# Effects of pre-weaning plane of milk replacer and feeding frequency on glucose metabolism in dairy calves

by

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**Abstract**

Feeding dairy calves large milk meal sizes at a low feeding frequency has been associated with reduced insulin sensitivity in previous literature. Therefore, the aim of this thesis was to investigate the influence of feeding an elevated plane of milk on glucose metabolism in calves pre- and post-weaning. To assess insulin sensitivity in calves in chapter 2 and 3, postprandial plasma glucose and insulin concentrations (pre-weaning), as well as insulin response to a glucose tolerance test (GTT; pre and post-weaning) were evaluated. In addition, in chapter 2 the rate of abomasal emptying was characterized pre-weaning to determine the extent of its control over glucose appearance in the blood. Results from chapter 2 where calves were fed a low (4 L/day) or a high (8 L/day) plane of milk twice daily indicated that calves fed either treatment did not experience hyperglycemia or hyperinsulinemia. Additionally, responses to a GTT were similar pre- or post-weaning suggesting both treatments had similar glucose tolerance at all ages. Abomasal emptying was reduced in calves fed a larger meal size (4 L) which indicates it can be used to modulate the appearance of glucose in the blood to prevent hyperglycemia. In chapter 3, feeding an elevated plane of milk (8 L/day) fed over four (4x; meal size 2 L) or two meals per day (2x; meal size 4 L) was compared. Neither treatment resulted in a state of hyperglycemia or hyperinsulinemia, and responses to the GTT were similar indicating similar glucose tolerance. Overall findings from this thesis suggest that calves fed an elevated plane of milk do not experience reduced insulin sensitivity when fed at differing frequencies of 2 or 4 times a day or when compared to calves fed a low plane of milk (4 L/day). These results have significant implications for the dairy industry as this means dairy operations can feed calves more milk, up to 8 L fed over two meals a day, allowing for greater pre-weaning growth without compromising glucose metabolism pre- or post-weaning.

**Preface**

This thesis encloses the original work of Jayden Ashley Rae MacPherson with international collaborations led by Dr. Michael Steele at the University of Alberta. International collaborations included work with Trouw Nutrition in Boxmeer, the Netherlands, where the study in chapter 2 was conducted. International co-authors for chapter 2 include Dr. Harma Berends, Dr. Leonel Leal, and Dr. Javier Martin-Tereso who contributed to the experimental design, experimental sampling, and manuscript preparation. Additionally, for chapter 2 Dr. John Cant from the University of Guelph in Guelph, Ontario collaborated on the statistical analysis of abomasal emptying data. Chapter 2 is published as: MacPherson, J. A. R., H. Berends, L. N. Leal, J. P. Cant, J. Martin Tereso, and M. A. Steele. 2016. Effect of plane of milk replacer intake and age on glucose and insulin kinetics and abomasal emptying in female Holstein Friesian dairy calves fed twice daily. Journal of Dairy Science. 99:8007-8017.

The research projects in this thesis received ethics approval from the University of Alberta and all the procedures performed on animals in each study were pre-approved by the Animal Care and Use Committee for Livestock at the University of Alberta and conducted according to the guidelines of the Canadian Council of Animal Care (Ottawa, ON, Canada, 2009). In addition, for chapter 2 all procedures performed on study animals were conducted in accordance with the Dutch Law on Experimental Animals, and the ETS123 (Council of Europe 1985 and the 86/609/EEC Directive) and were approved by the Animal Care and Use Committee from Utrecht University.

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**List of Abbreviations**

| | |
|---|---|
| ADG | Average Daily Gain |
| AUC | Area Under the Curve |
| BW | Body Weight |
| CCAC | Canadian Council of Animal Care |
| CF | Crude Fat |
| Cmax | Maximum Concentration |
| CP | Crude Protein |
| CR | Clearance Rate |
| CV | Coefficient of Variation |
| DM | Dry Matter |
| DMI | Dry Matter Intake |
| EDTA | Ethylenediaminetetraacetic acid |
| FSIGT | Frequently Sampled Intravenous Glucose Tolerance Test |
| GH | Growth Hormone |
| GLP-1 | Glucagon-Like-Peptide-1 |
| GLUT | Glucose Transporter |
| GTT | Glucose Tolerance Test |
| HIGH | Chapter 2 milk treatment of 8 L/day or 1.2 kg milk replacer |
| IGF | Insulin-like Growth Factor |
| ITT | Insulin Tolerance Test |
| LOW | Chapter 2 milk treatment of 4 L/day or 0.6 kg milk replacer |
| ME | Metabolizable Energy |

| | |
|---|---|
| MINMOD | Minimal Model computer program |
| MR | Milk Replacer |
| NEFA | Non-esterified Fatty Acids |
| OGTT | Oral Glucose Tolerance Test |
| SAS | Statistical Analysis System |
| SCFA | Short Chain Fatty Acids |
| Tmax | Time to maximum concentration |
| USD | United States Dollar |
| VFA | Volatile Fatty Acids |
| 2x | Chapter 3 milk feeding frequency treatment of twice a day |
| 4x | Chapter 3 milk feeding frequency treatment of four times a day |

# 1.0 Chapter 1: Literature Review

## 1.1 Introduction

There are many factors contributing to the success of a dairy operation. One of the most important is heifer rearing, as replacement heifers are the future milking herd. Heifer rearing is usually the second highest production cost after feed (Overton et al., 2013). The largest and one of the most crucial components of heifer rearing is calf nutrition (~ 60% the cost of production, Overton et al., 2013) as neonatal nutrition can be highly influential for development (Bartol et al., 2013). For example, recently feeding an elevated plane of milk or milk replacer (MR; ~8 L/day) during the pre-weaning period of heifer rearing is becoming increasingly popular in the dairy industry. Calves fed an elevated plane of milk demonstrate improved pre-weaning growth rates and have the potential to produce more milk when they enter lactation (Soberon et al., 2012). Current research still has many knowledge gaps when it comes to the influence of an elevated plane of milk on calf development including energy metabolism. Specifically, the effect of feeding more milk on glucose and insulin kinetics and whether there are any permanent consequences on glucose metabolism that can persist with age. Thus, the aim of this literature review is to discuss how feeding higher daily milk volumes (≥ 8 L/day) to dairy calves may influence glucose metabolism.

## 1.2 Calf Feeding Practices in the Dairy Industry

### 1.2.1 Conventional vs. Elevated Planes of Nutrition

In today's dairy industry there are two main feeding practices that are most often referred to as conventional or traditional, and intensive or elevated heifer rearing. In conventional rearing, dairy heifers are fed milk or milk replacer (MR) at 8-10% of the calf's birth body weight (BW),

which translates to ~4 L milk/day. Conventional rearing is viewed as a restricted milk feeding program designed to promote starter intake and therefore rumen development (Drackley, 2008). As milk is more costly to feed than solid feed, conventional feeding was originally adopted to promote early weaning due to increased rumen development and therefore reduce heifer rearing costs (Khan et al., 2011). However, increasing solid feed intake is achieved because calves fed conventional rations struggle to meet their growth energy requirements from the low plane of milk offered and therefore must compensate by increasing their starter intake. As a result, they are considered to be in a nutrient deprived state, with an average daily gain around 0.2 – 0.5 kg/day (Jasper and Weary, 2002), and often show visual signs of hunger and distress (Jensen, 2006; De Paula Vieira et al., 2008).

Intensive or elevated calf rearing consists of feeding a higher plane of milk (approximately double the volume of milk or MR compared to conventional) fed at 20% of the calf's birth BW, which translates to ~8 L/day. Calves raised on intensive feeding programs display many benefits including greater pre-weaning average daily gain (ADG; Jasper and Weary, 2002; Khan *et al*., 2007), fewer behavioural signs of hunger (Borderas *et al*., 2009; de Passillé *et al*., 2011; Miller-Cushon and DeVries, 2015), earlier onset of puberty (Bar-Peled et al., 1997), and the potential for greater milk production (Soberon et al., 2012). The average growth rate for calves fed this plane of nutrition is ~0.8 kg/day (Jasper and Weary, 2002) and a comprehensive meta-analysis by Soberon et al.(2012) showed that every additional kilogram of ADG during the pre-weaning period translated to 970 kg more milk in the first lactation.

A major concern the dairy industry has regarding implementing feeding an elevated plane of milk nutrition is the financial investment involved with feeding more milk. Previously, conventional feeding was perceived as the lower cost option, but a new study conducted in the

U.S.A. showed that feeding an elevated plane of milk results in a net value return of ~$180 per heifer after their first lactation (Overton et al., 2013). This calculation accounts for many components, some of the most significant being the higher feed costs ($1480 vs $1407 USD), lower morbidity (diarrhea and pneumonia 55% to 17%), lower mortality rates (3% and 7%), earlier breeding age (12.2 vs. 15.5 months), earlier age of pregnancy (15.1 vs 18 months), and higher future milk production of ~1700 lbs for calves fed an elevated plane of milk compared to those conventionally fed (Overton et al., 2013). Such increased monetary returns and positive benefits for both calf and cow health and performance are ultimately driving more operations towards feeding dairy calves more milk.

### 1.2.2 Feeding Frequency

Both conventional and elevated feeding regimens are commonly offered to calves twice daily on commercial dairy operations (Vasseur et al., 2010). However, this drastically contrasts how calves would naturally feed if they were allowed to suckle from their dam (Reinhardt and Reinhardt, 1981; Egli and Blum, 1998) or had ad libitum access to an automated calf feeder (de Passillé et al., 2014; Berends, 2014). In these scenarios, calves can consume around 12 L of milk/day spread out over 7-10 small meals. Without automation, mimicking natural feeding is challenging, and in most cases not practical for dairy operations to implement a feeding schedule which provides milk more than twice daily (Vasseur et al., 2010). Therefore, commercial dairies feeding an elevated plane of milk (≥ 8 L/day) will need to offer large meal sizes (≥ 4 L/meal) if limited to feeding twice a day. Knowledge gaps currently exist around the best way to adjust calf feeding to fit a commercial situation, it's currently unclear how feeding more milk practically twice a day will effect glucose metabolism and gastrointestinal function. These gaps are

disquieting, as feeding at a reduced feeding frequency of twice a day with large milk meal sizes has been linked to hyperglycaemia, hyperinsulinemia, glucosuria, and a decreasing insulin sensitivity in older veal calves (4 to 16 months old fed for ADG of 1.4 kg; Doppenberg and Palmquist, 1991; Palmquist et al., 1992a; Hostettler-allen et al., 1993; Hugi et al., 1997b; c, 1998a; b).

## 1.3 Glucose Metabolism in Calves

Glucose is a carbohydrate monosaccharide that is the main energy source for many organisms, including the pre-ruminant calf. Plasma glucose concentrations can be influenced by feed intake, endogenous glucose production from gluconeogenesis in the liver, and catabolism of glycogen stored in the liver, muscle, and adipose tissue (Aronoff et al., 2004). These metabolic pathways are highly regulated by endocrine control; therefore, proper endocrine regulation of glucose metabolism is crucial for glucose homeostasis (Kaneko, 1997). There are several hormones which can be involved in the regulation of glucose homeostasis including amylin, epinephrine, cortisol, insulin-like growth factor (IGF), growth hormone (GH), glucagon-like peptide (GLP), insulin, and glucagon (Aronoff et al., 2004). This thesis will focus largely on the action of insulin as it is one of the primary hormones regulating glucose concentrations and is the hormone associated with the metabolic disorder where calves experience decreased insulin sensitivity.

### 1.3.1 Insulin Action

Insulin is an anabolic hormone synthesized in and secreted from the β cells in the Islets of Langerhans located in the pancreas (Kaneko, 1997). The main purpose of insulin is to facilitate

the uptake of glucose across cell membranes primarily using glucose transporters (GLUT) (Kaneko, 1997). Secretion of insulin is stimulated by nutrients entering the small intestine causing a rise in plasma glucose concentration or hyperglycemia and is inhibited when concentrations return to the physiological basal concentration or if hypoglycemia occurs. The production and secretion of insulin depends highly on: 1) gastric emptying, which includes the rate nutrients appear in the small intestine, 2) feed composition, including carbohydrates and amino acids, and 3) neuroendocrine signalling to the pancreas to signal either stimulation or inhibition of insulin production (Aronoff et al., 2004). When stimulated, insulin action occurs in two phases: the first phase is an immediate release of preformed insulin and the second phase is continued synthesis of insulin based on plasma glucose concentrations (Aronoff et al., 2004). Eventually insulin will be catabolized by the liver and removed by the kidneys (Duckworth et al., 1998).

Insulin acts mainly on the liver, muscle, and adipose tissue which require abundant transport of glucose for energy and as a result, have an abundance of insulin receptors specific for each tissue (Khan and Pessin, 2002). In the liver, high concentrations of insulin act via the transporter GLUT-2 to increase glucose uptake and storage as glycogen via glycogenesis. In addition, low insulin concentrations coupled with glucagon action, another hormone secreted from the pancreas in the α cells, stimulates gluconeogenesis and breakdown of glycogen (Khan and Pessin, 2002). In skeletal muscle and adipose tissue insulin acts via the transporter GLUT-4 primarily to facilitate glucose uptake into the tissue by glycogenesis in muscle or lipogenesis in adipose tissues (Cross et al., 1997). These combined actions give insulin control over glucose transport and storage, triglyceride storage, inhibiting gluconeogenesis, glycogenolysis, glycogenesis, and hepatic ketogenesis (Kaneko, 1997). In addition, glucose may also be absorbed

independently by some select tissues, including the brain and mammary gland tissue, which use transporter GLUT-1 that does not require insulin action to function (Khan and Pessin, 2002).

#### *1.3.1.1 Insulin Sensitivity*

The inability of circulating plasma insulin to act as a modulator for glucose transport across the cell membranes of insulin dependent tissues is known as decreased insulin sensitivity (Defronzo et al., 1979). This is a physiological state in which a normal concentration of insulin does not produce a normal physiological response (Kahn, 1978). The cause of decreased insulin sensitivity may occur at different stages of insulin action: 1) before insulin binds to receptors from under production of insulin in the pancreas or increased degradation of insulin by the liver and kidneys, 2) at the receptor level from a decrease in binding affinity or reduced number of receptors, or 3) due to problems with the signal transduction within a cell (Kahn, 1978). This is a serious metabolic disorder that can lead to many metabolic problems including hyperglycemia, hyperinsulinemia, and glucosuria which compromise animal energy metabolism, health, production, and growth (Hayirli, 2006).

#### *1.3.1.2 Methods of Assessing Insulin Sensitivity*

There have been various methods developed to assess the extent of insulin sensitivity. The simplest and most common way to characterize insulin sensitivity is by looking at glucose to insulin ratios either pre- and/or postprandial and to follow these ratios with age. If the ratio of insulin to glucose is large or increasing this could indicate there is inefficient insulin action. Another test includes following glucose and insulin over a meal or an oral tolerance test (OGTT), which consists of ingesting a liquid meal with a high glucose concentration and measuring the

plasma insulin and glucose responses (Bergman, 1989). This test is very common in humans but can be confounded by gastric emptying rates and the rate of absorption in the small intestine (Bergman, 1989; Fraser et al., 1990).

Due to its non-invasive methodology and ability to yield good results, the intravenous glucose tolerance test (GTT) is currently the most popular test used by the dairy industry. A GTT consists of infusing glucose into the bloodstream to elicit an insulin response to hyperglycemia, followed by consecutive blood sample collection to plot the curve of insulin response (Bergman, 1989). This test measures the concentration of insulin required to maintain glucose homeostasis usually over the course of an hour, an elevated concentration of insulin indicates poor insulin sensitivity. While a GTT is the most popular choice, results may be influenced by the metabolic and physiologic state of the animal, as well as the rate of glucose utilization and production (Hayirli et al., 2001). For example, lactating dairy cows draw large quantities of glucose from the blood independent of insulin action for milk production, thus differentiating between insulin independent or dependent glucose utilization during a GTT for this physiological state would be difficult. In addition, glucose absorption from the small intestine in a post-absorptive state is dependent on gastric emptying as well as insulin secretion associated with a meal; to differentiate between insulin production and glucose utilization in response to a GTT or digestion would also be challenging.

In contrast, the insulin tolerance test (ITT) involves an infusion of insulin instead of glucose (Kaneko, 1997) and measures the animal's response to insulin-induced hypoglycemia. It is considered the least favorable insulin sensitivity test as it can be extremely dangerous to induce a hypoglycemic state in an organism. Insulin sensitivity is characterized by plasma

glucose concentrations failing to decrease below 50% of the basal concentrations or requiring more than 30 minutes to reach the maximum hypoglycemic concentration (Kaneko, 1997).

The final type of tolerance test is a combination of the glucose tolerance and insulin tolerance tests called the frequently sampled intravenous glucose tolerance test (FSIGT). The FSIGT consists of a glucose infusion followed by an insulin infusion roughly 20 minutes later (Bergman, 1989). When concentrations during this test are modeled using the minimal model computer program (MINMOD), which assesses multiple parameters for insulin and glucose concentrations during a FSIGT test, insulin sensitivity can be assessed (Bergman, 1989).

Alternative methods to measure the pancreatic β cell response to glucose and the sensitivity of insulin-dependent tissues to insulin action include several clamping methods (Defronzo et al., 1979; Bergman, 1989). The hyperinsulinemic-euglycemic clamp is considered the gold standard for measuring insulin sensitivity and consists of a dual infusion of both insulin and glucose to maintain a steady state of euglycemia under an imposed state of hyperinsulinemia. Decreased insulin sensitivity occurs when low concentrations of glucose are needed to maintain euglycemia indicating impaired glucose utilization or clearance and regulation of hepatic gluconeogenesis (Defronzo et al., 1979). A hyperglycemic clamp is another method which consists of infusing glucose to induce a constant state of hyperglycemia and measuring the rate of glucose utilization, where a reduced rate of utilization and elevated insulin concentrations indicate decreased insulin sensitivity (Defronzo et al., 1979). Both of these clamping methods require a minimal overnight fasting to prevent interference from post-absorptive digestion on insulin and glucose concentrations measured during the tests.

### 1.3.2 Glucose, Insulin, and Gastric Emptying

In relation to pancreatic insulin and glucagon action on glucose homeostasis, the rate at which digesta containing nutrients, such as glucose, empty into the small intestine plays a pivotal role in the regulation of plasma glucose concentrations (Aronoff et al., 2004; Tong and D'Alessio, 2014). In monogastric animals, a small delay in gastric emptying rate can influence postprandial glycaemia by reducing glucose appearance in the small intestine and therefore absorption into the blood (Fraser et al., 1990; Tong and D'Alessio, 2014). Young dairy calves do not yet have a functioning rumen, milk passes directly into the abomasum which behaves similar to a monogastric stomach via the reticular groove (Schaer et al., 2005). As a result, preweaning calves are what is called a pseudo-monogastric animal and abomasal emptying can be regarded as gastric empting. The volume of a fluid meal, the caloric content, the protein and fat content, the osmolarity, gastric hormones, as well as the duodenal pH are all factors that determine the rate of gastric emptying in calves (Sen et al., 2006). Studies have shown a meal with a high osmolarity and glucose concentration, such as milk, may lead to a lower gastric emptying rate (Sen et al., 2006). Gastric emptying rate is then highly related to insulin action and a crucial component for glucose metabolism.

#### *1.3.3.2 Methods of Assessing Gastric Emptying*

Gastric emptying can be investigated in a variety of ways; in dairy calves the most popular and least invasive methods are serial radiography of radiopaque meals (Ellingsen et al., 2016), nuclear scintigraphy (Marshall et al., 2004), ultrasonography (Wittek et al., 2005; Labussière et al., 2014), and using acetaminophen (Marshall et al., 2004; Schaer et al., 2005; Constable et al., 2009; Labussière et al., 2014). Of these methods, using acetaminophen as a

blood marker for gastric emptying is becoming one of the most popular methods in calves. When given orally, the concentration of acetaminophen in blood serum is directly dependent on the rate of gastric emptying (Marshall et al., 2004); acetaminophen is also poorly absorbed in the stomach, rapidly absorbed in the small intestine, and is easily metabolized in the liver and excreted in urine (Schaer et al., 2005). Acetaminophen also does not interfere with MR clotting factors (Constable et al., 2009) and has recently been successfully modeled with glucose and insulin appearance in blood when feeding a milk replacer meal in dairy calves (Stahel et al., 2016). These components make acetaminophen an ideal biomarker to quantify the influence of different milk or MR planes of nutrition and meal sizes on gastric emptying, and therefore glucose metabolism in calves.

### 1.4 Plane of Nutrition and Glucose Metabolism in Dairy Calves

To begin to evaluate glucose metabolism in dairy calves we must first acknowledge that calves do not have a functioning rumen during the first weeks of life and are therefore a "pseudo-monogastric" animal. Like monogastric species, pre-weaning calves' primary energy substrate is glucose, which is derived from lactose consumed from milk or MR until the calf begins consuming solid feed and fermenting volatile fatty acids (VFA) in the rumen (Drackley, 2008; Benschop and Cant, 2009). Endocrine regulation of glucose in young calves is one of the main mechanisms behind maintaining glucose homeostasis. This is done through adequate glucose absorption and utilization, storage as glycogen, and hepatic gluconeogenesis. In young pre-weaned ruminants, insulin is the primary hormone involved in glucose homeostasis and its secretion is stimulated by feed intake. Its response has been shown to vary when calves are fed different planes of nutrition at different ages indicating there is a strong relationship between calf

plane of nutrition and glucose metabolism (Hostettler-Allen et al., 1994; Hugi and Blum, 1997; Bach et al., 2013; Yunta et al., 2015).

### 1.4.1 Veal Calf Nutrition and Insulin Sensitivity

Veal calves are fed elevated MR diets with a high fat and lactose content until slaughter (18 – 35 weeks) to promote a high energy intake, high growth rates, and pale meat (Doppenberg and Palmquist, 1991). This type of feeding regimen, consisting of a high daily milk volume and low feeding frequency of twice a day for a prolonged period of time, is associated with the first reported incidence of decreased insulin sensitivity in calves. Towards the end of their growing periods, veal calves often experience hyperglycemia, hyperinsulinemia, glucosuria, and a decrease in insulin sensitivity which indicates an inefficient utilization of glucose (Doppenberg and Palmquist, 1991; Palmquist et al., 1992; Hostettler-Allen et al., 1994; Hugi et al., 1997b; c, 1998a; Kaufhold et al., 2000; Vicari et al., 2008). It is commonly believed that the cause of this disruption in glucose metabolism and homeostasis is the high daily lactose levels of MR coupled with a low feeding frequency which results in large meal sizes and increased postprandial blood glucose and insulin concentrations.

Evidence of reduced insulin sensitivity first came from studies conducted by Doppenberg and Palmquist (1991 and Palmquist et al., 1992) who compared glucose and insulin concentrations in both veal and ruminating calves. Holstein-Friesian bull calves were allocated to two treatments, with ruminating calves weaned at 6 weeks of age receiving 3% or 10% fat from a calf starter and grower and veal calves receiving 10% or 18% fat from a MR diet. Calves plasma insulin and glucose concentrations were assessed and calves were given a GTT at 8 and 16 weeks of age. They found basal concentrations of glucose and insulin were higher and clearance

of glucose was reduced in veal calves compared to ruminating calves. They connected these findings to diet impaired glucose utilization as the veal calves consumed a high daily content of lactose (>500 g/kg) per meal.

Soon after a comprehensive study was conducted by Hostettler-Allen et al., (1994) looking at veal calves 5 to 16 weeks of age fed MR twice daily to a level of 1.3 – 1.4 kg ADG. This study used multiple methods to evaluate insulin sensitivity including a hyperinsulinemic-euglycemic clamp, a hyperglycemic clamp, an OGTT, an intravenous insulin injection, a GTT pre- and postprandial, and postprandial insulin and glucose concentrations to assess insulin sensitivity. The study's euglycemic-hyperinsulinemic and hyperglycemic clamps were performed on calves twice at a body weight of 161 ± 3 kg and 181 ± 3 kg respectively, both demonstrated a state of reduced tissue sensitivity to insulin action. This was further demonstrated by elevated postprandial insulin and glucose concentrations which continued to increase with age throughout the trial and were often in an abnormal range for insulin (~1400 mU/L) and above the renal threshold for glucose (8.4 mmol/L) resulting in glucosuria. The high glucose concentrations postprandial were also minimally influenced by a postprandial insulin injection at a body weight of 141 ± 3 kg demonstrating reduced insulin action. The GTT conducted at a body weight of 131 ± 2 kg postprandial had exaggerated insulin responses compared to the pre-prandial GTT and glucose clearance was decreased. In addition, compared to studies conducted in conventionally fed ruminating calves, veal calves had higher concentrations of basal glucose. This study demonstrated that older calves fed high daily volumes of milk at a low feeding frequency had decreased insulin sensitivity.

Studies conducted by Hugi et al., (1997a; b; c, 1998a) examined plasma insulin and glucose in veal calves of a similar age, ADG (1.4 kg/day), and BW (70 – 200 kg) and also when

offering increased amounts of lactose (Hugi et al., 1997b, 1998a). These studies used methods including a glucose isotope infusion postprandial, an OGTT, a GTT and an ITT in both the pre- and postprandial states, and an euglycemic-hyperinsulinemic and hyperglycemic clamp. Calves in these studies demonstrated increased concentrations of both insulin, glucose, and insulin to glucose ratios with age resulting in chronic postprandial hyperglycemia and hyperinsulinemia which carried over to disturb glucose homeostasis in the unfed state of calves (Hugi et al., 1997b; a). Hyperglycemia was also increased when feeding more lactose but hyperinsulinemia was not affected by increasing lactose (Hugi et al., 1997b). These studies illustrate prolonged exposure to high daily volumes of milk replacer and large meal sizes decrease the calf's ability to cope with high oral glucose loads. They also reported a decrease in the number of insulin receptors, but not their affinity, in the soleus muscle of calves which during the growing period uses more glucose than all other organs (Hugi et al., 1998a). This is likely attributed to continued exposure to high insulin concentrations as calves age, triggering a down regulation of receptors which contributed to the reduced insulin dependent glucose utilization towards the end of the growth period.

One method proposed for improving glucose tolerance and preventing reduced insulin sensitivity with age in older veal calves is to increase feeding frequency. A study by Kaufhold et al., (2000) tested how metabolic and endocrine parameters could differ in older veal calves (6 – 16 weeks of age) fed six times a day over a 16 hour period by an automatic feeder or twice daily at fixed times by bucket. They found that feeding six times daily with the automatic feeder decreased plasma insulin and glucagon when compared to twice a day feeding by bucket. They also observed in calves fed twice a day a large increase in postprandial plasma glucose that instigated hyperinsulinemia which was not seen in calves fed the same amount of milk six times a day. Calves fed more frequently required lower levels of insulin to establish euglycemia and

the authors suggested that this may indicate that the calves had improved sensitivity to insulin when compared to calves fed twice a day. These findings were also confirmed by Vicari et al., (2008) who fed veal calves 10 to 22 weeks of age once, twice, or four times daily at a high feeding level of 2.5 times the metabolizable energy requirement for maintenance or a low feeding level of 1.5 times. They looked at postprandial glucose and insulin responses to a meal and found hyperinsulinemia in veal calves could be reduced by feeding more frequently and also at lower milk feeding levels. This study also concluded that veal calves fed more frequently may have an improved glucose homeostasis and anabolic capacity allowing for more efficient protein deposition (Van den Borne et al., 2006). Overall previous literature suggests that when calves are fed high planes of milk such as in veal at low feeding frequencies for a prolonged period of time there are complications associated with insulin sensitivity that arise such as hyperinsulinemia, hyperglycemia, glucosuria, and reduced muscle receptors which can negatively affect glucose metabolism.

### 1.4.2 Ruminating Calves and Glucose Metabolism

The plane of nutrition offered to ruminating calves and replacement heifers is significantly different from diets offered to veal calves. Calves fed conventionally with low milk or milk replacer intake and high starter consumption do not show decreased insulin sensitivity as seen in milk fed veal calves. In contrast, glucose and insulin concentrations decrease with age and increased starter consumption (Daniels et al., 1974; Depew et al., 1998; Bunting et al., 2000; Benschop and Cant, 2009). As calves consume solid feed and begin transitioning to becoming a functioning ruminant there are many changes that occur for energy metabolism. These changes encompass a shift from glucose absorbed from the small intestine as a primary energy source to

VFA from the rumen, which are short chain fatty acids (SCFA) produced by microbial fermentation of solid feed in the rumen (Baldwin et al., 2004). There are three main VFA used for energy including acetate, butyrate, and propionate which is converted to glucose in the liver via gluconeogenesis (McDonald et al., 2011). The microbial processes involved with producing VFA utilize dietary carbohydrates and as a result a very low amount of glucose reaches the small intestine, while carbohydrates ingested with milk or milk replacer flow directly through the abomasum to the small intestine (Huntington et al., 2006). Absorption of VFA from the rumen also provides energy to tissues at a much slower rate than rapidly absorbed glucose in the small intestine. Therefore, in addition to plane of milk, there may also be a strong relationship between glucose concentrations and metabolism with solid feed intake and rumen development in calves.

Feeding an elevated plane of nutrition of 20% birth weight (~8 L/day) is associated with feeding large milk meal sizes (Ellingsen et al., 2016) and reduced solid feed intake before weaning (Jasper and Weary, 2002). There is concern that the elevated feeding regimen when feeding twice daily will generate a similar decrease in insulin sensitivity to that observed in veal calves. A study by Bach et al. (2013) specifically investigated the influence of an elevated plane of nutrition (8 L/day) compared to a low plane of nutrition (4 L/day) on insulin responses to high plasma glucose in eight male Holstein calves from 2 – 10 weeks of age fed MR twice daily. On weeks 3, 5, and 8 of age calves were given a GTT 4 hours after the morning MR. This study found that all animals had a similar increase in plasma glucose levels and had similar clearance rates which indicated that all animals were able to control glycaemia. However, calves fed an elevated plane of nutrition needed significantly more insulin to control the suddenly high plasma glucose. In addition, the concentration of insulin needed to maintain glycaemia increased with age, which was not the case for the lower plane of nutrition. Calves fed 8 L/day also had a higher

maximum insulin level above the baseline measurements than low plane calves. Therefore, as calves aged and had longer exposure to large milk meal sizes (4 L MR fed twice a day) there seemed to be a decline in insulin sensitivity compared to calves fed smaller (2 L) MR meals. As a result, the insulin to glucose ratio in intensively fed calves was 3- fold greater. This was the first study to demonstrate that feeding 8 L/day in two meals per day during the pre-weaning period could disrupt insulin and glucose kinetics in a similar fashion to studies conducted in veal calves.

Currently it is unclear how feeding an elevated plane of milk pre-weaning influences glucose metabolism post-weaning when calves have transitioned to utilizing VFA from the rumen, not glucose absorbed from the small intestine, as the primary energy source. Only one study by Yunta et al., (2015) has investigated this concept where they conducted a GTT 4 hours postprandial on 42, 86, and 300 days of life in replacement heifers fed 2, 3, or 4 L meals of MR twice a day and weaned on 63 days of life. Calves fed 8 L/day were of younger age at first breeding, had increased serum insulin to glucose ratios, and impaired insulin sensitivity pre-weaning. However, insulin sensitivity was increased in these calves post-weaning at 300 days of life compared to calves fed lower planes of pre-weaning nutrition. This could indicate that there is no carry-over effect of decreased insulin sensitivity post-weaning when feeding an elevated plane of milk (8 L MR/day) twice a day, and warrants further investigation. Overall, studies so far have observed reduced insulin sensitivity pre-weaning when feeding intensively which may indicate poor glucose utilization and appears to be directly related to plane of nutrition and feeding practices where feeding large milk meal sizes at a low feeding frequency promotes reduced insulin sensitivity.

## 1.5 Knowledge Gap

Feeding calves more milk has been shown to have many production and developmental benefits. The challenge now is finding the best ways to feed more milk without compromising glucose metabolism and homeostasis in dairy calves. There are many factors that influence glucose and insulin differences in calves, two of the biggest being plane of nutrition and feeding frequency. For example calves fed conventionally and raised for breeding and milk production have a gradual decrease in plasma glucose as they age (Depew et al., 1998; Bunting et al., 2000; Hugi and Blum, 1997). In contrast, veal calves (Palmquist et al., 1992; Hostettler-Allen et al., 1994; Hugi et al., 1998a) or calves fed an elevated plane of milk (Bach et al., 2013; Yunta et al., 2015) at a low feeding frequency can develop decreased insulin sensitivity, hyperglycemia, and glucosuria. This indicates that there is a relationship between meal types, meal size, and feeding frequency but what is currently unknown is how that influences the metabolic capabilities of dairy calves pre- and post-weaning. The aim of this thesis is then to address the knowledge gap that exists regarding how feeding an elevated plane of milk pre-weaning can influence glucose and insulin kinetics in calves offered solid feed pre-weaning and post-weaning.

## 1.6 Objectives and Hypotheses

To address the above mentioned knowledge gap two studies were conducted. The objective of the first study was to investigate how feeding low and high planes of pre-weaning milk twice daily could influence abomasal emptying pre-weaning and insulin sensitivity pre- and post- weaning, when calves were offered solid feed and weaned. The hypotheses were: 1) Calves fed a high plane of nutrition would experience a decrease in insulin sensitivity pre-weaning but there would be no carry-over effect in the post-weaning period, and 2) abomasal emptying would

be delayed in calves fed larger meal sizes as a means to control glucose appearance in the small intestine and regulate glucose metabolism. The objective of the second study was to investigate the effect of increasing feeding frequency when calves were offered an elevated plane of milk with solid feed and weaned. The hypothesis was that by increasing feeding frequency, and therefore reducing meal size and spreading out the nutrient load evenly throughout the day, there would be improved glucose tolerance.

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## 2.0 Chapter 2: Effect of milk replacer intake and age on glucose and insulin kinetics and abomasal emptying in female Holstein Friesian dairy calves fed twice daily

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### 2.1 Abstract

The objective of this study was to investigate how pre-weaning plane of milk replacer intake and age can impact insulin and glucose kinetics, and abomasal emptying rate, in dairy calves fed twice a day. Twelve female Holstein Friesian calves were blocked by cow parity, paired by colostrum origin (same colostrum from one dam) and were randomly assigned to a high plane of milk replacer intake (HIGH; 8 L/d, 1.2 kg milk replacer/d; n = 6) or a low plane of milk replacer intake (LOW; 4 L/d, 0.6 kg milk replacer/d; n = 6). All calves received 4 L of colostrum over two meals (1 and 6 h after birth), then directly transferred to their assigned feeding plans until they were stepped-down from milk by 50% during wk 7 and weaned on wk 8. Milk replacer (24%cCrude protein, 18% crude fat) was fed at 150 g/L twice daily (0700h and 1700h) and all calves had *ad libitum* access to pelleted calf starter, chopped wheat straw, and water. Jugular catheters were placed in all calves at 4, 7 and 10 wks of age. Postprandial plasma glucose and insulin were measured for 420 min and abomasal emptying was determined using acetaminophen (supplied with the meal) the day following jugular catheter placement on wks 4 and 7. On the day following postprandial and emptying measurements and after jugular catheter

placement on wk 10, a glucose tolerance test (GTT) was conducted via the jugular catheter. At 4 and 7 wks of age, the rate constant (%/h) for abomasal emptying of the meal was lower in HIGH calves (0.21 ± 0.02 on wk 4; 0.27 ± 0.02 on wk 7) compared to LOW (0.34 ± 0.02 on wk 4; 0.47 ± 0.02 on wk 7). The postprandial plasma insulin area under the curve over 420 minutes was greater in HIGH calves (18,443 ± 7,329 μU/ml; LOW = 5,834 ± 739 μU/ml) compared to LOW. There were no differences in GTT kinetics between the HIGH and LOW dairy calves at 4, 7, or 10 wks of age. The findings from this study suggest that feeding dairy calves an elevated plane of nutrition in two meals of milk replacer per day does not decrease insulin sensitivity.

## 2.2 Introduction

Calf management programs have traditionally restricted milk or milk replacer (MR) fed to calves (~4 L or 10% birth weight) to promote starter consumption, rumen development, and to decrease replacement heifer raising costs (Khan et al., 2011). Current calf research is focusing on feeding elevated planes of nutrition to replacement heifers by increasing the volume of milk or MR to approximately double (~8L or 20% birth weight) the amount that traditionally has been fed. Calves raised on these programs have been shown to have increased average daily gain (ADG), earlier onset of puberty, as well as the potential for higher milk production sustained over multiple lactations (Soberon et al., 2012). In addition to improvements in production, feeding elevated planes have also been shown to decrease signs of hunger, which may improve animal welfare (Miller-Cushon and DeVries, 2015). These elevated feeding programs are often offered in a twice a day feeding plan on commercial dairy farms, which is in contrast to feeding patterns observed when the calf is allowed to suckle from the dam (Reinhardt and Reinhardt, 1981) or from an automated feeder (Berends et al., 2014), where they are consuming 7-10 meals

throughout the day. Feeding similar large volumes, but then twice daily, may have longstanding effects on digestion and metabolism.

One of the most common concerns when feeding larger volumes of milk to young calves at a low feeding frequency is the decrease of insulin sensitivity (Bach et al., 2013). Despite a generally delicate physiological regulation of glucose homeostasis, veal calves that are provided with large milk volumes up until 6 months of age often express problems characterized by postprandial hyperglycaemia, hyperinsulinemia, and glucosuria (Hostettler-Allen et al., 1994; Hugi et al., 1998a; Vicari et al., 2008). Impaired insulin sensitivity could lead to a reduced efficiency of protein and energy utilization (Van den Borne et al., 2006) and may predispose to metabolic diseases later in life (Quigley et al., 2006; Kaufhold et al., 2000). It has recently been shown that insulin sensitivity is affected by plane of MR intake in dairy calves, when calves are fed large meals twice daily during the preweaning period (Bach et al., 2013; Yunta et al., 2015). However, it is unclear to whether this can persist post-weaning when calves are transitioned to only solid feed.

Although insulin production in the pancreas and cellular glucose uptake have received most of the attention for mechanisms that control blood glucose, the rate of nutrient delivery to the lower gut after a meal may play a pivotal role in the regulation of blood glucose (Tong and D'Alessio, 2014). In calves the rate of abomasal emptying may control nutrient delivery to the small intestine (Schaer et al., 2005; Sen et al., 2006; Constable et al., 2009). Meals with high glucose concentrations have been shown to delay abomasal emptying when compared to electrolyte solutions which can lead to slower nutrient delivery (Wittek et al., 2005; Sen et al., 2006). When larger meals are fed, abomasal emptying may be slowed down as a means to decrease the rate of nutrient delivery that can help reduce dramatic increases in blood glucose

concentrations (Coradini et al., 2015). Therefore, abomasal emptying may be an important factor affecting plasma glucose levels and insulin action when feeding larger meals. However, effect of plane of nutrition on abomasal emptying in this scenario has not yet been investigated.

The objective of this study was to investigate the effect of feeding plane of milk replacer, when provided twice daily, on abomasal emptying pre-weaning, as well as insulin and glucose kinetics pre-weaning (wk 4 and 7 of age) and post-weaning (wk 10 of age). Our hypothesis was that an elevated plane of MR intake pre-weaning, fed two times a day, would decrease insulin sensitivity, but that there would be no carryover effects observed after weaning when calves were no longer consuming large milk meals. In addition, we expected that larger meal sizes of milk would result in a delayed abomasal emptying rate as an adaptive response to minimize large fluctuations in blood glucose levels.

## 2.3 Materials and methods

### *2.3.1 Animals*

Twelve Holstein Friesian female calves from Trouw Nutrition Ruminant Research facility (Boxmeer, The Netherlands) born between October 2014 and January 2015 were used for this study. They were housed in individual hutches (1.07 x 1.60 m) bedded with wheat straw and outfitted with a metal roof attachment to protect feed and water from precipitation. Procedures complied with the Dutch Law on Experimental Animals, and the ETS123 (Council of Europe 1985 and the 86/609/EEC Directive) and were approved by the Animal Care and Use Committee from Utrecht University.

*2.3.2 Feed Intakes and Body Weights*

All calves received 4 L of pasteurized colostrum that had been frozen and reheated from a bottle in two feedings of 2 L each at 1 and 6 h after birth. Calves were blocked in pairs by cow parity and colostrum origin (same colostrum for each pair of calves). Calves were randomly allocated to one of two treatments: 8 L MR/d for an elevated plane of MR intake (HIGH) and 4 L MR/d for the low plane of MR intake (LOW). Calves were fed MR (150 g/L; 24% crude protein, 18% crude fat, and 45% lactose; Sloten B.V. Deventer, The Netherlands) twice daily at 0700 and 1700 h via nipple buckets. The raw material inclusion of the calf MR was 50% skim milk powder, 20% sweet whey powder, 17% vegetable oils, 11% delactosed whey powder and 2% premix.

Low plane calves immediately began their treatment from birth after two colostrum feedings (Figure 2-1). Milk replacer volumes fed to calves in the HIGH treatment were gradually stepped-up in the first wk of life, consisting of two daily meals of 2.5 L for d 2 and 3, 3 L for d 4 and 5, and 3.5 L for d 6 and 7 (Figure 2-1). From d 8 onwards, calves were fed 4 L twice daily (8 L MR/d). Calves had *ad libitum* access to water, calf starter (18.2% crude protein, 11.2% crude fibre, 2.2 % crude fat, 3 mm pellet; AgruniekRijnvallei, Wageningen, The Netherlands), and wheat straw (3 cm chop length), each provided in separate buckets at the front of the hutch during the same time as morning milk feeding (0700h). Individual intakes of all feeds were recorded daily and weaning entailed a step-down reduction of the amount of MR in each meal by 50% on wk 7 and finished by wk 8. Body weights (BW) were recorded at birth, d 2, 7, 14, 21, 28, 35, 42, 49, 56, 63, 70 and/or on the day of jugular catheter placement. All weights were measured 2 to 4 h after the morning meal.

*2.3.3 Blood Samples*

Jugular catheters (Intraflon 2 13G, Ecouen France) were placed on wks 4, 7 and 10 of age and remained for ~48 h. The catheters were flushed with 2 ml heparinized saline (2% solution) before and after sample collection. Blood samples were collected using 20-ml syringes and then allocated into an EDTA Vacutainer™ for insulin and a sodium fluoride Vacutainer ™ (Becton Dickinson (BD), Franklin Lakes, NJ, USA) that contained a glycolysis inhibitor to quantify glucose. All blood samples were centrifuged immediately at 2800 x *g* for 30 min at 4ºC, and 1.5 ml of plasma was pipetted into 2 ml cryotubes and stored immediately at -20 °C.

*2.3.4 Postprandial Glucose, Insulin, and Abomasal Emptying*

To determine postprandial glucose and insulin patterns and abomasal emptying, 12 blood samples were collected at -30, 30, 60, 90, 120, 150, 180, 210, 240, 300, 360, and 420 min relative to feeding of the morning MR meal, 1 d after catheter placement on wks 4 and 7. The meal of 2 or 4 L for LOW or HIGH treatments, respectively, was consumed within 5 min and contained acetaminophen at 150 mg/kg $BW^{0.75}$ to estimate gastric emptying from kinetics of acetaminophen appearance in plasma. The method and dose for using acetaminophen in blood as a marker for gastric emptying is well established in calves (Schaer et al., 2005) and in many other species (Clements et al., 1978). During sampling, calves had no access to calf starter and chopped straw, but had access to water at all times. Calves did have access to their bedding however it was assumed that the intake was marginal and would not influence the precision of data collection in the study. Blood samples were analyzed for acetaminophen using the enzymatic Paracetamol (Acetaminophen) Assay Kit-K8002 (Cambridge Life Sciences Ltd, Ely, UK; validated by Berends, 2014), for glucose using the colorimetric EnzyChrom Glucose Assay Kit (BioAssay Systems, Hayward, USA; used by Zebeli et al., 2012), and for insulin using the Mercodia Bovine Insulin ELISA kit (Uppsala, Sweden; used by Bach et al., 2013) in the Trouw Nutrition R&D laboratory (Boxmeer, the Netherlands). For both glucose and insulin values quantified needed a CV below 5% for acceptance except if the values were to low to be detected by the kits, in this case a CV greater than 5 was accepted.

*2.3.5 Glucose Tolerance Test*

Calves were administered an intravenous glucose tolerance test (GTT) the day after abomasal emptying was evaluated. Since acetaminophen has been shown to have no long term effect on glucose metabolism (Basu et al., 2016), it was assumed that the dose a day prior to the GTT had no marginal influence over the results. The GTT encompassed an intravenous infusion of glucose (30% glucose solution, Eurovet, Bladel, the Netherlands) at a dose of 540 mg/kg of $BW^{0.75}$ via the jugular catheter (administered in one minute). The GTT occurred during the time of the morning feeding (0700 h) at 4, 7, and 10 wk of age. The dose was based on previous studies reported in calves (Bach et al., 2013; Yunta et al., 2015), therefore renal threshold was not evaluated for the dose in this study. Blood samples were collected at -15, 0, 5, 10, 20, 30, 45, 60, 90, 120, 180, and 240 min relative to the glucose infusion. To ensure basal plasma glucose levels were achieved prior to the infusion, calves were fasted overnight (12 h) with no access to starter and chopped straw, and their morning MR was delayed until sampling was finished (4 h). During sampling, calves were restricted from access to calf starter and chopped straw, but had access to water at all times. Blood samples were analyzed for glucose and insulin as previously described.

*2.3.6 Calculations and Statistics*

Body weight, ADG, and feed intake (weekly average) data were all summarized and analyzed as one measurement per wk and also over both the pre and post-weaning periods. For glucose and insulin during the GTT and postprandial measurements, time to reach maximum concentration (Tmax), maximum concentration (Cmax), ratio of Cmax/Tmax, basal levels, and

the change in concentration (delta change) were calculated from the raw data. The positive incremental area under the curve (AUC) for glucose and insulin was evaluated using the trapezoid rule for values above baseline and was calculated over 420 min for the postprandial responses and 240 min for the GTT. The clearance rate of glucose and insulin during the GTT was calculated using the method established in Pires et al. (2007).

$$\text{Clearance rate (\%/min)} = \{(\ln[ta] - \ln[tb])/(ta - tb)\} \times 100$$

Where [ta] and [tb] is the concentration of insulin or glucose at time (ta) or (tb), respectively. Furthermore, insulin sensitivity was estimated using a simplified model described by Christoffersen et al. (2009).

To calculate gastric emptying rate, it was assumed that absorption of acetaminophen prior to the small intestine was zero, outflow from the abomasum followed first-order kinetics according to the rate constant $k_{AB}$, absorption from the small intestine into blood was instantaneous, and elimination from blood plasma followed first-order kinetics according to the rate constant $k_{el}$. Another assumption was that MR did not enter the rumen. The differential equations describing acetaminophen mass in abomasum (A) and blood (B) are

$$\frac{dA}{dt} = -k_{AB}A, \qquad [1]$$

and

$$\frac{dB}{dt} = k_{AB}A - k_{el}B, \qquad [2]$$

respectively. Integrating these equations, and assuming a volume of distribution of acetaminophen of 90% of BW as previously recorded (Rawlins et al., 1977; Forrest et al., 1982), the concentration of acetaminophen in blood plasma at time t becomes

$$cB_t = \frac{dose \cdot k_{AB}}{0.9BW(k_{AB} - k_{el})}\left(e^{-k_{el}t} - e^{-k_{AB}t}\right). \qquad [3]$$

Estimates of $k_{AB}$ and $k_{el}$ were obtained by fitting Eq 3 to observed $cB_t$ curves from each calf at 4 and 7 wk of age using PROC NLIN of SAS (SAS Institute, 2004). It is important to acknowledge that calves were drinking different meals sizes as a proportion of their BW. However, according to the law of mass action embodied in eqns 1 and 2, the abomasal emptying rate constant, $k_{AB}$, and elimination rate constant, $k_{el}$, are independent of acetaminophen dose and concentration. Neither the dose of acetaminophen nor the intake of water and DM affect the ability to estimate these parameters with precision.

To examine the effect of treatment, data were analyzed using the MIXED procedure of the statistical analysis system (SAS Institute, 2004), with observations taken at different ages taken as a repeated measure. The model included the fixed effects of treatment, age, treatment by age interaction and block. For responses within a day such as individual time points of the postprandial and GTT data, the same model was utilized to compare between treatments at specific time points. The covariance structure with the minimum values of Akaike's Information Criterion (AIC) was determined to be compound symmetry and used for all variables. A Tukey test was used to correct for multiple comparisons and all values reported are least squares means. Significance was declared when $P \leq 0.05$ and trends were declared when $P < 0.10$ but $> 0.05$.

## 2.4 Results

### *2.4.1 Body Weights and Intakes*

The BW, ADG, and feed intake results are presented in Table 2-1 and the metabolizable energy of intake for each treatment are presented in Figure 2-2. Within the first wk of life HIGH calves were consuming their full allotment of MR. The HIGH calves had a higher ADG pre-weaning and the LOW calves consumed more starter prior to weaning (no differences in straw

intake detected). The BW at weaning was greater in HIGH calves and persisted as a trend until day 70 of the experiment.

*2.4.2 Postprandial Insulin, Glucose and Gastric Emptying*

The postprandial glucose and insulin concentrations over a meal were followed for 420 min in accordance with abomasal emptying measurements and are presented in Figure 2-3 and Table 2-2. The glucose baseline in HIGH calves was 27% higher during wk 4 and 12% higher at wk 7 compared to LOW calves. There were no age effects on postprandial measurements. The Cmax and maximum change in concentration for glucose were greater (18% and 21%, respectively) in HIGH calves compared to LOW calves. In addition, the Cmax and maximum change in concentration for insulin were greater (165% and 174%) in HIGH calves compared to LOW calves. The postprandial $AUC_{420}$ for insulin was 221% greater in HIGH calves while the $AUC_{420}$ for glucose was not different between treatments. There were no differences for Tmax and Cmax/Tmax for glucose and insulin and no difference for baseline insulin levels. For abomasal emptying, it was determined using plasma acetaminophen concentrations that the HIGH calves had 40% slower gastric emptying compared to LOW calves as shown in Figure 2 E,F. The rate constant (%/h) of abomasal emptying was positively correlated ($r = 0.41$; $P = 0.04$) with glucose $AUC_{420}$ and tended to be correlated with Tmax for glucose ($r = 0.37$; $P = 0.07$). No additional correlations between emptying rate and blood glucose and insulin kinetics were uncovered in postprandial data.

*2.4.3 Glucose Tolerance Test*

The results of the glucose tolerance test are displayed in Figure 2-4 and descriptions of the curves are in Table 2-3. Blood glucose levels for the GTT reached maximum concentration within 5 to 10 min after the infusion and Cmax levels ranged between 7.4 and 14.5 mmol/L and all calves returned to basal levels within an hour of the infusion. There was no significant difference between treatment and age or across ages for plasma glucose Tmax, Cmax, and insulin sensitivity index, respectively. The plasma glucose $AUC_{240}$ had a trend ($P = 0.08$) for an interaction between treatment and age, with the HIGH having a lower $AUC_{240}$ at wk 7, and $AUC_{240}$ increasing in the LOW group from wk 4 to wk 7. There was no significant difference in the change in glucose over the infusion or the basal glucose level before each infusion. Clearance rate of blood glucose decreased with age, with calves 10 wks of age having a slower clearance rate than 4-wk-old calves.

Insulin response to the GTT was highly variable across calves, with Cmax ranging from 12 - 129 μU/mL and Tmax ranging from 10 to 240 min. Neither Cmax nor Tmax was significantly different between treatment groups. $AUC_{240}$ ranged from 52 to 4064 μU/mL and was not different between treatments. The basal plasma insulin levels, the changes in insulin, and the insulin clearance rates were similar between treatment groups which may be due to the large variability in insulin sensitivity between calves. Overall there was no difference in blood insulin levels despite a large degree of variable insulin responses to a GTT.

## 2.5 Discussion

In recent years there has been a movement in the dairy industry to feed an elevated plane of milk (≥ 8 L MR/d) to dairy calves pre-weaning in order to improve growth and lifetime milk

production (Bar-Peled et al., 1997; Jasper and Weary, 2002; Soberon et al., 2012). Dairy farms typically feed calves manually twice per day (Vasseur et al., 2010), which dramatically contrasts the upwards of 7 to 10 meals per day that occur when a calf is left to suckle the dam (Reinhardt and Reinhardt, 1981) or from an automated feeder (Berends et al., 2014). Early-life feeding schemes have been shown to have a short- and long-term impact on insulin sensitivity in humans and rodents (Duque-Guimarães and Ozanne, 2013). However, in dairy calves the impact of common feeding strategies practiced in our industry on glucose metabolism pre- and post-weaning are largely undescribed. Thus, the objective of this study was to investigate the impact of elevated pre-weaning planes of milk offered in two meals a day on postprandial insulin and glucose kinetics and abomasal emptying pre-weaning, as well as insulin responsiveness to a GTT pre- and post-weaning.

In the current study, glucose metabolism was examined using postprandial measurements and a GTT simultaneously during sampling wks. In accordance with earlier studies (Kaufhold et al., 2000; Terré et al., 2009; Yunta et al., 2015), basal glucose and overall glucose concentrations were higher in calves fed an elevated plane of MR intake during postprandial measurements. This finding was expected as HIGH calves were fed a twofold greater level of dietary lactose, which has been shown to increase blood glucose levels (Palmquist et al., 1992). There was no effect of age on basal glucose which contrasts previous studies in veal calves showing that extended feeding of large volumes of milk for several months led to hyperglycemia and glucosuria (Hostettler-Allen et al., 1994; Hugi et al., 1997c, 1998a). There were, however, key discrepancies between these studies and the current study. In these studies (Hostettler-Allen et al., 1994; Hugi et al., 1997, 1998) the total feeding level of MR was greater than 2 kg/d (DM basis) with expected gains of greater than 1.4 kg/d (2x the HIGH calves and 4x the LOW calves

in this experiment). Furthermore, the majority of the other studies were conducted after the first two months of life, which constitutes a different experimental model compared to the current study. It has been well documented that insulin resistance develops with age when calves are maintained primarily on a diet consisting of milk for more than two months of life. This prolonged feeding of a predominantly milk diet, high in lactose in older calves is causally associated with reduced insulin receptor number in skeletal muscle as the calf ages past two months (Hugi et al., 1998). After two months of age specific gene expression changes occur in the rumen and liver to support the use of energy derived from ruminal microbial fermentation (Baldwin et al., 2004). These may be indicators that the calf may have a fixed time to utilize glucose derived from lactose in milk effectively.

Although the highest postprandial blood glucose and insulin concentrations, as well as the highest glucose delta (baseline to maximum concentration), were found in the calves fed an elevated plane of MR, the levels always returned to baseline by 360 min postprandial. No differences for the $AUC_{420}$ of glucose between treatments were found which suggests that the calves fed larger meals can regulate blood glucose concentrations as there was no evidence of hyperglycemia in this study. There was greater $AUC_{420}$ for insulin and greater variation in the concentration of insulin for HIGH calves compared to LOW calves, especially during wk 4 (Figure 2C) corresponding to differences in lactose load from the meal. Grutter and Blum (1991) showed that the mechanisms behind insulin secretion in the pancreas in response to blood glucose levels during the neonatal period are not fully developed until months after birth, which might explain the large variability in insulin concentration in HIGH and LOW calves.

The homeostasis of blood glucose relies on the interplay between pancreatic insulin and glucagon production, as well as gluconeogenesis and glucose utilization (Jones et al., 1995).

However, the role of gastric motility and emptying is often overlooked despite having a significant impact on the rate of glucose appearance in the bloodstream. Pre-weaning calves do not yet have a functional rumen and milk passes directly into the abomasum, which is similar to a simple stomach, effectively making the calf a pseudo-monogastric animal. The volume of a fluid meal, its caloric content, composition, and osmolality, as well as the abomasal and duodenal pH can alter the rate of abomasal emptying in a calf (Sen et al., 2006). In addition, the phenomenon of ruminal drinking where milk may leak into the rumen is commonly reported in veal calves, and could therefore have substantial influence on gastric emptying (Labussiere et al., 2014). However, it has recently been shown that calves between the ages of 19-23 days of life were able consume larger meal volumes compared to the current study without evidence of ruminal drinking before emptying into the small intestine (Ellingsen et al., 2016), therefore ruminal drinking was excluded as an influential factor for abomasal emptying rates.

In monogastrics it has been shown that minor delays in gastric emptying rate have a notable effect on the glycemic response after a meal (Tong and D'Alessio, 2014). For example, when gastric emptying is delayed, there is a small amount of glucose entering the blood as a means of reducing large spikes in blood glucose postprandially (Fraser et al., 1990). In the current study, the abomasal emptying rate for the entire meal was shown to be slower for the HIGH calves compared to LOW, however due to higher MR intake there was probably more lactose flowing into the small intestine per one unit of time in the HIGH calves. This suggests that in calves fed HIGH the emptying rate of the meal from the abomasum to the lower gut may be altered as a means of stabilizing blood metabolite levels. In accordance with our results, high glucose concentration and osmolality in a meal have been shown to lead to a slower gastric emptying rate in calves (Schaer et al., 2005; Sen et al., 2006). Traditionally, a reduced abomasal

emptying rate was considered unfavourable, as it was associated with reduced abomasal pH and ulcers (Ahmed et al., 2002). However, when large meals are fed twice daily, a delayed abomasal emptying may positively influence blood metabolite synchrony. It is well established in multiple species that larger meals are emptied more slowly from the stomach (Delgado-Aros et al., 2004; Jackson et al., 2004; Métayer et al., 2004). To our knowledge, we are the first to demonstrate this phenomenon in calves. From a mechanistic standpoint, the delay in meal empting may be related to detection of high nutrient concentrations in the ileum and large intestine, leading to glucagon-like peptide-1 (GLP-1) release and a slowdown of gastric emptying (Tong and D'Alessio, 2014).

Treatment differences were noted for postprandial glucose and insulin kinetics. However, no differences between HIGH and LOW calves were detected by a GTT pre- or post-weaning. These results suggest that feeding plane has little impact on insulin sensitivity and contrasts recent studies using a GTT to assess insulin sensitivity in preweaning calves fed similar 4 L MR/d and 8 L MR/d rations (Bach et al., 2013; Yunta et al., 2015). In these studies, calves had similar glucose AUC across treatment groups, indicating a tight regulation of blood glucose kinetics. However, calves fed 8 L MR/d needed higher levels of insulin to control blood glycaemia during a GTT performed pre-weaning, which suggests reduced insulin sensitivity.

These opposing results may be explained by several different factors. Firstly, the feeding regimen, including colostrum management, was implemented and controlled from the first hours of life in the current experiment as opposed to the second or third wk of life observed in the other studies (Bach et al., 2013; Yunta et al., 2015). Recent research in calves suggests that diet quantity and quality during the first days of life are positively associated with growth performance, development and health during the pre-weaning period (Blum and Hammon, 1999; de Passillé et al., 2014). It may be that metabolic programming occurs from dietary factors

present in the first days or wks of life (Soberon et al., 2012; Bartol et al., 2013). It may also be possible that HIGH calves had a larger window of time to adapt to larger meals, which decreased the risk of reduced insulin sensitivity compared to previous studies.

Another key difference between the current study and other studies evaluating glucose tolerance was that the GTT infusions were conducted in this study after a 12 h fast, whereas other studies conducted measurements 4 to 5 h after the morning meal (Bach et al., 2013; Yunta et al., 2015). As shown in Figure 2, the postprandial glucose and insulin concentrations took longer than four hours to return to basal levels, therefore a GTT at this time would impact the results in the GTT and be confounded with differences in abomasal emptying between treatments in the current study. In most human diabetes studies, patients are fasted overnight (~12 hours; Matsuda and DeFronzo, 1999) to establish basal glucose levels before a GTT, after which a higher basal glucose level and a high insulin response become indicators of impaired glucose tolerance (Fraser et al., 1990). When the GTT was performed pre-prandial in calves compared to postprandial, there were lower levels of glucose before and after the infusion, shorter glucose half-lives, lower insulin response levels, and Tmax for insulin was 15 and 60 min for pre- and postprandial infusions, respectively (Hostettler-Allen et al., 1994). It has been suggested that in the postprandial state, endogenous glucose production and glucose utilization are constantly being balanced, whereas in the pre-prandial or post-absorptive state, glucose is mostly utilized by insulin independent tissues (Bergman, 1989). As such, it is imperative that adequate hours fasted prior to a GTT be taken into consideration when interpreting results.

One final point to consider is that unlike most studies evaluating insulin sensitivity, we used female calves instead of bull calves (Bach et al., 2013). It has been shown in other species that mature females are less susceptible to developing decreased insulin responsiveness and

insulin sensitivity (Stubbins et al., 2012; Wintrob et al., 2014). In the study by Yunta et al. (2015), female calves fed 8 L MR/d demonstrated a moderate decrease in insulin responsiveness compared to calves fed 6 L MR/d and 4 L MR/d on day 42 but it did not show evidence of reduced insulin sensitivity post weaning, a finding which agrees with the current study. It is well characterized how specific hormones, such as leptin, can be regulated differently between pre-pubertal male and females which ultimately influences insulin sensitivity (Kennedy et al., 1996). Gender can clearly alter metabolism in early life and should be considered when interpreting calf metabolism results.

For all treatments and ages, calves in the current study were able to control glycaemia within an hour of the GTT infusion. The only age-related differences were found in LOW calves where plasma glucose $AUC_{240}$ and glucose clearance rate were reduced from 4 to 10 wk of age. This result agrees with previous reports which indicate that the ability to clear glucose decreases as the calf matures and is weaned (Palmquist et al., 1992; Yunta et al., 2015). The pre-weaning treatments caused no carry-over effects post-weaning which is in agreement with the measurements from Yunta et al. (2015), who found no treatment effect after weaning. Interestingly, the calves in that study that were fed 4, 6, and 8 L MR/d pre-weaning had an additional GTT on day 300 which noted that calves fed an elevated plane of milk intake (8 L MR/d) had greater insulin sensitivity than calves fed 4 and 6 L MR/d per day pre-weaning. These results highlight the discrepancy of information investigating pre-weaning plane of nutrition impacts on metabolism, especially post-weaning and beyond. In addition to plane of MR nutrition, factors such as the composition of the milk or MR and feeding frequency during the pre-weaning period can program metabolism later on in life (Bartol et al., 2013). More research

in this area is required for our dairy industry to properly assess and optimize the short and long-term implications of early-life nutrition on biological outcomes later in life.

## 2.6 Conclusion

In summary, these results suggest that feeding 8 L MR/d in two meals per day offers pre-weaning growth advantages compared to 4 L MR/d, but also has minimal impact on glucose metabolism and insulin sensitivity, in contrast to our first hypothesis. The calf's ability to slow down the delivery of large meals from the abomasum to the lower gut which as in agreement with our secondary hypothesis may have positively contributed to insulin sensitivity in calves fed more milk as observed from similar GTT concentrations.

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## 2.8 Tables and Figures

**Table 2-1**. Milk intake, body weight, starter intake, and average daily gain (ADG) of calves

| Item | Treatments | | SEM | *P*-value |
|---|---|---|---|---|
| | LOW | HIGH | | |
| Milk intake, (L/d; d 1 to 56) | 3.72 | 6.99 | 0.08 | <0.01 |
| Body weight (kg) | | | | |
| Initial, d 1 | 41.4 | 39.3 | 1.9 | 0.47 |
| Weaning, d 56 | 71.9 | 81.2 | 2.4 | 0.03 |
| Final, d 70 | 85.3 | 92.0 | 2.4 | 0.07 |
| Starter intake (g/d) | | | | |
| Pre-weaning (d 7 to 56) | 586.8 | 194 | 19 | <0.01 |
| Post-weaning (d 57 to 70) | 2526 | 2010 | 53 | 0.01 |
| Overall (d 7 to 70) | 1004 | 585 | 33 | <0.01 |
| ADG (kg/d) | | | | |
| Pre-weaning (d 1 to 56) | 0.61 | 0.77 | 0.04 | 0.01 |
| Post-weaning (d 57 to 70) | 0.94 | 0.79 | 0.07 | 0.27 |
| Overall (d 14 to 70) | 0.68 | 0.78 | 0.04 | 0.06 |

Milk intake, body weight, starter intake, and average daily gain (ADG) of calves fed a low plane of nutrition (LOW;4 L MR/d) and high plane of nutrition (HIGH; 8L MR/d). Data are means ± SEM, n = 6 per group.

**Table 2-2**. Effect of milk plane of nutrition on pre- and postprandial plasma glucose and insulin responses in dairy calves

| | Treatments[1] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | LOW | | HIGH | | | *P*-value[2] | | |
| Item[3] | Week 4 | Week 7 | Week 4 | Week 7 | SEM | T | W | T×W |
| Glucose Cmax (mmol/L) | 6.41 | 6.85 | 8.14 | 7.49 | 0.36 | <0.01 | 0.90 | 0.15 |
| Insulin Cmax (µU/mL) | 43.7 | 67.6 | 163 | 132 | 42.0 | 0.03 | 0.41 | 0.44 |
| Glucose Tmax (min) | 95.0 | 80.0 | 105 | 115 | 24.6 | 0.90 | 0.86 | 0.71 |
| Insulin Tmax (min) | 90.0 | 95.0 | 130 | 105 | 18.9 | 0.42 | 0.50 | 0.28 |
| Glucose Cmax/Tmax (mmol/L/min) | 0.08 | 0.09 | 0.13 | 0.11 | 0.03 | 0.65 | 0.90 | 0.57 |
| Insulin Cmax/Tmax (µU/ml/min) | 0.55 | 0.73 | 1.52 | 1.97 | 0.48 | 0.21 | 0.37 | 0.95 |
| Glucose AUC mmol/L x 420 min | 251 | 262 | 284 | 203 | 68.0 | 0.89 | 0.87 | 0.40 |
| Insulin AUC µU/ml x 420 min | 4,289 | 6,831 | 21,458 | 14,241 | 5,096 | 0.01 | 0.68 | 0.21 |
| Glucose Delta (mmol/L) | 1.84 | 2.06 | 2.33 | 2.05 | 0.32 | 0.40 | 0.99 | 0.49 |
| Insulin Delta (µU/mL) | 39.7 | 64.3 | 158.3 | 126.9 | 41.8 | 0.03 | 0.38 | 0.41 |
| Glucose Baseline (mmol/L) | 4.57 | 4.79 | 5.81 | 5.38 | 0.28 | 0.01 | 0.71 | 0.27 |
| Insulin Baseline (µU/mL) | 3.98 | 3.29 | 4.68 | 5.17 | 0.90 | 0.17 | 0.91 | 0.52 |

The effect of low plane of nutrition (LOW; 4 L MR/d) and high plane of nutrition (HIGH; 8L MR/d) on pre- and postprandial glucose and insulin responses in dairy calves.

[1]Treatments: LOW = Low plane of nutrition (4L MR/d); HIGH = High plane of nutrition (8L MR/d).

[2]Statistical comparisons: T effects = LOW vs. HIGH; W effects = week; T×W effects = treatment by time interaction.

[3]Cmax = maximum plasma concentration, Tmax = time of maximum concentration observed, AUC = area under the concentration-time curve, Delta = the maximum change from baseline.

**Table 2-3**. Effect of milk plane of nutrition on plasma glucose and insulin responses to a glucose tolerance test in dairy calves

| | Treatments[1] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | LOW | | | HIGH | | | | *P*-value[2] | | |
| Item[3] | Week 4 | Week 7 | Week 10 | Week 4 | Week 7 | Week 10 | SEM | T | W | T×W |
| Glucose Cmax (mmol/L) | 8.50 | 9.92 | 9.90 | 10.00 | 9.68 | 9.03 | 0.69 | 0.81 | 0.73 | 0.22 |
| Insulin Cmax (µU/mL) | 62.2 | 41.0 | 47.8 | 33.8 | 45.3 | 54.2 | 11.7 | 0.54 | 0.80 | 0.26 |
| Glucose Tmax (min) | 5.00 | 5.83 | 6.67 | 5.00 | 6.67 | 5.83 | 0.78 | 1.00 | 0.19 | 0.57 |
| Insulin Tmax (min) | 23.3 | 20.0 | 13.3 | 26.7 | 26.7 | 50.0 | 17.0 | 0.27 | 0.87 | 0.57 |
| Glucose Cmax/Tmax (mmol/L) | 1.72 | 1.77 | 1.68 | 2.00 | 1.62 | 1.68 | 0.20 | 0.78 | 0.61 | 0.54 |
| Insulin Cmax/Tmax (µU/mL) | 4.27 | 2.29 | 4.58 | 1.83 | 2.92 | 4.67 | 1.25 | 0.33 | 0.55 | 0.53 |
| Glucose AUC mmol/L x 240 min | 73.2 | 185 | 155 | 121 | 95.6 | 172 | 30.2 | 0.75 | 0.10 | 0.08 |
| Insulin AUC µU/ml x 240 min | 1398 | 614 | 1099 | 1129 | 810 | 1207 | 338 | 0.42 | 0.14 | 0.44 |
| Glucose Delta (mmol/L) | 4.30 | 5.43 | 5.15 | 4.88 | 4.62 | 5.10 | 0.59 | 0.85 | 0.63 | 0.50 |
| Insulin Delta (µU/mL) | 57.7 | 37.0 | 43.1 | 29.7 | 41.4 | 48.8 | 11.1 | 0.45 | 0.76 | 0.23 |
| Glucose Baseline (mmol/L) | 4.37 | 4.45 | 4.75 | 5.17 | 5.05 | 3.92 | 0.57 | 0.44 | 0.49 | 0.29 |
| Insulin Baseline (µU/mL) | 4.33 | 3.77 | 4.83 | 4.17 | 3.83 | 5.00 | 0.90 | 0.98 | 0.47 | 0.98 |
| Glucose Clearance Rate %/min | 3.23 | 1.72 | 1.37 | 2.12 | 2.43 | 1.30 | 0.61 | 0.70 | 0.05 | 0.29 |
| Insulin Clearance Rate %/min | 5.17 | 4.53 | 7.93 | 4.88 | 4.02 | 4.46 | 2.23 | 0.29 | 0.70 | 0.97 |
| Insulin Sensitivity Index, mmol/min × µU/mL | 0.31 | 0.79 | 0.56 | 0.96 | 0.53 | 0.51 | 0.08 | 0.21 | 0.34 | 0.43 |

The effect of feeding a low plane of nutrition (LOW; 4 L MR/d) and high plane of nutrition (HIGH; 8 L MR/d) on glucose and insulin responses to an I.V. glucose tolerance test in dairy calves.

[1]Treatments: LOW = Low plane of nutrition (4L MR/d); HIGH = High plane of nutrition (8L MR/d).

[2]Statistical comparisons: T effects = LOW vs. HIGH; W effects = week; T×W effects = treatment by time interaction.

[3]Cmax = maximum plasma concentration, Tmax = time of maximum concentration observed, AUC = area under the concentration-time curve, Delta = the maximum change from baseline.

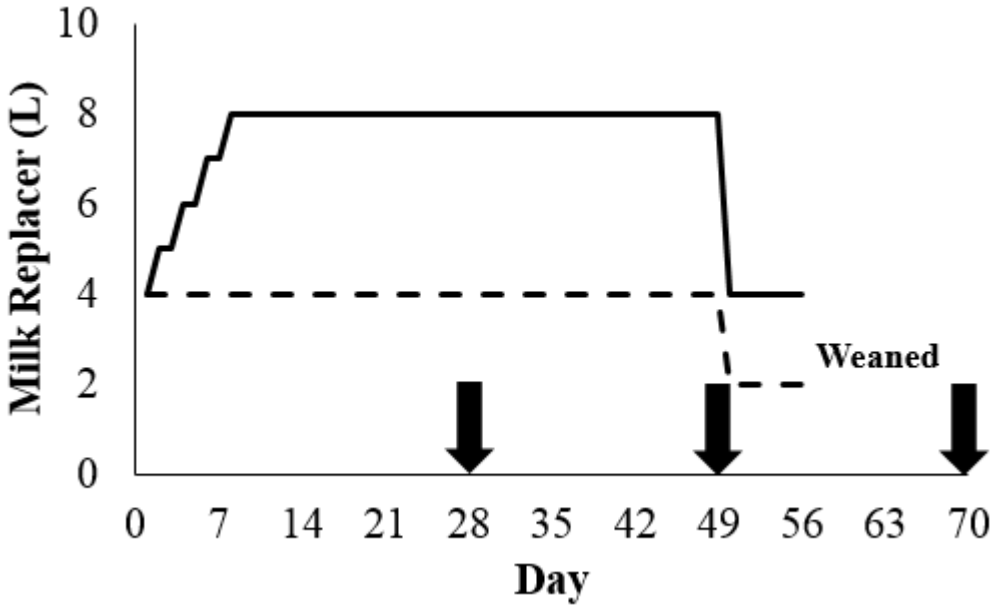


**Figure 2-1:** Feeding plane for calves fed a LOW plane of milk (dotted line) and calves fed a HIGH plane of milk (solid line). Arrows indicate blood sampling weeks 4, 7, and 10.

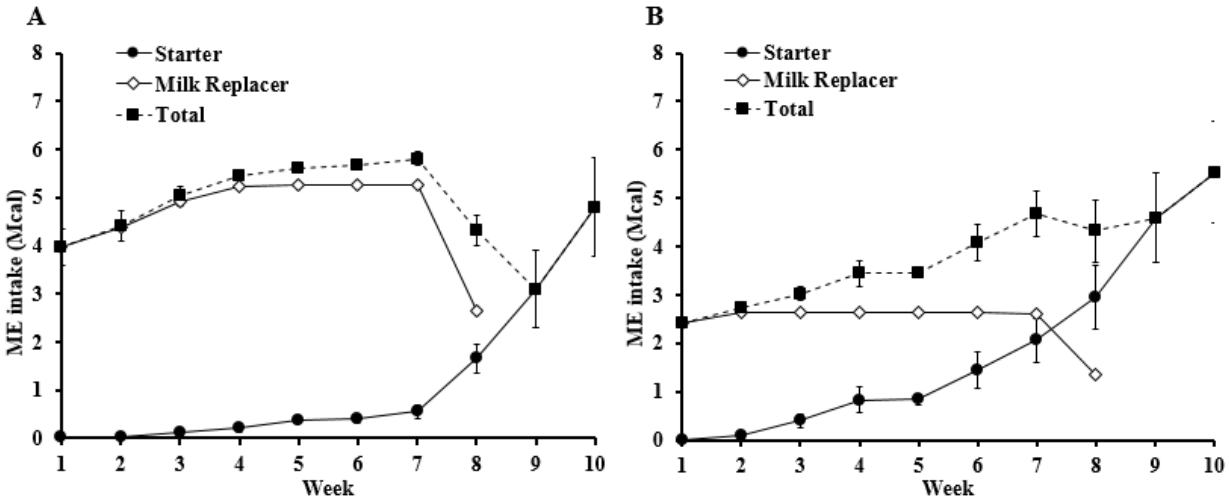


**Figure 2-2**: Weekly metabolizable energy from milk replacer (MR), starter, and both combined (total) of calves fed either a HIGH (A) or LOW (B) plane of MR. Data are least square means ± SEM, n = 6 per group.

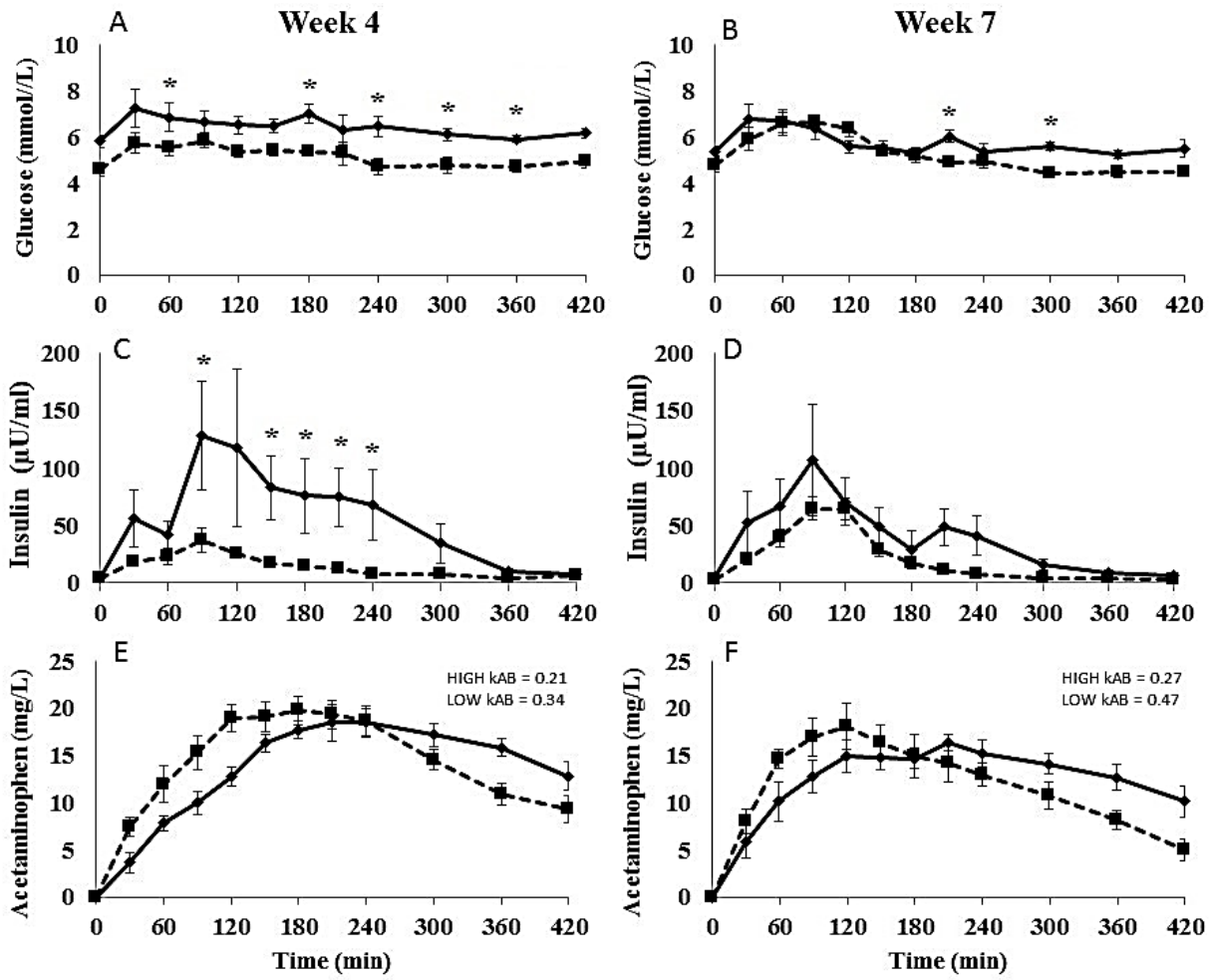


**Figure 2-3**: Effect of milk plane of nutrition on postprandial plasma glucose, insulin, and acetaminophen in calves. Postprandial blood glucose (A, B; mmol/L), insulin (C, D; µU/mL) and acetaminophen (E, F; mg/mL) in dairy calves at wks 4 and 7 of age for 420 min. The dotted line represents calves fed the LOW plane of nutrition and the solid line represents calves on the HIGH plane of nutrition. Data are least square means ± SEM, n = 6 per group. Differences between treatment groups at specific time points are denoted with *($P < 0.05$).

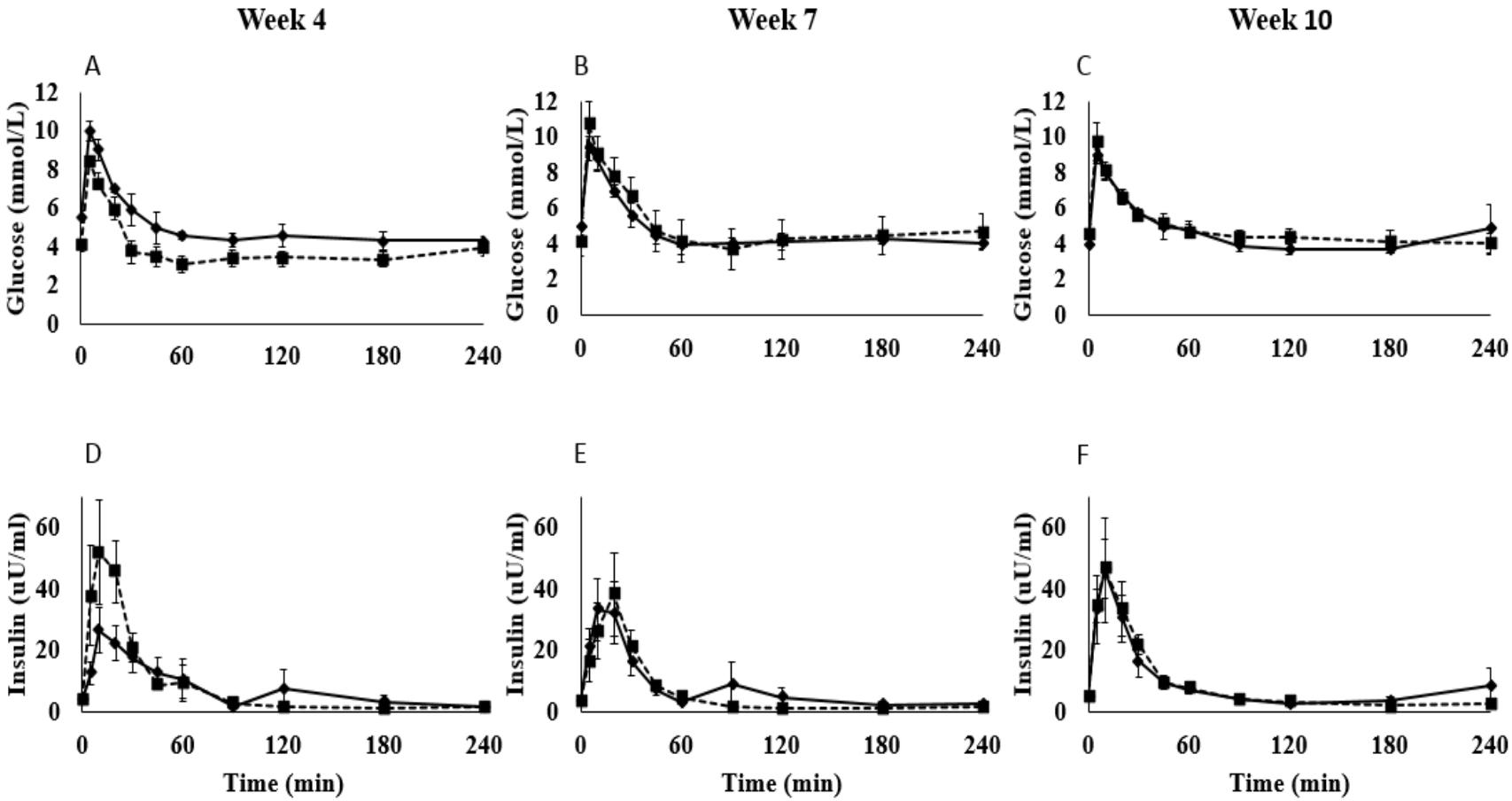


**Figure 2-4**: Effect of milk plane of nutrition on plasma glucose and insulin response to a glucose tolerance test. Blood glucose (A, B, C) and insulin (D, E, F) concentration relative to intravenous glucose tolerance test in calves at week 4, 7 and 10 of life in dairy calves placed on a LOW plane of nutrition (4 L MR/d; dotted line) and HIGH plane of nutrition (8 L MR/d; solid line). Data are least squares means ± SEM, n = 6 per group.

## 3.0 Chapter 3: Effects of milk replacer feeding frequency and calf age on performance and glucose and insulin kinetics in Holstein calves fed an elevated plane of milk supply

### 3.1 Abstract

The objective of this study was to investigate how an elevated plane of milk replacer (MR) pre-weaning, at different feeding frequencies, can influence calf performance as well as glucose and insulin kinetics both pre- and post-weaning. Ten male Holstein calves (42.2 kg ± 1.8 initial BW) were blocked by BW and randomly assigned to 2 treatments whereby calves were offered 8 L of milk replacer (150 g/L) per day in two (2x; meal size 4 L) or four feedings (4x; meal size 2 L) via an automated calf feeder. Calves were gradually stepped down by 1 L/d on week 8 and finished weaning by week 9 of the feeding trial. Water and a pelleted calf starter was offered *ad libitum* and intakes of milk replacer and starter were recorded daily and BW weekly. Jugular catheters were placed on weeks 4, 7, and 10 of the study to facilitate postprandial blood sampling and glucose tolerance tests. Statistical analysis was conducted using SAS Proc Mixed with repeated measures and any data not normally distributed were transformed logarithmically. Calf final BW and intakes did not differ between treatments. There were no interaction effects ($P > 0.1$) for postprandial plasma glucose concentrations indicating similar glycemic control by both treatments despite different meal sizes. Postprandial insulin $AUC_{240}$ tended to have a treatment by age interaction whereby concentrations were greater ($P = 0.09$) in 2x calves on week 7 however, insulin $AUC_{240}$ also decreased weekly with increasing calf age ($P < 0.01$). Additionally, there was no interaction ($P > 0.1$) effects between treatments and age observed for the glucose tolerance test, suggesting that feeding frequency in this study did not affect insulin

sensitivity as calves aged. These findings suggest that feeding an elevated plane of milk (8 L/d) twice or four times daily does not influence insulin sensitivity in Holstein dairy calves.

### 3.2 Introduction

Feeding dairy calves an elevated plane of milk (≥ 8L or 20% birth BW) is becoming increasingly popular in the dairy industry as replacement heifers raised on this feeding regimen have greater pre-weaning growth (Jasper and Weary, 2002; Khan *et al*., 2007), fewer signs of hunger (Borderas *et al*., 2009; de Passillé *et al*., 2011; Miller-Cushon and DeVries, 2015), and the potential for greater future milk production (Soberon *et al*., 2012). On farm, calves are typically fed twice per day (Vasseur *et al*., 2010), resulting in meal sizes of 4 L per feeding when offering an elevated plane of milk (8 L/d). This feeding regimen contrasts how calves would naturally feed if they were left to suckle from their dams (Reinhardt and Reinhardt, 1981; Egli and Blum, 1998) or had free access to an automated feeder, where they could feed in excess of 7-10 meals varying in their size throughout the day (de Passillé *et al*., 2011; Berends *et al*., 2014). A concern in the dairy industry is feeding larger meals pre-weaning at a low feeding frequency may have long-term metabolic consequences, as it has been linked to reduced insulin sensitivity in older veal calves (6 -19 weeks old) fed large meal sizes (>8 L/d) (Palmquist et al., 1992; Hostettler-Allen et al., 1994; Hugi et al., 1997c) and more recently in younger milk fed calves (1 - 9 weeks old) offered 8 L/d twice daily *(*Bach *et al., 2013*). Incidence of this metabolic disorder indicates impaired energy and protein metabolism which can result in reduced growth rates (Van den Borne *et al*., 2006; Vicari *et al*., 2008). This is an important issue when considering feeding calves more milk at a low feeding frequency pre-weaning as it is still unclear if there are carry over effects post-weaning on glucose metabolism when calves are offered solid feed and undergo

a weaning transition, whereby volatile fatty acids (VFA) absorption from the rumen become the primary energy source over glucose absorbed in the small intestine (Drackley, 2008; Benschop and Cant, 2009). Previous literature comparing a high vs low plane of milk pre-weaning in calves offered solid feed and weaned have noted minimal impact on post-weaning insulin and glucose kinetics when feeding twice daily (Yunta *et al*., 2015; MacPherson *et al*., 2016). However, the impact of pre-weaning feeding frequency of an elevated plane of milk feeding on both pre and post-weaning insulin sensitivity has not been studied. Current literature suggests that by increasing feeding frequency and decreasing meal size calves will demonstrate improved insulin sensitivity as the nutrient load is evenly spread across smaller meals throughout the day (Kaufhold *et al.*, 2000; Vicari *et al*., 2008).

Thus, the objective of this study was to investigate the effects of feeding an elevated pre-weaning milk replacer (MR) supply and calf age, on calf performance and glucose and insulin kinetics in male Holstein calves during the pre- and post-weaning periods. The overall hypothesis was that increasing feeding frequency could improve insulin sensitivity both pre- and post-weaning.

## 3.3 Materials and methods

### *3.3.1 Animals*

Ten male Holstein calves were collected from local dairies and brought to the University of Alberta Metabolic Unit. Calves were housed in a group pen and bedded with wood shavings. All procedures were pre-approved by the Animal Care and Use Committee for Livestock at the University of Alberta and conducted according to the guidelines of the Canadian Council of Animal Care (Ottawa, ON, Canada, 2009).

*3.3.2 Feed intakes, treatments, and body weights*

All calves received a minimum 4 L of colostrum in the first 24 h of life. Following colostrum, they were fed 2 L of a commercial MR (Grober Excel Pro Gro, Cambridge, Ontario) twice daily until calves were brought to the facility at 2.8 ± 0.49 days of age and initial BW of 42.2 kg ± 1.8. Once at the facility calves were fed via automated calf feeders for MR (Delaval Cambridge, Ontario, Canada) and calf starter (Forster Technik, Engen, Germany), and had *ad libitum* access to water. The MR had a metabolizable energy content of 4.7 Mcal/kg and contained 26% CP, 18% crude fat, 0.15% crude fibre, 7.5% Ash, 0.95% calcium, 0.70% phosphorus, 0.55% sodium, 40,000 IU/kg vitamin A, 4,000 IU/kg vitamin D3, 150 IU/kg vitamin E, and 0.3 mg/kg selenium. The pelleted calf starter was medicated with rumensin (60 mg/kg), had a metabolizable energy content of 2.8 Mcal/kg, and contained 19% CP, 4.7% crude fat, 6% crude fibre, 0.35% sodium, 0.9% calcium, 0.53% phosphorus, 30,000 IU/kg vitamin A, 4,400 IU/kg vitamin D3, 245 IU/kg vitamin E, and 0.3 mg/kg selenium (19% Calf Starter Pellet, Hi-Pro Feeds, Sherwood Park, Alberta).

Upon arrival, calves were blocked by body weight (BW) and randomly assigned to their feeding treatments. The feeding treatments consisted of two feeding groups whereby calves were fed 8 L MR/d (150 g milk replacer powder/L) over two (2x; meal size 4 L), or four (4x; meal size 2 L) meals per day. Before the feeding treatments began calves were given a 5-day adaptation period where they were fed at their feeding times and also trained to become familiar with the automated feeders and to transition calves up to consuming and elevated plane of milk of 8 L/day. Calves officially began their feeding treatments after the adaption period when calves were 8.8 ± 0.49 days of age. Milk entitlement times for the automated feeder were 1000 and

2000 h for the 2x treatment and 0500, 1000, 1500, and 2000 h for the 4x treatment. As calves were fed by a free-access automated feeder, exact feeding times varied for each calf but ranged within at least 9 h after the previous meal for the 2x treatment and 5 h after the previous meal for the 4x treatment. Calves remained on their milk treatments 7 weeks, when they were stepped down by 1 L MR/d during week 8 and weaned by week 9 of the study. Calf starter was offered *ad libitum* throughout and individual intakes for MR and starter were recorded daily via the automated feeding systems. Body weights were measured at arrival for initial BW (week 0) and every week afterwards at 1200 h except for sampling weeks (4, 7, 10) where calves were weighed at 0800 h to coincide with jugular catheter placement and reduce stress.

*3.3.3 Blood Sampling (Weekly and Postprandial Glucose and Insulin)*

Jugular catheters (using 36" Tygon Microbore tubing .015 inch and OD .070 inch; Saint Gobain Performance Plastics Corp, USA; via a 12 gauge 2" stainless steel needle; Jorgensen Labs, Loveland, Colorado) were placed on week 4, 7, and 10 of the study and remained for ~72 h for postprandial blood sample collection as well as for a glucose tolerance test (GTT). The catheters were flushed with 2 to 5 ml heparinized saline (10,000 IU/ml) after every sample collection. Blood samples were collected using 5 mL syringes and then transferred into a 4 ml lithium heparin Vacutainer™ (BD Canada, Mississauga, Ontario) on week 4 and a to 6 mL lithium heparin Vacutainer™ on weeks 7 and 10 that was immediately placed on ice. All blood samples were centrifuged immediately at 3000 x *g* for 15 min, and plasma stored at -20°C. Blood samples were analyzed for glucose on a 96 well plate using peroxidase glucose oxidase (PGO) enzyme and dianisdine dihydrochloride (Sigma, Missouri, USA; Sigma Technical Bulletin, 2003). Insulin was analyzed on a 96 well plate using a bovine insulin ELISA kit

(Mercodia,Bovine Insulin Elisa, 2014; Uppsala, Sweden; used by Bach et al., 2013). All plates were analyzed at a wavelength of 450 nm with a plate reader (SpectraMax 190, Molecular Devices Corp., Sunnyvale, CA). For both glucose and insulin values quantified needed a CV below 5% for acceptance.

To determine postprandial glucose and insulin concentrations following a MR meal on week 4 and 7, a total of 9 blood samples were collected at 0, 30, 60, 90, 120, 150, 180, 210, 240 min relative to the 1000 h MR feeding the day after catheters were placed. During sampling, calves had *ab libitum* access to water and starter at all times. A GTT was administered the day after postprandial sampling on week 4 and 7 then again separately post-weaning on week 10, consisting of an infusion of glucose (50% Dextrose, DMVet Inc, Quebec, Canada) via the jugular catheter at 1000 h at a concentration of 540 mg/kg of metabolic BW ($BW^{0.75}$; Bach et al., 2013; Yunta et al., 2015; MacPherson et al., 2016). Blood samples were collected at -15, 0, 5, 10, 20, 30, 45, 60, 90, 120, 180, and 240 min relative to the glucose infusion. To ensure basal plasma glucose levels were achieved before each infusion, calves were fasted overnight (12 h) and their morning MR meals were delayed until sampling was finished (4 h for 2x and 9 h for 4x) for week 4 and 7. During sampling calves were restricted from access to calf starter and water.

### *3.3.4 Calculations and Statistics*

Intake of metabolizable energy (ME), starter intake, and average daily gain (ADG) were analyzed separately for the pre-weaning (d 1 to 56 of the study), post-weaning (d 57 to 70), and overall (d 1 to 70) periods. For the GTT and postprandial measurements the time to maximum concentration (Tmax), the maximum concentration (Cmax), the baseline concentration, and the change in concentration were calculated from the raw data for both glucose and insulin. In

addition, the positive incremental area under the curve (AUC) was calculated using the trapezoidal rule for glucose and insulin concentrations above the baseline levels. The AUC was calculated over 240 min for both the GTT and postprandial measurements ($AUC_{240}$). For the GTT only, the clearance rate (CR) of glucose and insulin was also determined from the raw data using the method described in Pires et al., (2007).

$$\text{Clearance rate (\%/min)} = \{(\ln[ta] - \ln[tb])/(ta - tb)\} \times 100,$$

where [ta] and [tb] is the concentration of insulin or glucose at time (ta) or (tb), respectively. In addition, an insulin sensitivity index was calculated using the method described by Christoffersen et al., (2009).

Data were subjected to ANOVA using the MIXED procedure of SAS 9.1 (SAS, 2004) with time (week) as repeated measures for BW, MR intake, ME intake, starter intake, and ADG. Initial BW was considered a covariate for the final weight analysis. Calf was the experimental unit and random effect with the treatment, week, and interactions of treatment by week as the fixed effects. The model included age as a repeated measure and was run using the covariance structure with the lowest Akaike's Information Criterion (AIC) value which was either compound symmetry or auto regressive (AR(1)) depending on the variable, and the Kenward-Roger adjustment was used. Before analyses, all data were screened for normality using the UNIVARIATE procedure of SAS, any parameter that was not normally distributed was logarithmically transformed. When age and treatment interactions were detected, a Tukey test was used to separate means corrected for multiple comparisons between treatments by each week using the PDIFF statement in SAS. Significance was declared when $P \leq 0.05$ and trends were declared when $P < 0.10$.

## 3.4 Results

### *3.4.1 Body Weights and Intakes*

There were no differences for milk intake between treatments during the adaption period ($P > 0.05$). Final BW (kg; 2x = 112.1 ± 4.07 kg;4x = 109.7 ± 4.07 kg) and MR intake were similar ($P > 0.05$) between treatments. Overall metabolizable energy intake (2x = 6.25 ± 0.13 Mcal/d; 4x = 5.99 ± 0.13 Mcal/d), starter intake (2x = 0.82 ± 0.11 kg; 4x = 0.93 ± 0.11 kg), and ADG (2x = 0.92 ± 0.04 kg/d; 4x = 0.87 ± 0.04 kg/d) was also similar ($P > 0.05$) between treatments and there were no differences between pre-weaning, post-weaning, and overall periods from week 1 to 10.

### *3.4.2 Postprandial Glucose and Insulin*

Postprandial glucose (mmol/L) and insulin (μU/mL) concentration are presented in Table 3-1 and concentration curves are presented in Figure 3-1. There was no treatment by week interaction ($P > 0.05$) for any postprandial glucose measurements including the baseline, Tmax, Cmax, $AUC_{240}$, and change in glucose (delta; Table 3-1). The basal glucose concentration was greater (P = 0.02) in 4x calves as compared with that in 2x calves. However, glucose $AUC_{240}$ and glucose concentration change (delta) tended (P = 0.06) to be less in 4x calves as compared with that in 2x calves. In addition, week 7 had lower basal glucose and higher glucose concentration change (delta) as compared to week 4.

Postprandial insulin did not have an interaction between treatment and age for baseline, Tmax, Cmax, or change in concentration (delta; Table 3-1). There was a treatment by week tendency for insulin $AUC_{240}$ to be greater ($P = 0.09$) in the 2x calves on week 7. The $AUC_{240}$ also decreased ($P < 0.01$) as calves aged and was higher ($P = 0.01$) for 2x calves. Basal insulin

concentrations increased ($P < 0.01$) as calves aged and the Cmax concentrations tended to decrease ($P = 0.08$) with calf age. Both treatment groups reach similar Cmax but the Tmax was different ($P = 0.05$) between treatments with the 2x calves taking longer at around 114.00 ± 15.10 min to reach their Cmax compared to the 4x calves at 63.66 ± 16.40 min. Change in insulin tended to be greater ($P = 0.09$) for the 2x calves compared to the 4x calves and decreased ($P = 0.02$) as calves aged.

### *3.4.3 Glucose Tolerance Test*

The results for the GTT are presented in Table 3-2 and the curves are presented in Figure 3-2. Plasma glucose levels (mmol/L) for the GTT reached a Cmax of 8.79 ± 0.13 mmol/L within 5 to 10 min after the infusion and returned to baseline within 60 min of the infusion for all animals. For the GTT, there was no interaction between treatment and age for any of the measured parameters. The $AUC_{240}$ for plasma glucose was greater ($P = 0.03$) for 4x calves and was greater ($P < 0.01$) on week 10 compared to week 4 for both treatments. Basal glucose concentrations decreased ($P < 0.01$) with age and tended ($P = 0.09$) to be greater for 2x calves. In addition, the clearance rate (CR) for plasma glucose decreased ($P < 0.01$) with calf age.

There were no interactions between treatment and age for insulin response during the GTT for baseline, Tmax, Cmax, AUC, change in insulin (delta), and clearance rate (Table 3-2). In addition, insulin sensitivity index and insulin to glucose ratios were all similar between treatment groups. Therefore, overall there were no differences ($P > 0.10$) for plasma insulin response to the GTT.

### 3.5 Discussion

Recent research has demonstrated that feeding an elevated plane of milk or MR (≥8 L/d) pre-weaning compared to a low plane of milk (~4 L/day) can improve calf growth rates, decrease hunger behaviours, and potentially increase future milk production (Borderas et al., 2009; Soberon et al., 2012). However, farms typically feed twice a day, leading to larger meal sizes when feeding an elevated plane of milk, which has been linked to the development of hyperglycemia, hyperinsulinemia, glucosuria, and a decrease in insulin sensitivity in calves (Hostettler-Allen et al., 1994). Several studies on veal calves fed milk until 3 – 6 months of age have suggested that increasing the feeding frequency, and therefore decreasing meal size, can improve insulin sensitivity (Kaufhold et al., 2000; Vicari et al., 2008). It is still unclear how increasing feeding frequency of milk in calves younger than two months of age offered solid feed could influence glucose metabolism. Therefore, the objective of this experiment was to investigate feeding an elevated plane of milk fed at 8 L of MR over 2 or 4 meals per day and its effect on glucose and insulin kinetics both pre- and post-weaning.

To assess the influence of increasing the feeding frequency of an elevated plane of milk on glucose metabolism in calves we examined glucose and insulin kinetics after meals and conducted a GTT. As expected postprandial glucose and insulin concentrations decreased when increasing feeding frequency from 2 to 4 times/day. This result is in agreement with previous studies where veal calves older than two months demonstrated improved postprandial insulin sensitivity when feeding frequency was increased above two daily feedings (Kaufhold et al., 2000; Vicari et al., 2008). However, unlike these studies both insulin and glucose concentrations decreased with increasing calf age. Postprandial glucose and insulin also showed no difference between treatments for glycemic control, as illustrated by the notable aversion of hyperglycemia

or hyperinsulinemia. In addition, responses to the GTT were also similar between treatments, further demonstrating both treatments were adequately able to maintain glycaemia and showed no signs of decreased insulin sensitivity.

Postprandial hyperinsulinemia has been linked to developing hyperglycemia and a decrease in insulin sensitivity. One of the main driving mechanism for this is the high insulin concentrations sustained for a prolonged period which have been known to down regulate insulin receptors in muscle in veal calves older than two months of age fed at a low feeding frequency (Hostettler-Allen et al., 1994; Hugi et al., 1998). The calves in the current study were under two months of age which may have been a significant contributing factor as to why calves did not develop hyperglycemia or hyperinsulinemia postprandial. There are gene expression changes that occur in the liver and rumen around the age of weaning to support the use of VFA from microbial fermentation in the rumen as the main energy source (Baldwin et al., 2004). It is likely that glucose utilization is more efficient in young pre-weaning calves (Pantophlet et al., 2016), and this was demonstrated by the decreasing glucose clearance with age, which may be a factor for inefficient glucose utilization in veal calves fed only milk for a prolonged period.

Although insulin response in the current study was variable, postprandial plasma concentrations of both glucose and insulin decreased with age which contradicts findings from veal calves experiencing reduced insulin sensitivity when fed a high MR diet at a low feeding frequency (Hostettler-Allen et al., 1994; Kaufhold et al., 2000; Vicari et al., 2008). The decrease in concentrations of both insulin and glucose with age may be attributed to solid feed consumption and fermentation by microbes in the rumen which is lacking in milk-fed veal calves (Hammon *et al.,* 2002). Unlike ingestion of glucose from MR which is a absorbed rapidly in the small intestine, fermentation of carbohydrates from solid feed in ruminants generates VFA after

a lag phase of microbial digestion which delays nutrient absorption and prevents 75 – 80% of carbohydrates from reaching the small intestine (Huntington et al., 2006). Studies in ruminating calves (Webb et al., 1969; Khan et al., 2011) and lambs (Jarrett et al., 1964) looking at glucose concentrations report decreasing concentrations with increasing age. As dairy cows have a lower glucose concentrations than young ruminating calves (Hayirli, 2006) and veal calves (Hostettler-Allen et al., 1994) it has been suggested the presence of solid feed and switching to VFA as a primary energy source decreases glucose concentrations (Benschop and Cant, 2009). In addition, the milk meal size to calf body weight ratios decreased with increasing calf age in the current study as a result of feeding at a fixed rate of 8 L/day. It's likely this is another significant driving factor for the decrease in postprandial glucose and insulin concentrations as calves were better able to adapt to consuming 4 L. It's possible then feeding at a fixed rate of 8 L/day was not as challenging as expected as calves aged.

Meal size still influenced postprandial plasma concentrations for both insulin and glucose, with concentrations proving greater in 2x calves. This was expected and can be attributed to the 4 L meal size and to consuming two-fold the lactose concentration during a meal compared to 4x calves. However, postprandial plasma glucose concentrations for both treatments were still similar to the concentrations found in suckling (Egli and Blum, 1998) and weaned calves (Hugi and Blum, 1997), around the normal range of 4.4 – 6.9 mmol/L (Hayirli, 2006; Vicari et al., 2008). One possible mechanism for glycemic control is the calf's ability to adapt to low feeding frequency and larger meal sizes by slowing down the rate of nutrient delivery from the abomasum to the small intestine, or the abomasal emptying rate, as a means to modulate plasma glucose concentrations. Emptying rates and nutrient delivery are important for glucose homeostasis and triggering the function of gastric hormones including the secretion of insulin

from the pancreas (Aronoff et al., 2004). Abomasal emptying in calves has recently been shown to be delayed when feeding a large MR meal size of 4 L compared to 2 L (MacPherson et al., 2016). This suggests that calves are capable of modulating nutrient delivery rates to the small intestine and adapting to large meals to regulate plasma glucose concentration. In addition, the abomasum in calves 19 – 23 days old has a notable ability to distend, allowing for consumption of MR meals up to 7 L if the calf is allowed ad libitum access to milk (Ellingsen et al., 2016). Previously, feeding large meals was perceived to impair glucose metabolism and promote foregut disorders including ruminal drinking (Herrli-Gygi et al., 2008) and abomasal ulcers (Ahmed et al., 2002); however when feeding 4 L meal sizes at a low feeding frequency, delayed abomasal emptying may actually improve glucose metabolism.

The GTT also demonstrated that feeding frequency had little impact on insulin sensitivity in this study. Previous studies using a GTT to investigate insulin sensitivity in calves fed an elevated plane of milk focused on differences between high and low planes of milk. To our knowledge this is the first study using a GTT to evaluate the influence of feeding frequency when feeding an elevated plane of milk. In the current study both treatments had a similar capacity for glucose clearance and insulin sensitivity demonstrating that calves in both treatments had similar glucose tolerances at all ages. Current literature around feeding 4 L MR meals twice daily to calves younger than two months of age and using a GTT has conflicting results, with both decreased insulin sensitivity (Bach et al., 2013; Yunta et al., 2015) and unchanged insulin sensitivity (MacPherson et al., 2016) reported. However, these studies also use significantly different methodical approaches: studies reporting reduced insulin sensitivity (Bach et al., 2013; Yunta et al., 2015) conducted a GTT 4 hours postprandial in the post-absorptive state, whereas studies that do not report insulin issues (MacPherson et al., 2016),

including the current study, imposed 12-hour feed restrictions before testing. As such, the post-absorptive state of the calves likely influenced the GTT responses – for in the current study, 2x calves consuming 4 L had greater insulin concentrations 4 hours postprandial. These conflicting reports ultimately leave a knowledge gap for the dairy industry. Reduced insulin sensitivity indicates impaired protein and energy metabolism (Van den Borne et al., 2006), therefore it is important we do not offer a feeding regimen to pre-weaning calves that could compromise calf growth and future animal production.

Interestingly during the GTT 2x calves had a lower glucose AUC than 4x calves even though glucose clearance rates and insulin responses were not significantly different. This could potentially indicate that 2x calves had enhanced glucose tolerance. It has been suggested that intermittent fasting or time- restricted feeding periods (≥12 hours) between meals may improve glucose tolerance in humans and rodents (Romsos and Leveille, 1974; Mattson and Wan, 2005; Longo and Panda, 2016). It is possible that the extended fasts between milk meals for the 2x calves may have had a positive influence on glucose tolerance and could explain why 2x calves had similar insulin sensitivity to 4x calves both pre- and post-weaning. In agreement with the current study, no carryover effect from pre-weaning nutrition on the post-weaning calf has been reported (Yunta et al., 2015; MacPherson et al., 2016). Findings from this study then suggest feeding either a 2x or 4x feeding frequency when offering 8 L MR/day is a viable feeding regimen to increase calf ADG without impairing glucose and insulin kinetics. A positive effect of increasing milk feeding frequency, however, is noted in veal calves aged 10 – 22 weeks through the characterization of reduced postprandial glucose and insulin with increased feeding frequency (Kaufhold et al., 2000; Vicari et al., 2008). Studies conducted with older milk-fed veal calves that use a GTT to evaluate insulin responsiveness (Palmquist et al., 1992; Hostettler-Allen

et al., 1994; Hugi et al., 1997c) highlight that the age of calves (6 – 16 weeks) and target feeding plane (1.4 kg ADG) differ significantly from the current study and are likely the main driving factors behind reduced insulin sensitivity in veal calves. The decrease in plasma insulin concentrations as calves aged and their ability to maintain normal plasma glucose levels in this study indicates that there is no decrease in insulin sensitivity with increasing age in calves fed an elevated plane of MR at different feeding frequencies.

### 3.5 Conclusion

This aim of this study was to determine if increasing the feeding frequency from 2 to 4 meals per day of an elevated MR supply (8 L MR/d) in calves weaned at 2 months could improve glucose metabolism. Unexpectedly, there was also no effect of feeding frequency on glucose and insulin kinetics as demonstrated by the postprandial glucose and insulin patterns and the GTT where there was no decrease in insulin sensitivity. These findings suggest, that when calves are fed 8 L MR/d and undergo a weaning transition at two months of age, feeding frequency is not a factor influencing insulin sensitivity, and therefore both feeding regimens are plausible methods for feeding calves more milk without compromising glucose metabolism.

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### 3.6 Tables and Figures

**Table 3-1** Effect of feeding frequency on pre- and postprandial plasma glucose and insulin responses in dairy calves

| | Treatment[1] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 2x | | 4x | | | *P*-value[2] | | |
| Item[3] | Week 4 | Week 7 | Week 4 | Week 7 | SEM | T | W | T*W |
| Glucose | | | | | | | | |
| Baseline (mmol/L) | 5.56 | 4.88 | 5.92 | 5.18 | 0.13 | 0.02 | <0.01 | 0.84 |
| Tmax (min) | 84.00 | 66.00 | 105.19 | 66.00 | 17.02 | 0.50 | 0.12 | 0.54 |
| Cmax (mmol/L) | 8.40 | 8.76 | 7.97 | 7.58 | 0.47 | 0.12 | 0.89 | 0.20 |
| AUC mmol/L x 240 min | 335.76 | 431.26 | 248.64 | 244.73 | 62.21 | 0.06 | 0.12 | 0.32 |
| Delta (mmol/L) | 2.84 | 3.86 | 2.15 | 2.40 | 0.45 | 0.06 | 0.02 | 0.10 |
| Insulin | | | | | | | | |
| Baseline (µU/mL) | 4.90 | 15.16 | 6.95 | 25.92 | 3.98 | 0.34 | <0.01 | 0.55 |
| Tmax (min) | 114.00 | 114.00 | 73.31 | 54.00 | 25.99 | 0.05 | 0.72 | 0.72 |
| Cmax (µU/mL) | 180.04 | 105.30 | 96.50 | 48.30 | 40.27 | 0.11 | 0.08 | 0.67 |
| AUC (µU/mL) x 240 min | 20131.0$^{ab}$ | 7484.0$^{a}$ | 8442.7$^{ab}$ | 989.5$^{b}$ | 3985.7 | 0.01 | <0.01 | 0.09 |
| Delta (µU/mL) | 175.16 | 90.12 | 91.15 | 22.38 | 39.78 | 0.09 | 0.02 | 0.40 |

[1]Treatments: 2x = MR fed 2 times daily; 4x = MR fed 4 times daily.
[2]Statistical comparisons: T effects = 2x vs. 4x; W effects = week; T×W effects = treatment by time interaction.
[3]Cmax = maximum plasma concentration, Tmax = time of maximum concentration observed, AUC = area under the concentration-time curve, Delta = the maximum change from baseline.

**Table 3-2.** Effect of milk feeding frequency on plasma glucose and insulin responses to a glucose tolerance test in dairy calves.

| | Treatment[1] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 2x | | | 4x | | | | *P*-value[2] | | |
| Item[3] | Week 4 | Week 7 | Week 10 | Week 4 | Week 7 | Week 10 | SEM | T | W | T×W |
| Glucose | | | | | | | | | | |
| Baseline (mmol/L) | 5.69 | 5.18 | 4.63 | 5.16 | 4.63 | 4.46 | 0.25 | 0.09 | 0.01 | 0.60 |
| Tmax (min) | 8.00 | 5.00 | 5.00 | 11.39 | 6.00 | 6.00 | 1.41 | 0.06 | 0.02 | 0.65 |
| Cmax (mmol/L) | 9.61 | 8.86 | 8.50 | 8.74 | 8.32 | 8.67 | 0.31 | 0.21 | 0.04 | 0.12 |
| AUC (mmol/L) x 240 min | 77.87 | 67.80 | 133.16 | 90.96 | 99.20 | 172.01 | 16.41 | 0.03 | <0.01 | 0.78 |
| Delta (mmol/L) | 3.92 | 3.68 | 3.88 | 3.60 | 3.69 | 4.20 | 0.26 | 0.96 | 0.30 | 0.45 |
| Clearance rate (%/min) | 1.71 | 1.10 | 0.62 | 2.12 | 0.71 | 0.50 | 0.42 | 0.36 | <0.01 | 0.69 |
| Insulin | | | | | | | | | | |
| Baseline (μU/mL) | 3.79 | 1.34 | 2.06 | 1.11 | 1.14 | 2.54 | 0.88 | 0.34 | 0.22 | 0.13 |
| Tmax (min) | 10.00 | 8.00 | 7.00 | 11.68 | 15.00 | 7.00 | 4.20 | 0.28 | 0.52 | 0.69 |
| Cmax (μU/mL) | 27.91 | 14.98 | 28.97 | 37.63 | 19.64 | 20.36 | 9.39 | 0.71 | 0.15 | 0.31 |
| AUC (μU/mL) x 240 min | 465.61 | 357.13 | 451.94 | 575.41 | 366.69 | 409.10 | 136.35 | 0.82 | 0.26 | 0.69 |
| Delta (μU/mL) | 24.12 | 13.63 | 26.91 | 36.56 | 18.49 | 17.82 | 9.05 | 0.67 | 0.14 | 0.16 |
| Clearance rate (%/min) | 7.68 | 3.55 | 3.88 | 4.27 | 4.87 | 3.35 | 1.22 | 0.89 | 0.31 | 0.36 |
| Insulin to glucose ratio | 36.62 | 32.08 | 20.21 | 37.73 | 22.25 | 15.29 | 3.84 | 0.65 | 0.07 | 0.79 |
| Insulin sensitivity (mL/min×μU/mL) | 2.23 | 2.00 | 1.71 | 1.16 | 1.47 | 2.93 | 0.32 | 0.85 | 0.60 | 0.25 |

[1]Treatments: 2x = MR fed 2 times daily; 4x = MR fed 4 times daily.
[2]Statistical comparisons: T effects = 2x vs. 4x; W effects = week; T×W effects = treatment by time interaction.
[3]Cmax = maximum plasma concentration, Tmax = time of maximum concentration observed, AUC = area under the concentration-time curve, Delta = the maximum change from baseline.

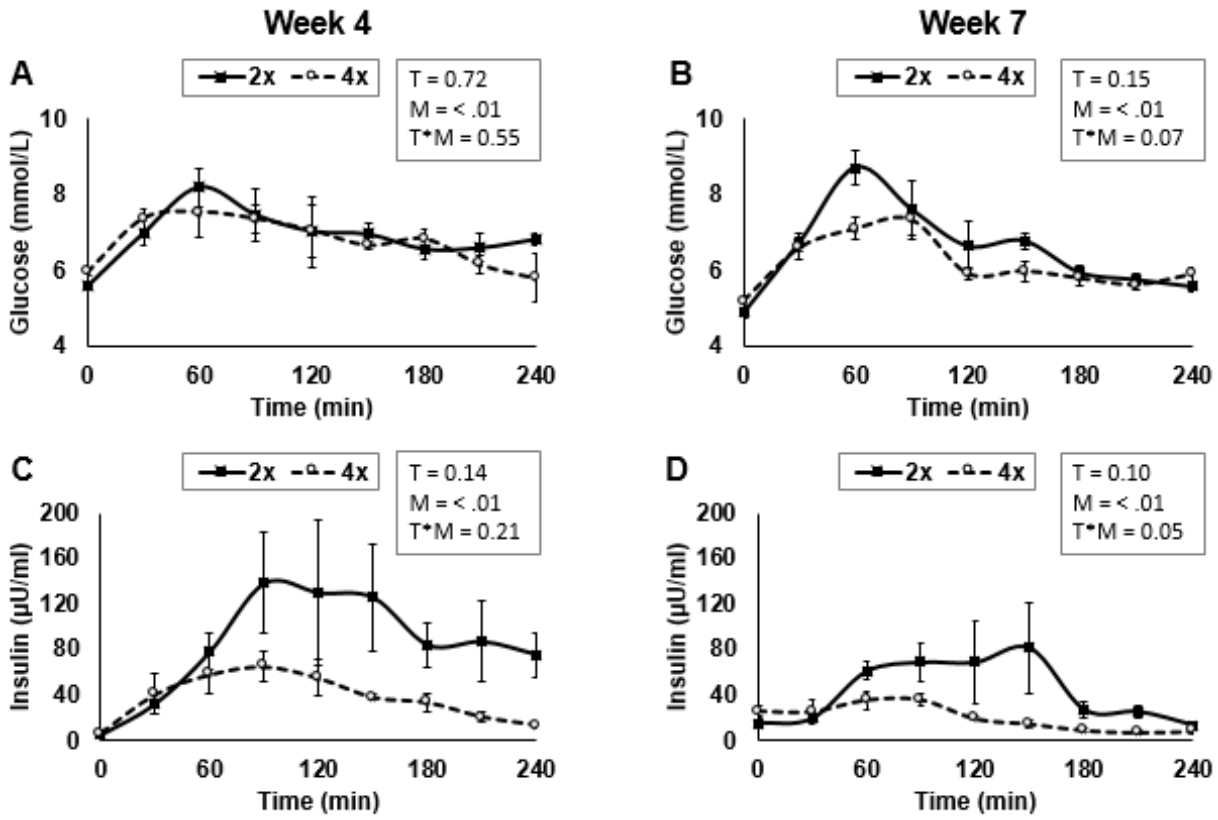


**Figure 3-1.** Effects of milk feeding frequency on postprandial plasma glucose and insulin in dairy calves. Postprandial plasma glucose (A, B; mmol) and insulin (C, D; uU/mL) at weeks 4 and 7 of study for 240 min in dairy calves fed milk replacer (MR) 2 or 4 times daily. The solid line represents calves fed MR twice daily and the dotted line represents calves fed MR 4 times daily. Data are means ± SEM, n = 5 per group. Means different between treatment groups are denoted with *($P < 0.05$). T = treatment, M = minute, T*M = interaction between treatment and minute.

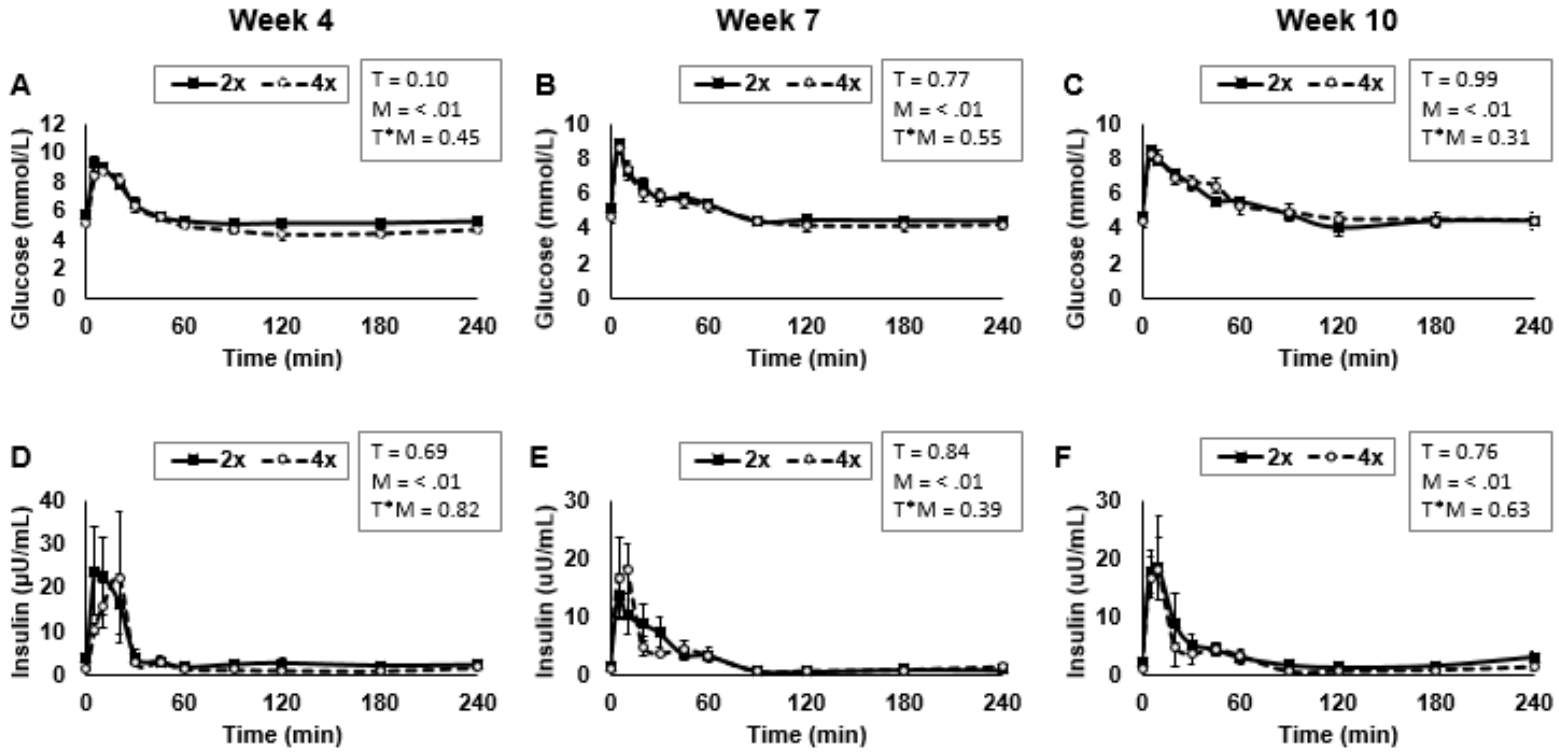


**Figure 3-2**. Effect of milk feeding frequency on plasma insulin and glucose during a glucose tolerance test in dairy calves. Plasma glucose (A, B, C) and insulin (D, E, F) concentration relative to intravenous glucose tolerance test at weeks 4, 7 and 10 of study in dairy calves fed milk replacer (MR) 2 or 4 times daily. The solid line represents calves fed MR twice daily and the dotted line represents calves fed MR 4 times daily. Data are means ± SEM, n = 5 per group. T = treatment, M = minute, T*M = interaction between treatment and minute.

## Chapter 4.0: General Discussion

The aim of this thesis was to determine how pre-weaning plane of milk and feeding frequency impacts insulin sensitivity and abomasal emptying in dairy calves. A potential concern arises when feeding calves more milk on farm as most dairy operations only feed calves twice daily, resulting in larger meal sizes (Vasseur et al., 2010). Feeding larger meal sizes at a low feeding frequency of twice daily has been linked to reduced insulin sensitivity in veal calves older than two months of age (Hostettler-Allen et al., 1994; Kaufhold et al., 2000), but also recently in pre-weaned calves younger than two months fed 8 L/day (Bach et al., 2013; Yunta et al., 2015). Prior to this thesis it was still largely unclear how feeding an elevated plane of milk (8 L/day) could influence glucose metabolism in dairy calves both pre- and post-weaning. Findings from chapter 2 and 3 where we investigated insulin sensitivity using postprandial blood glucose and insulin concentrations, as well as a GTT, suggest that calves fed an elevated plane of milk (8 L/day) compared to a low plane of milk (4 L/day), or fed an elevated plane of milk over two or four meals daily, show no signs of reduced insulin sensitivity or impaired glucose tolerance. In this discussion, the possible mechanisms driving the state of reduced insulin sensitivity in veal calves will be compared to the key findings from chapter 2 and 3 where glucose tolerance was not influenced. In addition, the novel finding from chapter 2 of slower abomasal emptying rates when feeding larger meals (4 L vs 2 L) as a means to control blood glucose concentrations will be discussed. Technical considerations and future directions for studies investigating insulin sensitivity and glucose metabolism in calves as well as abomasal emptying will also be explored.

## 4.1 Insulin Sensitivity

The first reports of reduced insulin sensitivity in calves were recorded around a quarter of a century ago in milk-fed veal calves older than two months fed for an ADG of ~1.4 kg/day (Doppenberg and Palmquist, 1991; Palmquist et al., 1992; Hostettler-Allen et al., 1994; Hugi et al., 1997b, 1998a). Calves in these studies experienced postprandial hyperglycemia, hyperinsulinemia, and glucosuria when fed large milk meals at a low feeding frequency of twice a day and demonstrated poor glucose tolerance when challenged by various insulin sensitivity tests (Hostettler-Allen et al., 1994; Hugi et al., 1998a; Kaufhold et al., 2000; Vicari et al., 2008). In chapters 2 and 3, postprandial glucose concentrations demonstrated no signs of sustained hyperglycemia and remained relatively stable indicating similar glycemic control for all calves, postprandial insulin concentrations decreased with age rather than approach hyperinsulinemia, and no differences between insulin responses to a GTT after a 12-hour fast were detected. The contrast between previous findings in veal calves and the current studies may be explained by the veal calf plane of nutrition and feeding practices, involving high daily allotments of milk replacer (~2.5 x metabolizable energy of maintenance), restricted or no access to solid feed, low feeding frequency of twice a day for milk meals, older calf rearing ages (18 – 35 weeks), and unknown colostrum intake and sources for calves. While the studies reported in this thesis had controlled colostrum intake, offered an elevated plane of nutrition by the first week of life, provided access to solid feed, and were weaned around 8 to 9 weeks of age.

In veal calves prolonged exposure to elevated postprandial concentrations of insulin or hyperinsulinemia is associated with the down regulation of insulin receptors in muscle tissue (Hugi et al., 1998a), which reduces the ability of insulin to facilitate glucose transport across cell membranes. Therefore, feeding a plane of nutrition consisting of large meals with a high rapidly

digestible carbohydrate content, such as milk or milk replacer, for a prolonged period of time is likely a significant factor behind the state of reduced insulin sensitivity observed in veal calves. In addition to the plane of nutrition and feeding practices, it has been suggested that insulin sensitivity in veal calves gradually decreases with age independent of diet or plane of milk nutrition from 3 weeks of age onwards (Hostettler-Allen et al., 1994; Hugi et al., 1998a; Pantophlet et al., 2016). This natural reduction could be attributed to biological changes in the liver to shift from glycolytic to gluconeogenic processes, as well as in the rumen to facilitate the use of VFA as an energy source instead of glucose (Baldwin et al., 2004). Studies looking at ruminating calves and lambs report that blood glucose concentrations decrease as they become a functioning ruminant (Jarrett et al., 1964; Depew et al., 1998; Bunting et al., 2000), which is reflected in adult ruminants who have a lower range of plasma glucose concentrations than young ruminants and non-ruminant species, ranging from 40 to 60 mg/dl compared to 80 to 120 mg/dl (Kaneko, 1997). This suggests that while growing calves are designed to drink milk, they may not be designed to continue deriving energy solely from milk and glucose absorbed in the intestine as they age. Therefore, when combining feeding large glucose loads per milk meal for upwards of six months of age in calves naturally experiencing reductions in insulin sensitivity it is logical to expect complications with glucose metabolism in veal calves towards the end of the fattening period.

Most studies investigating insulin sensitivity in veal calves also restrict access to solid feed while feeding a high plane of milk at a low feeding frequency. It is well known that ingesting solid feed facilitates rumen papillae development through continual VFA production from microbial fermentation which can be used as new energy sources aside from glucose derived from milk. The lack of solid feed in veal calves impedes this process which may be one

potential cause for the state of reduced insulin sensitivity observed in veal, as it has been suggested the concentration of blood glucose depends highly on the ratio of milk to solid feed (Benschop and Cant, 2009). For example, when calves are offered solid feed and weaned off milk they demonstrate lower glucose concentrations due to the increasing microbial use of dietary carbohydrates in the rumen and reduced glucose concentrations reaching the small intestine (Webb et al., 1969; Depew et al., 1998), while calves remaining on milk demonstrate rising glucose concentrations (Palmquist et al., 1992). In both chapters 2 and 3 the glucose clearance rate for the GTT decreased with increasing age and additionally, in chapter 3 the glucose baseline, Tmax, and Cmax decreased with age. With rumen fermentation of solid feed a continuous source of energy is produced when polysaccharides are broken down by microbes for production of the three main VFA, including propionate, acetate, and butyrate, of which propionate is absorbed by the rumen and converted to glucose by the liver via gluconeogenesis (McDonald et al., 2011). It is possible the shift to using microbial fermentation products as the main energy sources compared to rapidly digested simple carbohydrates absorbed in the small intestine may play a role in decreasing the incidence of compromised insulin sensitivity. Future studies may want to compare young milk fed calves to those offered solid feed to further our understanding of the influence of solid feed on young ruminant glucose metabolism.

Beyond studies in veal calves, there are two recent studies that investigated feeding young calves (1 – 84 days) an elevated plane of nutrition (8 L/day) twice daily, both of which demonstrated reduced insulin sensitivity using a GTT conducted four hours postprandial (Bach et al., 2013; Yunta et al., 2015). As illustrated in chapter 2 and 3, calves do not completely return to pre-prandial glucose and insulin concentrations within four hours after a 4 L milk replacer meal and calves fed larger meal sizes have greater postprandial insulin concentrations. Since the GTT

was conducted postprandial (Bach et al., 2013; Yunta et al., 2015), it is likely that a high percentage of the insulin reported can be attributed to the post-absorptive state of the calves and not the GTT. The greater insulin concentrations reported in these studies are then confounded by the greater insulin production already occurring in calves fed 4 L meal sizes. In Hostettler-Allen et al., (1994), a GTT was conducted pre- and post-prandial with results highlighting that the pre-prandial GTT had shorter half lives, lower insulin responses, earlier time to maximum concentrations for insulin, and lower glucose concentrations before and after the infusion. In human studies, tolerance tests are conducted in pre-prandial states to avoid confounding effects from endogenous production of glucose and insulin as well as differing rates of nutrient delivery from the stomach to the small intestine in a post-absorptive state (Fraser et al., 1990). Thus it is unclear whether the young calves in these studies truly experienced reduced insulin sensitivity. The current studies conducted the GTT after a 12 hour fast to reduce confounding effects of post-absorptive nutrient digestion, which is similar to studies conducted in humans (Fraser et al., 1990). However it could be beneficial to extend the fasting even further to 15 to 20 hours, or farther still (approx. 30 hours) if the calves are consuming a significant amount of starter, to ensure that the rumen is able to completely empty (Pantophlet et al., 2016). Taking this into consideration, the results from this thesis indicate that some previous insulin sensitivity results may be biased based on methodology and require consideration before interpretation.

In addition to the GTT, both of the current studies also evaluated postprandial insulin and glucose concentrations as older veal calves often experience both hyperglycemia and hyperinsulinemia (Hostettler-Allen et al., 1994; Kaufhold et al., 2000; Vicari et al., 2008). This was not the case for the calves in chapter 2 or 3, where interestingly, postprandial concentrations of insulin decreased with increasing age. Calves in both studies were limited to consuming 8 L

MR/day when they were fed the elevated plane of nutrition until their milk step down the week before weaning. This fixed feeding plane resulted in the body weight to meal size ratio significantly decreasing with increasing calf age, where total weekly metabolizable energy intake per kilogram of bodyweight from milk ranged from ~0.13 Mcal/kg on week 1 to ~0.09 Mcal/kg before the step downs. In addition, calves allowed to suckle from their dam or calves that have free access to an automated feeder can consume upwards of 12 L/day (Reinhardt and Reinhardt, 1981; de Passillé et al., 2014; Berends, 2014). As such, the calves may not have been challenged enough with respect to milk feeding plane of nutrition to approach a state of compromised glucose homeostasis and were able to adapt to the 4 L meal size with age.

The current studies are the first to use a GTT in addition to postprandial concentrations to assess insulin sensitivity in calves younger than two months of age fed an elevated plane of milk. Both of these methods are simple and relatively non-invasive. Developing calves rely heavily on insulin-dependent glucose utilization making the GTT method of measurement conducted in chapter 2 and 3 a reliable means of examining insulin response to high plasma glucose concentrations. However, in both studies, insulin responses measured over the GTT varied widely by individual calf. Measuring insulin response in calves could thus be difficult without high replication or using a different method. The hyperinsulinemic-euglycemic clamp is considered to be the gold standard for measuring insulin sensitivity because, unlike the GTT, hyperinsulinemic-euglycemic clamps can assess glucose utilization, however it is more invasive and requires more labour than a GTT (Bergman, 1989). A GTT still proved to be a valuable means of determining insulin sensitivity in calves in the current studies as it was conducted with a feed restriction of twelve hours allowing for both the physiological and absorptive states of the calves to be accounted for.

### 4.2 Abomasal Emptying and Insulin Sensitivity

The relationship between glucose and insulin depends highly on the rate at which nutrients are delivered to the small intestine which triggers pancreatic insulin secretion (Aronoff et al., 2004; Sen et al. 2006; Stahel et al., 2016). Previous studies assessing glucose tolerance in calves fed more milk linked reduced insulin sensitivity with feeding large milk meal sizes (Kaufhold et al., 2000; Vicari et al., 2008). This was assumed to result in high glucose concentrations being rapidly absorbed in the small intestine (Hostettler-Allen et al., 1994; Bach et al., 2013); however, no study prior to this thesis has actually measured abomasal emptying and its effect on glucose metabolism in calves. Results from chapter 2 indicated that abomasal emptying was actually delayed when feeding a larger meal size, likely as a means to control glycaemia (Tong and D'Alessio, 2014). Due to the high lactose concentration of the meal, more lactose may have entered the small intestine per unit of time following the meal. Nevertheless, our findings were able to debunk the misconception that large meals result in hyperglycemia in calves younger than two months of age fed 4 L meals. A recent study has also shown that the abomasum in calves 19 to 23 days of age has a large ability for distention where calves can comfortably consume close to 7 L in one meal without showing signs of distress or ruminal drinking (Ellingsen et al., 2016). Previous concepts of abomasal capacity and meal sizes influencing glucose metabolism are likely not up to date and should be investigated further.

### 4.3 Technical Considerations and Future Studies

To improve our understanding of insulin sensitivity in calves, there are some important knowledge gaps that can be investigated regarding the differences between veal calves and calves fed replacement dairy calf type diets. A recent study has suggested that insulin sensitivity

decreases with age independent of diet (Pantophlet et al., 2016), however the mechanisms behind this are not fully understood. Some studies have suggested changes in the liver and rumen to support VFA utilization may be associated with age (Baldwin et al., 2004). In addition, the pancreas may not be fully developed at birth (Grutter and Blum, 1991). Furthering our knowledge around the development of the liver, rumen, and pancreas, and associated insulin and glucagon secretory mechanisms, will ultimately improve our understanding of insulin secretion and action in calves.

Another direction for future work is to consider characterizing responses to multiple insulin sensitivity tests as there are currently no standardized methods for testing in calves. As an example, there has yet to be a study comparing a pre-prandial and postprandial GTT in pre-weaned calves or under different physiological conditions. Additionally, there is currently no standardization for fasting times before conducting a GTT (as discussed in section 4.1). Finally, the accuracy of various insulin sensitivity tests has mostly only been confirmed in other species, no current literature exists assessing the efficacy of various clamping methods with a GTT in pre-weaning calves. This is an important consideration for future work given that it is still unclear if one method is truly better than the other.

Current literature focusing on glucose metabolism and insulin sensitivity in replacement heifers has primarily only followed heifers up to six months of age (Yunta et al., 2015). Future work investigating insulin sensitivity in dairy calves should continue to quantifying carry over effects which may influence yearlings and future milking cows who display reduced insulin sensitivity during the transition period (i.e. three weeks before and after calving) (Bell, 1995; Drackley, 1999). The decrease in insulin sensitivity during the transition period coupled with a decrease in dry matter intake (DMI) immediately postpartum stimulates a negative energy

balance in lactating cows which compromises cow health and production (Drackley, 1999; Hayirli, 2006). To date no study has been completed that has linked pre-weaning nutrition with glucose metabolism associated issues in cows. Addressing this knowledge gap is an important future direction for dairy science. While the studies conducted in this thesis, and other literature (Yunta et al., 2015), do not indicate carry-over of reduced insulin sensitivity post-weaning, it is important to verify whether feeding an elevated plane of pre-weaning milk negatively or positively impacts lactating dairy cows.

### 4.4 Conclusions

In summary, the aim of this thesis was to assess how feeding an elevated plane of milk could influence glucose metabolism both pre- and post-weaning. Overall the two current studies suggest that, compared to a low plane of milk, feeding an elevated plane of milk twice or four times daily does not negatively affect glucose metabolism. The findings in this thesis contrast current literature conducted in veal calves fed only milk replacer at a low feeding frequency for gains of 1.4 kg/day who display hyperinsulinemia, hyperglycemia, glucosuria, and reduced insulin sensitivity. The difference is results may be attributed to feeding a fixed plane of nutrition of 8 L/day from the first week of life up until two months of age, offering solid feed, and calves undergoing a weaning transition. Findings from this study also contradict current literature in young calves fed 8 L twice daily where calves have been reported to experience reduced insulin sensitivity after a GTT conducted postprandial. The current studies used a GTT conducted after a 12-hour feed restriction to reduce confounding influences on glucose and insulin concentrations from meal digestion, previous literature using postprandial GTT should therefore be considered with caution. The findings from this thesis have significant importance for the dairy industry as

they suggest that dairy calves can be offered an elevated plane of milk pre-weaning under practical situations where dairy producers are feeding calves twice daily, without impairing glucose metabolism and still receiving all the benefits of feeding calves more milk.

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