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Effects of meal timing on anabolic hormone status and energy metabolism in neonatal dairy calves

Katherine Claire Simon

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EFFECTS OF MEAL TIMING ON ANABOLIC HORMONE STATUS AND ENERGY
METABOLISM IN NEONATAL DAIRY CALVES

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
In partial fulfillment of the
Requirements for the degree of
Master of Science

in

The Interdepartmental Program in Animal Sciences

By
Katherine Claire Simon
B.S., Louisiana State University, 2007
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ABSTRACT

Twelve neonatal Holstein bull calves (38.52 ± 5.87 kg) were fed milk replacer at a fixed meal time or a varied meal time to determine the effects of feeding time on metabolic hormone secretion, average daily intake, growth, and energy metabolism. Body weights were measured every two weeks from birth to 8 weeks. Rumen fluid was collected every two weeks from week 2 through 8. Serial blood collections were conducted every two weeks from week 2 through 8. Blood was collected, beginning at 0530, at 0, 15, 30, 45, 60, 75, 90, 105, 120, 135, and 150 minutes. Plasma was analyzed for ghrelin, leptin, growth hormone (GH), and insulin-like growth factor-1 (IGF-1). Treatment did not affect body weight or average daily intake. Mean plasma ghrelin, leptin, GH, and IGF-1 concentrations were not affected by treatment. A treatment by week interaction was observed for total plasma ghrelin concentrations. Total plasma ghrelin concentrations were higher at weeks 2 and 4 in control calves. Total plasma ghrelin concentrations decreased in all calves as they aged. A treatment by time interaction was observed for IGF-1, and a treatment by week by time interaction was observed for growth hormone and IGF-1. Growth hormone decreased as calves aged, while IGF-1 increased. There was no treatment effect or interactions of treatment and week on butyrate and propionate concentrations. However, both butyrate and propionate increased with age. Treatment and week effects were present for acetate, as well as a treatment by week interaction. Calves in the control group had a higher percentage of acetate. Acetate concentration increased in all calves as they aged. At weeks 4 and 8, intravenous glucose tolerance tests were performed to assess glucose clearance. A treatment effect was observed for glucose half life ($T_{1/2}$), glucose clearance rate (k), and insulin. Glucose half- life was higher for calves in the control group, while the clearance rate was lower for the control group. Peak insulin concentrations were higher for calves in the

treatment group. It is concluded that feeding time does not affect overall growth and feed intake, but does have an affect on the some of the regulatory mechanisms that control them.

CHAPTER 1

INTRODUCTION

Calves undergo tremendous physiological and metabolic changes from birth until weaning (NRC, 2001). During the pre-ruminant stage, metabolism and digestion are similar to those of non-ruminant animals in many respects. Investigating and understanding key hormonal regulation of metabolism and growth is important to ensuring proper development. In humans and rats, a stomach-derived hormone, ghrelin, was recently discovered to control appetite, feed intake, and some growth hormone (GH) secretory activity (Inui et al., 2004). The secretory pattern of ghrelin, along with hormones that interact with ghrelin, may be important in understanding the metabolism, feeding behavior, and rumen development of dairy calves.

Research has shown that a pre-meal increase in ghrelin may trigger the desire to eat in animals and humans by stimulating gastric motility and appetite, and inducing a positive energy balance to promote weight gain, in addition to stimulating GH (Inui et al., 2004). In humans, ghrelin levels have been shown to rise before a meal and fall afterwards (Cummings, et al., 2001). Humans undergoing a 24 hour fast, with no food-related ghrelin suppression, showed sudden increases in circulating ghrelin immediately prior to accustomed meal times. The ability of different feeding regimens to alter the number and magnitude of pre-prandial ghrelin surges indicates that ghrelin secretion is actively regulated, and is possibly a conditioned reflex (Mundiger et al., 2006). When sheep were placed on controlled feeding regimens with varying numbers of scheduled meals per day while maintaining the same caloric intake, ghrelin surges matched the number of habitual feedings (Sugino et al., 2003). Researchers have also shown that in sheep trained to eat at a specified time, a pulsatile surge in growth hormone followed a single surge in ghrelin (Sugino et al., 2002). If calves are accustomed to eating at specific meal times, a

ghrelin surge around feeding time may affect growth hormone secretion and nutrient metabolism.

While ghrelin appears to be involved in the stimulation of feed intake, leptin, a hormone produced by adipose tissue, is known to act as a satiety signal in animals. Research has shown that leptin and ghrelin act complementary to each other through stimulation or inhibition of other signals in the arcuate nucleus of the hypothalamus, particularly neuropeptide Y and agouti-related peptide (Inui et al., 2004).

Leptin levels rapidly decline with caloric restriction and weight loss and are associated with adaptive responses to starvation by increased appetite and decreased energy expenditure (Kershaw et al., 2004). Leptin also acts directly on peripheral tissues by stimulating lipolysis and inhibiting lipogenesis in adipose tissue, increasing insulin sensitivity and glucose utilization in muscles and increasing fatty acid oxidation in muscles, liver and adipose tissue (Chilliard et al., 2005). As rumen development takes place in Holstein calves, and the animal's digestive tract shows characteristics specific to ruminants, the gastric leptin expression disappears, leading to the assumption that the satiety mechanism changes during the weaning period (Yonekura et al., 2002).

It has also been speculated that ghrelin may play a role in glucose metabolism and insulin secretion. In mature Suffolk wethers, infused ghrelin enhanced insulin secretion when the secretion was induced by intravenously infused glucose (Takahashi et al., 2006). When ghrelin was infused into lactating dairy cows, a transient rise in insulin occurred. However, in pre-ruminant dairy calves, there was a decrease in insulin concentrations when ghrelin was infused, showing that a difference exists in the effects of infused ghrelin during different physiological states of dairy cattle. The effects of ghrelin secretion on glucose metabolism and pancreatic

hormone secretion are still unclear despite the fact that a relationship between ghrelin, insulin, and glucose metabolism is thought to exist (Itoh et al., 2005).

Most research concerning the interaction between ghrelin, leptin, and other metabolic hormones has been conducted in older animals. Therefore, investigating patterns of ghrelin and leptin secretion in relation to a routine feeding time, and its relation to metabolism in neonatal dairy calves can aid in understanding ruminant neonatal metabolism and development. Based on previous research showing that feeding time affects the secretion patterns of metabolic hormones, this project was designed to study the effects of a changing feeding time on the secretion patterns of ghrelin, leptin, and growth hormone. The effects of a varied feeding time on glucose metabolism and insulin secretion were also studied with the performance of an intravenous glucose tolerance test, based on research that ghrelin can affect insulin secretion and possibly glucose metabolism.

CHAPTER 2

REVIEW OF LITERATURE

Background

The nutritional status of a young animal determines whether proper growth and development will occur. This status is affected by many conditions including nutrient intake, disease, and, in newborn mammals, colostrum consumption. Understanding patterns of physiological hormones associated with feed intake and appetite stimulation, as well as satiety, are important in understanding feed intake and obtaining optimum growth in young animals.

A calf's rumen undergoes three stages of development. During the first stage, from birth until 3 weeks, the rumen is non-functional, and digestion is similar to a non-ruminant. From 3 to 8 weeks of age, the rumen begins to develop. After 8 weeks, the rumen becomes fully developed. As the animal ages, dry matter intake increases, followed by a change in the microbial population of the rumen leading to a more stable rumen environment (Lyford et al., 1988). Changes in dry matter intake may be influenced by changing hormone status.

Recent studies have shown that the hormone, ghrelin, produced by the stomach, stimulates appetite and feed intake. As the young calf grows, a changing gastrointestinal environment may affect hormone production in the calf's gastrointestinal system. A correlation between ghrelin and leptin, known to act as a satiety signal, has been shown in other animals. Current research emphasizes the need to investigate how ghrelin affects feed intake, appetite, nutrient utilization, and energy homeostasis in different physiological states in dairy calves.

Ghrelin

Ghrelin is a recently discovered peptide hormone that was first identified in rat and human stomachs, and is an endogenous ligand for the growth hormone (GH) secretagogue

receptor (Ueno et al., 2005). Ghrelin has been shown to stimulate GH secretion, in addition to appetite stimulation and feed intake. It also stimulates gastric motility, gastric acid secretion, and a positive energy balance which can lead to weight gain (Inui et al., 2004). Ghrelin is a 28-amino acid peptide that is modified at its third residue, a serine, by a middle-chain fatty acid- n-octanoic acid. It appears that the acylation at this particular amino acid residue is essential to its biological activity, especially in the binding and activation of the ghrelin receptor, which is formally known as the growth hormone secretagogue (**GHS**) receptor GHS-R (Kojima et al., 1999). It has been shown in rats that there are two isoforms of ghrelin precursor mRNA, in which one encodes for a 28- amino acid peptide and the other encodes for a 27- amino acid peptide. The 27- amino acid peptide accounts for about 10% of all immunoreactive ghrelin with the 28- amino acid form being the most prevalent. The 27- amino acid form is also able to stimulate increases in intracellular Ca^{2+} and GH secretion at the same degree as the 28- amino acid form (Hosoda et al., 2000). Ghrelin is produced in the chromogranin A- immunoreactive X/A-like endocrine cells located in the mucosal layer of the fundus. These specific cells are associated with the capillary networks that are used to transport ghrelin to the bloodstream to carry out its action (Anderson et al., 2005). Cells containing ghrelin have been found to be located primarily in the area of the abomasum in sheep, suggesting that the abomasum may be the major source of circulating ghrelin (Sugino et al., 2003). The GHS-R are distributed among the hypothalamus, pituitary, brain, stomach, intestine, kidney, pancreas, heart, and aorta in rats and humans (Gnanapavan et al., 2002).

Research shows that ghrelin may act as an appetite stimulating hormone through a pathway that involves neuropeptide Y and agouti-related peptide, both found in the hypothalamus. Neuropeptide Y is produced in the arcuate nucleus neurons that extend to the

adjacent hypothalamic areas such as the paraventricular nucleus, the lateral hypothalamic area, and the brainstem- all of which are major areas that control the regulation of feeding behavior, energy expenditure, and gastrointestinal function (Inui et al., 1999). It is likely that ghrelin, as well as the neuropeptide Y and agouti-related peptide systems, respond to states of fasting and negative energy balance (Inui et al., 2004).

Ghrelin Secretion

Inui et al. (2005) suggest that a pre-meal increase in ghrelin may trigger a desire to eat in animals and humans. Although it is clear that ghrelin secretion is controlled partly by nutrient status, it is possible that during the pre-meal period, other factors such as learned anticipation could contribute to the regulation of ghrelin secretion (Drazen et al., 2006). Concentrations have been shown to rise progressively during fasting and fall to a baseline within an hour of re-feeding (Inui et al., 2004). The ability of different feeding regimens to alter the number and magnitude of pre-prandial ghrelin surges shows that ghrelin secretion is actively regulated, and probably a conditioned response (Mundiger et al., 2006). Sugino et al. (2002) showed that a transient surge of ghrelin occurs just prior to a scheduled meal and pseudo-fed in 1-2 year old Suffolk rams. Sheep that were pseudo-fed were given a nylon bag containing the diet at the scheduled feeding time, but were not able to consume the feed inside. Earlier work by Sugino et al. (2002) showed that, when sheep are trained to being fed either twice or four times daily, ghrelin secretions reached significant peaks just prior to each meal and declined 1 hour after feeding. In the same experiment, sheep were fed ad libitum, and the change in ghrelin concentration was low, but relatively constant. These results demonstrate that ghrelin secretion may not be stimulated by direct contact with ingested feed or nutrients, but probably from psychological factors (Sugino et al., 2002). The transient surge of ghrelin was modified by the feeding regimen, demonstrating

that ghrelin secretion is not due to direct contact with ingested feed but cephalic responses explained as a conditioned physiological reflex (Sugino et al., 2003).

Natalucci (2005) observed the spontaneous 24 hour secretion pattern of ghrelin in fasted humans. Six volunteers, accustomed to eating three meals per day, were fasted from hour 0 of the first day to 0900 of the next day. At 0800 hours on day 2, an unexpected breakfast was served. Blood was collected every twenty minutes from 0800 on day 1 to 0900 on day 2. Results showed a substantial increase in plasma ghrelin concentrations around 4 time points- 0800, between 1200 and 1300, between 1700 and 1900 on day 1, and at 0800 on day 2. Plasma ghrelin concentrations decreased after the morning, noon, and evening peaks. This investigation concluded that plasma ghrelin concentrations have a characteristic diurnal pattern, with a rise and fall at customary mealtimes, and an overall decrease in levels during the 24 hour fast (Natalucci et al., 2005). In rats habituated to large meals compressed into 4 hours, ghrelin increased steadily over the 2 hours preceding the meal, and peaked half an hour before the time food was anticipated. In rats fasted for 4 hours, but not anticipating a meal, there was no pre-prandial rise in ghrelin (Drazen et al., 2006). One interpretation of the response is that the increased ghrelin contributes to the necessary metabolic adjustments the animals need to make sure to eat their full day's ration of calories over a short period (Woods et al., 1991, 1994), and that this anticipatory response becomes entrained over a 14 day period with a regimented meal pattern (Drazen et al., 2006). There is a learned, pre-meal increase of plasma ghrelin when individuals have been trained to eat food at a certain time (Drazen et al., 2006).

Inhibitory signals for ghrelin appear to be somatostatin, interleukin $I\beta$, GH, a high fat diet, and vagal tone. Fasting and low protein diets appear to increase the expression and concentrations of plasma ghrelin. Ghrelin and leptin have been shown to have antagonistic

activity in relation to gastrointestinal function. Ghrelin increases gastric acid secretion, motility, and emptying, while leptin decreases these functions. During starvation, leptin levels decline rapidly, while ghrelin concentrations increase. This increase leads to a stimulation of neuropeptide Y and agouti-related peptide production. In this sense, ghrelin and leptin work antagonistically of each other, demonstrating a type of feedback loop allowing the brain to know the nutritional and energy status of the animal (Inui et al., 2004).

Ghrelin-Leptin Interaction

Ghrelin is the first appetite stimulating signal to be derived from the stomach and is thought to antagonize the satiety signal of leptin (Ueno et al., 2005). Researchers have shown that leptin counteracts ghrelin by way of a phosphatidylinositol 3-kinase and phosphodiesterase-3 pathway. This interaction may play an important role in regulating arcuate nucleus neuropeptide-Y neuron activity and, thereby, feeding (Kohno et al., 2007).

Kohno et al. (2007) investigated the pathways of ghrelin, leptin, and their counteraction in rats. Activities of isolated neurons from the arcuate nucleus were monitored by measuring the cystolic Ca^{2+} concentrations, and the intracellular signaling mechanisms for leptin, ghrelin, and their interaction. Ghrelin and leptin were administered and amplitudes of cystolic Ca^{2+} increases in response to the infusions were calculated by subtracting pre-stimulatory basal levels from peak levels. Administration of ghrelin increased cystolic Ca^{2+} in neurons isolated from the arcuate nucleus. This cystolic Ca^{2+} increase was suppressed by the co-infusion of leptin. To further investigate the intracellular signaling pathway, Kohno first examined the involvement of phosphatidylinositol 3-kinase in the leptin suppression of the ghrelin responses. Pre-incubation with two PI3K inhibitors, LY294002 and wortmannin, abolished the leptin suppression of the increased cystolic Ca^{2+} in response to ghrelin. Kohno then investigated the involvement of

mitogen-activated protein kinase (MAPK), which is another type of signaling pathway for leptin. Pre-incubation with a MAPK inhibitor did not significantly change the suppression by leptin of the ghrelin-induced cytosolic Ca^{2+} increases. Because two downstream effectors of PI3K are considered to be PDE3 and K_{ATP} , Kohno investigated their involvement in leptin suppression of the ghrelin response. Pre-incubation with a PDE3 inhibitor, milrinone, removed the leptin suppression of the ghrelin induced cytosolic Ca^{2+} increase in the arcuate nucleus neurons. The same result occurred when a different PDE3 inhibitor, cilostamide, was used. K_{ATP} channel inhibitors did not significantly change the leptin suppression of the ghrelin response, and K_{ATP} activators did not mimic leptin by suppressing the ghrelin response. Results of this study are indicative of signaling mechanisms for ghrelin, leptin, and their interaction in the arcuate nucleus neuropeptide Y neurons. Ghrelin increases cytosolic Ca^{2+} via mechanisms dependent on the adenylate cyclase- cAMP-PKA and PLC pathways. Leptin counteracts ghrelin induced cytosolic Ca^{2+} increases by way of the PI3K- and PDE3- mediated pathway, whereas MAPK and K_{ATP} channels do not appear to play a significant role (Kohno et al., 2007).

Leptin

Leptin is a hormone produced primarily by adipose tissue that was first identified in rodents in 1994. It contains 167 amino acids and is secreted in direct proportion to adipose tissue mass in addition to nutritional status (Margetic et al., 2002). Although it is mainly produced in white adipose tissue and adipocyte- containing tissues, the leptin gene is also expressed in placental and fetal tissues, mammary gland, muscles, stomach, and brown adipose tissues in rodents and humans, but at low levels (Margetic et al., 2002). In ruminants, the leptin gene is shown to be expressed in adipose tissue (Chilliard et al., 2001), fetal tissues (Yuen et al., 2002; Ehrhardt et al., 2002; Muhlhausler et al., 2003), mammary gland (Bonnet et al., 2002; Smith et

al., 2002; Chelikani et al., 2003; Leury et al., 2003), rumen, abomasum and/or duodenum (Yonekura et al., 2002), and pituitary gland (Yonekura et al., 2003). It is involved in the central and/or peripheral regulation of body homeostasis; energy intake, storage and expenditure; and immune and fertility functions (Chilliard et al., 2005). Leptin and its responses to other factors are strongly linked to the quantity of body fatness (Chilliard et al., 2005). Leptin levels rapidly decline with caloric restriction and weight loss and are associated with adaptive responses to starvation by increased appetite and decreased energy expenditure (Kershaw et al., 2004). Leptin acts directly on peripheral tissues by stimulating lipolysis and inhibiting lipogenesis in adipose tissue; increases insulin sensitivity and glucose utilization in muscles; and increases fatty acid oxidation in muscles, liver and adipose tissue (Chilliard et al., 2005).

In growing dairy cattle, it has been suggested that leptin acts as a partial mediator to the effects of nutrition on reproductive and mammary development. Block (2003) conducted a study that focused on the developmental stage at which the plane of nutrition increases plasma leptin. In the study, neonatal calves were given milk replacer to support different rates of average daily gain. Weekly blood samples were collected between 1100 and 1300 h to monitor leptin levels. Results of the first study showed that plasma concentrations of leptin remained almost constant until one year of age, at which point there was an obvious increase in plasma leptin. This relationship between age and leptin reflects an increased rate of fat deposition as the animals reached a mature size. By the third week of the experiment, calves receiving a higher plane of nutrition had leptin concentrations that were beginning to increase. Those fed according to NRC recommendations (NRC, 2001) had constant adiposity and plasma leptin concentrations (Block et al., 2003). Little information of leptin secretion is available in the early postnatal life of the dairy calf. This information would be beneficial in growing dairy cattle because variations in the

plane of nutrition impact developmental processes that could be regulated by leptin (Block et al., 2003).

Factors Affecting Leptin Secretion

Yonekura et al. (2002) conducted a study to determine the effects of age and weaning on leptin mRNA expression. Leptin receptor mRNA was investigated using a reverse transcription polymerase chain reaction in the rumen, abomasum, and duodenum tissues in male Holsteins that were 3 week old pre-weaned calves, 13 week old post- weaned calves, and adult (1.5-2 yrs) animals. Leptin mRNA was expressed in the rumen and abomasum of 3 week old pre-weaned calves, but was not detected in 13 week old and adult animals. Leptin expression in the duodenum was observed in all age levels. In a second experiment, Yonekura (2002) studied the effect of weaning at 6 weeks, not weaning, or feeding a milk replacer with volatile fatty acids on leptin mRNA expression in the abomasum and rumen of Holstein bull calves. In milk fed animals, leptin mRNA was found in tissue samples of both the rumen and abomasum. In the weaned calves and those fed milk with volatile fatty acids, leptin mRNA was not detected in tissue samples of the rumen or the abomasum. Leptin mRNA was detected in tissue samples from the duodenum in all three groups. Researchers suggest that, although more research is required, gastric leptin in non-weaned pre-ruminant animals may act as a satiety factor as in non-ruminants. When the rumen had developed, and the calf's digestive tract showed characteristics specific to ruminants, the gastric leptin expression disappeared, leading to the assumption that the satiety mechanism changes during the weaning period of the calf (Yonekura et al., 2002).

Yonekura et al. (2003) studied the effects of acetate and butyrate on leptin and leptin receptor expression in the bovine pituitary of 13 week old male Holstein calves. Isolated anterior pituitary cells were added to mediums containing either acetate or butyrate at 1, 3, or 10

millimoles and were cultured for 12 to 48 hours. Leptin expression increased based on the concentration of acetate or butyrate in the medium. Treatment with 10 millimoles of acetate or butyrate also increased leptin expression in a time-dependent manner. The researchers also observed the ratio of the OB-Ra, the leptin receptor, to glyceraldehyde-3-phosphate dehydrogenase (G3PDH), in the control culture was 1.00 ± 0.09 . The anterior pituitary cells in the control culture were not exposed to acetate and butyrate, and were maintained in fetal bovine serum with Dulbecco's Modified Eagle's Medium. This ratio was significantly reduced in the culture treated with butyrate, but not acetate. The findings of this study show that leptin and leptin receptors are expressed in the bovine pituitary cells and are modulated by acetate and butyrate. This up-regulation of leptin expression exerted by acetate and butyrate suggest that in the bovine anterior pituitary, expression is regulated by nutrient availability (Yonekura et al., 2003).

Houseknecht (2000) studied the ability of GH to regulate leptin mRNA expression in bovine adipose tissue. Castrated male Angus steers (296.9 ± 27 kg body weight) were injected daily with either 200 $\mu\text{g/kg}$ body weight of saline or GH for three days. Then, a subcutaneous adipose tissue sample was collected to determine the effects of the injections on leptin gene expression in subcutaneous adipose tissue before a GH induced change in adiposity could occur. The steers were given 7 days to recover from the first experiment, and then the experiment was conducted again, but the treatments were alternated. These researchers also investigated the effect of GH treatment on insulin and dexamethasone stimulated leptin expression. Varying concentrations and combinations of insulin, dexamethasone, and GH were included in the culture medium, and the regulation of leptin expression in tissue culture was determined. Results showed that both insulin and dexamethasone at high concentrations significantly stimulated

bovine leptin mRNA abundance when compared to basal levels. There was no additive effect of insulin and dexamethasone. GH alone did not increase leptin gene expression. However, when combined with high concentrations of insulin, dexamethasone, or both, GH attenuated the stimulatory effects of insulin and dexamethasone shown previously. To expand the previous experiment, Houseknecht (2000) examined the effect of daily GH administration (200 µg/kg body weight) on leptin gene expression. Calves that had an increase in IGF-1 mRNA in adipose tissue in response to the GH were used as a positive control to ensure that the animals were eliciting a known biological response to the GH. In the animals used, GH caused a significant increase in IGF-1 mRNA abundance in adipose tissue. GH treatment increased leptin mRNA abundance significantly in those exhibiting the positive IGF-1mRNA response. Those animals in negative energy balance, with no response to the GH administration, had a significant down-regulation of leptin expression. Results indicated that concentrations of leptin mRNA were highly correlated with adipose tissue IGF-1 mRNA concentration during both positive and negative energy balance. Other researchers have reported a negative effect of IGF-1 on leptin gene expression, thus more research is needed to clarify if the effects of GH on leptin gene expression are mediated via effects on IGF-1 (Houseknecht et al., 2000).

Growth Hormone

GH is a 191 amino acid polypeptide that is synthesized by somatotrophs in the anterior pituitary. It acts by inducing protein synthesis and nitrogen retention and impairs glucose tolerance by antagonizing insulin's action. GH stimulates lipolysis, which causes an increase in circulating fatty acid levels, and enhances lean body mass. The secretion of GH is known to be controlled only by GH releasing hormone (GHRH) and by somatostatin, produced in the hypothalamus and transported to the anterior pituitary gland (Guyton and Hall, 2006). However,

with the recent discovery of ghrelin and its ability to bind to a GH secretagogue receptor, there is an increasing interest in the effects of ghrelin on GH secretion.

Effects of Ghrelin on Growth Hormone Secretion

Itoh et al. (2005) conducted a study to determine the differences of GH secretion to ghrelin and GHRH in growing dairy cattle. On the day of the experiment, either ghrelin (0.3 nmol/kg BW), GHRH (0.3 nmol/kg BW), or 2% bovine serum albumin saline were intravenously infused into suckling (10 day old) and weanling (111 days old) Holstein calves. Both GHRH and ghrelin stimulated an increase in GH concentrations in all animals, while the control injection of saline had no effect regardless of the physiological state. In suckling calves, 7.5 to 15 minutes after the ghrelin injection, there was a brief rise in GH. However, there was a significant rise in GH in response to the GHRH injection at this same time. The GH response to GHRH was greater than the response from the ghrelin injection. Similar results were observed in the 3-month old weanling calves (Itoh et al., 2005).

ThidarMyint et al. (2008) conducted a similar study to investigate the effects of ghrelin, GHRH, and a combination of the two hormones on plasma GH, IGF-1, insulin, glucose, and non-esterified fatty acids (NEFA) in Holstein bull calves. Basal levels of plasma insulin, and NEFAs were higher during the pre-weaning period, but concentrations of active and total ghrelin, GH, and IGF-1 were similar for pre- and post-weaning periods. In the pre-weaning period, GH levels peaked within 5 minutes of the injection. Responses to ghrelin and GHRH alone were similar, but the response to the combined administration was greater than the sum of ghrelin and GHRH alone. In the post-weaning period, plasma GH increased immediately following the injections, and peaked at 10 minutes. Levels of GH gradually returned to baseline values. Injection with

ghrelin, GHRH, or a combination of ghrelin and GHRH did not alter plasma insulin, IGF-1, glucose or NEFA concentrations (ThidarMyint et al., 2008).

Hashizume et al. (2005) conducted a study using three male Holstein calves, aged 8-9 months, to determine the effects of an intra-hypothalamic injection of ghrelin. On the days of the experiment, calves were fasted until after blood was collected. A unilateral guide cannula was surgically implanted into the medial basal hypothalamus three weeks prior to the experiment. Catheters for blood collection were inserted 1 day prior to collection. On the day of the experiment, a micro-infusion cannula was inserted through the implanted cannula. Following the cannula insertion, 10 nmol of ghrelin in 200 μ l of saline or 200 μ l of saline was infused over a period of 30 seconds. Blood samples were collected every 10 minutes beginning 50 minutes before infusion and continuing to 5 hours post-infusion. Results showed that mean plasma concentrations of GH were not significantly altered with the saline infusion. However, GH concentrations began to increase just following the infusion of ghrelin and were significantly higher at 20, 30, and 40 minutes after the infusion when compared to the saline control (Hashizume et al., 2005). Hashizume (2005) concluded that ghrelin stimulates GH release in vivo in ruminants.

Wertz et al. (2005) conducted a study to determine the effects of an intravenous infusion of ghrelin on GH concentrations. Six beef steers were used in a crossover designed experiment. Saline or bovine ghrelin was infused through a jugular catheter at 1200h and 1400h. Times for ghrelin infusion were chosen when the steers were normally not consuming feed so as to assure them being in a satiated state. A serial blood collection was conducted beginning at 0600 h and continuing to 1800 h, with samples being collected every 15 minutes. Ghrelin was infused to reach a concentration of 1000 pg/ml, based on previous research that indicated a peak of this

concentration in fasted steers. Steers were allowed a 5 day rest between the first and second treatment periods, and then the treatments were switched and the treatment/sampling period was repeated (Wertz et al., 2005). Results showed that infused bovine ghrelin elevated plasma GH concentrations at the initial sampling after the first infusion time. After the second infusion, GH concentrations tended to be higher, but were not different from the control steers. The magnitude of increase of GH was smaller when compared to the first infusion. Wertz (2005) suggests that, although a clear explanation for these results cannot be established, it is possible that the attenuated GH response after the second infusion could be a result of a feedback mechanism in response to the ghrelin surges.

Effects of Ghrelin on Insulin Secretion and Glucose Metabolism

Insulin is a polypeptide hormone that is secreted by the pancreas and consists of two chains, A and B, composed of 21 and 30 amino acids, respectively. Insulin is synthesized in the beta cells of the pancreas. The release of insulin is affected by glucose, mannose, leucine, and other amino acids, ketone bodies, and fatty acids, with blood glucose being the primary regulator of both insulin release and synthesis (Kaneko et al., 1997).

Insulin is as essential as GH because of its requirement in the synthesis of proteins. The administration of either insulin or GH causes almost no growth; however, it appears that the hormones function synergistically to promote growth (Guyton and Hall, 2006).

Itoh (2006) studied the effects of infusing ghrelin on plasma glucose and insulin concentrations. Pre-ruminant calves (10 days old) were infused with either ghrelin or a control buffer solution, followed by a serial blood collection; calves were allowed three days to readjust from the treatments before being infused with the opposite treatment. The first blood sample was collected 3-4 hours after the calves were fed. Ghrelin infusion had no effect on glucose

concentration, but insulin concentrations were lower from 45-180 minutes after the ghrelin infusion when compared to basal concentrations. There was no change in insulin after the control infusion. The effects of ghrelin on glucose metabolism and insulin secretion are still unclear even though a relationship between them is believed to exist (Itoh et al., 2006).

Similar results were reported in an experiment designed to determine if a relationship between ghrelin, insulin, and glucose concentrations existed in fed versus fasted steers. Two year old beef steers were divided into one of two groups, either fed or fasted. The steers were acclimated to a climate controlled environment for 11 days prior to the experimental period. Steers were fed once a day and allowed feed for a specific time period. On the day of blood collection, for the steers on the “fed” treatment, feed was given at the adapted time. Feed was not offered to the fasted calves for a 48 hour period, and blood was collected from all steers during this period. Plasma ghrelin concentrations were higher in the fasted steers than those that were fed, and concentrations continued to increase throughout the length of the fast. Plasma insulin concentrations were greater in the steers that were fed as opposed to those that were fasted. However, glucose concentrations were similar despite the nutritional state of the steer (Wertz et al., 2006).

Effects of Nutritional Status and Feed Intake

Wertz (2006) conducted a study to determine the effects of pulsed intravenous bovine ghrelin infusions on dry matter intake in beef steers. Two year old beef steers were acclimated for 10 days in a climate controlled facility to a specific feeding schedule. Dry matter intake was measured using a digital load cell, with the feeder weight data logged at 20 second intervals. Data was used to calculate the length of time spent feeding and dry matter intake, with measurements beginning 2 days before treatment, and occurring the day of treatment, and 1 day

after treatment. Bovine ghrelin was infused at 1200 and 1400 hours to establish a peak concentration of 1000 pg/mL. This concentration was previously determined to be the concentration in fasted steers. Saline was infused into the control steers. The length of time spent feeding and dry matter intake were similar between the groups prior to treatment with bovine ghrelin. Dry matter intake was not different between the groups at each of the 1 hour post infusion periods. However, when the data for the two 1 hour post infusion periods were combined, the length of time spent feeding was greater in those infused with ghrelin than those infused with saline (Wertz et al., 2006).

Bohan (2007) investigated the effects of feeding a no growth versus low growth versus a high growth milk replacer on plasma ghrelin levels in pre-ruminant calves. No growth calves were targeted to gain 0.0 kg daily, low growth was targeted to gain 0.55 kg daily, and high growth was targeted to gain 1.2 kg daily. Twenty-four Holstein bull calves were acquired over a 2 week period. Prior to the trial, calves were fed a 20% crude protein/20% fat milk replacer twice a day. Calves were approximately 9 days old on the first day of the experiment. Weights were taken weekly, and milk replacer offered was adjusted each week to allow for the changing body weights. Blood was collected weekly prior to morning feeding. Body weights were similar prior to the initiation of treatments, but by week one, a difference was measured between the three treatment groups, and continued throughout the experiment. Calves on the no growth diet had higher concentrations of active ghrelin than those on the low and high growth diets. Calves on the low growth diet had higher concentrations of active ghrelin than calves on the high growth diet, but the difference was not as pronounced after the 3rd week of the experiment. There was a decrease in active ghrelin at weeks 4 and 6 of the experiment among all groups. There was also no difference in total ghrelin concentrations between the low and high groups; however the ratio

of total to active ghrelin concentrations was higher in the no growth calves than in the low or high growth calves. Calves on the no and low growth diets had higher active ghrelin than those on the high growth diet, but the total amount secreted is the same in response to energy intake. Researchers suggest that changing the ratio of active to des-acyl ghrelin may be a possible route by which calves regulate the physiological effects of active ghrelin in response to energy intake (Bohan et al., 2007). Des-acyl ghrelin lacks the n-octanoic acid modification on the third serine which appears to be necessary for the activation of cells with GHS receptors (Hosoda et al, 2000).

Based on research previously described, the following experiment was designed to study the effects of meal timing on specific anabolic hormones, overall growth, and glucose metabolism. Researchers have found that, in animals trained to eat at regularly scheduled times, ghrelin concentrations increased in anticipation of a meal. When infused, researchers have also found that ghrelin caused an increase in GH, and in neonatal dairy calves, caused a decrease in insulin secretion. The results from the previously discussed studies indicate that ghrelin may have an affect on growth and metabolism. Researching patterns of normal ghrelin secretion and its effects on growth and metabolism is important to understand for dairy calf management purposes.

CHAPTER 3

MATERIALS AND METHODS

Animals and Dietary Treatments

Twelve neonatal Holstein bulls (38.52 ± 5.87 kg) were used in an eight week experiment to assess the effects of feeding time on anabolic hormone status and glucose metabolism. All animals were born and raised on the LSU Agricultural Center's Dairy Research and Teaching Farm. Calves were born between August and September, 2008. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the LSU Agricultural Center.

In the experiment, calves were separated from their dams within 3 hours of birth, weighed, and moved to individual calf hutches measuring 2.5 m^2 with an additional 2.8 m^2 wire enclosure on rock bedding. Calves were vaccinated orally for Rotavirus and Coronavirus (Calf Guard, Pfizer Animal Health, Lenexa, KS) prior to receiving colostrum. For the first day of life, calves received 4-6 liters of first milking colostrum, which was followed by transition milk on days 2 and 3. On day 4 of life, calves were given milk replacer (20% protein, 20% fat; Nutra Blend LLC, Neosho, MO) (MR), reconstituted to 15% solids and containing decoquinat, at 10% of their birth weight and trained to drink from a bucket. Water was offered ad libitum. At 2 weeks of age, following the first serial blood collection, calves were offered a starter diet (19% CP; Table 1) ad libitum. Starter intake was measured twice a day. Calves were abruptly weaned from the MR at 6 weeks of age.

At birth, calves were assigned to one of two treatment groups. Calves in the regularly fed group (**REG**) were offered MR at 0630 every morning. Those fed irregularly (**IRR**) were offered milk replacer at 0600, 0830, 0715, 0900, 0630, 0800, and 0730 on Monday, Tuesday, Wednesday, Thursday, Friday, Saturday, and Sunday, respectively.

Sample Collection

Body weights were recorded bi-weekly from birth through 8 weeks. Starter intake was measured daily from week 3 through 8 at 0600 and 1530. Rumen fluid was collected via esophageal tube 4 hours post feeding at 2, 4, 6, and 8 weeks of age for rumen pH and volatile fatty acid analysis.

Serial blood collections were conducted via a 14 g x 17 cm jugular catheter (MILA International, Inc.; Erlanger, KY) at 2, 4, 6, and 8 weeks of age beginning at 0530, and at 15 minute intervals, through 150 minutes. Calves were fasted for the duration of the collection time. Blood was collected 10 mls at a time and divided between K₂EDTA and potassium oxalate /sodium fluoride tubes (Kendall Medical, St. Louis, MO). Aprotinin (Sigma Chemical, St. Louis MO) (500 KIU per ml) was added to the K₂EDTA tubes that were to be later assayed for ghrelin. Blood samples were centrifuged within 30 minutes of collection, and plasma was stored frozen until analysis.

At 4 and 8 weeks of age, an intravenous glucose tolerance test (IVGTT) was performed at 0700 hour. Calves were fasted for the duration of the glucose tolerance test. A 50% glucose solution (Kaneko, 1997) was infused (0.5g/kg body weight) through a jugular catheter at time 0. Blood was collected in potassium oxalate/sodium fluoride (Kendall Medical, St. Louis, MO) tubes at -10 and 0 minutes pre-glucose infusion and 5, 10, 15, 25, 35, 45, and 60 minutes post- glucose infusion. Samples were centrifuged within 30 minutes of collection and plasma was stored frozen for analysis of glucose and insulin.

Table 1. Calf Starter Composition

<u>Ingredient</u>	<u>% As Fed</u>
Rolled or Cracked Corn	35.00
Kentwood Custom Heifer-R ¹	1.50
Pro-Lak	2.50
Friends HI-Fat 14-5	10.00
Rumensin/Vitamin E Premix ²	1.00
Cargill Pellet Milk +	2.50
DDG	9.00
Sweet Stuff	5.00
Protein Pellets	12.25
Oats, rolled or crimped	17.50
Molasses	3.75
Chemical Analysis	
Dry Matter (%)	89.89
TDN, (%DM) ³	79.6
Crude Protein, (% DM)	19.12
Netural Detergent Fiber, (% DM)	19.49
<u>Acid Detergent Fiber, (%DM)</u>	<u>7.86</u>

¹Kentwood Custom Heifer-R contains Monensin 2,400 g/ton, Calcium(Min) 15.00%, Calcium(Max) 18.00%, Phosphorus(Min) 5.75%, Salt(Min) 18.00%, Salt(Max) 21.00%, Magnesium(Min) 2.60%, Potassium(Min) 0.90%, Sulfur(Min) 1.00%, Cobalt(Min) 25 ppm, Copper(Min) 800 ppm, Iodine(Min) 80 ppm, Manganese(Min) 2,700 ppm, Selenium(Min) 20 ppm, Zinc(Min) 2,750 ppm, Vitamin A(Min) 200,000 IU/lb, Vitamin D-3(Min) 45,000 IU/lb, Vitamin E(Min) 1,000 IU/lb

²Rumensin/Vitamin E Premix contained 94.5% dried distiller's grain, 0.5% Rumensin, and 5% Vitamin E

³TDN= Total Digestible Nutrients calculated according to NRC (2001).

Laboratory Procedures

Plasma was analyzed for total ghrelin concentrations using radioimmunoassay kits (Human Ghrelin (Total) RIA; Millipore, St. Charles, MO) (Appendix A). The intra- and inter-assay coefficients of variation are 1.898% and 1.945%, respectively. Plasma was analyzed for leptin concentrations by radioimmunoassay (Cartmill et al., 2003). The intra- and inter-assay coefficients of variation for the leptin assay were 6.44 % and 8.85%, respectively (Appendix B). Plasma was analyzed for GH concentrations using radioimmunoassay procedures as described by Granger et al. (1989). The intra- and inter-assay coefficients of variation were 9.69% and 11.10%, respectively (Appendix C). Plasma was analyzed for IGF-1 concentrations using a radioimmunoassay procedure as modified for cattle by Sticker et al. (1995). The intra- and inter-assay coefficients of variation were 1.46% and 2.01%, respectively (Appendix D). Plasma was analyzed for glucose using an oxidase reagent set (Glucose Oxidase Reagent Set; Pointe Scientific, Lincoln Park, MI) (Appendix E). Plasma was analyzed for insulin using commercial radioimmunoassay kits (Coat-A-Count; Diagnostic Products Corporation, Los Angeles, CA) (Appendix F). Rumen fluid was analyzed for volatile fatty acids by gas-liquid chromatography using a Shimadzu GC 2010 equipped with a 15- m EC 1000 column, having an internal diameter of 0.53 mm and a film thickness of 1.2 μm (Alltech Associates, Inc.; Deerfield, IL). The procedures for reagent preparation and the temperature gradient for the VFA analysis were adapted from Grigsby et al. (1992) and Bateman, et al. (2002) (Appendix G).

Statistical Methods and Calculations

Starter intake data, reduced to weekly means prior to analysis, and bi-weekly body weights were analyzed by ANOVA using a mixed model (Littell et al., 1998). For the experiment, the model used included terms for the fixed effects of treatment, week, and

treatment by week interaction. The random variable was calf nested within treatment. Week was the repeated term that was assumed to be correlated within calf using a compound symmetry covariance structure.

Hormone data were analyzed by ANOVA using a mixed model (Littell et al., 1998). The model included fixed effects of treatment, week, time, and the interaction of treatment, week, and time. The random variables were calf nested within treatment and week by calf nested within treatment. Week and time were modeled as the repeated terms that were assumed to be correlated within calf with a compound symmetry covariance structure.

Plasma glucose response to the intravenous glucose tolerance test was evaluated by calculating the fractional turnover rate (k) and the half life ($T_{1/2}$) (Kaneko, 1997; Forbes et al., 1998) for the period from 5 to 60 minutes after glucose infusion. The peak insulin concentration was determined for the same period, and the time at which the peak concentration occurred was recorded. Week and sample time were modeled as repeated terms for the analysis of insulin and glucose concentrations, and both were assumed to be correlated within calf with a compound symmetry covariance structure.

Effects and interactions of all measured parameters were determined to be significant if $P < 0.10$.

CHAPTER 4

RESULTS AND DISCUSSION

Performance Data

Least squares means of body weights for calves fed MR at regular versus irregular feeding times are presented in Figure 1. Least squares means for average daily starter intake for calves are presented in Figure 2. There was no feeding time by week interaction for body weight or starter intake ($P > 0.1$) nor were there main effects of feeding time on body weight or starter intake ($P > 0.1$). Body weight and starter intake increased as the calves aged ($P < 0.05$). As expected with normal growth, body weights increased as starter intake increased.

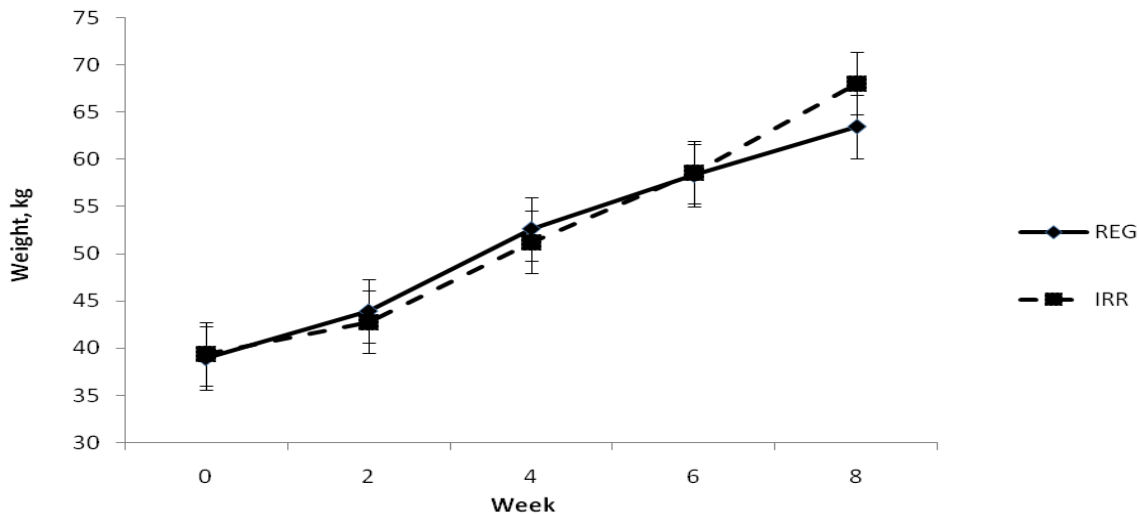


Figure 1. Least squares means of body weights for calves fed MR at regular (**REG**) versus irregular (**IRR**) feeding times. There was a main effect of week ($P < 0.05$).

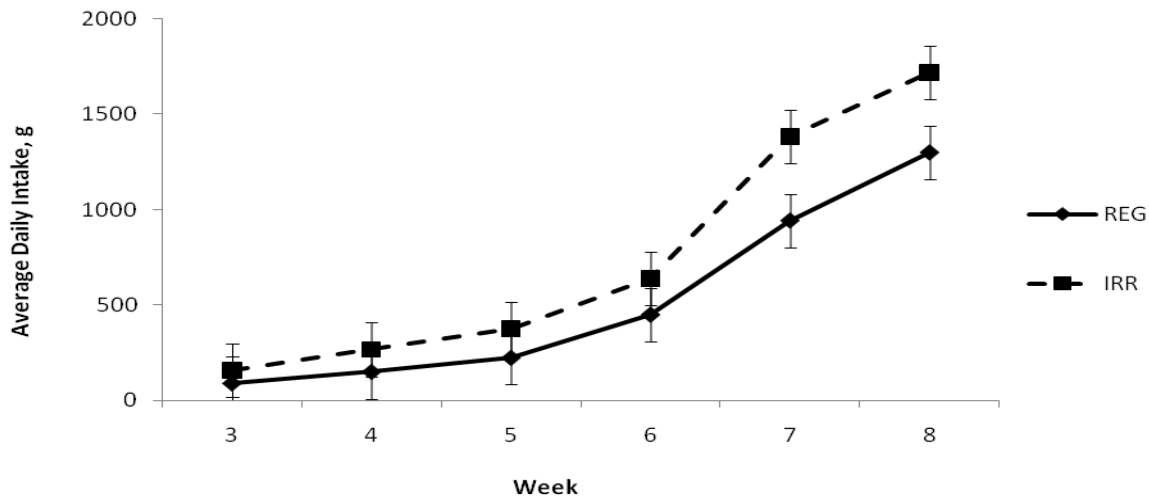


Figure 2. Least squares means of average daily starter intake for calves fed MR at regular (**REG**) versus irregular (**IRR**) feeding times. There was a main effect of week ($P < 0.05$).

Hormone and Metabolite Data

Least squares means for weekly plasma ghrelin are presented in Figure 3. There was a treatment by week interaction ($P < 0.05$). At weeks 2 and 4, calves fed at a regular feeding time had a higher concentration of plasma ghrelin than those with an irregular feeding time. However, when calves were weaned at week 6 and were no longer being fed milk replacer at a regular time, ghrelin concentrations did not differ. A main effect of week ($P < 0.05$) was present for calves in both groups, with an overall decrease in plasma ghrelin concentrations as the calves aged. Least squares means of total plasma ghrelin by minute for calves fed MR at regular versus irregular times are presented in Figure 4. Least squares means of plasma ghrelin by minute for all calves is presented in Figure 5. A main effect of minute ($P < 0.05$) was observed. Concentrations of plasma ghrelin for all calves varied from time point to time point during the sample collection period. Calves fed MR regularly showed a slight increase in ghrelin prior to regular feeding time ($t = 60$ min); however, calves fed irregularly demonstrated no pattern

surrounding regular feeding time. Sugino et al. (2002) found that, in mature sheep fed twice a day, plasma ghrelin concentrations were higher than in those fed four times a day. In mature sheep fed ad libitum, plasma ghrelin concentrations remained low, and were relatively constant (Sugino, et al., 2002). Bohan et al. (2007) observed a decrease in total plasma ghrelin as neonatal dairy calves aged, regardless of their plane of nutrition. Results of the current experiment show that, while calves were being fed milk replacer at a regularly scheduled time, total plasma ghrelin increased just prior to normal feeding time. However, calves fed at irregular feeding times showed no increase in total plasma ghrelin prior to the time of regular feeding.

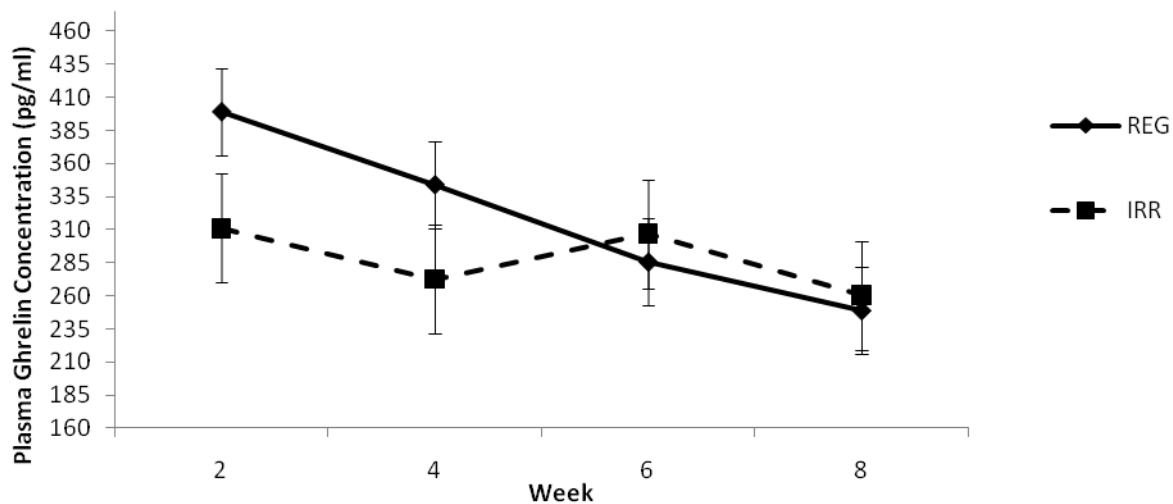


Figure 3. Least squares means of plasma ghrelin by week in calves fed MR at regular (**REG**) versus irregular (**IRR**) feeding times. There was a treatment by week interaction, and a main effect of week ($P < 0.05$).

Least squares means for weekly plasma leptin concentrations are presented in Figure 6. Plasma leptin concentrations were not affected by fluctuating MR feeding times ($P > 0.10$). Least squares means for leptin concentrations by minute for REG versus IRR are presented in Figure 7. There were no main effects or interactions ($P > 0.10$). These results were expected,

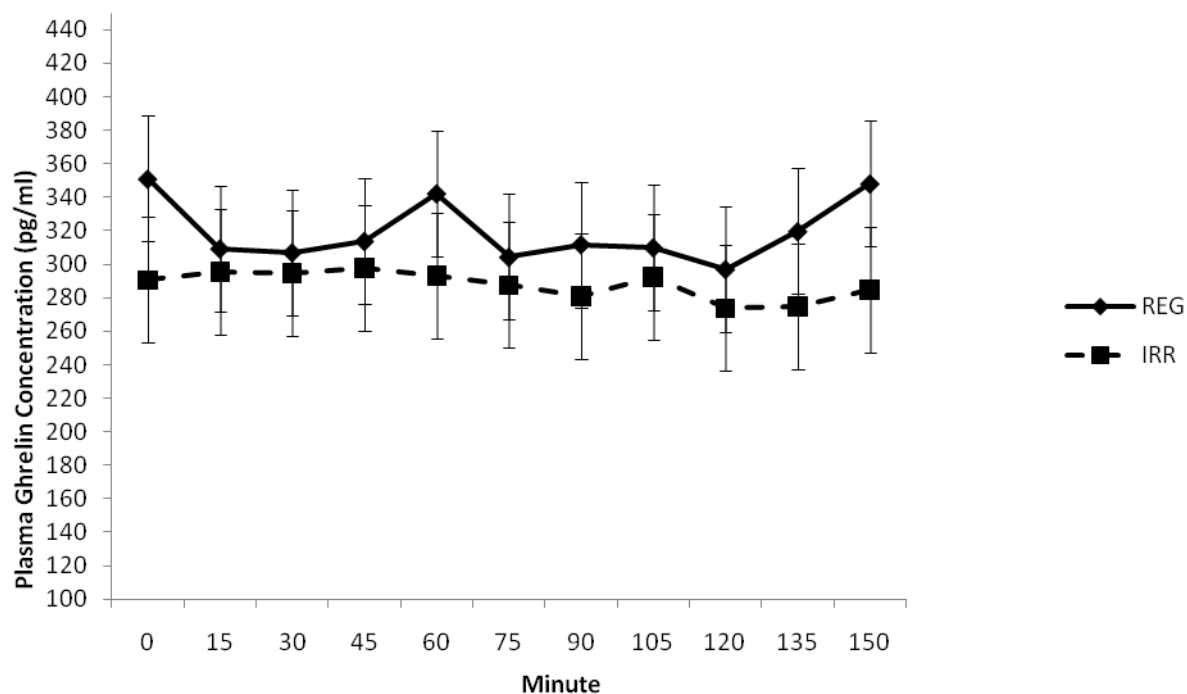


Figure 4. Least squares means of plasma ghrelin by minute in calves fed MR at regular (**REG**) versus irregular (**IRR**) feeding times. Regular feeding time was at t= 60 minutes. There was a main effect of minute ($P < 0.05$).

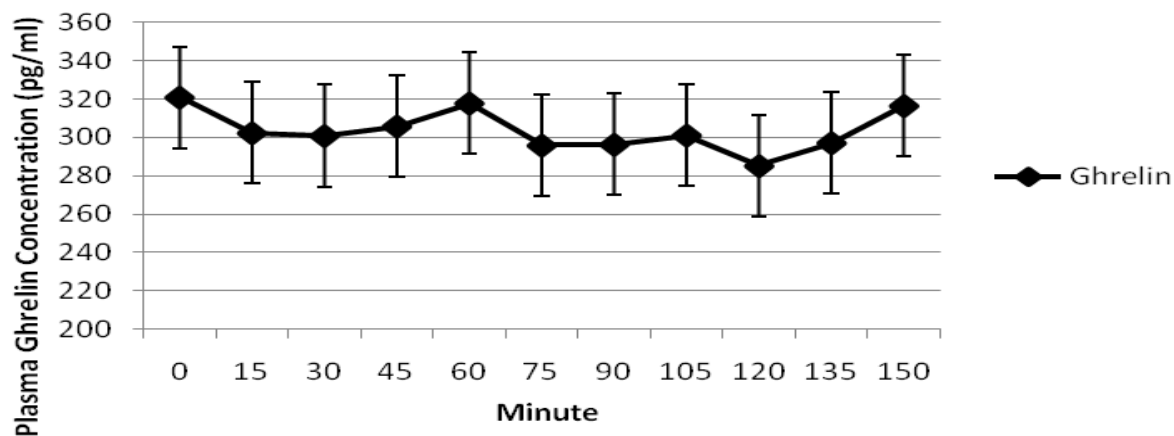


Figure 5. Least squares means of plasma ghrelin by minute for all calves. There was a main effect of minute ($P < 0.05$).

considering that the concentration of plasma leptin is dependent upon amounts of adipose tissue and tends to increase post-prandially. Block et al. (2003) found that leptin concentrations remained constant until one year of age in neonatal Holstein bulls and heifers. Dairy calves fed according to NRC recommendations (NRC, 2001) maintained constant leptin concentrations as opposed to those fed a higher plane of nutrition whose leptin concentrations increased (Block et al., 2003). Calves in the current experiment were fasted for the duration of the serial blood collection. Also, because of age, adipose tissue deposition was very small, indicating that there should be low levels of plasma leptin.

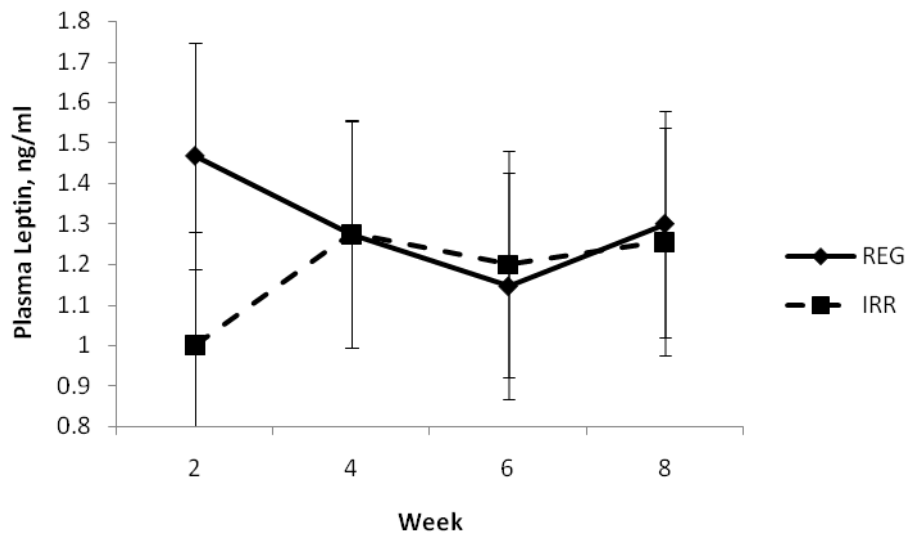


Figure 6. Least squares means of leptin concentrations in calves fed MR at regular (**REG**) versus irregular (**IRR**) feeding times.

Least squares means of plasma GH concentrations at each week for REG versus IRR calves are shown in Figure 8. There were no overall main effects of treatment on plasma GH concentrations ($P > 0.10$). There was a main effect of week ($P < 0.05$), with a decrease in GH in both groups over time. Least squares means for plasma GH at each minute are presented in Figure 9. A treatment by time interaction was present ($P < 0.10$). Prior to regular feeding time

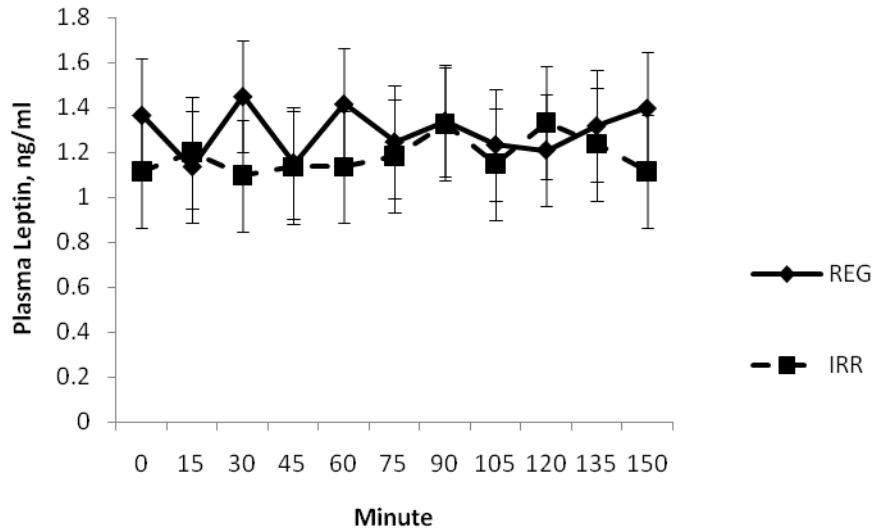


Figure 7. Least squares means of plasma leptin by minute in calves fed MR at regular (**REG**) versus irregular (**IRR**) feeding times. Regular feeding time was at t= 60 minutes.

(t=60), IRR calves had higher GH than REG calves. Figure 10 shows least squares means for GH concentrations at each minute at each week. There was an overall treatment by week by minute interaction ($P < 0.05$). Calves fed MR at irregular times had higher GH concentrations after week 4. Calves fed MR at regular times had higher GH concentrations at weeks 2 and 4, but these concentrations decreased at weeks 6 and 8. In contrast to the overall decrease in plasma GH, Buonomo & Baile (1991) found that nutritional restriction in pigs leading to growth arrest was associated with an increase in plasma GH concentration. During times of reduced growth rates, such as fasting, starvation, or poor nutrition, animals have increased concentrations of plasma GH (Brameld, 1997). When ghrelin was infused into growing dairy cattle, GH increased following the infusion (Itoh, et al., 2005; Hashizume et al., 2005; Wertz et al., 2005). Research also showed that, in rats, GH administration decreased plasma ghrelin levels significantly leading to the idea that GH exerts a negative feedback action on stomach-derived

ghrelin (Qi et al., 2003). Results of the present study show that as physiologic ghrelin decreased with age, GH also decreased indicating a possible relationship between their secretions.

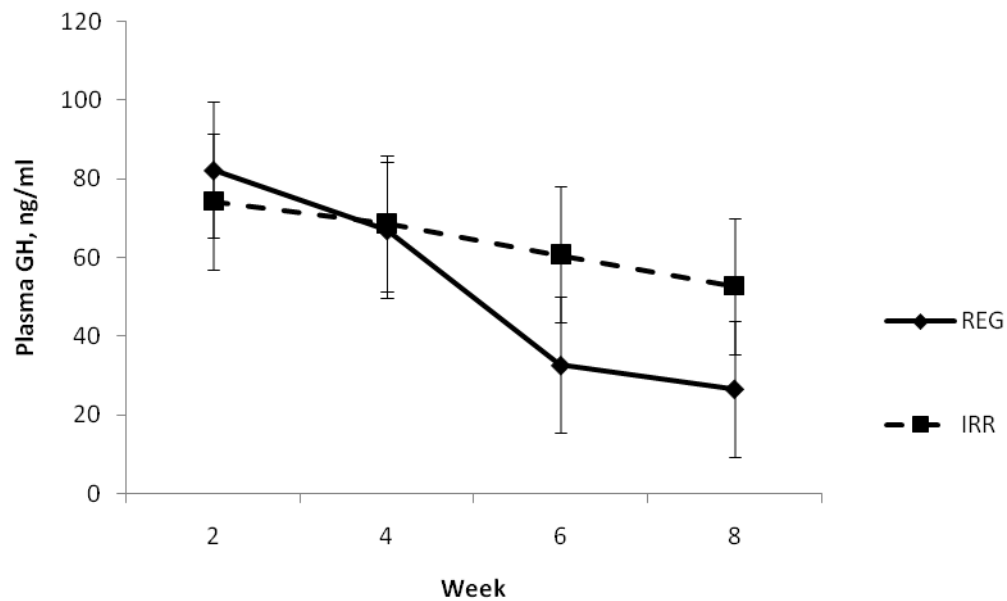


Figure 8. Least squares means of plasma GH at each week in calves fed MR at regular (**REG**) versus irregular (**IRR**) feeding times. There was a main effect of week ($P < 0.05$).

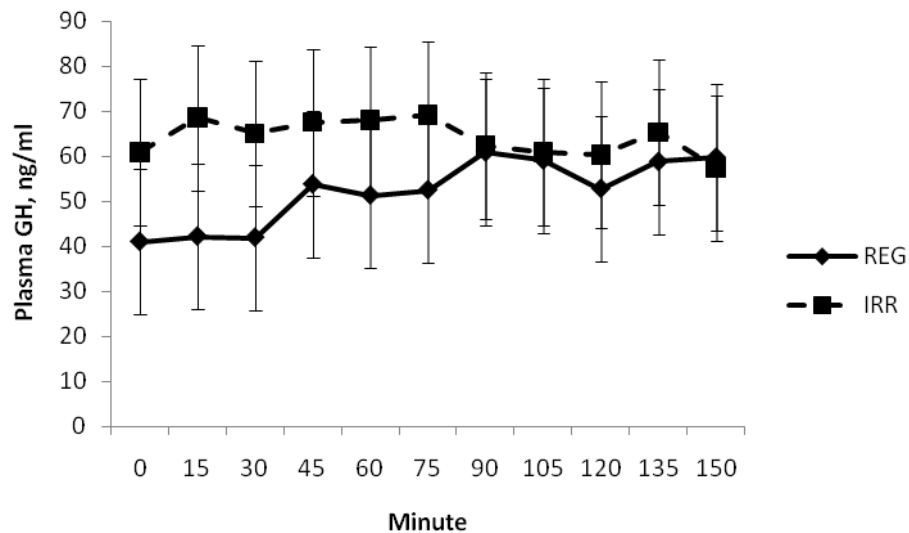


Figure 9. Least squares means of plasma GH at each minute in calves fed MR at regular (**REG**) versus irregular (**IRR**) feeding times. Regular feeding time was at $t = 60$ minutes. There was a treatment by time interaction ($P < 0.10$).

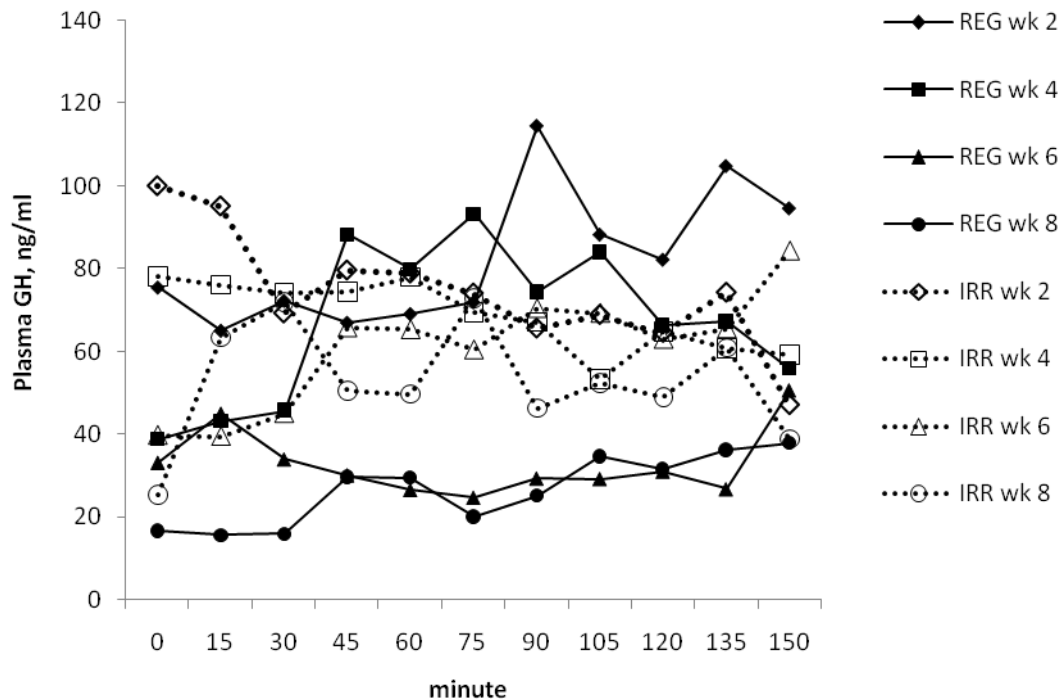


Figure 10. Least squares means of plasma GH at each minute for each week in calves fed MR at regular (**REG**) versus irregular (**IRR**) feeding times. Regular feeding time was at t= 60 minutes. A treatment by week by time interaction ($P < 0.05$) was present. SEM= 20.77

Least squares means for plasma IGF-1 concentrations at weeks 2, 4, 6, 8 are shown in Figure 11. Plasma IGF-1 concentrations were not affected by treatment or minute ($P > 0.10$). There was a main effect of week ($P < 0.05$), with IGF-1 concentrations increasing as calves aged. Calves fed regularly had significantly higher IGF-1 concentrations at week 6, but concentrations at weeks 2, 4, and 8 were not significantly different. Least squares means for IGF-1 concentrations at each minute are presented in Figure 12. Results showed significant treatment by time ($P < 0.10$) interaction between calves fed MR at a regular time versus irregular time. IGF-1 concentrations tended to be higher for calves fed regularly versus irregularly, particularly at t= 30 and t= 150 minutes. Figure 13 represents least squares means for plasma IGF-1 concentrations at each minute of each week for calves fed MR regularly versus irregularly.

Plasma IGF-1 concentrations increased over time, and were higher in the regularly fed calves at week 6, prior to weaning, and week 8. In swine, reduced blood concentrations of IGF-1 were associated with times of reduced growth rates, such as starvation, fasting, or poor nutrition (Brameld, 1997). Buonomo and Baile (1991) found that, after 48 hrs of feed restriction, circulating IGF-1 concentrations were significantly decreased in swine. However, results of this study showed that IGF-1 concentrations increased as the calves aged, leading to the idea that an irregular feeding pattern does not equate to poor nutrition.

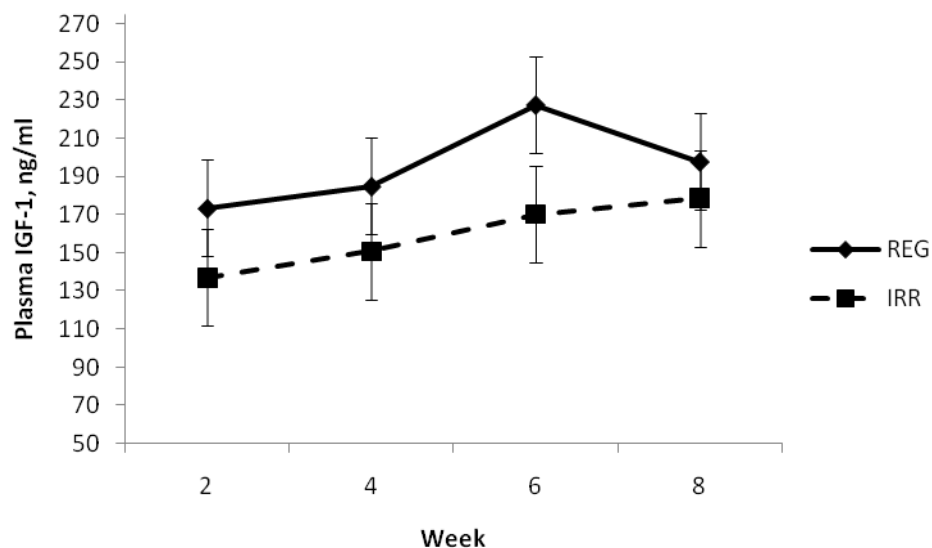


Figure 11. Least squares means of plasma IGF-1 for each week in calves fed MR at regular (**REG**) versus irregular (**IRR**) feeding times. There was a main effect of week ($P < 0.05$).

Intravenous Glucose Tolerance Test (IVGTT)

Least squares means for glucose clearance rate (k), half life ($T_{1/2}$), peak insulin concentration and peak insulin time at weeks 4 and 8 presented in Table 2. Glucose clearance rate, k , was effected by feeding time ($P < 0.05$). Calves fed MR at irregular times had a faster glucose clearance rate than those fed MR regularly. However, no effect of week or treatment by week interaction ($P > 0.10$) was observed. Glucose half life was affected by MR feeding time

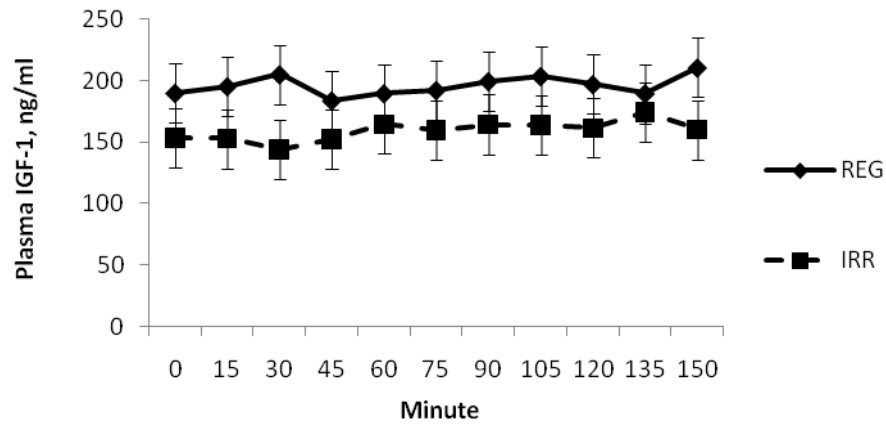


Figure 12. Least squares means of plasma IGF-1 by minute in calves fed MR at regular (**REG**) versus irregular (**IRR**) feeding times. Regular feeding time was at t= 60 minutes. There was an interaction between treatment and time ($P < 0.10$).

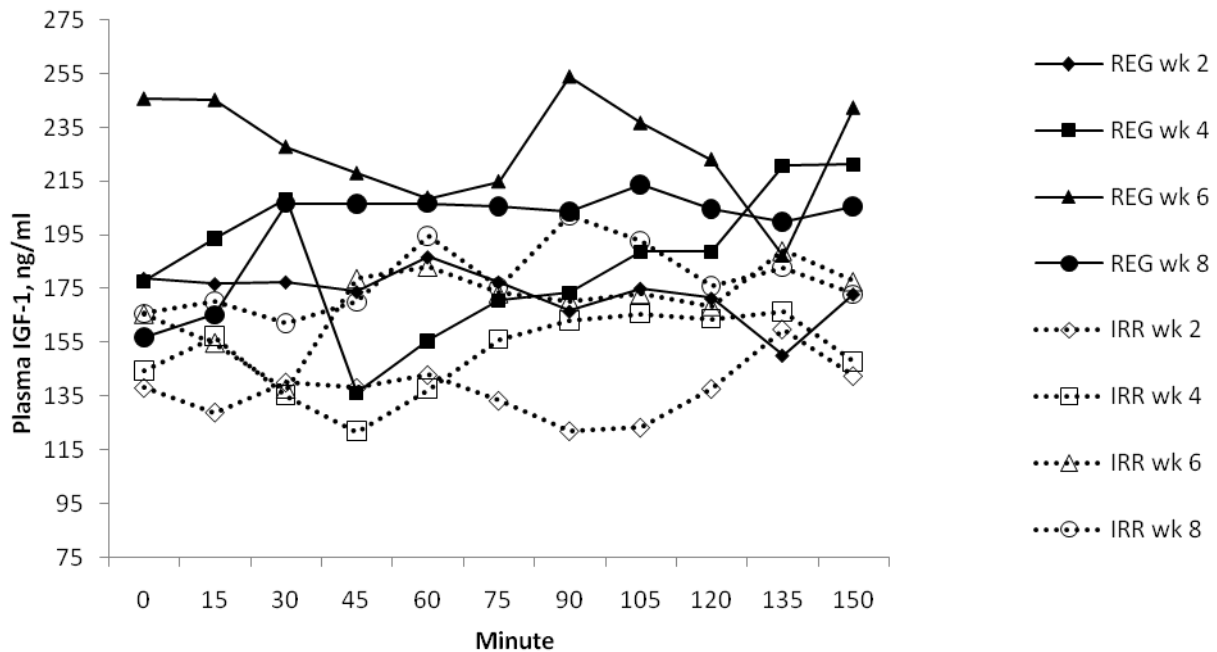


Figure 13. Least squares means of plasma IGF-1 by minute of each week in calves fed MR at regular (**REG**) versus irregular (**IRR**) feeding times. Regular feeding time was at t= 60 minutes. There was a treatment by week by time interaction ($P < 0.10$). SEM=28.02.

($P < 0.05$), where calves fed MR regularly had a significantly greater glucose half life ($P < 0.05$) than those fed MR irregularly. There was also a main effect of week on glucose half life ($P < 0.10$) with $T_{1/2}$ increasing as the calves aged. Figure 14 demonstrates peak insulin concentrations after the glucose infusion at each week for calves fed MR regularly versus irregularly. Peak insulin concentration was greater for calves fed MR at irregular times versus regular times ($P < 0.05$). There were no treatment by week interaction and no effects of week ($P > 0.10$) observed. Figure 15 presents least squares means for insulin concentrations over time during the IVGTT. Least squares means for glucose concentrations over time during the IVGTT are presented in Figure 16. Calves fed MR at irregular times had greater insulin concentrations while glucose concentrations did not differ. It appears that calves fed MR at irregular times required more insulin to promote glucose uptake during the IVGTT. Insulin responses were similar to results reported by Stanley et al. (2002), who found that the acute insulin response was greater in dairy calves fed MR twice a day versus once a day. Stanley et al. (2002) also reported that acute insulin response increased with age. In contrast, results of the present study showed that insulin response in calves fed MR at regular times decreased with age, while insulin response in calves fed MR at irregular times increased with age. Itoh et al. (2006) found that intravenous ghrelin injection decreased plasma insulin concentrations in pre-ruminant calves. In contrast, Takahashi et al. (2006) found that ghrelin enhanced glucose-induced insulin secretion in adult sheep. Although the role of plasma ghrelin in insulin secretion and glucose metabolism are still unclear, results of the present study show that an irregular MR feeding time has an effect on insulin secretion and glucose metabolism, which may be induced by changes in physiological plasma ghrelin concentrations.

Table 2. Least squares means for IVGTT variables by week in calves fed MR at regular (**REG**) versus irregular (**IRR**) feeding times.

	REG		IRR		SEM ¹
Week	4	8	4	8	
Glucose					
k, %/min ²	1.51	1.24	2.01	1.76	.18
T _{1/2} , min ³	48.20	61.33	35.85	43.44	6.03
Insulin					
Peak, µUI/ml	25.99	15.66	40.92	56.12	10.76
Peak time, min	30.83	34.17	28.22	26.67	7.96

¹SEM = Standard error of the mean. ²k= fractional turnover rate. ³T_{1/2}= half life. There was a main effect of feeding time for k, T_{1/2}, and peak insulin (P < 0.05). A main effect of treatment (P < 0.05) was also present for peak insulin.

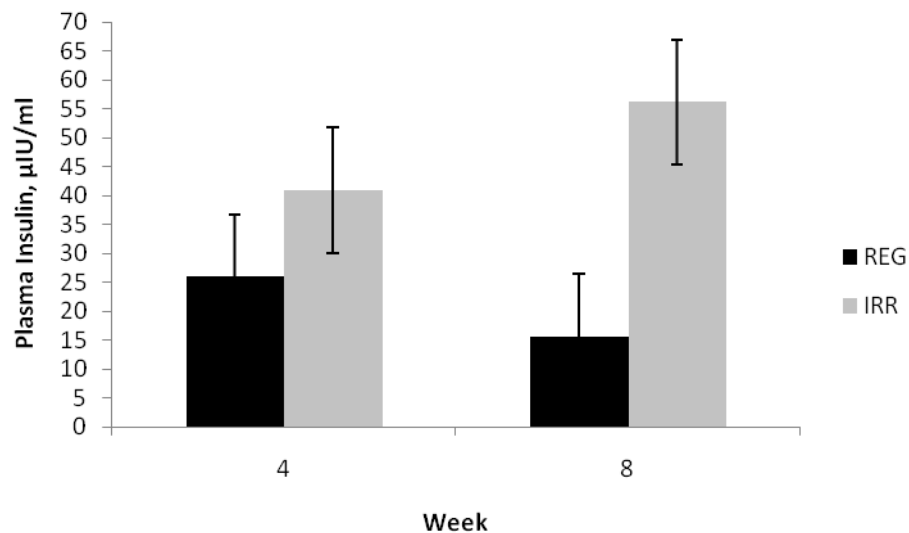


Figure 14. Least squares means of peak insulin concentration in calves fed MR at regular (**REG**) versus irregular (**IRR**) feeding times. There was a main effect of irregular feeding time (P < 0.05).

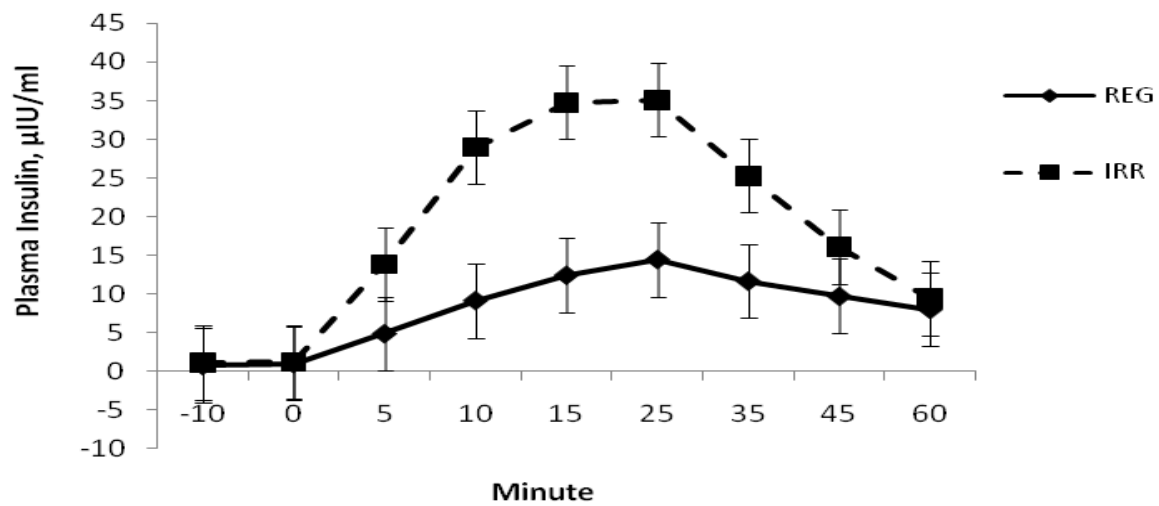


Figure 15. Least squares means of insulin concentrations at each minute in calves fed MR at regular (**REG**) versus irregular (**IRR**) feeding times. There was an main effect of irregular feeding times ($P < 0.10$). Glucose infusion was at $t = 0$.

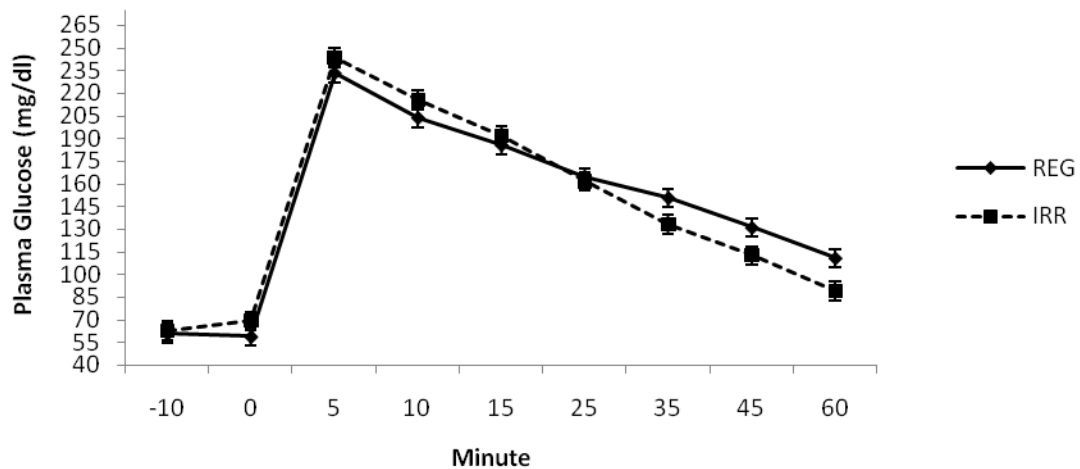


Figure 16. Least squares means of glucose concentrations in response to insulin during the IVGTT in calves fed MR at regular (**REG**) versus irregular (**IRR**) feeding times.

Rumen Development Data

Least squares means for acetate, butyrate, and propionate concentrations are presented in Figures 17, 19, and 21, respectively. A main effect of week ($P < 0.05$) was observed for concentrations of acetate, butyrate, and propionate. Concentrations of these VFAs increased as the animals aged, paralleling the increase in starter consumption. A treatment by week interaction ($P < 0.10$) was also present for butyrate concentrations, with calves that were fed MR at regular times having higher butyrate concentrations at week 8. Results of the present study for overall VFA concentrations are comparable with Laborde (2008) and Doescher (2010) who reported increased VFA concentrations as calves aged. Least squares means for molar percentages of acetate, butyrate, and propionate are shown in Figures 18, 20, and 22, respectively. There was a treatment by week interaction ($P < 0.10$) for molar percentage of acetate. Calves fed MR at a regularly scheduled time had a higher molar percentage of acetate at weeks 2 and 4; however, there was no difference at weeks 6 or 8 (post-weaning), thus indicating no carry over effects. There was also a main effect of week with a decrease in molar percentage of acetate as the calves aged. There was no treatment by week interaction or a main effect of treatment ($P > 0.10$) present for molar percentages of butyrate or propionate. There was, however, a main effect of week ($P < 0.05$), with molar percentages of butyrate and propionate increasing in both groups as the calves aged. Doescher (2010) reported similar results of an increase in molar percentages of butyrate and propionate, and a decrease in molar percentage of acetate as neonatal Holstein calves aged. Least squares means for total VFAs are presented in Figure 23. There was no treatment by week interaction or a main effect of treatment for total VFA production. There was a main effect of week ($P < 0.05$) with total VFAs increasing as the calves aged.

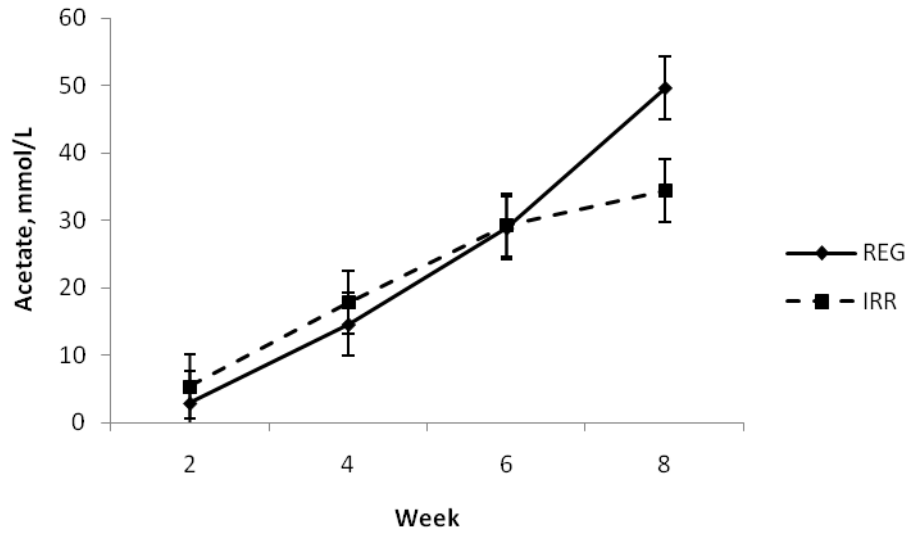


Figure 17. Least squares means of acetate concentrations in calves fed MR at regular (**REG**) versus irregular (**IRR**) feeding times. There was a main effect of week ($P < 0.05$).

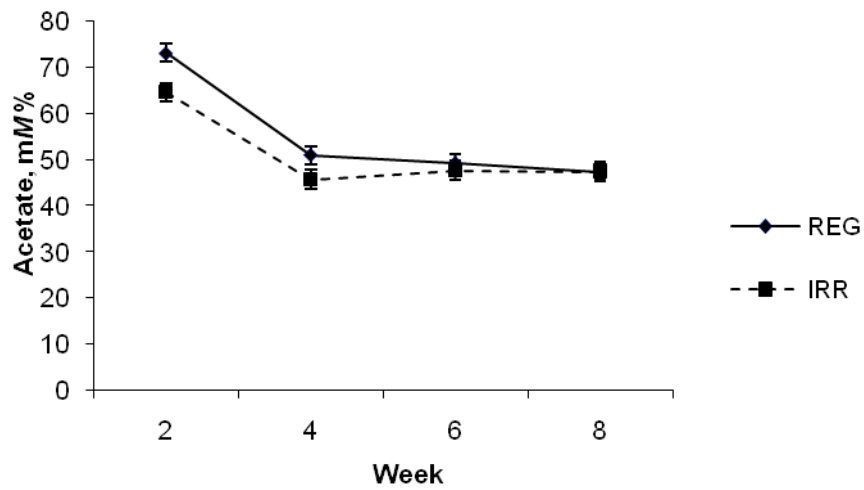


Figure 18. Least squares means of molar percentages of acetate in calves fed MR at regular (**REG**) versus irregular (**IRR**) feeding times. There was an main effect of feeding time, and a main effect of week ($P < 0.10$).

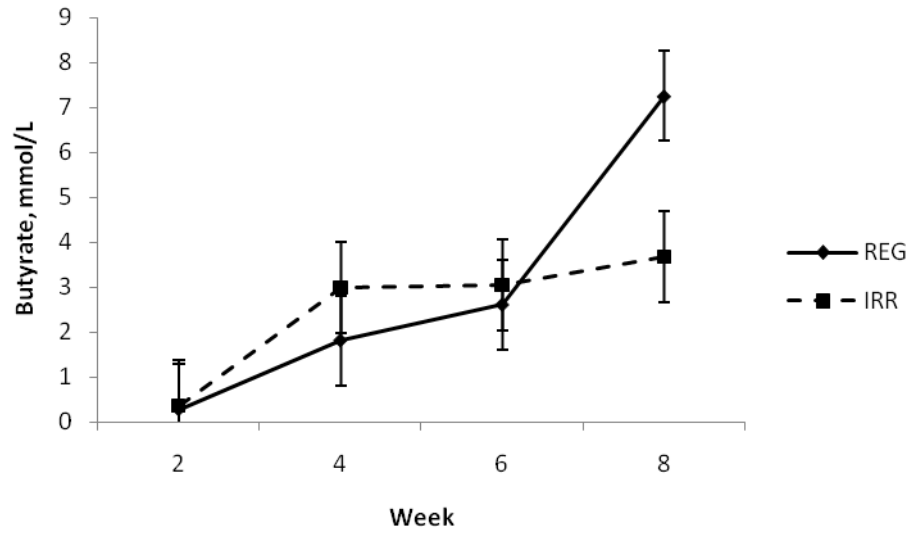


Figure 19. Least squares means of butyrate concentrations in calves fed MR at regular (**REG**) versus irregular (**IRR**) feeding times. There is treatment by week interaction ($P < 0.10$) and a main effect of week ($P < 0.05$).

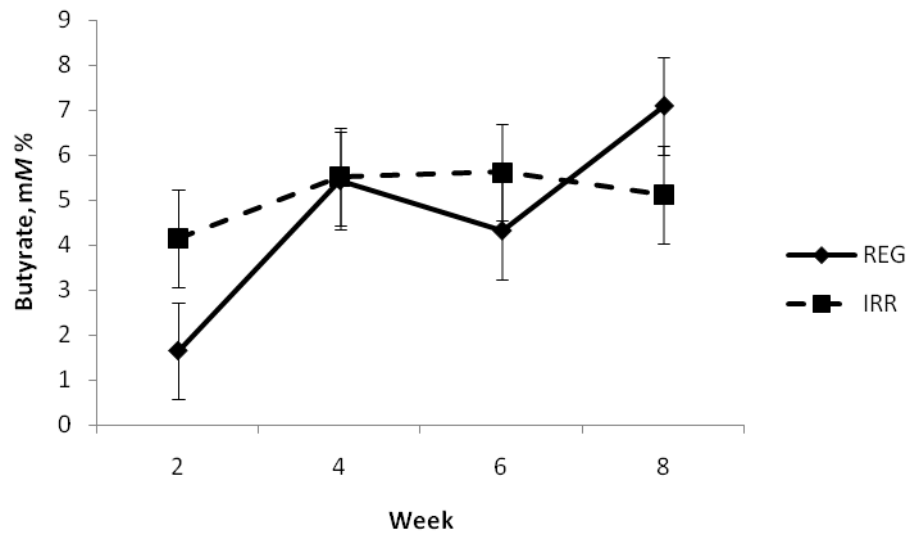


Figure 20. Least squares means of molar percentages of butyrate in calves fed MR at regular (**REG**) versus irregular (**IRR**) feeding times. There was a main effect of week ($P < 0.05$).

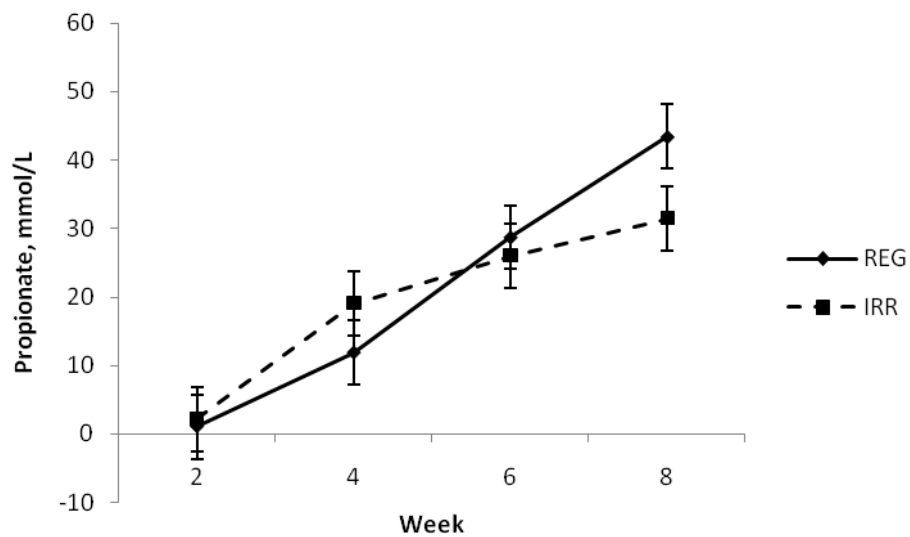


Figure 21. Least squares means of propionate in calves fed MR at regular (**REG**) versus irregular (**IRR**) feeding times. There was a main effect of week ($P < 0.05$).

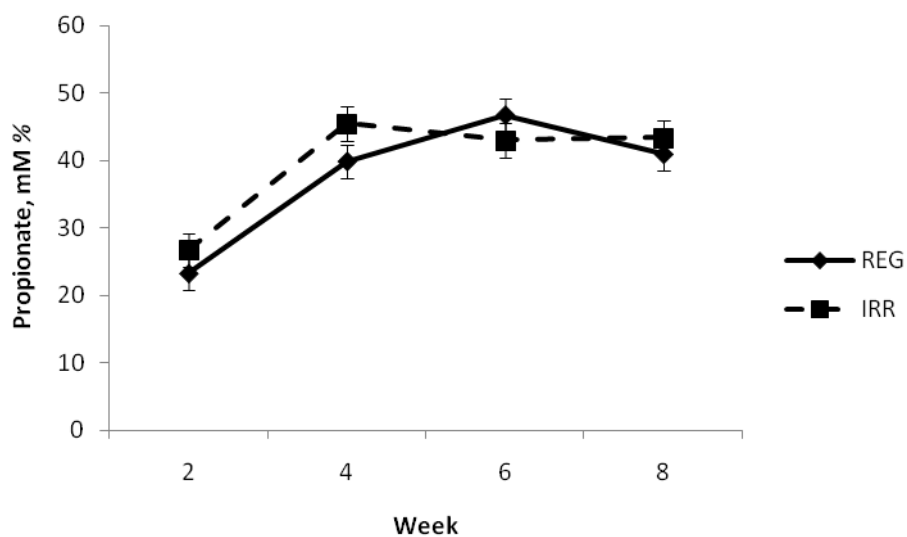


Figure 22. Least squares means of molar percentages of propionate in calves fed MR at regular (**REG**) versus irregular (**IRR**) feeding times.

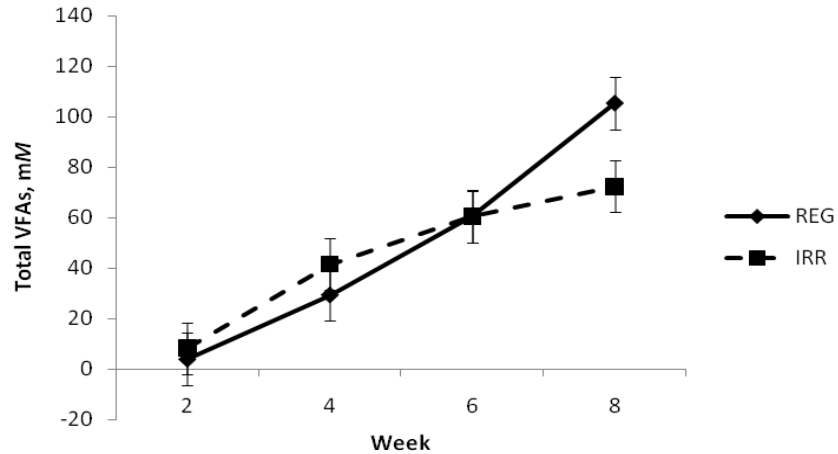


Figure 23. Least squares means of total VFA production in calves fed MR at regular (**REG**) versus irregular (**IRR**) feeding times. There was a main effect of week ($P < 0.05$).

Least squares means for rumen pH for calves fed MR at regular versus irregular feeding times are presented in Figure 20. Results showed that there were no treatment by week interaction and no effect of treatment ($P > 0.10$). There was a main effect of week ($P < 0.05$), with pH decreasing until week 6, and then beginning to increase post-weaning. The resulting changes in rumen pH in the present study were expected as the calves aged. These results are similar to Laborde (2008), who reported that the rumen pH was influenced by the age of the calf.

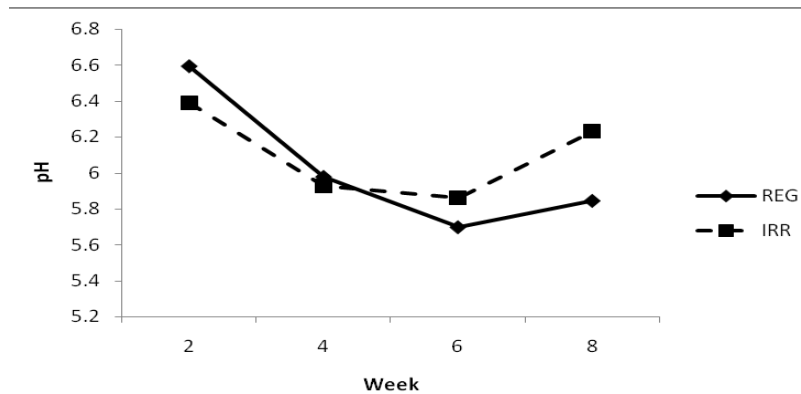


Figure 24. Least squares means of rumen pH in calves fed MR at regular (**REG**) versus irregular (**IRR**) feeding times. There was a main effect of week ($P < 0.05$). SEM = 0.18.

CHAPTER 5

SUMMARY AND CONCLUSION

Summary

This study aimed to determine whether feeding calves milk replacer (MR) at irregular times affected normal growth and metabolism. Twelve neonatal Holstein bulls were utilized in this 8 week study. Calves were randomly divided into two groups. Regularly fed calves were offered MR at 0630 h. Irregularly fed calves were offered MR at 0600, 0830, 0715, 0900, 0630, 0800, and 0730 h on Monday, Tuesday, Wednesday, Thursday, Friday, Saturday, and Sunday, respectively.

Body weights were collected and rumen fluid was obtained at weeks 2, 4, 6, and 8. Starter intake was measured twice daily for weeks 3, 4, 5, 6, 7, and 8. Serial blood collections were conducted at weeks 2, 4, 6, and 8, beginning at 0530 h and continuing through 150 minutes. Plasma was analyzed for ghrelin, leptin, GH, and IGF-1. At weeks 4 and 8, an IVGTT was conducted, and plasma was collected for analysis of glucose and insulin.

Results showed that there were no overall effects of an irregular feeding pattern on body weight or starter intake ($P > 0.10$). There were age effects present for body weight and starter intake ($P < 0.05$). Body weight and starter intake increased over time as expected.

Hormone and metabolite results demonstrate that there were no overall effects of an irregular feeding pattern on plasma ghrelin, leptin, GH, or IGF-1 concentrations ($P > 0.10$). Main effects of age were present for ghrelin, GH, and IGF-1. Plasma ghrelin and GH decreased as calves aged, while IGF-1 increased as calves aged. A treatment by week interaction was present for total plasma ghrelin ($P < 0.05$). Calves fed MR regularly maintained higher plasma ghrelin concentrations at weeks 2 and 4, but differences between the groups were reduced

following weaning. A treatment by week by minute interaction was present for GH and IGF-1 ($P < 0.10$), and a treatment by minute interaction was present for plasma IGF-1 ($P < 0.10$).

Effects on insulin and glucose metabolism were determined by calculating the glucose clearance rate (k), half-life ($T_{1/2}$), peak insulin concentrations, and time of peak insulin concentrations. Glucose half-life and clearance rate, as well as peak insulin concentrations were significantly affected by irregular feeding times ($P < 0.05$). Glucose half-life was higher in calves fed MR regularly, while clearance rate was higher in calves fed irregularly. Calves fed irregularly had higher peak concentrations, as well as overall secretion, of insulin in response to glucose infusion. Insulin secretion in response to the infused glucose decreased over time in calves that were fed MR regularly; however, insulin secretion increased over time in calves fed MR irregularly.

Total production of VFAs, as well as butyrate and propionate were not affected by an irregular feeding pattern. As expected, there was an overall age effect ($P < 0.05$). Concentrations of butyrate and propionate increased as a percentage of total VFAs as the calves aged as expected. No treatment by week interactions were present for any of the VFAs. Feeding time and age effects were present for molar percentage of acetate ($P < 0.10$). Overall, acetate production increased as the calves aged, which was expected.

Conclusion

Feeding MR to neonatal calves at irregular times does not affect their overall growth. However, there appears to be an effect on the regulatory mechanisms of feed intake in relation to the time milk replacer is offered, as well as overall effect on energy metabolism, specifically glucose metabolism.

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APPENDIX A

PLASMA GHRELIN (TOTAL) RADIOIMMUNOASSAY

This assay utilizes ^{125}I -labeled Ghrelin and a Ghrelin antiserum to determine the level of total ghrelin in serum, plasma or tissue culture media by the double antibody/PEG technique.

Assay Procedure

Day One

1. Pipette 300 μl of Assay Buffer to the Non-Specific Binding (NSB) tubes (3-4). Pipette 200 μl of Assay Buffer in the Reference (Bo) tubes (5-6). Pipette 100 μl of Assay Buffer to tubes seven through the end of the assay.
2. Pipette 100 μl of Standards and Quality Controls in duplicate (see assay flow chart).
3. Pipette 100 μl of each sample in duplicate. (NOTE: Smaller volumes of sample may be used when Ghrelin concentrations are anticipated to be elevated or when sample size is limited. Additional Assay Buffer should be added to compensate for the difference so that the volume is equivalent to 100 μl (e.g., when using 50 μl of sample, add 50 μl of Assay Buffer).
4. Pipette 100 μl of Ghrelin Antibody to all tubes except Total Count tubes (1-2) and NSB tubes (3-4).
5. Vortex, cover, and incubate overnight (20-24 hours) at 4°C.

Day Two

6. Hydrate the ^{125}I -Ghrelin tracer with 13.5 ml of Label Hydrating Buffer. Gently mix. Pipette 100 μl of ^{125}I -Ghrelin to all tubes.
7. Vortex, cover and incubate overnight (22-24 hours) at 4°C.

Day Three

8. Add 1.0 ml of cold (4°C) Precipitating Reagent to all tubes except Total Count tubes (1-2).
9. Vortex and incubate 20 minutes at 4°C.
10. Centrifuge, at 4°C, for 20 minutes at 2,000-3,000 xg. Note: If less than 2,000 xg is used, the time of centrifugation must be increased to obtain a firm pellet (e.g. 40 minutes). Multiple centrifuge runs within an assay must be consistent. Conversion of rpm to xg:xg

$= (1.12 \times 10^{-5}) (r) (rpm) 2r$ = radial distance in cm (from axis of rotation to the bottom of the tube) rpm = revolutions per minute.

11. Immediately decant supernatant from all centrifuged tubes except Total Count tubes (1-2). Drain tubes for 15-60 seconds (be consistent between racks), blot excess liquid from lip of tubes and count pellet using the gamma counter according to the manufacturer's instructions.

Calculations

1. Average duplicate counts for Total Count tubes (1-2), NSB tubes (3-4), Total Binding tubes (reference, Bo) (5-6), and all duplicate tubes for standards and samples to the end of the assay.
2. Subtract the average NSB counts from each average count (except for Total Counts). These counts are used in the following calculations.
3. Calculate the percentage of tracer bound (Total Binding Counts/Total Counts) X 100
This should be 35-50%.
4. Calculate the percentage of total binding (%B/Bo) for each standard and sample
 $\%B/Bo = (\text{Sample or Standard}/\text{Total Binding}) \times 100$.
5. Plot the % B/Bo for each standard on the y-axis and the known concentration of the standard on the x-axis using log-log graph paper.
6. Construct the reference curve by joining the points with a smooth curve.

Determine the pg/ml of Ghrelin in the unknown samples and controls by interpolation of the reference curve. [NOTE: When sample volumes assayed differ from 100 μ l, an appropriate mathematical adjustment must be made to accommodate for the dilution factor (e.g., if 50 μ l of sample is used, then calculated data must be multiplied by 2).]

APPENDIX B

PLASMA LEPTIN RADIOIMMUNOASSAY

Principle

This assay is composed of a solid-phase radioimmunoassay with ¹²⁵I-labeled leptin competing for a fixed time with non-labeled leptin in the sample for sites on a leptin specific antibody. A gamma counter is then used to yield a number, which by means of a calibration curve conversion table, measures the leptin present in the sample.

Radioimmunoassay Procedure

Day 1: Set-up

1. Protocol should include the following tubes: 2 total count (TC), 3 normal rabbit serum (NRS), 3 buffer control (BC), a high quality control, a low quality control, samples in duplicate, a high quality control, a low quality control, 3 NRS, 3 BC, and 2 TC tubes.
2. Add 200 μ L of PBS-gel/0.05% TritonX-100 (Leptin Assay buffer) to the NRS, and BC tubes; add 100 μ L to all other tubes except TC.
3. Pipette samples at 100 μ L to appropriate duplicate tubes.
4. The standard is porcine recombinant leptin at 84 ng/mL. Make 7 serial dilutions of the original:
 - a. Label 8 tubes 1-8.
 - b. Put 300 μ L of PBS-gel in tubes 1-7 and 600 μ L of the standard in tube #8.
 - c. Transfer 300 μ L of the standard from tube 8 to tube 7 and mix thoroughly.
 - d. Transfer 300 μ L of the mixture in tube 7 to tube 6 and mix thoroughly.
 - e. Repeat the process through tube #1 which will have 300 μ L because no other dilutions are made.
 - f. Pipette 100 μ L of the appropriate standard in duplicate to the assay tubes.
 - g. The residual solutions can be discarded.
5. Pipette the 2 pools in duplicate (100 μ L).
6. Pipette 200 μ L of NRS #3 1:105 into NRS tubes.
7. To all other tubes except TC and NRS add 200 μ L antibody solution (KLHRed, 1:8000 in 1:105 nR #3 in 50% PBS-EDTA/50% PBS gel). Mix all tubes thoroughly, incubate in the refrigerator for 2 days.

8. Day 3: Hot Hormone Addition

9. Add 200 μL of ^{125}I -human recombinant leptin (about 30,000 cpm) to all tubes, mix thoroughly. Incubate in the refrigerator for 2 days.

10. Day 5: ARGG precipitation

11. Add 200 μL of “4X” ARGG (currently 1:4.5) to all tubes except TC, mix thoroughly. Incubate in the refrigerator for at least 2 days.

12. Day 7: Pour-off

13. Routine pour-off is two spins. Centrifuge, 4°C , all tubes (except for total count tubes) for 30 minutes at 2,000-3,000 $\times g$. (spin first with no buffer, pour-off supernatant gently, add 1 mL cold PBS, re-spin, and pour-off supernatant).

Calculations

1. Average duplicate counts for TC, NRS tubes, Maximum Binding tubes and remaining tubes.
2. Subtract the average NSB counts from each average count.
3. Calculate the percentage of tracer bound $[(\text{BC} / \text{TC}) \times 100]$. This should be 35-50%.
4. Calculate the percentage of maximum binding $\%B / B_o = (\text{Sample or Standard} / \text{BC}) \times 100]$.
5. Plot the $\%B / B_o$ for each standard on the y-axis and the known concentration of the standard on the x-axis.
6. Construct the reference curve by joining the points with a smooth curve.
7. Determine the pg/mL of leptin in the unknown samples by interpolation of the reference.

A. H. 1. Plasma Leptin Radioimmunoassay Standard Curve

Standard Concentration (pg/mL of leptin)

1	0.6565
2	1.3125
3	2.6525
4	5.25
5	10.5
6	21
7	42
8	84

APPENDIX C

PLASMA GROWTH HORMONE RADIOIMMUNOASSAY

Volumes of reagents to pipette:

1. Anti-oGH 200 μ L
2. 1:105 NRS #3 200 μ L (1:42 + 50% horse serum) already made or add 200 μ L 1:42 NRS + 100 μ L LE per NRS tube
3. 1:45 pARGG #3 200 μ L
4. 125I-bGH 200 μ L or amount on bottle
5. PBSG 200 μ L *this volume changes for different standard tubes*
6. LE2 200 μ L
7. HB35ng/mL 200 μ L
8. Sample 200 μ L *If this volume change, make up to 200 μ L With*
 1. PBSG and change LE & HB accordingly
9. STD (bgh 100ng/mL) 1.6, 3.2, 6.2, 12.5, 25, 50, 100, 200 μ L
10. STD (PBSG) 198.4, 196.8, 193.8, 187.6, 175, 150, 100, 0 μ L

What Goes in Each Tube

1. TC 125I-bGH (HH)
2. NRS PBSG (200 μ L) + #3 1:105 NRS + HH + 1:45 (4X)pARGG
3. BC PBSG (200 μ L) + Anti-oGH (1:600)+HH+1:45(4X)pARGG
4. STD PBSG (follow the curve) + bGH 35ng/mL + Anti-oGH + HH + pARGG
5. LE LE + Anti-oGH + HH + pARGG
6. HB HB + Anti-oGH + HH + pARGG
7. Sample Sample + Anti-oGH + HH + pARGG

APPENDIX D

PLASMA INSULIN-LIKE GROWTH FACTOR-1 RADIOIMMUNOASSAY

Day 1 – Sample Preparation

1. Create a protocol for the sample extraction in Excel:
 - a. The samples are in single. Put sample number (1,2,3...end) in column A, put sample ID in column B.
 - b. Samples 1-3 are to be LE2, Low Bovine, and Blank.
 - c. Follow with your samples.
 - d. End with LE2, Low Bovine, and Blank (there will be a total of N+6 tubes).
2. Label tubes for sample extraction (1, 2...N+6).
3. Add 300 μ L of glycine-HCl (pH=3.2) mixture to all tubes.
4. Pipette 200 μ L of each sample INTO the glycine-HCl mixture in the appropriate tube (do NOT dribble down the side of the tube).
5. When all samples are pipette, vortex the tubes gently (but thoroughly mix) for 5 seconds
6. Cover, Label, and place in fridge for 24 hours.

Day 2 – Sample Neutralization and Assay Set-up

7. To each sample extraction tube, add 2.6 mL of PBS-EDTA.
8. Vortex thoroughly (several repetitions of 3-5 seconds each).
9. Create an assay protocol per normal (2TC, 3 NSB, 3BC, 8 STD), sample 1 in duplicate, sample 2, in duplicate,...sample N + 6 in duplicate, 3 NSB, 3 BC, 8 STD, 2 TC.
10. Label assay tubes (total number will be $2(N+6) + 32 = 2N + 52$).
11. *Sample-standard Phase:* Pull the TC, NSB, BC, and STD tubes and set aside. Into all sample tubes, pipette 150 μ L of IGF-1 Assay buffer (PBSG:PBS-EDTA 1:100). Then into appropriate duplicate tubes, pipette 50 μ L of the sample. Pipette directly down into the buffer.
12. Pipette 200 μ L of IGF-1 assay buffer into all NSB and BC tubes. For the STD, first pipette the appropriate amount of IGF-1 assay buffer to make the difference between the 8 standard volumes and 200 μ (that is 198, 196, 192, 185, 175, 150, 100, and 0 μ L), Then pipette the appropriate amount of standard (50ng.mL) into the tubes (2, 4, 8, 15, 25, 50, 100, 200 μ L, respectively) Pipette standard directly into the buffer.

13. *Antibody Phase:* Put all tubes except TC and NSB back into the racks in numerical order.

To all other tubes, add 200 μ L of IGF-1 Antiserum (1:60,000). To the NSB tubes, add 200 μ L of IGF-1 NSB solution (PBSG:PBS-EDTA: protamine sulfate 100mL:100mL:0.2mg/mL).

14. Place the NSB and TC tubes in racks and vortex all. Cover, label, and incubate 24-48 hr in fridge (48 is ideal but 24 is okay if in a hurry).

1. Day 4 (or 3) – Hot Hormone Addition:

15. Remove all racks from the fridge. Add 200 μ L of ¹²⁵I-IGF-1 solution to all tubes (this solution should be between 40,000 and 60,000cpm/200 μ L)

16. Cover and vortex. Incubate in fridge 24-48 hours (48 is ideal...).

2. Day 6 (or 5 or 4) – Second Antibody Precipitation:

17. Remove racks from fridge. To all tubes except TC, add 200 μ L of 4 X NRS (1:105).

Follow this with 200 μ L of 4 X pARGG (1:45). Vortex all tubes and incubate in fridge over night (24 hrs is ideal; however precipitation likely is complete in 12-18 hrs.)

3. Day 7 – pour off:

18. Load all tubes except TC into centrifuge carriers. Spin at 3,000 rpm for 30 minutes.

Immediately when centrifuge stops, remove, decant and drain and blot on paper towels.

Add 1 mL cold PBS to all tubes, and repeat centrifuge-decant-drain-blot.

19. Count all tubes and save on disk. Adjust raw cpm's in DETECTOR.XLS. Save adjusted Data. Copy into current RIANAL.123. The following standard doses should be used 62, 124, 65, 775, 1550, 3100, 6200 ng.

APPENDIX E

PLASMA GLUCOSE ASSAY

(REF: Glucose Oxidase Reagent Set, Pointe Scientific, INC. 1025 Papalas Drive, Lincoln Park, Michigan 48146 USA)

Principle

Glucose is first oxidized to gluconic acid and hydrogen peroxide (via Glucose Oxidase), with the latter reacting with 4-aminoantipyrine and p-hydroxybenzene sulfonate (via peroxidase) to form a quinoneimine dye that has a maximal absorbance at 500 nm. The intensity of the color produced is directly proportional to the concentration of glucose in the sample.

Reagents

1. Glucose Trinder Reagent: Sigma # 315-500 (5x 500 mL). Store at 4°C before and after reconstituting with distilled/deionized water; however, use at room temperature.
2. Glucose Standard: Sigma # 16- 100 (100 mL). A combined Glucose (100mg/dL = 5.56 mmol/L) and Urea-N (10 mg/dL = 3.57 mmol/L). Store refrigerated (4°C).

Assay Procedure

1. Turn spectrophotometer (505 nm) on to warm up (~ 30 min). Set the absorbance reading to 0.00 against distilled water.
2. Label borosilicate glass tubes (12 X 75 mm).
3. Pipette 6.25 µL (right syringe) of standards and samples, and 1,250 µL (left syringe) of the Glucose Trinder Reagent.
4. Vortex tubes and incubate at room temperature for 18 minutes.
5. Read on spectrophotometer at 505nm.

Note: Use the “Timed Assay Sheet” to insure samples are read on spectrophotometer exactly 18 minutes after adding Trinder Reagent.

Calculations

Plasma Glucose concentration = Abs sample x C standard = C sample (mg/dl) Abs standard

APPENDIX F

PLASMA INSULIN RADIOIMMUNOASSAY

(REF: Diagnostic Products Corporation, Coat-A-Count Insulin Kit, Diagnostic Products Corporation, 5700 West 96th Street, Los Angeles, CA 90045-5597)

Principle

This kit is composed of a solid-phase radioimmunoassay with ¹²⁵I labeled insulin competing for a fixed time with a non-labeled insulin in the sample sites on an insulin-specific antibody. The antibody is immobilized to the tube wall. Decanting the supernatant terminates the competition and isolates the antibody-bound fraction of the radiolabeled insulin. A gamma counter is then used to yield a number which, by means of a calibration curve conversion table, measures the insulin present in the sample.

Radioimmunoassay Procedure

1. Plain Tubes: Label four plain (uncoated) 12 x 75 mm polypropylene tubes T (total counts) and NSB (nonspecific binding) in duplicate. Coated Tubes: Label fourteen Insulin AB-coated Tubes A (maximum binding) and B through G in duplicate. Label AB-coated tubes, also in duplicate, for controls and test samples.
2. Pipette 200μL of the zero calibrator A into the NSB and A tubes, and 200μL of each remaining calibrator, control and test sample into the tubes prepared. Pipette directly to the bottom of the tube.
3. Add 1.0mL of ¹²⁵I Insulin to every tube. Vortex.
4. Incubate for 18-25 hours at room temperature (15-18°C).
5. Decant thoroughly.
6. Count for one minute in a gamma counter.

Standard Curve

Calibrator Approximate $\mu\text{IU/mL}$

A (MB)	0
B	5
C	15
D	50
E	100
F	200
G	400

Preparation of Reagent Solutions

1. 125Insulin: Add 100mL of distilled or deionized water to each vial of concentrated iodinated insulin. Mix by gentle inversion. Store refrigerated. Stable at 2-8°C for 30 days after preparation.
2. Insulin Calibrators: At least 30 minutes before use, reconstitute the zero calibrator A, with 6.0mL of distilled or deionized water. Reconstitute the remaining calibrators B through G with 3.0mL each of distilled or deionized water. Use volumetric pipettes and mix gently by swirling. Store frozen. Stable at -20°C for 30 days after reconstitution.

Calculations

1. Average duplicate counts for Total Count tubes, NSB tubes, Maximum Binding and remaining tubes.
2. Subtract the average NSB counts from each average count.
3. Calculate the percentage of tractors bound $[(\text{Maximum Binding Counts} / \text{Total}) \times 100]$. This should be 35-50%.
4. Calculate the percentage of maximum binding $\%B/B_0 = (\text{Sample or Standard} / \text{Maximum Binding}) \times 100$.
5. Plot the $\%B/B_0$ for each standard on the y-axis and the known concentration of the standard on the x-axis.
6. Construct the reference curve by joining the points with a smooth curve.
7. Determine the pg/mL of insulin in the unknown samples by interpolation of the reference curve.

APPENDIX G

ANALYSIS OF VOLATILE FATTY ACIDS IN RUMINAL FLUID

Based on preparation procedures described in Grigsby et al., 1992. J. Anim. Sci. 70:1941-1949, and temperature gradient program described in Bateman et al., 2002. Prof. Anim. Sci. 18:363-367.

Reagents

1. 25% (wt/vol) metaphosphoric acid (fluka #79615) acid solution containing 2 g/L of 2-ethyl butyric acid (216.5 μ L 2-EB to 100 mL m-phos acid solution; Aldrich #10, 995-9).
2. VFA standard: Add the following volumes of acids to a 100-mL volumetric flask and fill volume with dH₂O. Store in refrigerator when not in use.

MW	Acid	Volume (μ L)	Conc (g/L)	Conc (mM)
60.06	Acetic	330	3.46	57.62
74.08	Propionic	400	3.97	53.59
88.10	Isobutyric	30	0.29	3.29
88.10	Butyric	160	1.53	17.37
102.13	Isovaleric	40	0.375	3.67
102.13	n-Valeric	50	0.471	4.61

Sample and Standard Preparation

- 1) Centrifuge strained ruminal fluid at 30,000 x g for 20 min (this step may be skipped).
- 2) Mix 4 mL of rumen fluid supernatant with 1 mL of m-phosphoric acid solution containing 2-EB.
- 3) Allow to stand in ice bath for 30 min (this step may be skipped).
- 4) Centrifuge at 30,000 x g for 20 min.
- 5) Remove the supernatant for GC analysis.
- 6) To insure that standard is prepared in the same manner as the samples, treat the mixed sample from step A-2 above as a sample. Remember to correct the dilution factor from the m-phos solution when calculating the final VFA concentrations (4mL fluid mixed with 1 mL acid provide a correction factor of 1.25). For use on Shimadzu GC, samples should be in 2 mL autosampler vials. The optimal vials

that we have used are ordered from Cole-Parmer. They are Target autosampler vials (#A98810-00). These are a screw cap vial so you also need caps, and the septa color is important. The autosampler recognizes white as the color of the septa (#A98801-23).

Temperature Gradient Program

- 7) The column temperature at the beginning of the program is 115°C and is held there for 0.1 min.
- 8) It is then increased at a rate of 10°C/min to 150°C and held there for 0.1 min.
- 9) It is then further increased at a rate of 11°C/min to 170°C and held there for 1 min.
- 10) The injector of the chromatograph is held at 250°C and the detector is held at 275°C.
- 11) Peak detection is by a flame ionization that uses a H₂/ air flame.
- 12) Helium is used as the carrier gas with a splitless injection at a flow of 60 mL/min.**

VITA

Katherine Claire Simon was born in Metairie, Louisiana, to Brent and Margaret Simon. Katie graduated from Archbishop Chapelle High School in May of 2003, and began her undergraduate degree at Louisiana State University in August of 2003. Katie completed her Bachelor of Science degree in animal science in May of 2007. In August of 2007, she began working on her Master of Science in ruminant nutrition and physiology. In August of 2009, she attended her first year of veterinary school at Louisiana State University. In August of 2010, she will graduate with her master's degree.