0800h) when fresh feed was delivered daily or on alternate days at 0800h (Phillips and Rind, 2001). Evening feed delivery has been shown to improve average daily gain (ADG) and feed:gain ratio in beef cattle (Knutsen et al., 1995; Kennedy et al., 2004; Schwartzkopf-Genswein et al., 2004). Evening feeding was thought to benefit beef cattle as it allowed the heat increment of feeding to coincide with the lower nightly temperatures (Kennedy et al., 2004). The greatest improvement of ADG occurred during winter backgrounding periods which included the lowest nightly temperatures (Schwartzkopf-Genswein et al., 2004). Recently, a trend for increased milk fat yield was observed in 8 dairy cows fed at 2100h compared to 0900h (Nikkhah et al., 2005).

This experiment was designed to test the effects of evening feeding on milk production parameters and plasma glucose and insulin levels in dairy cows using a larger treatment group than that of Nikkhah et al. (2005). My hypothesis is that evening feeding will increase productivity in dairy cows as demonstrated by improved body conditioning and higher milk fat content.

4.3 MATERIALS AND METHODS

4.3.1 Animals and Housing

Twenty-eight Holstein cows (10 primiparous and 18 multiparous) were chosen from the Glenlea Dairy herd and had an average milk yield of 43 kg/d and days in milk of 87 ± 46 DIM at the beginning of the experiment. The cows were housed individually in tie-stalls with individual feeders and had free access to water using shared waterers between two cows. Cows were allowed 2h of exercise outdoors at the half-way point of the experiment on a non-sampling day. The darkness period was from 2230h to 0345h and dim red light was used to obtain samples and check on cows during that time. Cows were milked twice daily starting at 0400h and 1600h. Cows were cared for in accordance with the Canadian Council on Animal Care (CCAC, 1993) guidelines.

4.3.2 Experimental Procedure

The experimental design was a randomized complete block with cows assigned to either 0900h-fed or 2100h-fed treatment for 6wk. The treatment groups were balanced for DIM, milk production and parity after one week adaptation (Pre-treatment week) to the diet (feed delivery at 0900h). All cows were fed a total mixed ration (forage:concentrate 46:54; Tables 7 and 8) *ad libitum* (allowing for 5 to 10% orts) at either 0900h (0900h-fed) or 2100h (2100h-fed). After the Pre-Treatment week, on Day 1 of treatment, the cows assigned to the 2100h-fed group were fed half their ration in the morning (0900h) and the remaining half in the evening (2100h). Thereafter, the 2100h-fed cows received their entire ration in the evening (2100h) and no change was made to

Table 7. Nutrient composition of the ingredients included in the experimental diet.

Ingredient	% Dry Matter
Alfalfa hay	46
Energy supplement	45
Protein supplement	9
Forage:concentrate	46:54

Chemical component	Alfalfa hay	Energy supplement	Protein Supplement	TMR
DM %	87	90	90	84.8
CP, % DM	17.5	19.3	32.4	18.2
SP, % DM	6.1	4.0	4.8	5.9
NDF, % DM	36.2	27.2	38.6	35.8
ADF, % DM	27.9	14.4	13.3	25.4
Ca, % DM	1.42	1.07	2.66	1.4
P, % DM	0.29	0.84	1.00	0.4
K, % DM	3.19	0.97	0.99	2.9
Mg, % DM	0.35	0.42	0.30	0.4

Table 8. Ingredient composition of energy supplement and protein supplement (%).

Ingredient	Energy supplement	Protein supplement[1]
Rolled barley	55.8	-
Luprosil salt (Calcium propionate)	0.2	-
Dairy supplement[2]	40.0	-
Vegetable oil	4.0	
Dried distillers grain	-	42.0
Fish meal	-	7.0
Canola meal	-	22.8
Soybean meal	-	20.0
Beet molasses	-	3.0
Niacin	-	0.3
Sodium bicarbonate	-	5.0

[1]Protein pellets contain 46.1% soybean meal, 2.6% wheat shorts, 40.0% canola meal, 5.0% oat hulls, 0.3% pellet binder, 1.0% cane molasses, and 5.0% corn gluten meal.
[2]Dairy supplement contains 0.13% vitamin ADE premix (Vit A, 16800 IU/kg; Vit D, 2215 IU/kg; Vit E, 75 IU/kg, DM basis), 0.13% trace mineral premix, 2.6% soybean meal, 0.06% selenium, 39.1% wheat shorts, 5.0% distillers grain, 17.5% canola meal, 15.0% ground wheat, 1.7% dicalcium phosphate, 1.6% salt, 2.0% dynamite, 0.3% pellet binder, 1.0% cane molasses, 3.7% calcium carbonate, and 10.0% corn gluten meal.

feeding time for the 0900h-fed cows for the 6wk experiment. The TMR was mixed each morning (0800h) using a Data Ranger Mixer (American Calan, Northwood, NH) with a Weigh Tronix head (model 1000, American Calan, Northwood, NH) for all cows and placed either directly into the feeders of the 0900h-fed cows or into plastic tubs for the 2100h-fed cows; on alternate days the order of feeding was reversed. The plastic tubs were placed out of sight until feed delivery at 2100h when they were emptied into the feeders for each cow.

Blood samples were taken from the coccygeal vein while the cows remained in their stalls twice a day (1100h and 2300h) for two consecutive days (Days 3 & 4) every week of the trial (including the pre-treatment week). Two 10mL plasma vacutainers (sodium heparin, Fisher Scientific, Fairlawn, NJ) were filled at each sampling. Sampling of all 28 cows was completed within 30min. Plasma was separated and aliquoted to two 2mL cryovials (VWR International, Edmonton, AB) and frozen (-20°C) for later analysis.

Cows were weighed twice a day (0800h and 2000h) for two consecutive days (Days 3 & 4) during the Pre-Treatment week and weeks 1, 3, 5, and 6. Body condition scores (BCS) were recorded based on a 5-point scale (Edmonson et al., 1989) once weekly (Day 3 or 4) during all weeks. Back and hip subcutaneous fat was measured using ultrasound (Aloka, model SSD500 with 5 MHz probe head, model # UST-588U-5 linear probe, Wallingford, CT) at the beginning and end of the trial. Hip measurements were made at the midline between the front of hook and back of pin; back measurements were made between the 12^{th} and 13^{th} ribs (Small et al., 2004; Schröder and Steufenbiel, 2006). To avoid between operator variation, the same operator made the measurements before and after the trial.

Dry matter intake (DMI) was measured by weighing the orts daily and ort and feed samples were collected once a week (Day 3 or Day 4 if no orts were available on Day 3) for dry matter and particle size analysis using Penn State Particle Separator (PSPS). The PSPS had 3 screens with respective diameters of 19, 8, and 1.18 mm, and a bottom pan (Heinrichs, 1996).

Milk yield was recorded daily using TruTest regulation meters (Westfalia Surge, Mississauga, ON) and 4 samples from consecutive milkings (afternoon milk Day 2, morning and afternoon milk Day 3, and morning milk Day 4) were collected each week of the experiment and sent to Dairy Farmers of Manitoba (Winnipeg, MB) for component analysis by near infrared using Milk-O-Scan 303AB (Foss Electric, Hillerod, Denmark).

4.3.3 Analysis

Blood was analysed for glucose (Stat Profile Critical Care Express, Nova Biomedical, Waltham, MA) and insulin (Coat-a-Count RIA kit, Diagnostic Products Corporation, Los Angeles, CA). Milk samples were analysed for protein and fat content (Dairy Farmers of Manitoba, Winnipeg, MB). Weekly TMR samples and TMR components were dried at 60°C over 48h and sent for analysis (Table 4.3) (Norwest Labs, Lethbridge, AB) (AOAC 934.01, 1990). Particle sizes of ort and TMR (as fed) samples were determined using PSPS. Approximately 200g of sample were placed on the top screen and then the PSPS was shaken 40 times (5 times in each direction, twice) (Heinrichs, 1996). The content of each tray was weighed and the percentage of each fraction was calculated (Figure 15). The ort and TMR samples were then dried at 60°C over 48h to determine dry matter content. Weekly DMI was calculated by subtracting the

products of the percentage dry matter of the TMR and orts by their respective wet weights (as fed) and deducting the orts dry matter from the amount of TMR dry matter fed.

4.3.4 Statistics

All data were tested for normality and then log transformed and analyzed using the PROC MIXED procedure with repeated measures in SAS (SAS, 1999) with the exception of subcutaneous fat measurements. Figures and tables display the non-transformed data but all *P*-values were determined using the transformed data. The pre-treatment week was tested for significant differences for all data and was found to be equal in the 0900h-fed and 2100h-fed cows and therefore was removed from the data set. The model for insulin and glucose included feeding time, parity, hours post-feeding and week of experiment and the repeated measure was week. The model for DMI, milk parameters, BCS and body weight were feeding time, parity and week of experiment and the repeated measure was week. The effect of parity, feeding time and hours post-feeding was considered fixed. The effect of cow within parity was considered random. Tukey's mean comparison test was used to compare mean values when the main or interaction effect was significant (Steel et al., 1997). The difference between before and after experiment measurements of subcutaneous fat was determined to minimize the amount of zeros in the data set. The difference was then analysed by non-parametric test WILCOXON procedure in SAS (SAS, 1999) after averaging the difference for both back and hip locations. The analysis was run separately for parity and feeding time.

4.4 RESULTS & DISCUSSION

4.4.1 Dry Matter Intake

Alfalfa hay was used as a forage source and incorporated into the TMR. The cows were able to sort the ration because it was dry and, as Sutton et al. (1988) have shown, cows that consume concentrate and forage at different times have depressed milk fat. Evidence for sorting was observed during the experiment in that orts had more large particles (top two screens) than the TMR during the pre-trial week and weeks 1, 2, and 3 of the trial (Figure 15).

Feeding time (FT) did not affect DMI (Table 9), which agrees with previous reports of unaltered daily consumption with evening feeding compared to morning feeding in dairy cows (Ominski et al., 2001; Aharoni et al., 2005; Nikkhah et al., 2005).

Multiparous cows had higher DMI ($P < 0.01$) than the primiparous cows (Table 9). The DMI declined ($P < 0.001$) in week 6 and the decline was less pronounced (P x FT x W, $P < 0.05$) in the 0900h-fed multiparous cows compared to the other three groups (Figure 16). The higher DMI by multiparous cows compared to primiparous cows was likely related to higher milk yield (45.6 kg/d vs 38.1 kg/d, $P < 0.01$), milk protein yield (1.4 kg/d vs 1.2 kg/d, $P < 0.05$), milk fat yield (1.0 kg/d vs 0.8 kg/d, $P < 0.05$) and body weight (662 kg vs 601 kg, $P < 0.05$) in multiparous compared to primiparous cows as has also been shown by others (Dado and Allen, 1994, Grant and Albright, 1995).

Figure 15. Percentage of particles remaining in each of four screens of the PSPS from weekly samples of the TMR (as fed) and weighbacks (WB) from 0900h-fed and 2100h-fed cows.

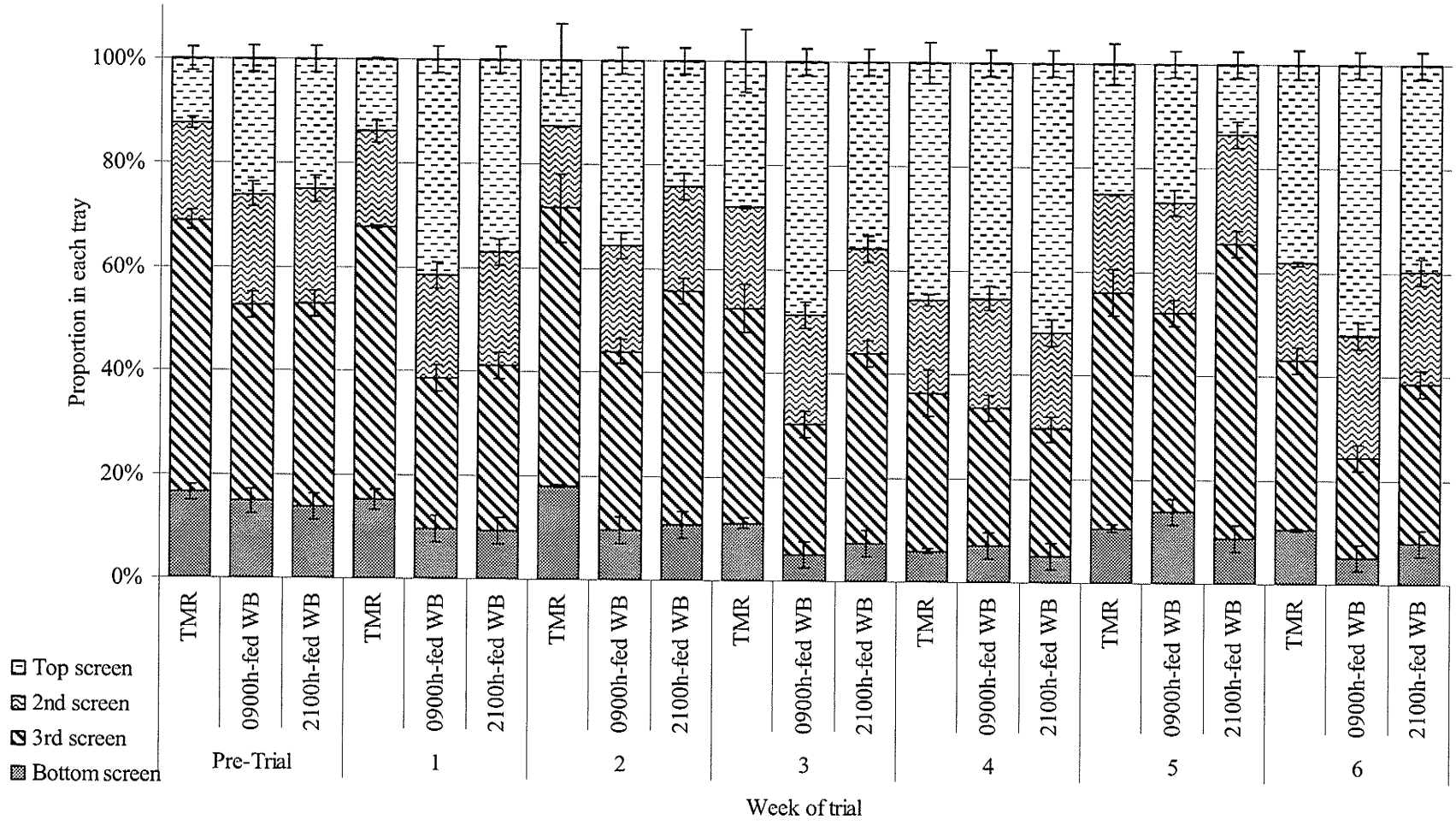
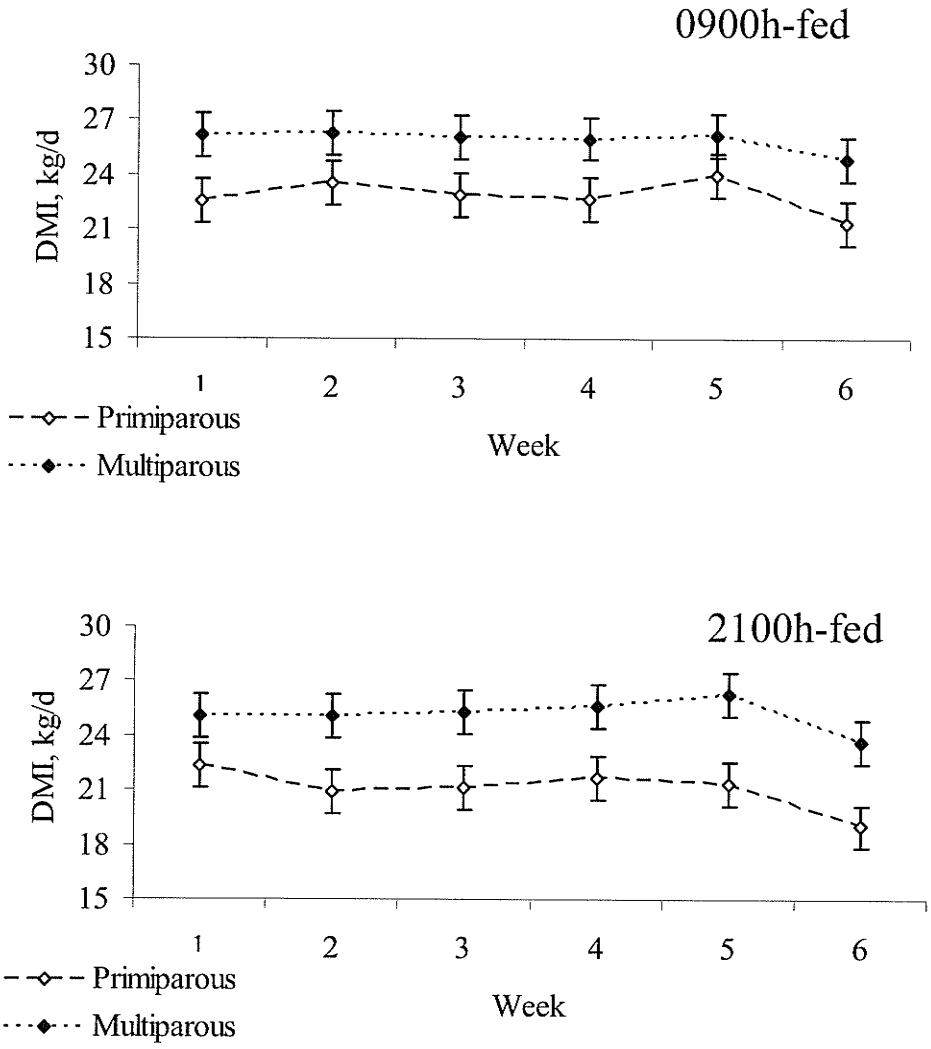


Table 9. Effect of feed delivery time and parity on production parameters of dairy cows.

	Feed Time			Parity			*P* value[1]						
	0900	2100	SEM	Primi-parous	Multi-parous	SEM	FT[2]	W[3]	FT x W	P[4]	P x FT	P x W	P x FT x W
DMI, kg/d	24.4	23.3	0.8	22.1	25.6	0.8	NS	***	NS	**	NS	NS	*
Milk yield, kg/d	43.8	39.8	1.8	38.0	45.6	1.6	NS	***	NS	**	NS	NS	NS
Milk protein, %	3.16	3.22	0.06	3.28	3.10	0.05	NS	***	NS	†	NS	**	NS
Protein yield, kg/d	1.37	1.27	0.05	1.23	1.40	0.05	NS	NS	NS	*	NS	NS	NS
Milk fat, %	2.17	2.00	0.10	1.99	2.18	0.10	NS	***	NS	NS	NS	†	NS
Fat yield, kg/d	0.95	0.81	0.07	0.75	1.00	0.06	†	***	NS	**	NS	**	NS
BW, kg	645	627	17	605	667	16	NS	***	NS	*	NS	NS	NS
BCS	2.8	2.9	0.1	2.9	2.8	0.1	NS	**	NS	NS	NS	*	NS
Change in Subcutaneous fat[2], mm	0.6	1.2	0.2	1.0	0.8	0.3	NS	-	-	NS	-	-	-

[1] NS = $P > 0.10$, † = $P < 0.10$, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$
[2] Feedtime
[3] Week
[4] Parity
[5] end of trial – start of trial subcutaneous fat

Figure 16. Interaction ($P < 0.1$) of dry matter intake (DMI) of dairy cows, feed delivery time (0900h-fed - □, 2100h-fed - ■) and week.



4.4.2 Body Condition Score, Body Weight and Subcutaneous Fat

All cows gained BCS ($P < 0.01$) and body weight ($P < 0.001$) throughout the experiment (Table 9). Primiparous cows tended to gain BCS faster ($P < 0.1$) than multiparous cows each week of the experiment (Figure 17). Gallo et al. (1996) explain that because multiparous cows produce more milk, they are in greater negative energy balance and therefore require more energy intake than primiparous cows for the same increase in BCS. Although multiparous cows had higher DMI (Figure 16; Nikkhah et al., 2004; Nikkhah et al., 2005), this may not have been sufficient to achieve a BCS increase similar to that of primiparous cows.

Body weight and BCS were not affected by FT possibly because the treatment effect was small and these methods are quite inaccurate. Body weight in cattle can vary greatly depending on gut fill, stage of pregnancy, and height of the animal therefore it is not thought to be an accurate measure of condition in cattle (Jaurena et al., 2005). Each BCS unit increase equates to approximately 21Kg of body weight in lactating cows and 35Kg of body weight in dry cows and 10mm of subcutaneous fat (Jaurena et al., 2005). Changes in BCS and body weight also reflect changes in muscle diameter and therefore are not simply measures of body fat stores (Jaurena et al., 2005).

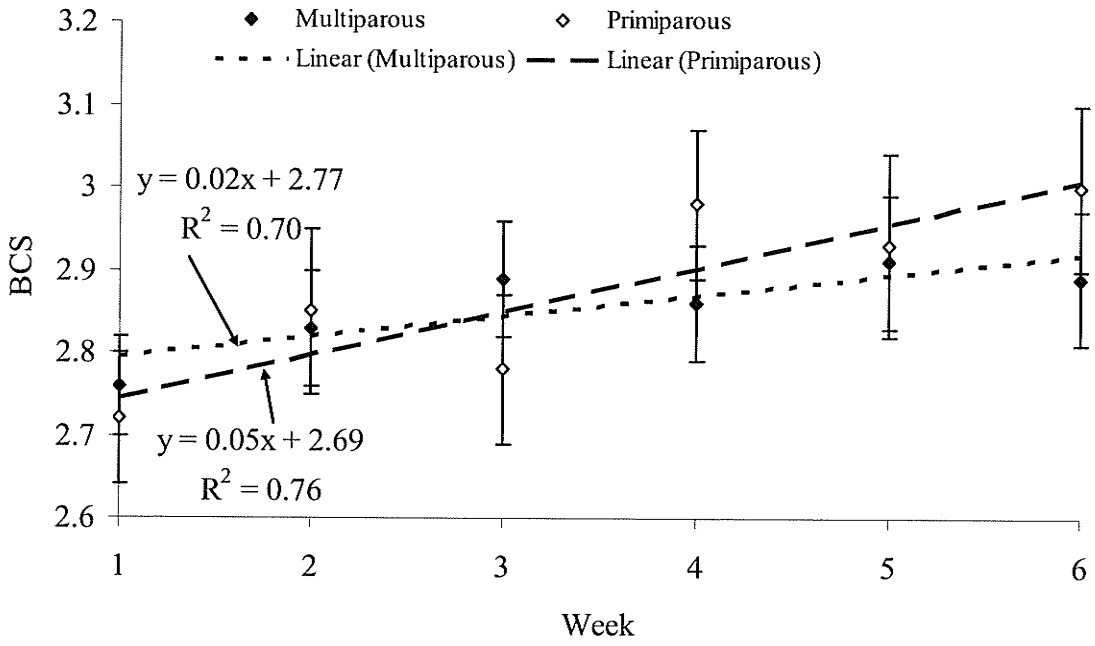
Ultrasonic measurements of subcutaneous fat have been shown to accurately reflect actual subcutaneous fat thickness (Bruckmaier et al., 1998). Since 10mm of subcutaneous fat (one BCS unit) is a large change for a high producing dairy cow that typically has very low body fat reserves (1.0 to 2.0mm) (Jaurena et al., 2005), ultrasonic measurements may be a more sensitive indicator of energy status in Holsteins (Jaurena et al., 2005).

The average subcutaneous fat measurement before and after the experiment were 0.27 ± 0.81 mm and 1.15 ± 1.52 mm, respectively. Although the change in subcutaneous fat of 2100h-fed cows (1.2 mm) was twice that of 0900h-fed cows (0.6 mm), the treatment effect was not significant ($P = 0.17$, Table 9). Parity also did not affect changes in subcutaneous fat thickness (Table 9). A larger sample size may have brought the treatment differences closer to significance in the present trial. Failure to demonstrate a difference between 0900h-fed and 2100h-fed cows may have been due to the inherent errors associated with ultrasonic measurement (Houghton and Turlington, 1992; Jaurena et al., 2005) compared to the very low level of subcutaneous fat found in the cows of this study. The numerically larger increase in subcutaneous in this trial in the 2100h-fed cows may have been related to the tendency for greater milk fat depression in 2100h-fed cows (see below).

4.4.3 Milk Yield, Milk Fat and Milk Protein

Feeding time did not have an effect on milk yield, milk protein content or yield, or milk fat content (Table 9). The 0900h-fed cows tended ($P < 0.1$) to have 17% greater milk fat yield than the 2100h-fed cows (Table 9). This contradicts other reports of increased milk fat yield with evening (2100h) feeding (Nikkhah et al., 2005; Nikkhah et al., 2006) and midnight (0030h) provision of protein supplement (Robinson et al., 1997) and may have been a result of the dry ration (see DMI data above). Zimmerman et al. (1991) reported an increased severity in milk fat depression when cows were switched from a diet containing alfalfa hay as the forage source to a low fibre diet; the cows in this experiment experienced a 39% decrease in milk fat yield by the end of the experiment

Figure 17. Interaction ($P < 0.1$) of weekly body condition score (BCS) and parity in dairy cows.



(Figure 23). Therefore, any benefits from evening feeding may have been masked by the extreme milk fat depression seen in this experiment caused by the use of alfalfa hay and sorting. That the 2100h-fed cows may have had increased subcutaneous fat may suggest that the increased availability of nutrients in 2100h-fed cows could not be used for improved milk fat production. The tendency for an increase in subcutaneous fat by the end of the experiment in both groups (Table 9) is consistent with milk fat depression where lipolysis is inhibited due to reduced demand for fatty acids (Bauman and Griinari, 2003).

Milk yield, fat content and fat yield decreased ($P < 0.001$); whereas, milk protein content and protein yield increased ($P < 0.001$) throughout the experiment (effect of week) (Table 9). The increase in milk protein yield and content may have been related to the increase ($P < 0.001$) in plasma insulin throughout the experiment (Figure 23). McGuire et al. (1995) found a significant increase ($P < 0.05$) in milk protein yield and trend toward higher ($P < 0.09$) milk protein content during a 4d hyperinsulinemic-euglycemic clamp in Holsteins. McGuire et al. (1995) noted that insulin promotes amino acid uptake and use by the mammary gland and increases in circulating insulin gradually increases milk protein secretion. Milk protein secretion does not change with acute changes in insulin level but rather requires extended periods of elevated insulin level as seen in the 4d clamp by McGuire et al. (1995) and the current 6wk study.

Primiparous cows had lower milk yield ($P < 0.01$), protein yield ($P < 0.05$), fat yield ($P < 0.01$) and tended to have higher protein content ($P < 0.1$) than multiparous cows (Table 9). With the exception of the higher protein content, these results are typical of primiparous and multiparous cows in early to peak lactation (Stanton et al., 1992).

Milk protein content increased ($P < 0.001$) from Weeks 3 through 6 in primiparous cows; whereas, protein content only tended to increase ($P < 0.1$) in Weeks 3 and 4 and significantly increased ($P < 0.05$) in Week 6 in multiparous cows. Primiparous cows tended to have higher milk protein content than the multiparous cows during Weeks 5 and 6 of the experiment (Figure 18).

Parity x week tended to be significant ($P < 0.1$) for milk fat content (Table 9) as the primiparous cows began the experiment with numerically higher milk fat content than multiparous cows but had numerically lower milk fat content by Week 2 of treatment (Figure 18). Milk fat yield was significantly reduced ($P < 0.001$) from Week 2 in primiparous cows but only tended to be reduced ($P < 0.1$) in Week 2 in multiparous cows (Figure 19). Milk fat yield was not significantly reduced until Week 5 in multiparous cows at which point yield was significantly higher ($P < 0.05$) in multiparous cows than primiparous cows. These results contradict the expectation of greater milk fat depression with greater demand from the mammary gland. Multiparous cows have greater milk yields than primiparous cows and therefore should rely more heavily on the mobilization of body stores for milk fat production (Gallo et al., 1996); as such, a greater reduction in milk fat yield and content was expected for the multiparous cows.

Figure 18. Interaction of weekly milk protein content ($P < 0.05$) of primiparous (□) and multiparous (■) cows and milk fat content ($P < 0.1$) of primiparous (Δ) and multiparous (▲) cows. Means within a treatment differ from Week 1 by † = $P < 0.10$, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$. Means within a week differ by a = $P < 0.1$ between parities.

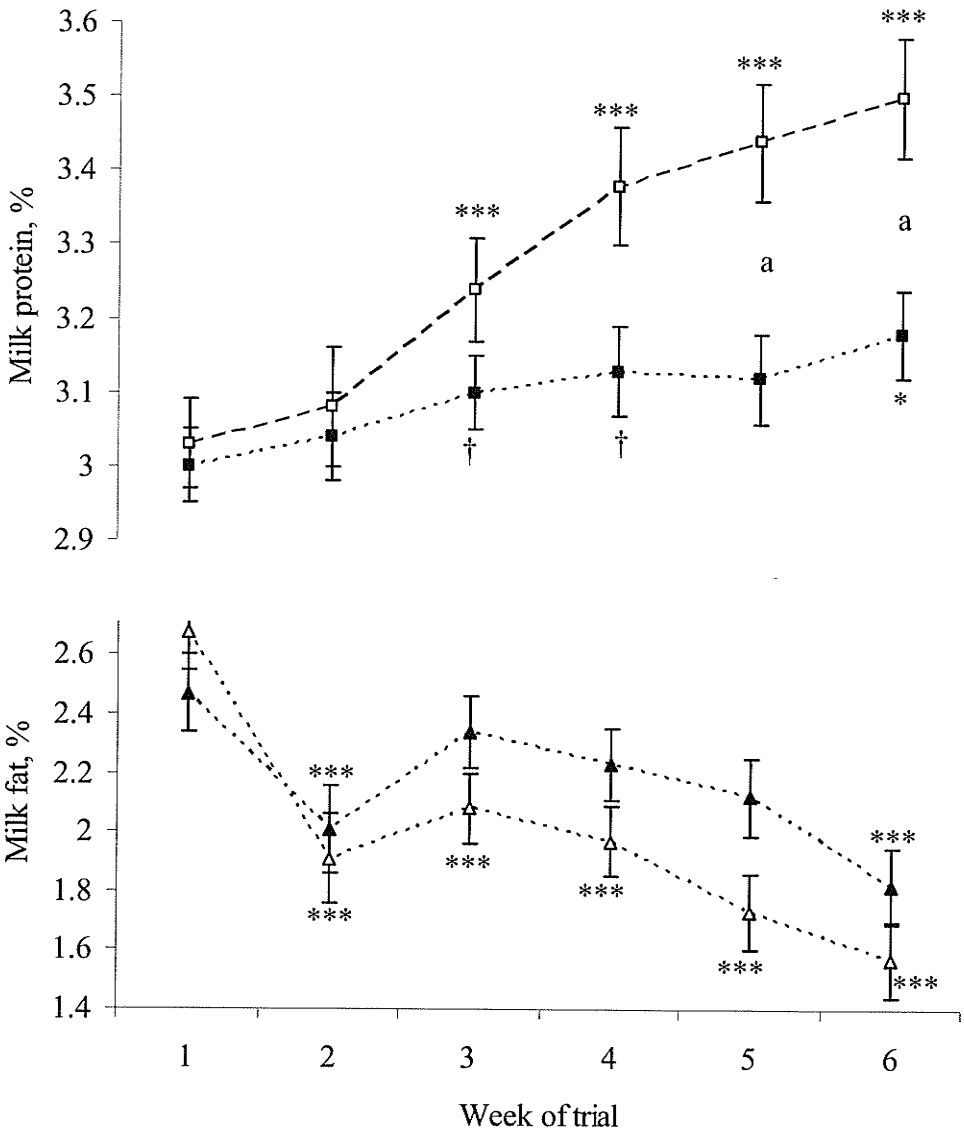
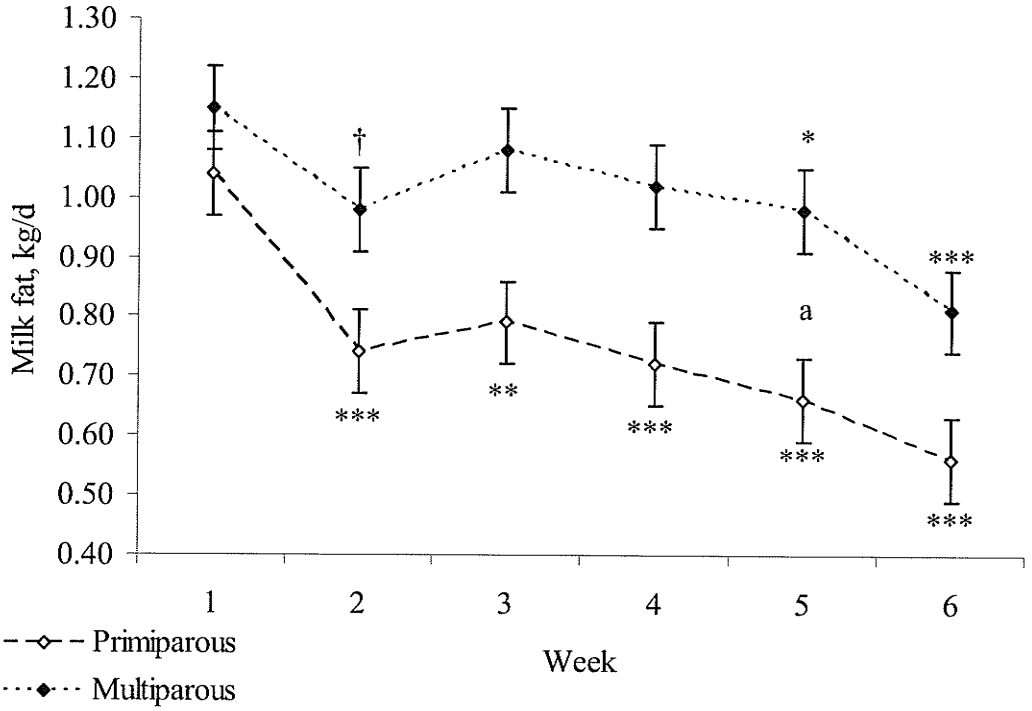


Figure 19. Interaction ($P < 0.01$) of parity and weekly milk fat yield in dairy cows. Means within a treatment differ from Week 1 by † = $P < 0.10$, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$. Means within a week differ by a = $P < 0.05$ between parities.



4.4.4 Glucose and Insulin

Feed delivery time did not have an effect on daily plasma glucose and insulin levels; however, feed delivery time interacted with hours post feeding for both glucose ($P < 0.01$) and insulin ($P < 0.001$) (Table 10). The interaction of FT, hours post-feeding and week was significant for glucose ($P < 0.01$) and neared a trend for insulin ($P = 0.11$) (Table 10). Both treatment groups had higher glucose level at 14h than 2h after feed delivery (Figure 20) likely as a result of increased gluconeogenesis from propionate and glucogenic amino acids provided by the diet (Bergman, 1973). Glucose level at 2h post-feeding has been shown to be lower than at any other time post-feeding in cattle fed once daily (Bassett, 1974b; Study 1).

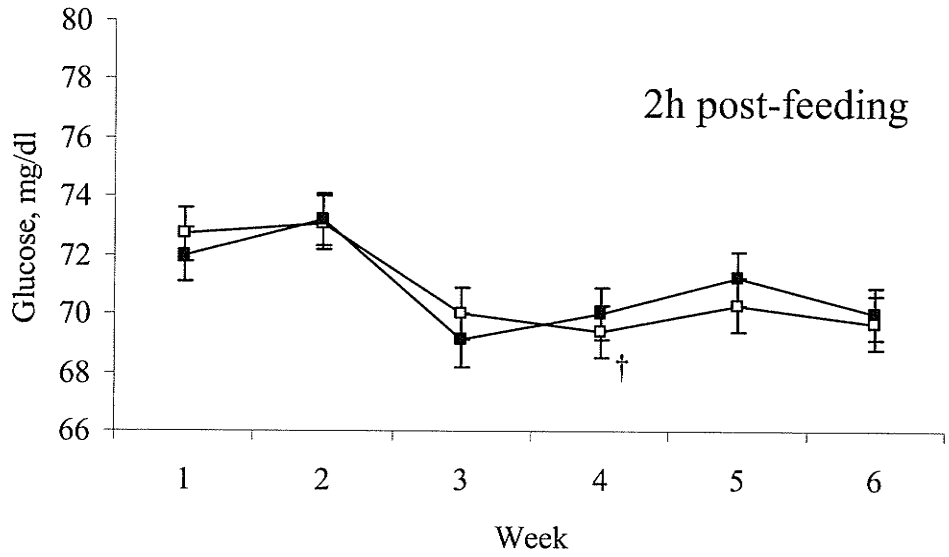
Plasma insulin level was highest at 2300h for both treatments which corresponds to 14h post feeding in 0900h-fed cows and 2h post feeding in 2100h-fed cows (Figure 21). This suggests a diurnal variation in plasma insulin level that is not related to feed intake. In Study 1, it was found that the plasma insulin peak in dairy cows occurred earlier post-feeding when cows were 2100h-fed compared to 0900h-fed and this might be related to differences in feeding behaviour of the two treatment groups as 2100h-fed cows ate 6 to 10% more feed in the first 3h post-feeding (Nikkhah et al., 2005; Nikkhah et al, 2006). In addition, peak plasma insulin level in 0900h-fed cows was observed from 10h to 12h post-feeding whereas peak plasma insulin level in 2100h-fed cows was from 2h to 6h post-feeding when blood samples were taken every 2h in Study 1. This could explain the higher plasma insulin level at 14h post-feeding in the 0900h-fed cows compared to 2h post-feeding in the 2100h-fed as well as the higher plasma insulin level at 2h versus 14h post-feeding in the 2100h-fed cows.

Table 10. Effect of feeding time and parity on plasma insulin and glucose levels in cows receiving fresh TMR at either 0900h (0900h-fed) or 2100h (2100h-fed).

	Feed Time (FT)		Parity (P)			*P* value[1]							
	0900h	2100h	Primi-parous	Multi-parous	SEM	FT	P	FT x P	H[2]	FT x H	P x H	W[3]	FT x W x H
Glucose, mg/dL	72.4	73.3	74.1	71.6	0.6	NS	**	NS	***	**	†	***	**
Insulin, uIU/mL	15.3	16.3	17.2	14.5	1.3	NS	NS	NS	NS	***	**	***	NS

[1] NS = $P > 0.10$, † = $P < 0.10$, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$
[2] Hours post-feeding
[3] Week of experiment

Figure 20. Interaction ($P < 0.01$) of week, hours post-feeding and treatment group (0900h-fed - □, 2100h-fed - ■) on plasma glucose level in dairy cows. Means within a figure within a treatment differ from Week 1 by † = $P < 0.10$, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$. Means within a figure within a week differ between treatments by a = $P < 0.05$.



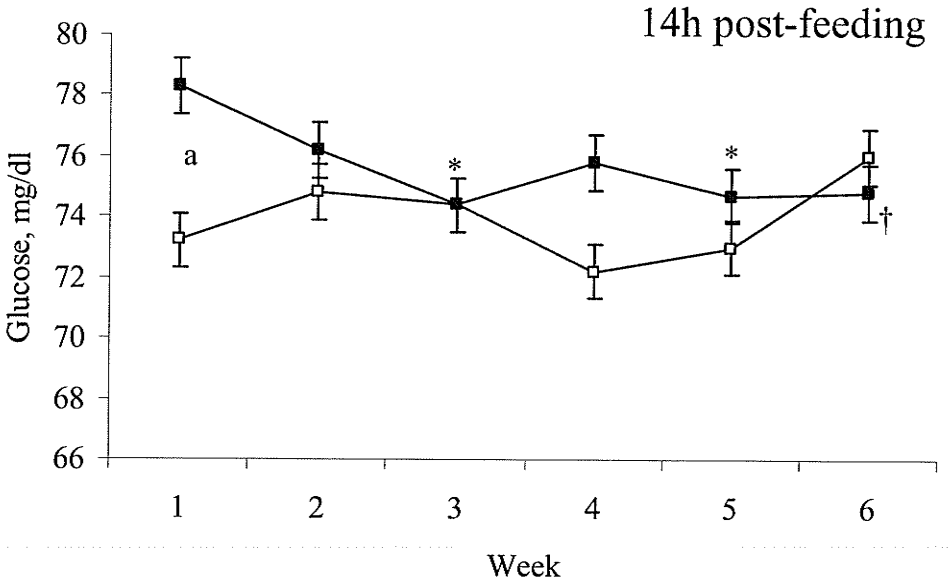
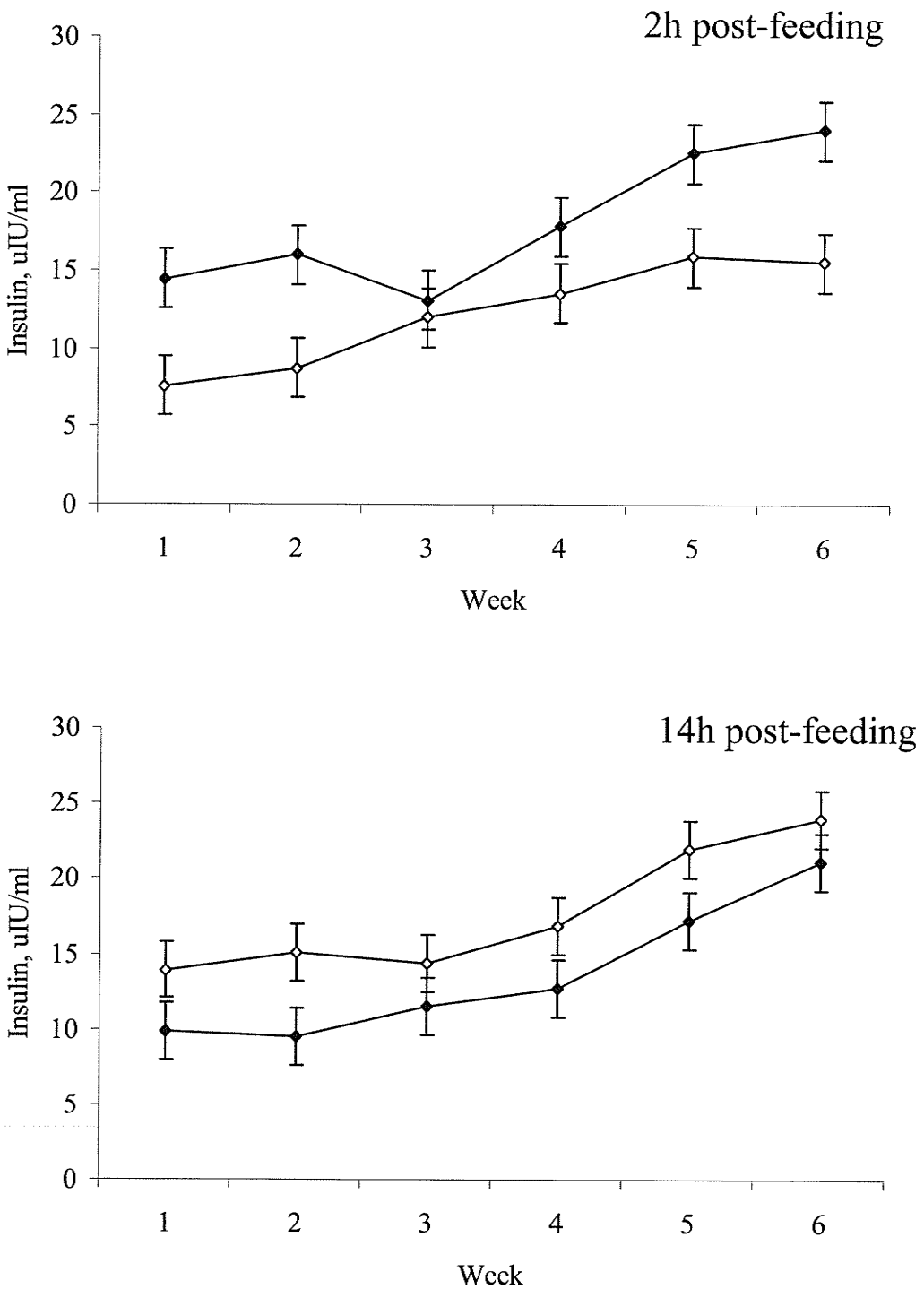


Figure 21. Interaction ($P = 0.11$) of week, hours post-feeding and treatment group (0900h-fed - ◊, 2100h-fed - ♦) on plasma insulin level in dairy cows.

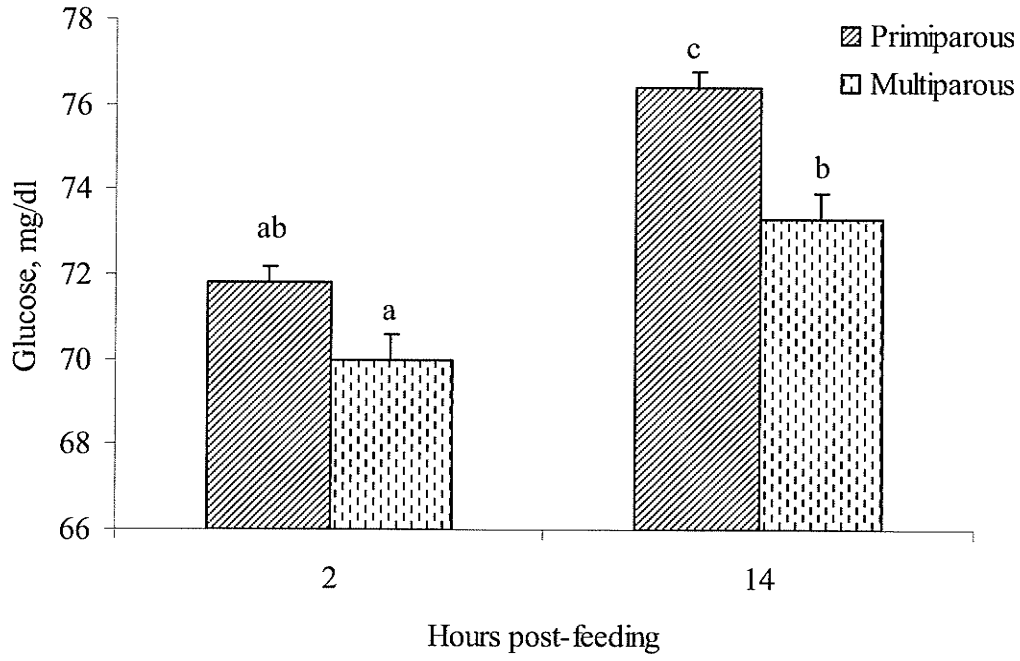


Parity influenced the post-feeding level of plasma glucose ($P < 0.1$) and insulin ($P < 0.01$) (Table 10). Plasma glucose level was higher ($P < 0.05$) at 14h post-feeding in both parities and primiparous cows had higher ($P < 0.05$) plasma glucose level than multiparous cows at 14h post-feeding (Figure 22). This is likely a result of less demand for glucose by the lower producing mammary gland in primiparous cows compared to multiparous cows (Dorshorst and Grummer, 2002).

Plasma insulin level was similar in multiparous cows at 2h and 14h post-feeding but tended to be higher in primiparous cows at 14h compared to 2h post-feeding (Figure 22). The differences in post-feeding plasma insulin level between parities were not observed in previous experiments involving primiparous and multiparous cows (Study 1). The difference between studies could relate to diet. Analysis of blood samples for other metabolites such as NEFA or other hormones such as glucagon may have allowed more detailed interpretation of the current data.

Week had a significant effect on plasma glucose and insulin levels regardless of treatment (Table 10). Plasma glucose decreased ($P < 0.001$) and plasma insulin increased ($P < 0.001$) by the end of the trial and at the same time milk fat yield also decreased by 39% by the end of the experiment (Figure 23). Although the diet was balanced for the appropriate level of production, the dryness of the diet allowed the cows to sort and select for smaller particle sizes and, as mentioned, caused a depression in milk fat.

Figure 22. Plasma glucose and insulin levels at 2h and 14h post-feeding in primiparous and multiparous dairy cows. Means with different letters differ by $P < 0.05$ (glucose) or $P < 0.1$ (insulin).



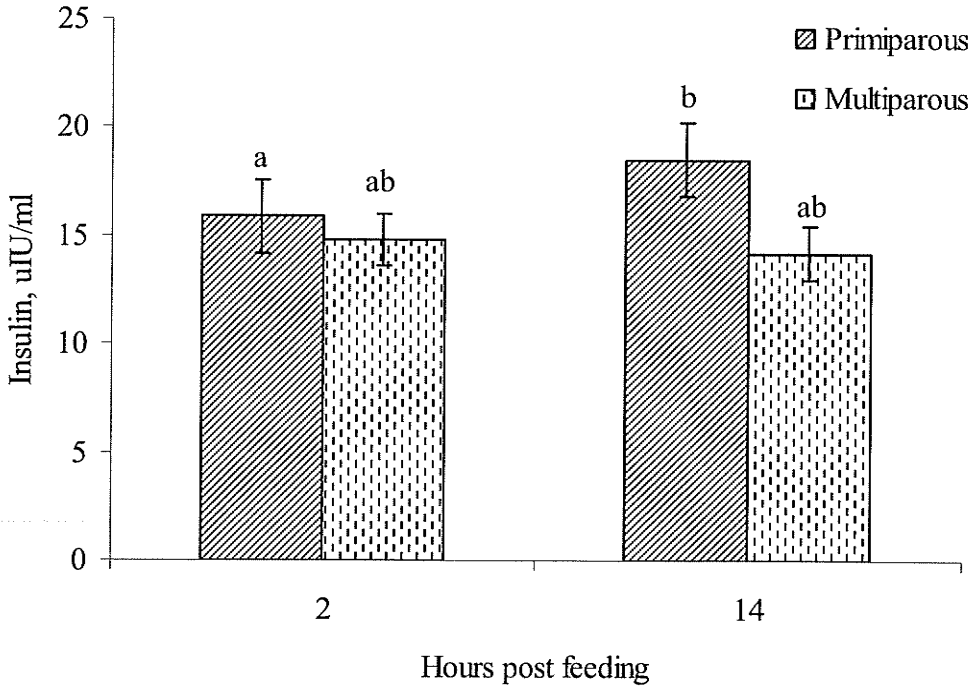
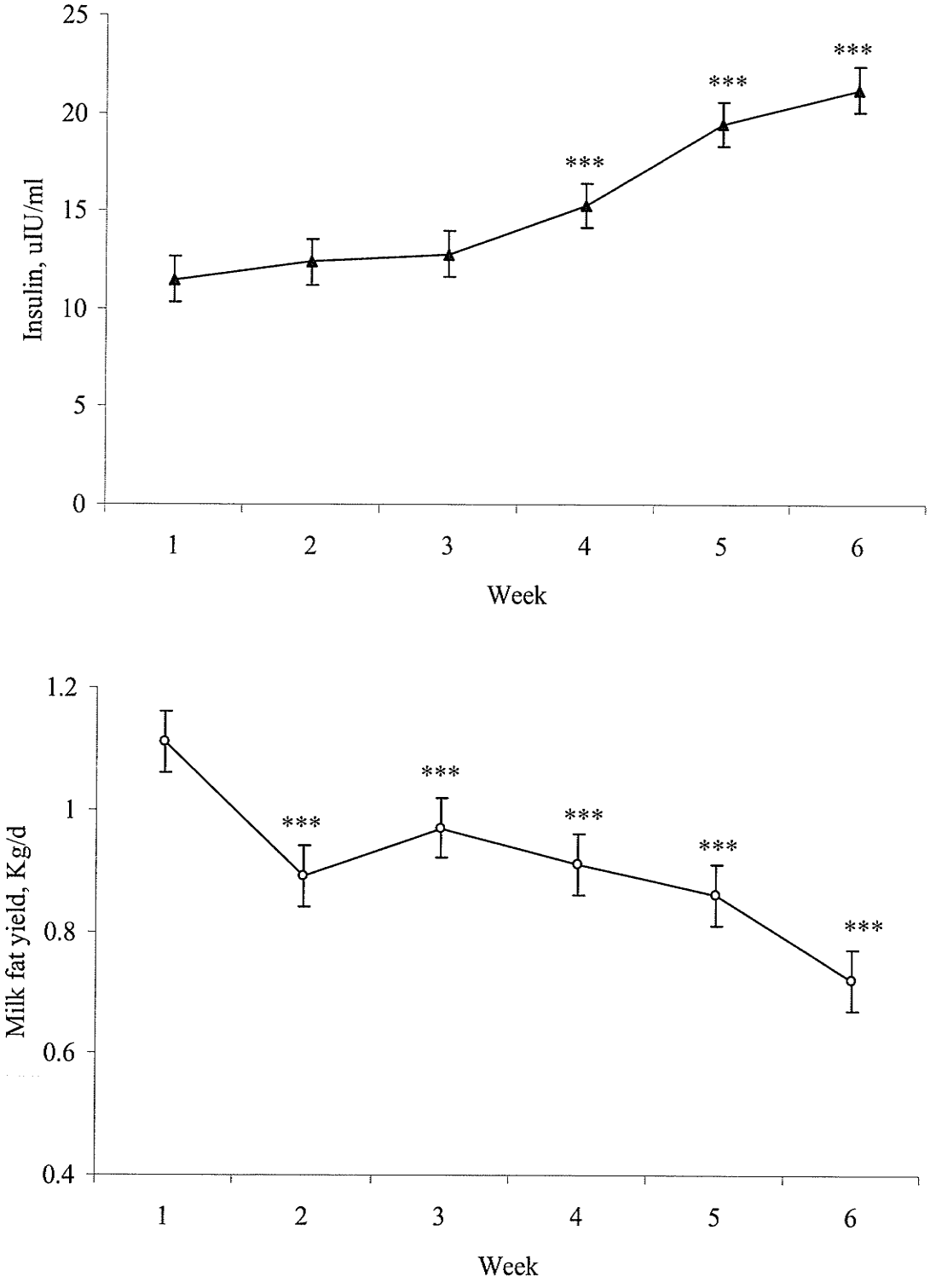


Figure 23. Weekly changes in plasma insulin and milk fat yield in dairy cows throughout the experiment. Symbols denote significant differences from Week 1 within the same parameter by † = $P < 0.10$, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.



4.5 CONCLUSION

Providing fresh TMR at 2100h instead of 0900h did not result in an increase in milk fat yield as seen in previous studies. The severe milk fat depression observed during this study may have masked any beneficial effects of evening feeding. Cows fed at 2100h had a greater insulin response at 2h post-feeding than 0900h-fed cows. Other studies have shown a higher rate of feed intake in 2100h-fed cows in the first few hours after feed delivery that coincides with a larger insulin response at 2h post-feeding compared to 0900h-fed cows which have a larger insulin response at 14h post-feeding. Therefore, the same feeding behaviour was likely a factor in the 2h and 14h plasma glucose and insulin levels seen in the current study.

5.0 GENERAL DISCUSSION and CONCLUSIONS

Evening feeding of beef cattle improved ADG and feed:gain ratio when compared to morning feeding (Schwartzkopf-Genswein, 2004; Kennedy et al., 2004). Night-time (0030h) feeding of a protein supplement to dairy cows improved milk fat yield when compared to morning (0830h) provision (Robinson et al., 1997). Evening feeding of fresh TMR to dairy cows improved milk fat yield in the two experiments of Study 1 as reported by Nikkhah et al. (2005) and Nikkhah et al. (2006). In order for differences between evening fed and morning fed animals to occur, changing the time of feed delivery must also exact changes on the physiology and metabolism of the animal in the manner and efficiency of nutrient absorption and use.

Daily rhythms in plasma glucose and insulin have been observed in cattle fed twice daily (Sutton et al., 1988; Blum et al., 1985; Andersson, 1982), or once daily (Ross and Kitts, 1973). The objective of the first study was to determine the effects of a 12h shift of feeding time on plasma hormone (insulin and melatonin), enzyme (AST, ALP, GGT and GLDH) and metabolite (glucose and cholesterol) levels and glucose tolerance in high-producing dairy cows. Shifting the delivery of fresh TMR to 2100h from 0900h resulted in a shift and increased the variability of the daily rhythms of plasma glucose, insulin, cholesterol, and liver enzymes in Experiment 1 and plasma insulin and glucose tolerance in Experiment 2. More feed was consumed in the first 3h post-feeding by 2100h-fed cows in Study 1 as reported by Nikkhah et al. (2005) and Nikkhah et al. (2006) and the increased nutrient absorption and availability likely resulted in the differences between treatments in the early hours post-feeding.

Hugi et al. (1997) concluded that insulin resistance in veal calves is a postprandial condition and the differences in insulin resistance observed in the current experiments were also significantly associated with hours post-feeding. However, others have observed a reduction of insulin sensitivity during i.v. GTT performed on sheep at feeding time when feed is delivered at 1200h compared to 0800h and 1600h (Sasaki et al., 1984). Although not statistically significant ($P = 0.18$), the 2100h-fed cows in the current experiments had numerically higher insulin responses when early post-feeding coincided with night (2400h) and early morning (0700h), suggesting we may not conclusively rule out a period of reduced insulin sensitivity at night in dairy cows. The energy balance of the animal, the amount of energy in the diet and the number of hours post-feeding seem to be determinants of insulin resistance in dairy cows. The 2100h-fed cows consumed feed at a higher rate in the first 3h post-feeding and this resulted in an exaggeration of the postprandial insulin resistant state that appears to occur in ruminants.

A 39% decrease in milk fat yield was observed in Study 2, due to sorting of the TMR by the cows and this likely masked any beneficial effects of evening feeding. However, post-feeding glucose and insulin levels were similar to those found in the two experiments of Study 1 in that plasma glucose was higher at 14h post-feeding than at 2h post-feeding in both treatment groups but plasma insulin was higher at 2h post-feeding in the 2100h-fed cows and higher at 14h post-feeding in the 0900h-fed cows. As such, the higher rate of feed consumption within 3h of feed delivery by 2100h-fed cows, as found previously in the two experiments of Study 1 (Nikkhah et al., 2005; Nikkhah et al., 2006), was likely also occurring in Study 2. Although not significant ($P = 0.17$), evening feeding increased subcutaneous fat deposition in dairy cows when compared to morning

feeding in agreement with previous findings of improved ADG and feed:gain ratio in beef cattle (Schwartzkopf-Genswein, 2004; Kennedy et al., 2004).

Researchers investigating circadian rhythms often have a blood sampling schedule of every 15min in order to properly assess the fluctuations in plasma levels (Van Cauter, 1992; Bitman et al., 1990). Research has shown that the cephalic phase of insulin secretion, within 15min of feeding, is an important determinant of post-feeding plasma glucose and insulin levels in sheep (Godden and Weekes, 1981; Herath et al., 1999) and rats (Del Prato, 2003). Therefore, since the first postprandial sample was 2h after food delivery in our studies it is possible that we may have missed important treatment differences.

Further research is still required in order to determine the exact causes of the improved performance (milk fat yield, condition, etc.) seen with evening feeding in dairy cows. For example, monitoring changes in plasma glucagon, because of a close relationship with insulin and glucose, could have allowed more in depth analysis of the energy balance of dairy cows throughout a 24h period or across weeks of treatment. More frequent blood sampling, at least in the first 2h of feed delivery, may also indicate reasons behind the observed differences in post-feeding plasma glucose and insulin levels.

In conclusion, these studies have shown that evening feeding does result in post-feeding patterns in glucose and insulin that are characteristic of insulin insensitivity as insulin level was elevated soon after feeding in the 2100h-fed cows. Plasma glucose and insulin responses to an i.v. glucose load were more elevated at night likely because of insulin insensitivity. Milk fat production might be optimal at 10h post-feeding because

glucose sparing is taking place for milk production when glucose is most abundant, and this effect is enhanced with evening feeding. Therefore, evening feeding may provide a natural management way to control glucose sparing that is available to Canadian farmers.

6.0 LITERATURE CITED

Aharoni, Y, A. Brosh, & Y. Harari. 2005. Night feeding for high-yielding dairy cows in hot weather: effects on intake, milk yield and energy expenditure. Livest. Prod. Sci. 92: 207-219.

Ahren, B., & H. Larsson. 2001. Impaired glucose tolerance (IGT) is associated with reduced insulin-induced suppression of glucagon concentrations. Diabetologia. 44: 1998-2003.

Alvarez, J. D. 2004. Genetic basis for circadian rhythms in mammals. Pages 93-140 in Molecular Biology of Circadian Rhythms. Sehgal, A., ed. John Wiley & Sons, New Jersey, U.S.A.

Andersson, L. 1982. Quantitative measurements of ketone bodies in blood and milk as indicators of subclinical ketosis. Pages 420-434 in Proceedings of the 12th World Congress on Diseases of Cattle.

Annison, E. F., R. Bickerstaffe, & J. L. Linzell. 1974. Glucose and fatty acid metabolism in cows producing milk of low fat content. J. Agr. Sci. Camb. 82: 87-95.

AOAC. 1990. Association of Official Analytical Chemists. Official methods of analysis. 15th ed. AOAC, Arlington, Virginia, U.S.A.

Armstrong, S. M., V. M. Cassone, M. J. Chesworth, J. R. Redman, & R. V. Short. 1986. Synchronization of mammalian circadian rhythms by melatonin. J. Neural Transm. 21 (Suppl): 375-394.

Basset, J. M. 1974a. Early changes in plasma insulin and growth hormone levels after feeding in lambs and adult sheep. Aust. J. Biol. Sci. 27: 157-166.

Bassett, J. M. 1974b. Diurnal patterns of plasma insulin, growth hormone, corticosteroid and metabolite concentrations in fed and fasted sheep. Aust. J. Biol. Sci. 27: 167-181.

Bauman, D. E. 1999. Bovine somatotropin and lactation: from basic science to commercial application. Domest. Anim. Endocrinol. 17: 101-116.

Bauman, D. E. & J. M. Griinari. 2003. Nutritional regulation of milk fat synthesis. Annu. Rev. Nutr. 23: 203-227.

Bauman, D. E., & R. G. Vernon. 1993. Effects of exogenous bovine somatotropin on lactation. Annu. Rev. Nutr. 13: 437-461.

Bell, A. W., & D. E. Bauman. 1997. Adaptations oucose metabolism during pregnancy and lactation. J. Mammary Gland Biol. 2: 265-278.

Bergman, E. N. 1973. Glucose metabolism in ruminants as related to hypoglycemia and ketosis. Cornell Vet. 63: 341-382.

Bergman, R. N., D. T. Finegood, & M. Ader. 1985. Assessment of insulin sensitivity *in vivo*. Endo. Rev. 6(1): 45-86.

Berthoud, H. R. 1984. The relative contribution of the nervous system, hormones, and metabolites to the total insulin response during a meal in the rat. Metabolism. 33(1): 18-25.

Bitman, J., D. L. Wood, & A. M. Lefcourt. 1990. Rhythms in cholesterol, cholesteryl esters, free fatty acids, and triglycerides in blood of lactating dairy cows. J. Dairy Sci. 73: 948-955.

Bizot-Espiard, J. G., A. Double, B. Guardiola-Lemaitre, P. Delagrange, A. Ktorza, & L. Penicaud. 1998. Diurnal rhythms in plasma glucose, insulin, growth hormone and melatonin levels in fasted and hyperglycaemic rats. Diabetes Metab. 24: 235-240.

Blum, J. W., R. M. Bruckmaier, & P.-Y. Vacher. 1999. Insulin-dependent whole-body glucose utilization and insulin-responses to glucose in week 9 and week 19 of lactation in dairy cows fed rumen-protected crystalline fat or free fatty acids. Domest. Anim. Endocrinol. 16: 123-134.

Blum, J. W., F. Jans, W. Moses, D. Frohli, M. Zemp, M. Wanner, I. C. Hart, R. Thun, & U. Keller. 1985. Twentyfour-hour pattern of blood hormone and metabolite concentrations in high-yielding dairy cows: effects of feeding low or high amounts of starch, or crystalline fat. Zbl. Vet. Med. A. 32: 401-418.

Boulos, Z., A. M. Rosenwasser, & M. Terman. 1980. Feeding schedules and the circadian organization of behavior in the rat. Behav. Brain. Res. 1: 39-65.

Brockman, R. P. 1990. Effect of insulin on the utilization of propionate in gluconeogenesis in sheep. Br. J. Nutr. 64: 95-101.

Brockman, R. P., & B. Laarveld. 1986. Hormonal regulation of metabolism in ruminants; a review. Livest. Prod. Sci. 14: 313-334.

Bruckmaier, R. M., E. Lehmann, D. Hugi, H. M. Hammon, & J. W. Blum. 1998. Ultrasonic measurement of longissimus dorsi muscle and backfat, associated with metabolic and endocrine traits, during fattening of intact and castrated male cattle. Livest. Prod. Sci. 53: 123-134.

Bubenik, G. A., R. R. Hacker, G. M. Brown, & L. Bartos. 1999. Meltonin concentrations in the luminal fluid, mucosa, and muscularis of the bovine and porcine gastrointestinal tract. J. Pineal Res. 26: 56-63.

Bubenik, G. A., S. F. Pang, J. R. Cockshut, P. S. Smith, L. W. Grovum, R. M. Friendship, & R. R. Hacker. 2000. Circadian variation of portal, arterial and venous

blood levels of melatonin in pigs and its relationship to food intake and sleep. J. Pineal Res. 28: 9-15.

Bubenik, G. A., S. F. Pang, R. R. Hacker, & P. S. Smith. 1996. Melatonin concentrations in serum and tissues of porcine gastrointestinal tract and their relationship to the intake and passage of food. J. Pineal Res. 21: 251-256.

Bucolo, R. J., R. N. Bergman, D. J. Marsh, & F. E. Yates. 1974. Dynamics of glucose autoregulation in the isolated, blood-perfused canine liver. Am. J. Physiol. 227(1): 209-217.

Cailotto, C., S. E. la Fleur, C. V. Heijningen, J. Wortel, A. Kalsbeek, M. Feenstra, P. Pevet, & R. M. Buijs. 2005. The suprachaismatic nucleus controls the daily variation of plasma glucose via the autonomic output to the liver: are the clock genes involved? Eur. J. Neurosci. 22: 2531-2540.

Canadian Council on Animal Care. 1993. Guide to the Care and Use of Experimental Animals. Vol. 1. E. D. Olfert, B. M. Cross, and A. A. McWilliam, eds. CCAC, Ottawa, ON, Canada.

Challet, E., P. Pevet, B. Vivien-Roels, & A. Malan. 1997. Phase-advanced daily rhythms of melatonin, body temperature, and locomotor activity in food-restricted rats fed during daytime. J. Biol. Rhythm. 12(1): 65-79.

Chelikani, P. K., D. H. Keisler, & J. J. Kennelly. 2003. Response of plasma leptin concentration to jugular infusion of glucose or lipid is dependent on the stage of lactation of Holstein cows. J. Nutr. 133: 4163-4171.

Coon, S. L., L. A. Zarazaga, B. Malpaux, J.-P. Ravault, L. Bodin, P. Voisin, J. L. Weller, D. C. Klein, & P. Chemineau. 1999. Genetic variability in plasma melatonin in sheep is due to pineal weight, not to variations in enzyme activities. Am. J. Physiol. 277: E792-E797.

Corl, B. A., S. T. Butler, W. R. Butler, & D. E. Bauman. 2006. Short communication: Regulation of milk fat yield and fatty acid composition by insulin. J. Dairy Sci. 89: 4172-4175.

Critser, J. K., T. M. Block, B. W. Kirkpatrick, M. J. Lindstrom, & E. R. Hauser. 1988. The effect of photoperiod on diurnal rhythms of serum gonadotrophins, prolactin and melatonin in ovariectomized heifers. Domest. Anim. Endocrinol. 5: 23-34.

Daan, S. & J. Aschoff. 2001. The entrainment of circadian systems. Pages 7-43 in Handbook of Behavioral Neurobiology. Volume 12. Circadian Clocks. Takahashi, J.S., F. W. Turek & R. Y. Moore ed. Kluwer Academic/Plenum Publishers New York, U.S.A.

Dado, R. G., & M. S. Allen. 1994. Variation in and relationships among feeding, chewing, and drinking variables for lactating dairy cows. J. Dairy Sci. 77: 132-144.

Dahl, G. E., B. A. Buchanan, & H. A. Tucker. 2000. Photoperiodic effects on dairy cattle: A review. J. Dairy Sci. 83: 885-893.

Deacon, S., & J. Arendt. 1994. Posture influences melatonin concentrations in plasma and saliva in humans. Neurosci. Lett. 167: 191-194.

Debras, E., J. Grizard, E. Aina, S. Tesseraud, C. Champredon, & M. Arnal. 1989. Insulin sensitivity and responsiveness during lactation and dry period in goats. Am. J. Physiol. 256: E295-E302.

de Jong, A. 1981. The effect of feed intake on nutrient and hormone levels in jugular and portal blood in goats. J. Agric. Sci., Camb. 96: 643-657.

Del Prato, S. 2003. Loss of early insulin secretion leads to postprandial hyperglycemia. Diabetologia. 46 (Suppl. 1): M2-M8.

Devlin, P. F. 2002. Signs of the time: Environmental input to the circadian clock. J. Exp. Bot. 53(374): 1535-1550.

DeVries, T. J., & M. A. G. von Keyserlingk. 2005. Time of feed delivery affects the feeding and lying patterns of dairy cows. J. Dairy Sci. 88: 625-631.

DeVries, T. J., M. A. G. von Keyserlingk, & K. A Beauchemin. 2005. Frequency of feed delivery affects the behaviour of lactating dairy cows. J. Dairy Sci. 88: 3553-3562.

Domecq, J. J., A. L. Skidmore, J. W. Lloyd, & J. B. Kaneene. 1995. Validation of body condition scores with ultrasound measurements of subcutaneous fat of dairy cows. J. Dairy Sci. 78: 2308-2313.

Dorshorst, M. E., & R. R. Grummer. 2002. Effects of day relative to parturition and dietary crude protein on rumen fermentation in prepartum transition cows. J. Dairy Sci. 85: 2290-2298.

Dühlmeier, R., A. Hacker, A. Widdel, W. von Engelhardt, & H.-P. Sallman. 2005. Mechanisms of insulin-dependent glucose transport into porcine and bovine skeletal muscle. Am. J. Physiol. 289: R187-R197.

Dühlmeier, R., K. Sammet, A. Widdel, W. von Engelhardt, U. Wernery, J. Kinne, & H.-P. Sallmann. 2007. Distribution patterns of glucose transporters GLUT4 and GLUT1 in skeletal muscles of rats (*Rattus norvegicus*), pigs (*Sus scrofa*), cows (*Bos taurus*), adults goats, goat kids (*Capra hircus*), and camels (*Camelus dromedaries*). Comp. Biochem. Physiol. A. 146: 274-282.

Edmonson, A. J., I. J. Lean, L. D. Weaver, T. Farver, & G. Webster. 1989. Body condition scoring chart for Holstein dairy cows. J. Dairy Sci. 72: 68–78.

Evans, E., J. G. Buchanan-Smith, G. K. MacLeod, & J. B. Stone. 1975. Glucose metabolism in cows fed low- and high-roughage diets. J. Dairy Sci. 58: 672-677.

Ferguson, J. D., D. T. Galligan, & N. Thomsen. 1994. Principal descriptors of body condition score in Holstein cows. J. Dairy Sci. 77: 2695-2703.

Gabris, J. & A. Duran. 1988. Changes in ALT, AST, and alkaline phosphatase activities in the blood serum of dairy cows. Veter. Med. 33: 469-474.

Gallo, L., P. Carnier, M. Cassandro, R. Mantovani, L. Bailoni, B. Contiero, & G. Bittante. 1996. Change in body condition score of Holstein cows as affected by parity and mature equivalent milk yield. J. Dairy Sci. 79: 1009-1015.

Garvey, W. T., L. Maianu, J. A. Hancock, A. M. Golichowski, & A. Baron. 1992. Gene expression of GLUT4 in skeletal muscle from insulin-resistant patients with obesity, IGT, GDM, and NIDDM. Diabetes. 41: 465-475.

Garvey, W. T., L. Maianu, T. P. Huecksteadt, M. J. Bimbaum, J. M. Molina, & T. P. Ciaraldi. 1991. Pretranslational suppression of a glucose transporter protein causes insulin resistance in adipocytes from patients with non-insulin-dependent diabetes mellitus and obesity. J. Clin. Invest. 87: 1072-1081.

Gaynor, P. J., R. A. Erdman, B. B. Teter, J. Sampugna, A. V. Capuco, D. R. Waldo & M. Hamosh. 1994. Milk fat yield and composition during abomasal infusion of *cis* or *trans* octadecenoates in Holstein cows. J. Dairy Sci. 77: 157-165.

Gaynor, P. J., D. R. Waldo, A. V. Capuco, R. A. Erdman, L. W. Douglass, & B. B. Teter. 1995. Milk fat depression, the glucogenic theory and trans-$C_{18:1}$ fatty acids. J. Dairy Sci. 78: 2008-2015.

Giebultowicz, J. M. 2004. Multiple oscillators. Pages 213-229 in Molecular Biology of Circadian Rhythms. Sehgal, A., ed. John Wiley & Sons, New Jersey, U.S.A.

Godden, P. M. M., & T. E. C. Weekes. 1981. Insulin, prolactin and thyroxine responses to feeding, and to arginine and insulin injections during growth in lambs. J. Agric. Sci., Camb. 96: 353-362.

Goodyear, L. J., & B. B. Kahn. 1998. Exercise, glucose transport, and insulin sensitivity. Annu. Rev. Med. 49: 235-261.

Grant, R. J., & J. L. Albright. 1995. Feeding behaviour and management factors during the transition period in dairy cattle. J. Anim. Sci. 73: 2791-2803.

Griinari, J. M., D. A. Dwyer, M. A. McGuire, D. E. Bauman, D. L. Palmquist, & K. V. V. Nurmela. 1998. *Trans*-octadecenoic acids and milk fat depression in lactating dairy cows. J. Dairy Sci. 81: 1251-1261.

Griinari, J. M., M. A. McGuire, D. A. Dwyer, D. E. Bauman, & D. L. Palmquist. 1997. Role of insulin in the regulation of milk fat synthesis in dairy cows. J. Dairy Sci. 80: 1076-1084.

Grill, H. J., K. C. Berridge, & D. J. Ganster. 1984. Oral glucose is the prime elicitor of preabsorptive insulin secretion. Am. J. Physiol. 246: R88-R95.

Grunberg, W., D. E. Morin, J. K. Drackley, & P. D. Constable. 2006. Effect of rapid intravenous administration of 50% dextrose solution on phosphorus homeostasis in postparturient dairy cows. J. Vet. Intern. Med. 20: 1471-1478.

Hampton, S. M., L. M. Morgan, J. A. Tredger, R. Cramb, & V. Marks. 1986. Insulin and C-peptide levels after oral and intravenous glucose; contribution of enteroinsular axis to insulin secretion. Diabetes. 35: 612-616.

Hanson, R. W., & F. J. Ballard. 1967. The relative significance of acetate and glucose as precursors for lipid synthesis in liver and adipose tissue from ruminants. Biochem. J. 105: 529-536.

Hart, I. C. 1983. Endocrine control of nutrient partition in lactating ruminants. Proc. Nutr. Soc. 42: 181-194.

Hart, I. C., P. M. E. Chadwick, A. Coert, S. James, & A. D. Simmonds. 1985. Effect of different growth hormone-releasing factors on the concentrations of growth hormone, insulin and metabolites in the plasma of sheep maintained in positive and negative energy balance. J. Endocrinol. 105: 113-119.

Hayirli, A., D. R. Bremmer, S.J. Bertics, M. T. Socha, & R. R. Grummer. 2001. Effect of chromium supplementation on production and metabolic parameters in periparturient dairy cows. J. Dairy Sci. 84: 1218-1230.

Heinrichs, J. 1996. Evaluating particle size of forages and TMR using the Penn State Particle Size Separator. Penn State, University Park, PA.

Herath, C. B., G. W. Reynolds, D. D. S. MacKenzie, S. R. Davis, & P. M. Harris. 1999. Vagotomy suppresses cephalic phase insulin release in sheep. Exp. Physiol. 84: 559-569.

Herbein, J. H., R. J. Aiello, L. I. Eckler, R. E. Pearson, & R. M. Akers. 1985. Glucagon, insulin, growth hormone, and glucose concentrations in blood plasma of lactating dairy cows. J. Dairy Sci. 68: 320-325.

Hoffman, R. M., R. C. Boston, D. Stefanovski, D. S. Kronfeld, & P. A. Harris. 2003. Obesity and diet affect glucose dynamics and insulin sensitivity in thoroughbred geldings. J. Anim. Sci. 81: 2333-2342.

Hofmann, R. R. 1988. Anatomy of the gastro-intestinal tract. Pages 14 to 43 in The Ruminant Animal: Digestive Physiology and Nutrition. Church, D. C., ed. Prentice Hall, New Jersey, U.S.A.

Holst, J. J. 1992. Role of classical and peptidergic neurotransmitters in insulin secretion. Pages 23-39 in Nutrient Regulation of Insulin Secretion. Flatt, P., ed. Portland Press, London, U.K.

Holst, J. J., R. Gronholt, O.B. Schaffalitzky de Muckadell, & J. Fahrenkrug. 1981. Nervous control of pancreatic endocrine secretion in pigs. I. Insulin and glucagon responses to electrical stimulation of the vagus nerves. Acta Physiol. Scand. 111: 1-7.

Holtenius, K., S. Agenas, C. Delavaud, & Y. Chilliard. 2003. Effects of feeding intensity during the dry period. 2. Metabolic and hormonal responses. J. Dairy Sci. 86: 883-891.

Houghton, P. L., & L. M. Turlington. 1992. Application of ultrasound for feeding and finishing animals: a review. J. Anim. Sci. 70: 930-941.

Hugi, D., R. M. Bruckmaier, & J. W. Blum. 1997. Insulin resistance, hyperglycemia, glucosuria, and galactosuria in intensively milk-fed calves: dependency on age and effects of high lactose intake. J. Anim. Sci. 75: 469-482.

Jaurena, G., J. M. Moorby, W. J. Fisher, & R. Cantet. 2005. Association of body weight, loin longissiumus dorsi and backfat with body condition score in dry and lactating Holstein dairy cows. Anim. Sci. 80: 219-223.

Kalsbeek, A., S. la Fleur, C. Van Heijningen, & R. M. Buijs. 2004. Suprachiasmatic GABAergic inputs to the paraventricular nucleus control plasma glucose concentrations in the rat via sympathetic innervation of the liver. J. Neurosci. 24: 7604-7613.

Kalsbeek, A., & J. H. Strubbe. 1998. Circadian control of insulin secretion is independent of the temporal distribution of feeding. Physiol. Behav. 63(4): 553-558.

Kennedy, A. D., R. D. Bergen, T. J. Lawson, J. A. Small, & D. M. Veira. 2004. Effects of evening feeding and extended photoperiod on growth, feed efficiency, live animal carcass traits and plasma prolactin of beef heifers housed outdoors during two Manitoba winters. Can. J. Anim. Sci. 84: 491-500.

Kennelly, J. J., B. Robinson, & G. R. Khorasani. 1999. Influence of carbohydrate source and buffer on rumen fermentation characteristics, milk yield, and milk composition in early-lactation Holstein cows. J. Dairy Sci. 82: 2486-2496.

King, P.A., J. J. Betts, E. D. Horton, & E. S. Horton. 1993. Exercise, unlike insulin, promotes glucose transporter translocation in obese Zucker rat muscle. Am. J. Physiol. 265: R447-R452.

King, P. A., E. D. Horton, M. F. Hirshman, & E. S. Horton. 1992. Insulin resistance in obese Zucker rat (fa/fa) skeletal muscle is associated with a failure of glucose transporter translocation. J. Clin. Invest. 90: 1568-1575.

Knutsen, J.S., J. J. Vetos, & R. H. Pritchard. 1994. Effect of morning, evening, or twice daily feeding on yearling steer performance. SDSU Animal/Range Sci. Cattle 94-14: 49-53.

Kritz-Silverstein, D., E. Barrett-Conner, & D. L. Wingard. 1989. The effect of parity on the later development of non-insulin-dependent diabetes mellitus or impaired glucose tolerance. New Engl. J. Med. 321(18): 1214-1219.

la Fleur, S. E. 2003. Daily rhythms in glucose metabolism: suprachiasmatic nucleus output to peripheral tissue. J. Neuroendocrinol. 15: 315-322.

la Fleur, S. E., A. Kalsbeek, J. Wortel, & R. M. Buijs. 1999. A suprachiasmatic nucleus generated rhythm in basal glucose concentrations. J. Neuroendocrinol. 11: 643-652.

Lawson, T. J. 2000. Endocrine and winter performance responses to supplemental light and evening feeding in heifers. Master Thesis. Universtiy of Manitoba, Winnipeg, MB.

Lawson, T. J., & A. D. Kennedy. 2001. Inhibition of nighttime melatonin secretion in cattle: threshold light intensity for dairy heifers. Can. J. Anim. Sci. 81: 153-156.

Lefcourt, A. M., J. B. Huntington, R. M. Akers, D. L. Wood, & J. Bitman. 1999. Circadian and ultradian rhythms of body temperature and peripheral concentrations of insulin and nitrogen in lactating dairy cows. Domest. Anim. Endocrinol. 16: 41-55.

Lemosquet, S., & P. Faverdin. 2001. A dynamic model to analyse intravenous glucose and insulin tolerance tests performed on dairy cows. Brit. J. Nutr. 86: 359-369.

Lemosquest, S., N. Rideau, H. Rulquin, P. Faverdin, J. Simon, & R. Verite. 1997. Effects of duodenal glucose infusion on the relationship between plasma concentrations of glucose and insulin in dairy cows. J. Dairy Sci. 80: 2854-2865.

Lemosquet, S., S. Rigout, A. Bach, H. Rulquin, & J. W. Blum. 2004. Glucose metabolism in lactating cows in response to isoenergetic infusions of propionic acid or duodenal glucose. J. Dairy Sci. 87: 1767-1777.

Lima, F. B., U. F. Machado, I. Bartol, P. M. Seraphim, D. H. Sumida, S. M. F. Moraes, N. S. Hell, M. M. Okamoto, M. J. A. Saad, C. R. O. Carvalho, & J. Cipolla-Neto. 1998. Pinealectomy cause glucose intolerance and decreases adipose cell responsiveness to insulin in rats. Am. J. Physiol. 275: E934-E941.

Lomax, M. A., G. D. Baird, C. B. Mallinson, & H. W. Symonds. 1979. Differences between lactating and non-lactating dairy cows in concentration and secretion rate of insulin. Biochem. J. 180: 281-289.

Marks, V., E. Samols, & J. Stagner. 1992. Intra-islet interactions. Pages 41-57 in Nutrient Regulation of Insulin Secretion. Flatt, P. R., ed. Portland Press, London, U.K.

McCarthy, J. P., A. Faulkner, P. A. Martin, & D. J. Flint. 1992. Changes in the plasma concentration of gastric inhibitory polypeptide and other metabolites in response to feeding in sheep. J. Endocrinol. 134: 235-240.

McClymont, G. L., & S. Vallance. 1962. Depression of blood glycerides and milk-fat synthesis by glucose infusion. P. Nutr. Soc. 21: xli-xlii.

McGuire, M. A., J. M. Griinari, D. A. Dwyer, & D. E. Bauman. 1995. Role of insulin in the regulation of mammary synthesis of fat and protein. J. Dairy Sci. 78: 816-824.

Mir, P. S., G. J. Mears, C. M. Ross, S. D. Husar, W. M. Robertson, S. D. M. Jones, & Z. Mir. 1998. Insulin responses during glucose tolerance test in steers with increasing wagyu genetic influence. Can. J. Anim. Sci. 78: 233-235.

Mistlberger, R. E. 1994. Circadian food-anticipatory activity: Formal models and physiological mechanisms. Neurosci. Biobehav. Rev. 18(2): 171-195.

Moore, R. Y. 1978. Central neural control of circadian rhythms. Pages 185-206 in Frontiers in Neuroendocrinology. Ganong, W. F., & L. Martini, eds. 5th ed. Raven Press, New York.

Moore, R. Y., R. K. Leak. 2001. Suprachiasmatic nucleus. Pages 141-179 in Handbook of Behavioral Neurobiology. Volume 12. Circadian Clocks. Takahashi, J.S., F. W. Turek & R. Y. Moore ed. Kluwer Academic/Plenum Publishers New York, U.S.A.

Morgan, L. M. 1992. Insulin secretion and the entero-insular axis. Pages 1-22 in Nutrient Regulation of Insulin Secretion. Flatt, P., ed. Portland Press, London, U.K.

Moshtaghi Nia, S. A., P. H. Robinson, M. Gill, J. R. Ingalls, & J. J. Kennelly. 1995. Influence of feeding a rapidly rumen degraded dietary protein at night or with a basal mixed ration on performance of dairy cows. Can. J. Anim. Sci. 75: 575-582.

Muthuramalingam, P., A. D. Kennedy, & R. J. Berry. 2006. Plasma melatonin and insulin-like growth factor-1 responses to dim light at night in dairy heifers. J. Pineal Res. 40: 225-229.

Nauck, M., F. Stockmann, R. Ebert, & W. Creutzfeldt. 1986. Reduced incretin effect in Type 2 (non-insulin-dependent) diabetes. Diabetologia. 29: 46-52.

Nikkhah, A., J. C. Plaizier, C. J. Furedi, & A. D. Kennedy. 2005. Effects of time of feeding and forage to concentrate ratio on rumen fermentation and productivity of lactating dairy cows. J. Dairy Sci. 88 Suppl. 1: 186.

Nikkhah, A., J. C. Plaizier, C. J. Furedi, & A. D. Kennedy. 2006. Impact of providing total mixed ration at evening vs morning on feed intake, rumen pH and productivity of lactating Holsteins. J. Dairy Sci. 89 Suppl. 1: 296-297.

Ominski, K. H., A. D. Kennedy, K. M. Wittenberg, & S. A. Moshtaghi Nia. 2002. Physiological and production responses to feeding schedule in lactating dairy cows exposed to short-term, moderate heat stress. J. Dairy. Sci. 85: 730-737.

Pere, M. C., M. Etienne, & J. Y. Dourmad. 2000. Adaptations of glucose metabolism in multiparous sows: Effects of pregnancy and feeding level. J. Anim. Sci. 78: 2933-2941.

Phillips, C. J. C., & M. I. Rind. 2001. The effects of frequency of feeding a total mixed ration on the production and behaviour of dairy cows. J. Dairy Sci. 84: 1979-1987.

Piccione, G., G. Caola, & R. Refinetti. 2003. Circadian rhythms of body temperature and liver function in fed and food-deprived goats. Comp. Biochem. Phys. A. 134(3): 563-572.

Picinato, M. C., E. P. Haber, A. R. Carpinelli, & J. Cipolla-Neto. 2002a. Daily rhythm of glucose-induced insulin secretion by isolated islets from intact and pinealectomized rat. J. Pineal Res. 33: 172-177.

Picinato, M. C., E. P. Haber, J. Cipolla-Neto, R. Curi, C. R. de Oliveira Carvalho, & A. R. Carpinelli. 2002b. Melatonin inhibits insulin secretion and decreases PKA levels without interfering with glucose metabolism in rat pancreatic islets. J. Pineal Res. 33: 156-160.

Piperova, L. S., B. B. Teter, I. Bruckental, J. Sampugna, S. E. Mills, M. P. Yurawecz, J. Fritsche, K. Ku, & R. A. Erdman. 2000. Mammary lipogenic enzyme activity, *trans* fatty acids and conjugated linoleic acids are altered in lactating dairy cows fed a milk fat-depressing diet. J. Nutr. 130: 2568-2574.

Plaizier, J. C., A. M. Fairfield, P. A. Azevedo, A. Nikkhah, T. F. Duffield, G. H. Crow, R. Bagg, P. Dick, & B. W. McBride. 2005. Effects of monensin and stage of lactation on variation of blood metabolites within twenty-four hours in dairy cows. J. Dairy Sci. 88: 3595-3602.

Porte, D., Jr. 1967. A receptor mechanism for the inhibition of insulin release by epinephrine in man. J. Clin. Invest. 46(1): 86-94.

Reist, M., D. Erdin, D. von Euw, K. Tschuemperlin, H. Leuenberger, C. Delavaud, Y. Chilliard, H. M. Hammon, N. Kuenzi, & J. W. Blum. 2003. Concentrate feeding strategy in lactating dairy cows: metabolic and endocrine changes with emphasis on leptin. J. Dairy Sci. 86: 1690-1706.

Rhoads, D. B., D. H. Rosenbaum, H. Unsal, K. J. Isselbacher, & L. L. Levitsky. 1998. Circadian periodicity of intestinal Na^+/glucose cotransporter 1 mRNA levels is transcriptionally regulated. J. Biol. Chem. 272: 9510-9516.

Robinson, P. H., M. Gill, & J. J. Kennelly. 1997. Influence of time of feeding a protein meal on ruminal fermentation and forestomach digestion in dairy cows. J. Dairy Sci. 80: 1366-1373.

Rohner-Jeanrenaud, F., E. Bobbioni, E. Ionescu, J. Sauter, & B. Jeanrenaud. 1983. Central nervous system regulation of insulin secretion. Pages 193-220 in CNS Regulation of Carbohydrate Metabolism. Szabo, A. J., ed. 10th ed. Academic Press, New York.

Ross, J. P. & W. D. Kitts. 1973. Relationship between postprandial plasma volatile fatty acids, glucose and insulin levels in sheep fed different feeds. J. Nutr. 103: 488-493.

Sano, H., M. Nakai, T. Kondo, & Y. Terashima. 1991. Insulin responsiveness to glucose and tissue responsiveness to insulin in lactating, pregnant, and nonpregnant, non-lactating beef cows. J. Anim. Sci. 69: 1122-1127.

SAS Institute, Inc. 1999. User's Guide, Version 6.12 for Windows. SAS Institute, Inc., Cary, North Carolina, U. S. A.

Sasaki, Y., H. Takahashi, H. Aso, K. Hikosaka, A. Hagino, S. Oda. 1984. Insulin response to glucose and glucose tolerance following feeding in sheep. Br. J. Nutr. 52: 351-358.

Sasaki, Y., H. Takahashi, H. Aso, A. Ohneda, & T. E. C. Weekes. 1982. Effects of cold exposure on insulin and glucagon secretion in sheep. Endocrinology. 111: 2070-2076.

Scheepers, A., H.-G. Joost, & A. Schurmann. 2004. The glucose transporter families SGLT and GLUT: molecular basis of normal and aberrant function. J. Parenter. Enter. Nutr. 28: 365-372.

Schröder, U. J., & R. Staufenbiel. 2006. Methods to determine body fat reserves in the dairy cow with special regard to ulrasonographic measurement of backfat thickness. J. Dairy Sci. 89: 1-14.

Schwartzkopf-Genswein, K. S., K. A. Beauchemin, T. A. McAllister, D. J. Gibb, M. Streeter, & A. D. Kennedy. 2004. Effect of feed delivery fluctuations and feeding time on ruminal acidosis growth performance, and feeding behavior of feedlot cattle. J. Anim. Sci. 82: 3357-3365.

Sehgal, A. 2004. General Concepts. Pages 3-16 in Molecular Biology of Circadian Rhythms. Sehgal, A., ed. John Wiley & Sons, New Jersey, U.S.A.

Sitren, H. S., & N. R. Stevenson. 1978. The effects of meal-feeding at different times of the day on daily changes in serum insulin, gastrin and liver enzymes in the rat. J. Nutr. 108: 1393-1401.

Small, J. A., A.D Kennedy, D. M. Veira, W. P. McCaughey, & D. R. Ward. 2004. Time of feeding and growth promotant effects on the winter growth performance and carcass traits of steers. Can. J. Anim. Sci. 84: 133–144.

Stanton, T. L., L. R. Jones, R. W. Everett, & S. D. Kachman. 1992. Estimating milk, fat, and protein lactation curves with a test day model. J. Dairy Sci. 75: 1691-1700.

Steel, R. G., J. H. Torrie, & D. A. Dickey. 1997. Principles and Procedures of Statistics: A Biometrical Approach. 3rd ed. WCB/McGraw-Hill, New York, U.S.A.

Stephan, F. K. 2001. Food-entrainable oscillators in mammals. Pages 223-246 in Handbook of Behavioral Neurobiology. Volume 12. Circadian Clocks. Takahashi, J.S., F. W. Turek & R. Y. Moore ed. Kluwer Academic/Plenum Publishers New York, U.S.A.

Stojevic, Z., J. Pirsljin, S. Milinkovic-Tur, M. Zdelar-Tuk, & B. Beer Ljubic. 2005. Activities of AST, ALT, and GGT in clinically healthy dairy cows during lactation and in the dry period. Vet. Arhiv. 75: 67-73.

Stricklin, W. R., & C. C. Kautz-Scanavy. 1984. The role of behaviour in cattle production: a review of research. Appl. Anim. Ethol. 11: 359-390.

Subiyatno, A., D. N. Mowat, & W. Z. Yang. 1996. Metabolite and hormonal responses to glucose or propionate infusions in periparturient dairy cows supplemented with chromium. J. Dairy Sci. 79: 1436-1445.

Sutton, J. D., I. C. Hart, S. V. Morant, E. Schuller, & A. D. Simmonds. 1988. Feeding frequency for lactating cows: diurnal patterns of hormones and metabolites in peripheral blood in relation to milk-fat concentration. Br. J. Nutr. 60: 265-274.

Takahashi, J. S., & M. Zatz. 1982. Regulation of circadian rhythmicity. Science. 217: 1104-1111.

Tappy, L., E. Jequier, & P. Schneiter. 2000. Autoregulation of glucose production. News Physiol. Sci. 15: 198-202.

Unger, R. H., & M. D. Dallas. 1971. Glucagon and the insulin:glucagon ratio in diabetes and other catabolic illnesses. Diabetes. 20(12): 834-838.

Unger, R. H., & A. M. Eisentraut. 1969. Entero-insular axis. Arch. Intern. Med. 123: 261-266.

Van Cauter, E., & O. M. Buxton. 2001. Circadian modulation of endocrine secretion. Pages 685-714 in Handbook of Behavioral Neurobiology. Volume 12. Circadian Clocks. Takahashi, J.S., F. W. Turek & R. Y. Moore ed. Kluwer Academic/Plenum Publishers New York, U.S.A.

Van Cauter, E., D. Desir, C. Decoster, F. Fery, & E. O. Balasse. 1989. Nocturnal decrease in glucose tolerance during constant glucose infusion. J. Clin. Endocrinol. Metab. 69: 604-611.

Van Cauter, E., E. T. Shapiro, H. Tillil, & K. S. Polonsky. 1992. Circadian modulation of glucose and insulin responses to meals: Relationship to cortisol rhythm. Am. J. Physiol. 262: E467-E475.

Vilsboll, T., T. Krarup, S. Madsbad, & J. J. Holst. 2002. Defective amplification of the late phase insulin response to glucose by GIP in obese Types II diabetic patients. Diabetologia. 45: 1111-1119.

Watson, R. T., & J. E. Pessin. 2001. Intracellular organization of insulin signalling and GLUT4 translocation. Recent Prog. Horm. Res. 56: 175-193.

Wild, S., G. Roglic, A. Green, R. Sicree, & H. King. 2004. Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. Diabetes Care 27: 1047-1053.

Wolfe, R. R., T. F. O'Donnell Jr., M. D. Stone, D. A. Richmand, & J. F. Burke. 1980. Investigation of factors determining the optimal glucose infusion rate in total parenteral nutrition. Metabolism. 29(9): 892-900.

W.H.O. 1999. Definition, diagnosis and classification of diabetes mellitus and its complications. Part1. Diagnosis and classification of diabetes mellitus. World Health Organization. Geneva.

Xing, G. Q., D. D. S. Mackenzie, S. N. McCutcheon, & B. W. Wickham. 1993. Pancreatic insulin responses to exogenous glucose in Friesian heifers of low or high genetic merit for milk-fat yield. Anim. Prod. 56: 171-178.

Zanquetta, M. M., P. M. Seraphim, D. H. Sumida, J. Cipolla-Neto, & U. F. Machado. 2003. Calorie restriction reduced pinealectomy-induced insulin resistance by improving GLUT4 gene expression and its translocation to the plasma membrane. J. Pineal Res. 35: 141-148.

Zhao, F.-Q., W. M. Moseley, H. A. Tucker, & J. J. Kennelly. 1996. Regulation of glucose transporter gene expression in mammary gland muscle, and fat of lactating cows by administration of bovine growth hormone and bovine growth hormone-releasing factor. J. Anim. Sci. 74: 183-189.

Zimmerman, C. A., A. H. Rakes, R. D. Jaquette, B. A. Hopkins, & W. J. Croom, Jr. 1991. Effects of protein level and forage source on milk production and composition in early lactation dairy cows. J. Dairy Sci. 74: 980-990.

Zucker, I. 2001. Circannual rhythms: mammals. Pages 509-528 in Handbook of Behavioral Neurobiology. Volume 12. Circadian Clocks. Takahashi, J.S., F. W. Turek & R. Y. Moore ed. Kluwer Academic/Plenum Publishers New York, U.S.A.

Appendix 1. Standard curve of melatonin kit and dilutions of cattle plasma and internal standard.