2013

Effects of meal timing on growth hormones, ghrelin, and insulin sensitivity in male Holstein calves

Erica Lynn Chartier
Louisiana State University and Agricultural and Mechanical College, echart1@lsu.edu

EFFECTS OF MEAL TIMING ON GROWTH HORMONE, GHRELIN, AND INSULIN SENSITIVITY IN MALE HOLSTEIN 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
The School of Animal Sciences

by
Erica Lynn Chartier
B.S., Louisiana State University, 2010
December 2013

ACKNOWLEDGEMENTS

First and foremost my sincere appreciation goes to my major professor Dr. Cathy Williams for giving me the opportunity and resources to accomplish this research. Her motivation, enthusiasm, immense knowledge, humor, and friendship have been more than I could ask for. Many thanks to her for going above and beyond to help me achieve my goal, and I attribute the completion of my thesis and my success to her guidance and encouragement.

For their support and willingness to serve as my committee members, I would like to thank Dr. Bruce Jenny, Dr. Charles Hutchison, and Dr. Donald Thompson. Special thanks go to Dr. Donald Thompson for his continuous help and assistance in the RIA lab. His knowledge and assistance setting up, interpreting, and computing assays were essential to the completion of my thesis. I would also like to extend my gratitude and thank Ashley Dolejsiova for her time and patience while teaching and assisting me with laboratory procedures. Also, thank you to Dr. Michael Kearney, for his assistance with statistics.

I would like to thank Mr. Randy Morell for his helpfulness and the LSU Dairy Research and Teaching Farm for allowing me to conduct my research. To my fellow graduate students Jake Anderson and Steven Blair I am especially grateful. Thank you for the countless mornings of getting up at 4 A.M. to help with sample collection, feeding, and weighing calves. Such an undertaking could not have been achieved without your help. Also thank you to, Christie Burke for her time feeding and taking care of the calves.

I would like to thank the Dairy Nutrition lab workers Alex Levin, Reid Rabalais, Stacey Vignes, and Jennifer West for their hard work in the lab and at the farm. I am

particularly grateful for the hundreds of test tubes they labeled and the time they spent inputting data into the computer.

Lastly I would like to express my gratitude to my parents, Michael and Susan, and my sister Shawntelle, for always being there while I pursue my goals. I cannot thank them enough for their continuous encouragement, support, understanding, love, and advice. If it were not for their endless support through my college career, I would not be where I am today.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Eighteen neonatal Holstein bull calves (38.85 ± 4.71 kg) were assigned to one of two treatments at birth to determine the effect of feeding time on growth, nutrient intake, metabolic hormone secretion, and energy metabolism. Regularly fed calves (n = 9) were fed MR daily at 0630 h, and irregularly fed calves (n = 9) at 1030, 0800, 0630, 0830, 0530, 0930, and 0730 Monday through Sunday. Body weights were measured weekly from birth to 9 weeks. Water intake, fecal scores, and starter intake were measured daily. Serial blood collections were conducted at 2, 4, 6, and 8 weeks for ghrelin and GH concentrations. Blood sample collection began one hour prior to regular feeding time (0530 h) and ended one hour post regular feeding time (0730 h), at time points 0, 15, 30, 45, 50, 55, 60, 65, 70, 75, 90, 105, and 120 minutes. An IVGTT was performed at weeks 3, 6, and 9 to assess glucose metabolism. Water intake increased ($P < 0.05$) in irregularly fed calves at weeks 1 and 3. A treatment by week interaction and a main effect of week were observed for ghrelin concentrations ($P < 0.05$), and regularly fed calves exhibited increased ghrelin concentrations at week 4 ($P < 0.10$). Plasma ghrelin concentrations increased with age until weaning at week 6 then decreased at week 8. An increase in GH concentrations were observed at time points t= 75 ($P < 0.05$), 90 ($P < 0.10$), and 120 ($P < 0.10$) min. A treatment by week interaction and a main effect of week were observed for GH concentrations ($P < 0.0001$). Regularly fed calves had higher GH concentrations at weeks 2 and 4 ($P < 0.05$). No differences were observed ($P > 0.10$) for glucose concentrations. Peak insulin concentrations ($P < 0.05$) and AUC for insulin ($P < 0.10$) increased as calves aged, indicating that calves become less sensitive to insulin as they

develop. Results indicated that feeding time does not have an overall effect on growth, feed intake, and glucose metabolism, but does affect growth hormone concentrations.

CHAPTER I
INTRODUCTION

In order to raise domestic animals efficiently it is critical to understand and have proper management of feed intake and growth. In recent discoveries the hormone, ghrelin, has been shown to affect feeding behavior, feed intake, and growth in farm animals. Young calves undergo considerable changes in digestive and physiological mechanisms around the time of weaning (Katoh et al., 2004). Calves must adjust from a liquid diet to solid feedstuffs, thus requiring them to undergo changes in their metabolic and digestive functions. Understanding the regulation of feed intake and behavior is therefore essential to understanding the growth and development of the young ruminant.

Ghrelin has been found to work as an appetite stimulator through neuropeptides in the hypothalamus (Kalra et al., 2003), play a role in energy and glucose homeostasis (Toshinai et al., 2006), and act as a growth hormone (GH) stimulant (Zhang et al., 2008). Sugino et al. (2004) observed a surge in circulating ghrelin concentrations in sheep prior to scheduled feeding, followed by a decrease in ghrelin concentration after feeding. Research has shown plasma ghrelin concentrations to increase with acute feed deprivation in cattle and sheep (Sugino et al., 2002; Wertz-Lutz et al., 2006). Wertz-Lutz et al. (2006) observed increased time feeding and a tendency towards increased dry matter intake (DMI) when ghrelin injections were administered in cattle. Increases in ghrelin and GH secretion were observed in lactating dairy cows in a negative energy state, resulting in the assumption that ghrelin could enhance GH secretion in this metabolic state (Bradford and Allen, 2007). In studies with dairy calves, Fukumori et al. (2013) observed that not only physiological changes around the time of weaning but also

nutritional status could affect ghrelin and GH secretion. It is suggested that ghrelin action on glucose and insulin may depend on the physiological stage of life in the ruminant (Itoh et al., 2006; Fukumori et al., 2013).

Previous research has shown that at various physiological stages ghrelin plays a role in feeding behavior, GH secretion, insulin secretion, and glucose metabolism. However, studies on the regulatory effects of gastrointestinal hormones in growth of ruminants are limited. Previous research conducted at Louisiana State University by Simon (2010) demonstrated an effect on the regulatory mechanisms of feed intake in relation to the time milk replacer was offered. This study was designed as a continuation of previous work by Simon (2010) to show how alterations in feeding times can affect the secretion of ghrelin and GH in pre- and post-weaned dairy calves. An intravenous glucose tolerance test (IVGTT) was performed pre and post weaning to evaluate the effects of varied feeding times on glucose metabolism and insulin secretion.

CHAPTER II
REVIEW OF LITERATURE

Background

Nutrition is essential to proper development and growth of a young animal. Calves undergo numerous physiological changes from birth until weaning. During the first 2-3 weeks of life the dairy calf's digestive tract is developing rapidly (Guilloteau et al., 2009). During the first 3 weeks of a calf's life, the pre-ruminant stage, digestion and metabolism are similar to a monogastric animal. The abomasum makes up about 50 % of the total tissue weight of the stomach and is the main compartment. Rumen development begins around 3 to 8 weeks of life, and calves are encouraged to intake dry feed early on for proper rumen development (Preston, 1963). Proper rumen development is essential for the absorption of volatile fatty acids (VFA), and the VFA butyrate and propionate are major stimulators for ruminal epithelial tissue development (Sander et al., 1959). The microbial population is well established around 6-9 weeks of life, and the segmented compartments of the stomach attain proportions similar to a mature bovine around 12-16 weeks of life (Bryant et al., 1958; Lyford et al., 1988).

Pre-weaned ruminants digest feeds in the small intestine and absorb nutrients across the epithelium. Post-weaning ruminants ferment consumed plant material and absorb VFA across the rumen epithelium wall for use as a main energy source (Flatt et al., 1958). It has been hypothesized since weaning signifies changes in regulatory metabolism, changes in regulatory hormone levels should occur at this time. Weaning is a critical period in a calf's life and major changes occur not only in the digestive tract but also throughout the endocrine system (Fukumori et al., 2013).

Ghrelin

Ghrelin is a 28-amino acid peptide modified at the third residue, serine (Ser), by a middle chain fatty acid -n-octanoic acid. The octanoyl group on Ser at the 3rd position in the amino acid chain gives the peptide hormone biological activity (Kojima et al., 1999). Two isoforms of circulating ghrelin have been purified from rat stomachs, ghrelin and des-Gln^{14}-ghrelin, and both bind to the growth hormone secretagogue - receptor (GHS-R). Ghrelin, a 28-amino acid peptide, and des-Gln^{14}-ghrelin are identical other than a deletion of a glutamine in des-Gln^{14}-ghrelin. This results in a 27-amino acid peptide. Acyl-ghrelin is modified with medium chain fatty acids, and des-acylated-ghrelin lacks side chain modification (Hosoda et al., 2000). Acyl-ghrelin has been classified into four groups from acylation on the Ser ^{3}. The groups consist of non-acylated, octanoylated (C8:0), decanoylated (C10:0), and possibly decenoylated (C10:1) (Hosoda et al., 2003). Monogastrics produce both forms of ghrelin, however ruminants have been shown to possess only des-Gln^{14}-ghrelin (Dickin et al., 2004). In rodents, ghrelin is mostly produced in the stomach by neuroendocrine X/A-like cells, while in humans it is produced in P/D1 cells (Rindi et al., 2002). In ruminants, specifically the bovine and ovine, the hormone is produced mainly in the oxyntic (parietal) glands of the abomasum (Grouselle et al., 2008). Ghrelin has been shown to have numerous functions with effects on feeding behavior, acting as a GH secretagogue, and playing a role in energy and glucose homeostasis. (Nakazato et al., 2001; Toshinai et al., 2006; Zhang et al., 2008).

Ghrelin Secretion

Blood concentrations of ghrelin increase during fasting, hunger, and prior to scheduled meal times, and are normally suppressed by satiation (Wertz-Lutz et al., 2006;

Bradford and Allen, 2007). It has been shown that ghrelin may be regulated by a pathway involving neuropeptide Y. Produced in the arcuate nucleus of the hypothalamus, neuropeptide Y acts on the central nervous system as an appetite stimulator. It is controlled through feedback action of ghrelin from the stomach and leptin from the adipose tissue (Kalra et al., 2003). Ghrelin antagonizes leptin, through the neuropeptide Y receptor pathway, with leptin promoting satiety and ghrelin stimulating nutrient intake (Kalra et al., 2003). Other physiological effects of ghrelin include adiposity in rodents, increased gastric acid secretion in rats, increased gastric motility in rats, and hyperglycemia in humans (Masuda et al., 2000; Tschop et al., 2000; Gauna et al., 2004). During fasting and negative energy balance, ghrelin stimulates appetite directly through increased gut motility and the release of neuropeptide Y (Masuda et al., 2000). Ghrelin is currently the only known hormone to readily stimulate feeding, thus reducing fat oxidation and stimulating adiposity after administration (Kalra et al., 2003).

Sugino et al. (2002) observed a surge in circulating ghrelin concentrations prior to feeding that was followed by a decrease in ghrelin concentrations after feeding in 1-2 year old Suffolk rams. Plasma ghrelin concentrations peaked within 1 h prior to feeding when sheep were feed 2 or 4 times daily. This was immediately followed by a decrease in ghrelin concentrations 1 h after feeding. A surge was not observed, and ghrelin levels stayed relatively constant when animals had constant access to food. Sugino et al. (2002) also found that a ghrelin surge occurred in sheep that were pseudo-fed. The experiment consisted of sheep given a nylon bag containing feed at scheduled feeding times but animals were unable to consume the feed. This research suggested that the ghrelin surge

around a conditioned meal time is due to a conditioned physiological reflex, and direct contact with feed is not necessary (Sugino et al., 2002).

Drazen et al. (2006) observed that ghrelin levels increase during fasting and decrease with re-feeding in rats. Rats on a fixed feeding schedule had an increase in ghrelin prior to each feeding. They concluded that along with the effects that fasting and feeding have on ghrelin concentrations, it could be possible that the expectancy of feeding may contribute to ghrelin secretion. To differentiate between the two possible regulatory influences, an experiment was conducted to determine ghrelin levels in freely fed rats and meal-fed rats trained to consume a feeding in a 4 h period in the light phase. Plasma ghrelin levels increased to a peak of 778 +/- 95 pg/ml in freely fed rats. Meal-fed rats were fed one of two diets, rat chow or a high-fat Ensure® plus liquid diet. Plasma ghrelin increased 2 h prior to the meal to peaks of 2192 +/- 218 and 2075 +/- 92 pg/ml, in rats fed rat chow or Ensure® plus, respectively. Freely fed rats were then fasted for a time equivalent to meal-fed rats, and no peak of plasma ghrelin was observed. The researchers concluded that ghrelin levels in the rat are influenced by the anticipation of eating as well as nutritional status (Drazen et al., 2006).

Growth Hormone

Growth hormone is a 191 amino acid polypeptide synthesized, stored, and secreted by somatotrophs of the adenohypophysis. The anabolic hormone is essential for the metabolism of proteins, carbohydrates, fats, and minerals in mammals, and for long bone growth, muscle growth, and energy homeostasis. The pulsatile pattern of GH secretion is stimulated by GH releasing hormone (GHRH) and is inhibited by

somatostatin (Tannenbaum et al., 1998; Frohman et al., 2000). Although GHRH and somatostatin are the two main regulators of GH secretion, other physiological stimulators and inhibitors play a role in GH secretion. Stimulators of GH include sleep, hypoglycemia, exercise, dietary protein, short-term fasting, and arginine. Inhibitors of GH secretion are hyperglycemia, chronic glucocorticoid use, estradiol, and circulating concentrations of insulin-like growth factor-1 (IGF-1) through negative feedback on the hypothalamus (Alba Roth et al., 1988; Veldhuis, 2003; Zizzari and Bluet-Pajot, 2004). In 1999, the endogenous ligand of the GHS-R, ghrelin, was purified and also considered a stimulant of GH (Kojima et al. 1999).

Effects of Ghrelin on Growth Hormone Secretion

The name ghrelin comes from a proto-Indo-European origin, *ghre*, which means growth (Kojima et al., 1999). Ghrelin has been identified as the endogenous ligand for the pituitary GHS-R (Briatore et al., 2003). The occurrence of the 28-amino acid peptide in both humans and rats indicated that GH release from the pituitary could also be regulated by ghrelin and not only by the hypothalamic GHRH. The 27-amino acid peptide also has the ability to stimulate GH secretion as well as the 28-amino acid peptide (Kojima et al., 1999). Ghrelin is found in the gastrointestinal tract of non-ruminants and ruminants, and acts as a GH secretagogue (GHS) (Kojima et al., 1999; Takaya et al., 2000). GHS receptors are dispersed throughout the body, including the rumen, intestine, pancreas, immune system, brain, pituitary, and in the accurate nucleus of the hypothalamus (Hayashida et al., 2001; Gentry et al., 2003; Geary, 2004; Sugino et al., 2004).

Sugino et al. (2002) confirmed that a momentary ghrelin surge occurs prior to feeding in sheep on a scheduled meal feeding program. No changes in plasma ghrelin were observed in sheep with constant access to feed. A surge in plasma GH followed the plasma ghrelin surge in the scheduled meal fed sheep. Growth hormone levels peaked during feeding and rapidly declined at the end of feeding, then gradually increased with periodic secretion for the rest of the day (Sugino et al., 2002).

In an infusion study by Itoh et al. (2005), ghrelin and GHRH were injected intravenously into suckling calves, weanling calves, early lactation cows, mid lactation cows, and non-lactating cows. The peak levels observed for ghrelin induced GH release were found in early lactation cows. GHRH caused a greater GH response than ghrelin in both suckling and weanling calves. Plasma GH levels were higher in suckling calves and early lactation cows compared to non-lactating cows. The data showed that GHRH induces GH release in growing calves, and that ghrelin contributes to the increase in plasma GH in early lactation cows. The study concluded that ghrelin may act as a nutrient state dependent signal for GH release (Itoh et al. 2005).

Similar results were observed in a study conducted by Bradford and Allen (2007) with high producing dairy cows in early and late lactation to assess the effects of energy balance on plasma hormones. Cows were fed a diet once daily at 1130 h, and refusal was weighed from 1000 h to 1130 h, during which time cows had no access to feed. Blood collection started at 1000 h on day 4 for 24 h. A ghrelin surge prior to the conditioned meal time was observed and was followed by a GH surge in the early lactation cows. Early lactation cows were found to be in negative energy balance, and their net energy intake was shown to be significantly lower than late lactation cows. This suggests that

lactating dairy cattle may display a meal induced increase in ghrelin and GH secretion due to being in a negative energy state. It was postulated that ghrelin could enhance GH secretion in this metabolic state (Bradford and Allen, 2007).

ThidarMyint et al. (2008) investigated the effects of ghrelin, GHRH, and the combination of the two on GH in Holstein bull calves. Growth hormone levels peaked within 5 min of the injection of ghrelin, GHRH, or the combination of the two in the pre-weaning phase. Ghrelin and GHRH had similar GH responses, but the GH response from combined administration was greatest. In the post weaning phase plasma GH peaked at 10 minutes, and then gradually returned to baseline (ThidarMyint et al., 2008). A similar study was conducted by Takahashi et al. (2009) when investigating how ghrelin affects GH secretion in response to GHRH in fed and fasted sheep. Experiment 1 was conducted 2.5 h to 5 h after feeding, and experiment 2 was conducted 47.5 to 50 h after feeding. All animals in each experiment received 12 treatments of 0, 3.3 (low), and 6.6 (high) µg ghrelin/kg body weight (BW), with or without 0.25 µg GHRH/kg BW. A ghrelin peak was observed 10 minutes after all ghrelin injections, with ghrelin levels being maintained longer in the fasting state. Basal GH levels were observed to be higher in the fasting state than the satiety state. Low and high ghrelin infusions stimulated GH secretion more in the satiety state then in the fasting state. Similarly, GHRH, in addition to low and high ghrelin, also stimulated GH secretion greater in the satiety state than the fasting state. It was observed that ghrelin and GHRH had a combined effect in the satiety state but not in the fasting state. Overall the study indicated that depending on the sheep's energy status, ghrelin controls GH secretion response differently than GHRH (Takahashi et al., 2009).

Digestive and physiological mechanisms change around the time of weaning in young calves. Growth hormone responses to GHRH have been shown to decrease in post-weaned calves when compared to pre-weaned calves (Katoh et al., 2004). Fukumori et al. (2013) postulated that ghrelin's action may change around weaning due to the quality of the diet or from changed endocrine functions due to aging. A study was then carried out to evaluate the effects of intravenous ghrelin injection on plasma GH in calves at weaning. Holstein bull calves were fed whole milk, and ad libitum calf starter, grass hay, and water twice a day starting 5 days after birth at 0900 h and 1700 h. On day 49, calves were weaned and fed calf starter and grass hay. Intravenous ghrelin injections were given 4 h after morning feeding at weeks 1, 2, 4, 6, 7, 9, 11 and 13. Blood samples were collected at -10, 0, 1.5, 3, 6, 9, 12, 15, 20, 25, 30, 40, 50, and 60 min, with injection time after the 0 min blood sample. Growth hormone levels were elevated after ghrelin injection at all experimental weeks, but the GH response did not differ between pre and post weaning, except for week 7 (weaning). The ghrelin-induced GH response was highest at week 7, and may have resulted from calves not obtaining adequate energy from solid feed (Fukumori et al., 2013). Studies conducted with sheep found that certain stressors, such as restricted feeding or decreased feeding frequency, tend to increase plasma GH levels (Thomas et al., 1991). Fukumori et al. (2013) believe that the increase in ghrelin action on GH at weaning could be influenced by nutritional status and not the physiological changes that occur at weaning. They concluded that there is an increase in plasma ghrelin levels in a nutrient deficient state, and this may alter ghrelin action on GH (Fukumori et al., 2013).

Effects of Ghrelin on Insulin Secretion and Glucose Metabolism

Insulin is a 51 amino acid peptide hormone produced in the β cells of the pancreas. It is comprised of an A and a B chain, composed of 21 and 30 amino acids, respectively (Kaneko, 1997). Insulin is secreted in response to hyperglycemia and increases glucose transport across cell membranes using glucose transporters. The release of insulin is inhibited by hypoglycemia and somatostatin. As an anabolic hormone that stimulates energy storage, insulin stimulates triglyceride storage and glycogen synthesis, and inhibits glycogenolysis, gluconeogenesis, and hepatic ketogenesis (Wilcox, 2005).

Ghrelin has been found to not only affect growth hormone secretion but also is suggested to regulate pancreatic islet function (Date et al., 2002). In non-ruminant species ghrelin has been shown to be both inhibitory and stimulatory. Intravenous infusions of ghrelin have led to mixed results. In weanling pigs, no differences in plasma glucose concentrations were observed after ghrelin infusion, but increases in plasma insulin concentrations were observed (Salfen et al., 2004). Fasted humans have shown an increase in plasma glucose concentrations, and a decrease in plasma insulin concentrations post ghrelin infusion (Broglio et al., 2001). Intravenous infusion and ingestion of dextrose have shown to acutely decrease ghrelin concentrations in rats (Tschop et al., 2000; McCowen et al., 2002). Similarly, in humans it has been found that both intravenous and oral administration of glucose caused a significant decrease in plasma ghrelin concentrations. The study concluded that ghrelin secretion may be suppressed by increased plasma glucose in humans (Nakagawa et al., 2002).

Itoh et al. (2006) studied the effects of ghrelin infusion on glucose and insulin in dairy cattle at various physiological stages of life. The physiological stages consisted of pre-ruminant calves (~10 d of age), non-lactating cows, and lactating cows. Lactating cows experienced continuous hyperglycemia after the ghrelin infusion as compared to saline infused cows. In non-lactating cows glucose concentrations increased at several time points post infusion, and pre-ruminant calves showed no significant difference from saline infused calves in glucose concentration. Momentary increases in insulin were observed in lactating cows at 15 and 30 min post ghrelin infusion, in non-lactating cows at each time point post ghrelin infusion except for 7.5 and 60 min. In pre-ruminant calves insulin concentrations decreased at time points 45 to 180 minutes post ghrelin infusion. This decrease in insulin post ghrelin infusion has been observed in earlier studies with monogastric animals such as rats (McCowen et al., 2002), mice (Reimer et al., 2003), and humans (Broglio et al., 2001). Itoh (2006) concluded that ghrelin has different effects on plasma glucose and insulin at different physiological stages in the dairy cow.

Kobayashi et al. (2006) studied the effects of suckling milk from the dam on plasma hormone concentrations in 4-week old Shiba-goats. Kids were separated from their dams at 0900 h, then allowed to suckle for 10 min at 1300 h. Blood samples were collected 30 minutes prior to feeding, 0 minutes, and then in 15 minute intervals after feeding till 120 minutes. Results showed plasma insulin levels are significantly increased by suckling, although no change was observed in plasma ghrelin levels except for one value at 120 minutes after suckling. In a second experiment, the effect of intravenous injection of D-glucose on plasma hormone concentrations was conducted. Plasma insulin levels significantly increased 15 minutes post infusion and returned to baseline levels

approximately 45 min post infusion. In contrast plasma ghrelin levels were not significantly changed by the D-glucose infusion (Kobayashi et al., 2006).

Wertz-Lutz et al. (2006) studied the effects of intravenous pulse injections of bovine ghrelin on glucose and insulin concentrations in 2 year old crossbred steers. Ghrelin was injected at 1200 and 1400 h, but no difference was found in plasma glucose concentrations. These results reflect similar work in which a greater plasma glucose response post ghrelin infusion was observed only in lactating cows, while non-lactating and suckling calves were less responsive to the ghrelin injection (Itoh et al., 2006). These researchers proposed that the response in the lactating cows was a result of negative energy balance. Bovines in negative energy balance display increased gluconeogenesis and decreased glucose uptake, allowing glucose to be spared for vital functions (Bauman, 1999). Wertz-Lutz et al. (2006) believe that the lack of plasma glucose response was due to steers being in a similar physiological state as a non-lactating cow. Similarly, no differences in plasma insulin levels were observed in these steers post ghrelin injection (Wertz-Lutz et al., 2006).

Krueger and Melendez (2012) researched the effect of ghrelin on metabolites in 9 month old lambs. Lambs were assigned to one of two treatments including either an intramuscular ghrelin injection or an intramuscular saline injection. The injections were conducted 3 h after feeding for 4 d. Serum samples were collected 1 h after the injection on days 1, 3, 4, and 5. Feed was offered at 0830 h every morning and remained for ad libitum feeding for 24 hr. Residual feed was weighed each morning and fresh feed was offered. Ovine ghrelin showed a tendency to decrease plasma insulin at d 5 but did not affect glucose levels in lambs. This response is controversial as no ghrelin effect was

observed on insulin in 2 year old crossbred steers (Wertz-Lutz et al., 2006), while plasma insulin concentrations increased after dose dependent intravenous ghrelin injections in Holstein heifers (ThidarMyint et al., 2006). The inconsistent results suggest that ghrelin prompts responses for different metabolites depending on the physiological stage and species (Krueger and Melendez, 2012).

Fukumori et al. (2012) conducted a study to evaluate the effect of weaning on glucose concentrations, and the effect of intravenous glucose infusion on blood ghrelin concentrations in pre- and post-weaned Holstein bull calves. Calves were fed whole milk (10% of birth BW) and ad libitum calf starter and grass hay twice a day at 0900 h and 1700 h. On day 49 calves were weaned and fed calf starter and grass hay. In Experiment 1, blood samples were collected once a week for 13 weeks in 10 minute intervals relative to the morning feeding time (0900 h). Feed was provided after blood sampling at the 0 min. As expected basal glucose levels decreased at weaning, and gradually declined as calves aged. Basal ghrelin levels also decreased as the calves aged. The post feeding glucose response changed with age as 13 week old calves exhibited a decreased response compared to 6 week old calves. Baseline blood acetate levels increased with age from 2 to 13 week in calves. Fukumori et al. (2012) suggest that at the time of weaning the absorbed energy source changes from glucose to VFA in the calf. In the second experiment, a glucose infusion was conducted after a 16 h fast in Holstein bull calves at 2, 4, 6 (pre-weaning), 9, 11, and 13 (post-weaning) weeks of age. Serial blood samples were collected at time points -10, 0, 1.5, 3, 6, 9, 12, 15, 20, 25, 30, 40, 50, and 60 min with glucose infused after the 0 minute collection. Blood ghrelin concentrations after glucose infusion at all weeks showed no significant change when compared to basal

ghrelin levels. Overall, ghrelin levels after glucose infusion were found to be lower in post-weaned calves. Researchers suggest that the lower ghrelin levels observed post-weaning may be due to weaned ruminants absorbing VFA as their primary energy source, since increased blood acetate levels were observed as calves aged (Fukumori et al., 2012).

Fukumori et al. (2013) studied the effect of intravenous ghrelin injection on plasma insulin and glucose concentrations in Holstein bull calves at weaning. Calves were fed and weaned, and infusions were conducted as described previously. At all weeks of age ghrelin concentrations reached a peak 1.5 min after injection and returned to basal levels within 40-60 min. Plasma insulin and glucose responses to ghrelin injections did not differ as calves aged. An earlier study by Fukumori et al. (2011) reported increased plasma insulin and glucose concentrations in lactating dairy cows, after ghrelin injection, but this response was not observed in non-lactating dairy cows or pre-ruminant dairy calves. In their more recent study, Fukumori et al. (2013), noted that calves at week 7 (weaning) had a temporary decline in their digestible energy intake per metabolic body weight (DEI/MBW). Due to calves possibly being in a nutrient deficient state at weaning, similar to an early lactation cow, it was hypothesized that a similar response to the ghrelin challenge would be observed in 7 week old calves. However, there was no observed increase in plasma insulin and glucose in 7 week old calves. The study concluded that the ghrelin action on plasma insulin and glucose levels may change depending on the physiological state of the animal (Fukumori et al., 2013).

Effects of Ghrelin on Nutritional Status and Feed Intake

Previous studies have indicated a negative correlation with body energy balance and ghrelin levels (Shiiya et al., 2002) and have shown that feed deprivation increases circulating ghrelin concentrations (Cummings et al., 2001; Sugino et al., 2002). In a study conducted by Kobayashi et al. (2006), plasma ghrelin concentrations after feeding in pre-weaned 4 week old milk replacer fed goats were compared to those from post-weaned 13 week old alfalfa hay cube free choice fed goats. An increase in plasma ghrelin concentrations were observed in 4 week old milk replacer fed goats after feeding. However, there was no change in plasma ghrelin concentrations in 13 week post weaned goats. The results were thought to result from 13 week old post-weaned goats having free choice access to hay cubes, thus leaving them in a positive energy balance. Researchers suggested this may explain the decreased ghrelin concentrations post weaning (Kobayashi et al., 2006).

Wertz –Lutz et al. (2006) conducted a study comparing fasted and fed 2 year old beef steers, with the fasted steers having feed withheld for 48 h. Blood collections began at 22 h after initiation of the fasting period and continued through the end of the 48 h fast. Plasma ghrelin concentrations were elevated prior to feeding then declined after feeding in the fed steers. In the fasted steers plasma ghrelin concentrations remained elevated and increased throughout the sampling period as compared to the fed steers. Non esterified fatty acid (NEFA) concentrations were also elevated for fasted steers as compared to fed steers. The authors concluded that plasma ghrelin concentrations fluctuate in cattle depending on their nutritional status, and ghrelin may serve as a signal for energy balance in ruminants. In a second experiment, steers were given two ghrelin injections in the

treatment group and two saline injections in the control group at 1300 and 1500 h. Blood samples were collected in 15 min intervals from 0600 to 1800 h, with 0800 h indicating feeding time for fed steers or 36 h of fasting for fasted steers. Dry mater intake (DMI) and length of time spent eating was calculated 2 d prior to injection, the day of the injection, and 1 d after the injection. The time spent eating and DMI prior to the ghrelin injection were similar for experimental treatment and control steers. At each of the 1 h post injection periods the length of time and DMI did not differ between treatment groups. When the 1 h post injection periods were combined, the length of time spent eating was greater and there was a tendency for DMI to be greater for ghrelin infused steers (Wertz-Lutz et al., 2006).

Wertz-Lutz et al. (2008) used a crossover design to determine the effects of prolonged moderate nutrient restriction on nutritional status and plasma ghrelin concentrations in 3 year old Angus crossbred steers. Steers were placed on a high grain diet at 240% of the intake needed for BW maintenance (2.4 X M). After a period of acclimation, 2 steers continued on the 2.4 X M diet and 2 steers were placed on a diet at 80% of the intake needed for BW maintenance (0.8 X M). After the first 21-d period another acclimation period was initiated, and steers were then placed on opposite treatments for a second 21-d period. Serial blood samples were collected on day 7, 14, and 21 in 15 min intervals at 0700 to 1145, 1300 to 1345, 1600 to1645, and 1800 to 1845. Over the 21-d period plasma ghrelin levels were elevated in steers on the 0.8 X M diet. The results support previous research indicating that ghrelin concentrations are elevated in ruminants in moderate yet prolonged nutrient restriction (Wertz-Lutz et al., 2008).

Wertz-Lutz et al. (2010) conducted another study to demonstrate how plasma ghrelin concentrations are affected in 2 year old Angus crossbred steers fed a similar amount of dietary energy but with different feedstuffs. Experiment 1 consisted of one treatment group receiving 50% hay-50% concentrate (HAY) diet offered at an amount to meet the steer's NEm requirement and were offered an additional 3.5 Mcal of NEg daily. The second treatment group received a diet of 10% hay: 90% concentrate (LFC) but were limit fed to reach an energy intake similar to the HAY diet. The study was conducted as a crossover design composed of two 21-d periods. On d 21 serial blood samples were collected in 15-min intervals at the beginning, middle, and end of a 12 h feeding period. Experiment 2 was conducted similarly to experiment 1 except the steers were in a catabolic state and supplied with 80% of the NEm required. In both experiments, the decreased energy density of the first diet resulted in greater DMI as compared to the steers on the second diet. No differences were observed in plasma ghrelin levels in either treatment group. They concluded that DMI does not affect plasma ghrelin concentrations in steers when energy intake is similar (Wertz-Lutz et al., 2010).

Krueger and Melendez (2012) researched the effect of ghrelin on feed intake in 9 month old lambs. Lambs were assigned to a treatment group receiving an intramuscular ghrelin injection or an intramuscular saline injection. The injections were conducted 3 h after feeding for 4 d. Serum samples were collected 1 h after the injection on days 1, 3, 4, and 5. Feed was offered ad libitum at 0830 h every morning. Residual feed was weighed each morning and fresh feed was offered. Increased feed intake was observed on d 3, and it was concluded that ghrelin injections minimally increased feed intake in lambs (Krueger and Melendez, 2012).

Fukumori et al. (2012) as stated previously, studied the effect of weaning on circulating ghrelin concentrations in dairy calves. Blood samples were collected from 1 week of age to 13 weeks of age, with calves being weaned at 7 weeks of age. Basal concentrations of ghrelin decreased with age after weaning. Digestible energy intake per kg metabolic body weight increased with age throughout the 13 weeks, with a decline only at weaning on week 7. The decrease in plasma ghrelin concentrations post weaning was thought to be related to having a greater digestible energy intake (Fukumori et al., 2012).

The studies previously described indicated that ghrelin may have an effect on growth, metabolism, and feed intake. However, studies on gastrointestinal hormones in ruminants are still limited, and further research is essential. The following experiment was designed to study the effect of meal time feeding on growth, feed intake, ghrelin, growth hormone, and glucose metabolism in male Holstein calves.

CHAPTER III
MATERIALS AND METHODS

Animals and Dietary Treatments

Eighteen neonatal Holstein bull calves (38.85 ± 4.71 kg) were used in a nine week experiment to assess the effects of feeding time on anabolic hormone status and glucose metabolism. All animals were born and raised on the Louisiana State University Agricultural Center's Dairy Research and Teaching Farm. Calves were born between September through November, 2012. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the LSU Agricultural Center.

Calves were separated from their dams within 3 hours of birth, weighed, and moved to individual calf hutches measuring 2.5 m^2 with a 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 colostrum, which was followed by transition milk on days 2 and 3. On day 4 of life, calves were moved into elevated metal calf crates (0.62 m^2) under a covered free stall barn. The calves were given milk replacer (MR) containing lasalocid (20% protein, 21% fat; ADM Alliance Nutrition, Inc., Quincy, IL) at 10% of their birth weight and trained to drink from a bucket. MR was mixed at 15% solids. Body weights were recorded weekly, and MR was increased to 10% of BW until 28 days of age. Water was offered ad libitum and measured once daily. At 28 days of age, following a serial blood collection, calves were offered KC Premium calf starter containing lasalocid ad libitum (19.25% CP, 1.65% CF; Kentwood Co-Op, Kentwood, LA) in addition to milk replacer. 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 once daily at 0630 every morning. Those fed irregularly (IRR) were offered MR once daily at 1030, 0800, 0630, 0830, 0530, 0930, and 0730 on Monday, Tuesday, Wednesday, Thursday, Friday, Saturday, and Sunday, respectively. These feeding times were continued until weaning at 6 wk of age.

Sample Collection

Body weights were recorded weekly from birth through 9 weeks. Water intake was measured daily from weeks 1 through 9, and starter intake from week 4 through 9. Fecal scores (fluidity) were recorded daily based on a physical appearance scale: 1= normal, 2= soft, 3= runny, 4= watery (Larson et al., 1977).

Blood was collected at 24 to 48 h of birth for serum IgG concentration via jugular venipuncture in 10 mL Vacutainer® tubes. Blood was centrifuged for twenty minutes, and serum was separated and stored frozen at -20ºC until 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 h, with blood collected at 0, 15, 30, 45, 50, 55, 60, 65, 70, 75, 90, 120 minutes. Calves were fasted for the duration of the collection time. Blood was collected in 10 mL aliquots and divided between K_2EDTA 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 K_2EDTA tubes and plasma was later analyzed for ghrelin. Blood samples were centrifuged within 30 minutes of collection, and plasma was then removed and stored at -20°C until analysis.

At 3, 6 and 9 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 (0.5g/kg BW) (Kaneko, 1997) was infused 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 at -20°C for later analysis of glucose and insulin.

Laboratory Procedures

Serum IgG concentrations were measured using a turbidimetric method (MBC QT II ^{TM} Bovine Serum IgG; Midland Bioproducts Corporation Boone, IA) (Appendix A). Plasma was analyzed for total ghrelin concentrations using radioimmunoassay kits (Human Ghrelin (Total) RIA; Millipore, St. Charles, MO) (Appendix B). The intra- and inter-assay coefficients of variation were 6.13% and 4.46%, respectively. 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 2.25% and 1.17%, respectively (Appendix C). Plasma was analyzed for glucose using a glucose oxidase reagent set (Glucose Oxidase Reagent Set; Pointe Scientific, Lincoln Park, MI) (Appendix D), and for insulin using commercial radioimmunoassay kits (Coat-A-Count; Diagnostic Products Corporation, Los Angeles, CA) (Appendix E).

Statistical Methods and Calculations

All performance data were analyzed using the MIXED procedure (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 termed that was assumed to be correlated within calf using an autoregressive covariance structure.

Hormone and metabolite data were analyzed using the MIXED procedure (Littell et al., 1998). The model included fixed effects of treatment, week, time, and a three way 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 an autoregressive covariance structure.

Plasma glucose response to the IVGTT was evaluated by calculating the fractional turnover rate (k) and the half-life ($T_{1/2}$) (Kaneko, 1997) for the period from 10 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. Responses of plasma glucose and insulin to the IVGTT were also evaluated by calculating areas under the response curves (AUC) relative to the basal levels using trapezoidal geometry to time periods 5-60 minutes after glucose infusion. Data were analyzed using the MIXED procedure (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 an autoregressive

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

CHAPTER IV
RESULTS AND DISCUSSION

Performance Data

IgG concentrations for all calves ranged from 12.38-54.04 g/L. All calves exceeded the recommended 10 g/L blood IgG concentration at 48 h of age for passive transfer of immunity (Bovine Alliance on Management and Nutrition, 1995).Least squares means for average daily body weight gain for regular versus irregular fed calves are presented in Table 1. There was a main effect of week on average daily gain (ADG) ($P<0.0001$). A treatment by week interaction was not observed for ADG, nor was there a main effect of treatment ($P>0.10$). Least squares means of weekly body weights, starter intake, and water intake for calves fed MR at regular versus irregular feeding times are presented in Figure 1, 2, and 3, respectively. There was no main effect of treatment on weekly body weight, starter intake, or water intake ($P>0.10$). A treatment by week interaction for weekly body weight or starter intake ($P >0.10$) was not observed, but there was a treatment by week interaction for water intake ($P < 0.10$). During weeks 1 and 3 water intake was greater in irregularly fed calves ($P=0.028, 0.045$). As expected, body weight, and starter intake increased as the calves aged ($P < 0.05$). Water intake increased with age after weaning ($P < 0.05$). Growth results observed in the current experiment displayed similar results to those observed by Simon (2010), who also studied meal timing in neonatal dairy calves. The increase in water intake by irregularly fed calves is suggested to occur from calves compensating during the 0630 feeding when other calves received MR. At 6 weeks of age water intake steadily increased as expected after weaning when calf starter is the sole feed source offered. Least squares means of average weekly fecal scores are presented in figure 4. A treatment by week interaction for fecal

scores was not observed (P>0.10). Fecal scores during the experimental period were within normal range for healthy calves, and an improvement in fecal score was observed as animals aged (P<0.0001).

Table 1. Least squares means for ADG for calves fed MR at regular (REG) versus irregular (IRR) feeding times.

	Treatment			
	REG	IRR	SEM[1]	P-Value
ADG, kg/d[2]				
Birth to wk 6	0.41	0.40	0.09	0.946
Wk 6 to wk 9	0.87	0.72	0.09	0.134
Birth to wk 9	0.56	0.49	0.09	0.431

[1]SEM = Standard error of the mean.
[2]There was a main effect of week on ADG (P<0.0001).

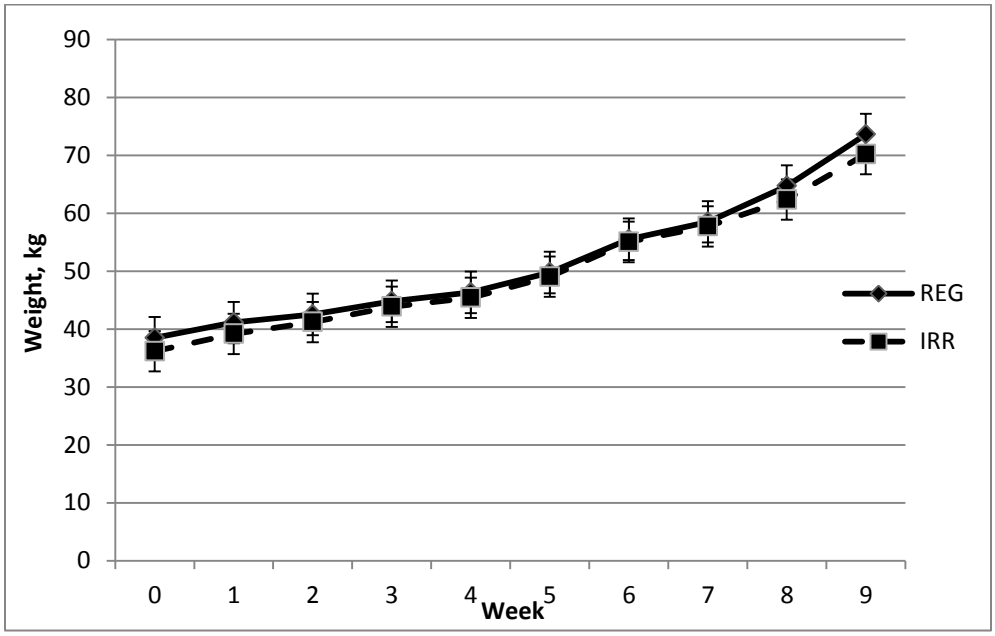

Figure 1. Least squares means of body weights by week for calves fed MR at regular (REG) versus irregular (IRR) feeding times. There was a main effect of week (P < 0.05).

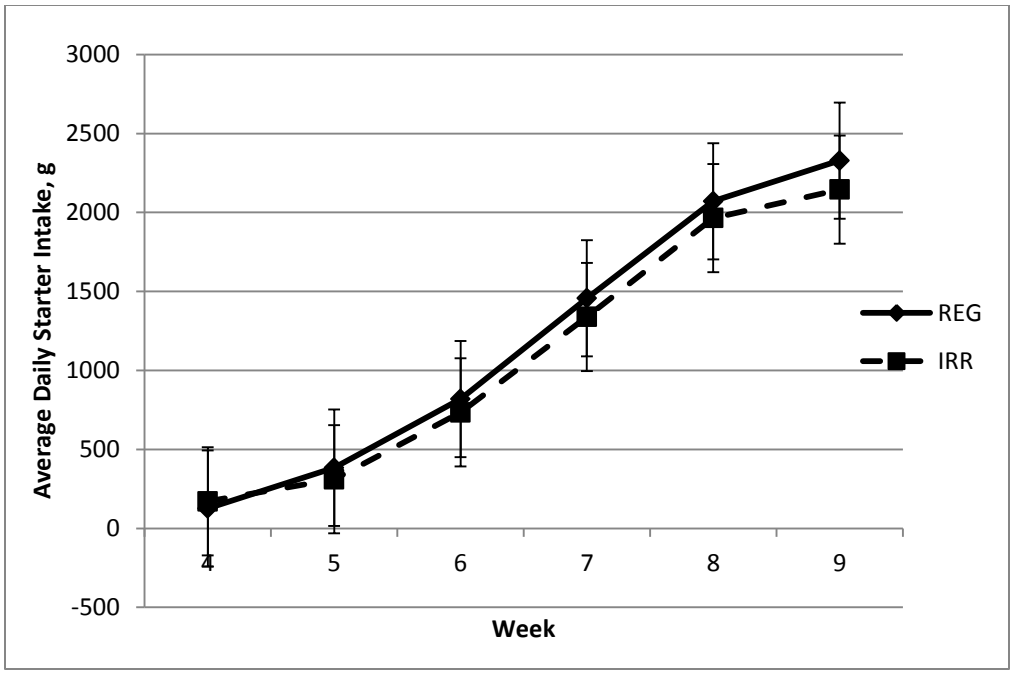

Figure 2. Least squares means of average daily starter intake by week for calves fed MR at regular (REG) versus irregular (IRR) feeding times. There was a main effect of week ($P < 0.05$).

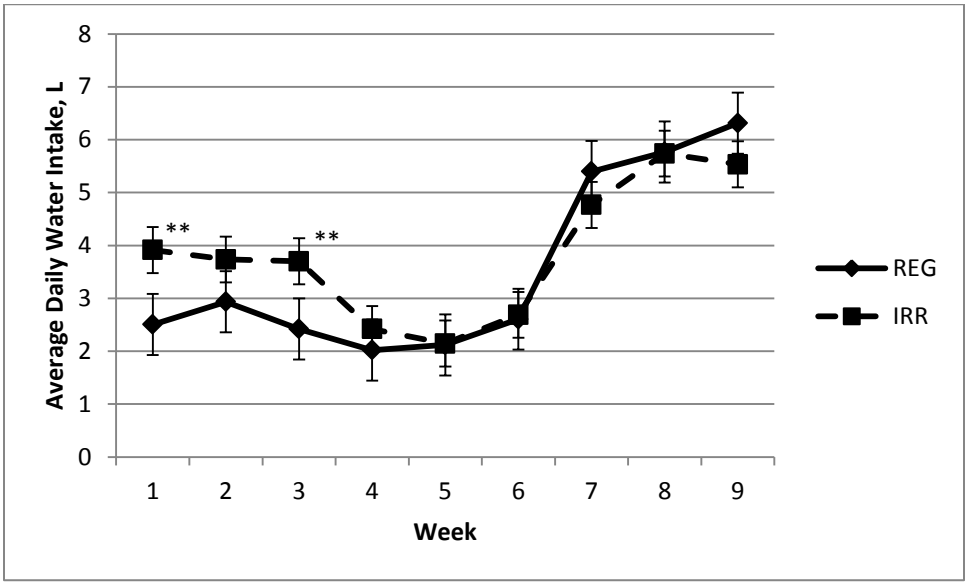

Figure 3. Least squares means of average daily water intake by week for calves fed MR at regular (REG) versus irregular (IRR) feeding times. There was a treatment by week interaction ($P < 0.10$) and a main effect of week ($P < 0.05$). **, Significantly different from regularly fed ($P<0.05$).

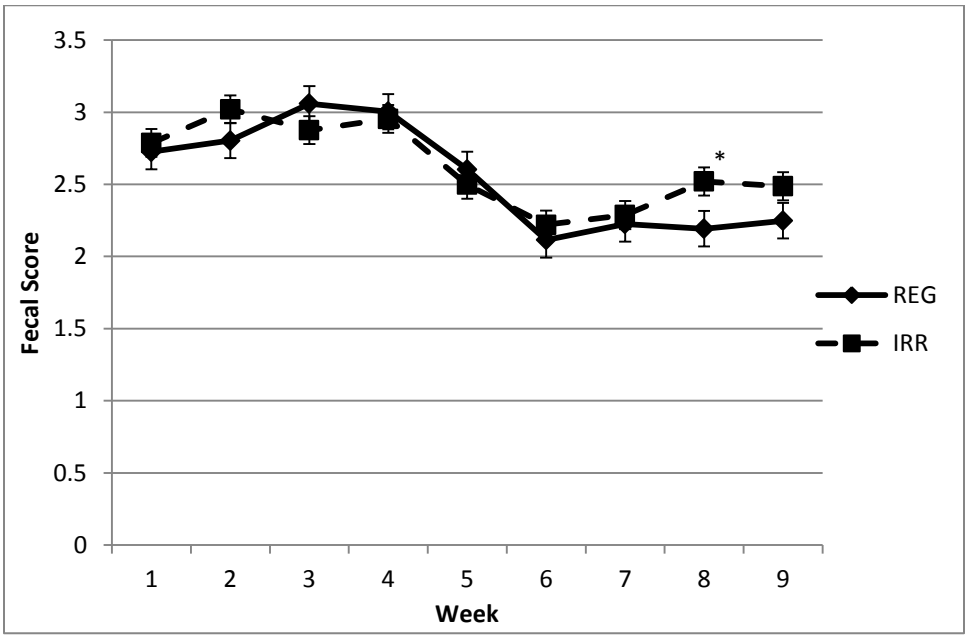

Figure 4. Least squares means of average weekly fecal score by week for calves fed MR at regular (REG) versus irregular (IRR) feeding times. There was a main effect of week (P<0.0001). *, Significantly different from REG fed calves (P<0.10).

Hormone and Metabolite Data

Least squares means for weekly plasma ghrelin are presented in Figure 5. There was a treatment by week interaction (P < 0.05), and a main effect of week (P<0.05). At week 4, calves fed at a regular feeding time had higher plasma ghrelin concentrations then those fed at irregular times (P<0.05). However, when calves were weaned at week 6, both regular and irregular fed calves showed increased plasma ghrelin concentrations followed by decreased plasma ghrelin concentrations at 8 weeks post weaning. Fukumori et al. (2012) also observed increased plasma ghrelin concentrations at weaning, followed by decreased plasma ghrelin concentrations through week 13 in Holstein bull calves. The decline in plasma ghrelin concentrations post weaning was thought to be related to post-

weaning calves having greater digestible energy intake (Fukumori et al. 2012). Results of the current study are similar, as ADG was greater during the post weaning period when ghrelin concentrations had declined.

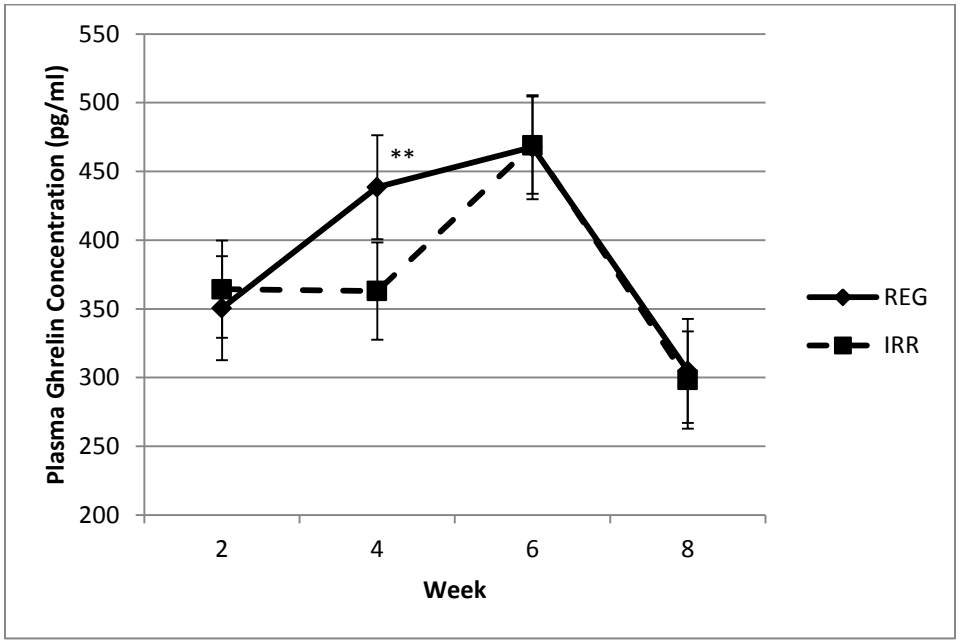

Figure 5. Least squares means of plasma ghrelin by week in calves fed MR at regular (REG) versus irregular (IRR) feeding times. There was a treatment by week interaction, and a main effect of week ($P<0.05$). **, Significantly different from IRR fed calves ($P<0.05$).

Figure 6 illustrates least squares means of total plasma ghrelin by minute for calves fed MR at regular versus irregular feeding times. No main effect of time nor a treatment by time interaction were observed to be significant ($P > 0.10$). These results are in contrast to previous research conducted in ruminants. Sugino et al. (2002) confirmed that a momentary ghrelin surge occurs prior to feeding in sheep on a scheduled meal feeding program, whereas no change in plasma ghrelin was observed in sheep with constant access to feed. Plasma ghrelin concentrations peaked within 1 h prior to feeding when sheep were regularly feed 2 or 4 times daily. This was then immediately followed

by decreased ghrelin concentrations 1 h after feeding (Sugino et al. 2002). Wertz –Lutz et al. (2006) conducted a study comparing fasted and fed 2 year old beef steers. Plasma ghrelin levels were elevated prior to feeding then declined after feeding in the fed steers. In the fasted steers plasma ghrelin concentrations remained elevated and increased throughout the sampling period as compared to the fed steers (Wertz-Lutz et al. 2006).

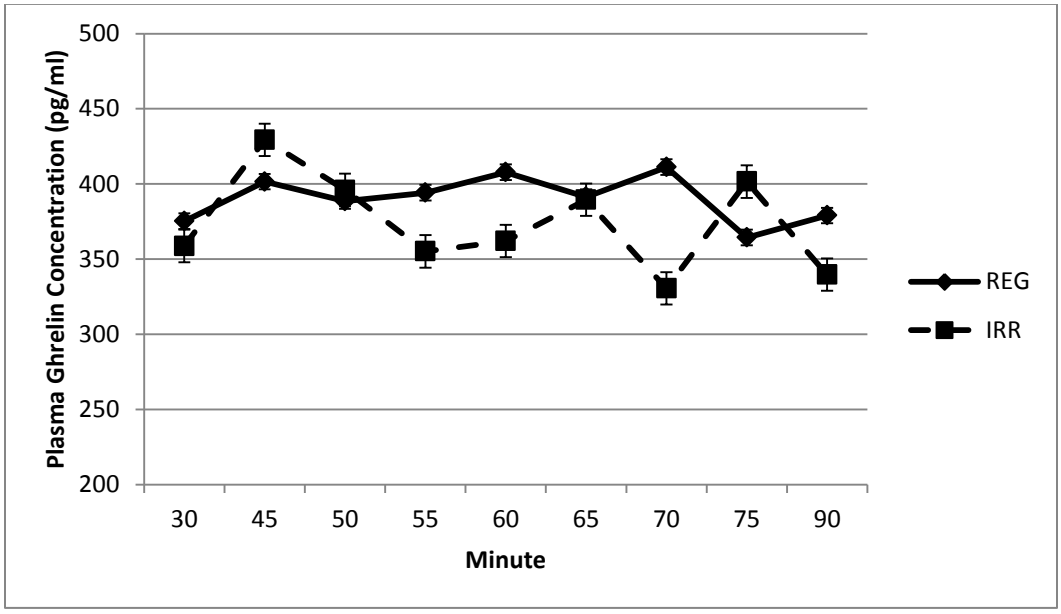

Figure 6. Least squares means of plasma ghrelin by minute in calves fed MR at regular (REG) versus irregular (IRR) feeding times. Regular feeding time was at t = 60 minutes. There was no main effect of time or trt, or a treatment by time interaction ($P > 0.10$).

Least squares means of plasma GH concentrations at each week for calves fed MR at regular versus irregular times are presented in Figure 7. A treatment by week interaction was present as well as a main effect of week ($P<0.0001$). Regularly fed calves exhibited significantly higher GH concentrations at weeks 2 and 4 ($P<0.05$). At week 6,

as calves were weaned, GH concentrations did not differ (P>0.10). The increased GH concentration at week 4 corresponds with the increased ghrelin concentration at week 4 in the regularly fed calves. At weaning at week 6, meal timing was no longer occurring, resulting in similar GH and ghrelin concentrations between treatment groups. At week 8 regularly fed calves exhibited numerically lower GH concentrations than irregularly fed calves, however no significance was observed (P>0.10). Regularly fed calves had an overall decrease in plasma GH concentrations post-weaning. Ghrelin concentrations in regularly fed calves were lower at week 8. Katoh et al. (2004) observed that a GH decreases in post-weaned calves when compared to pre-weaned claves.

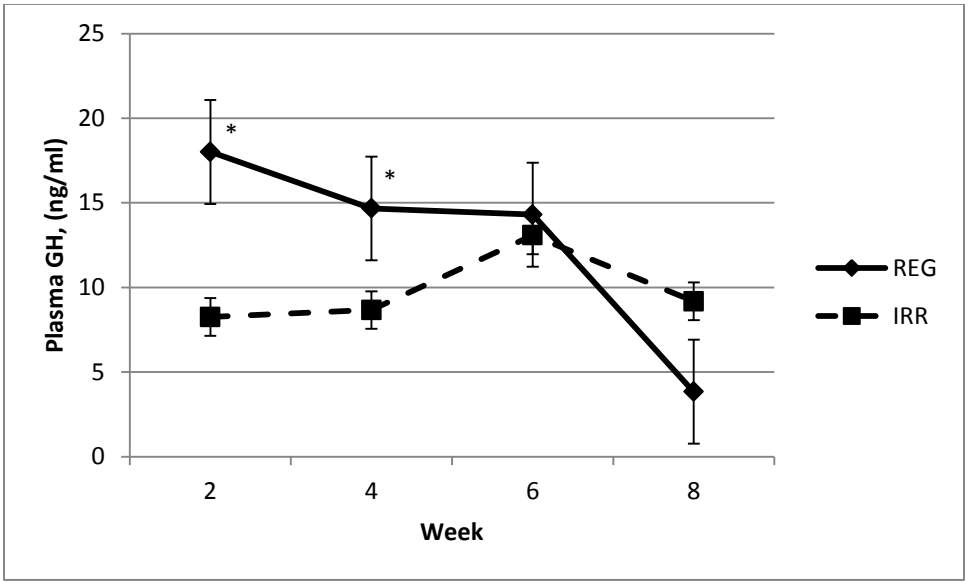

Figure 7. Least squares means of plasma GH concentrations by week in calves fed MR at regular (REG) versus irregular (IRR) feeding times. There was a treatment by week interaction, and a main effect of week (P<0.0001).

Least squares means of plasma GH at each minute in calves fed MR at regular versus irregular feeding times are shown in Figure 8. No main effects of treatment or time were observed (P>0.10), nor was there a treatment by time interaction (P>0.10). There were differences between treatments at time points t= 75, 90, and 120 min (P=0.0313, 0.0576, 0.0787, respectively). Sugino et al. (2002) confirmed that a momentary surge in plasma GH was found to follow the plasma ghrelin surge in scheduled meal fed sheep. Growth hormone levels peaked during feeding and rapidly declined at the end of feeding, then gradually increased with periodic secretion for the rest of the day (Sugino et al. 2002). In the current study, the regularly fed calves displayed an increase in plasma GH at time points which tended to follow the plasma ghrelin pattern. A notable GH concentration difference was observed when comparing GH concentrations in the current experiment to a previous experiment by Simon (2010), who reported higher GH concentrations in irregularly fed neonatal Holstein calves. Simon (2010) began feeding calf starter at 2 weeks of age, and MR was not increased weekly as calves grew. Therefore, calves may not have consumed enough starter to obtain adequate energy from solid feed resulting in an increase in plasma GH concentrations. Thomas et al. (1991) found that certain stressors, such as restricted feeding or decreased feeding frequency, tended to increase plasma GH levels. Fukumori et al. (2013) observed no difference in GH response in pre- versus post-weaned calves, except for week 7 (weaning). The ghrelin-induced growth hormone response was highest at week 7, and was thought to occur due to calves not receiving adequate energy from solid feed. They concluded that the increase in ghrelin action on GH at weaning could be influenced by nutritional status at weaning and not physiological changes that occur at this time (Fukumori et al. 2013).

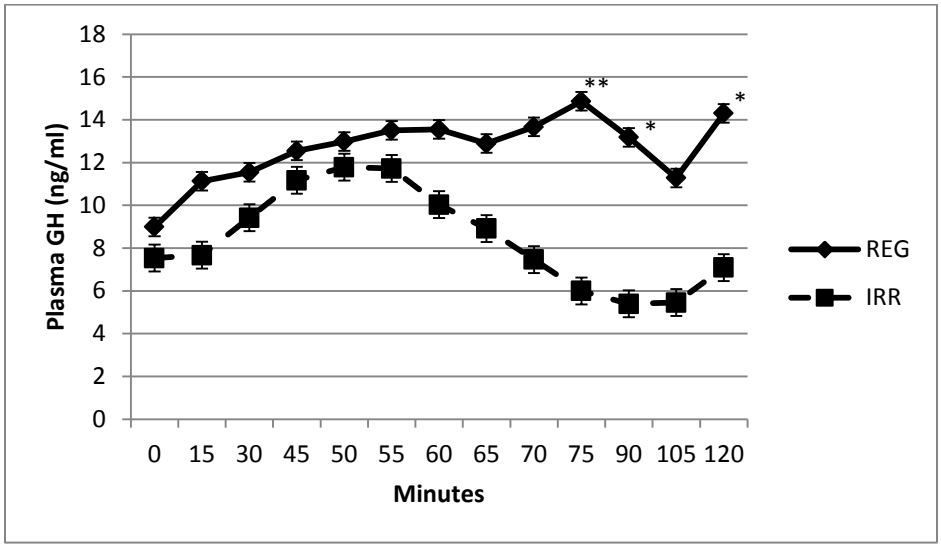

Figure 8. Least squares means of plasma GH by minute in calves fed MR at regular (REG) versus irregular (IRR) feeding times. Regular feeding was at t=60 minutes. **, Significantly different from IRR fed (P<0.05); *, Significantly different from IRR fed (P<0.10).

Intravenous Glucose Tolerance Test

Least squares means for glucose clearance rate (k), half-life ($T_{1/2}$), peak insulin concentration, and peak insulin time at weeks 3, 6, and 9 are presented in Table 2. There was no main effect of week or treatment, nor was a treatment by week interaction observed (P>0.10), for k, $T_{1/2}$, or peak insulin time point. There was a main effect of week on peak insulin concentration (P<0.05), but a treatment by week interaction was not observed (P>0.10). Least squares means for glucose concentrations over time during the IVGTT are displayed in figure 9. As expected, there was a main effect of time (P<0.05) as glucose concentrations increased 5 min post infusion. Least squares means for glucose concentrations over time during the IVGTT for weeks 3, 6, and 9 are displayed in figure 10. A treatment by week interaction (P<0.0001), and a main effect of week and of time (P<0.05) were observed.

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

	REG			IRR			SEM[1]
Week	3	6	9	3	6	9	
Glucose							
k, %/min[2]	0.96	0.78	1.05	0.73	0.85	0.68	0.18
$T_{1/2}$, min[3]	91.20	133.00	99.25	130.03	113.54	138.69	26.95
Insulin							
Peak, µU/ml	10.02	15.15	17.83	12.60	19.22	17.67	4.07
Peak time, min	29.32	22.65	37.10	26.00	28.22	32.11	4.90

[1]SEM =Standard error of the mean. [2]k = fractional turnover rate. [3]$T_{1/2}$ = half-life. There was a main effect of week for peak insulin ($P<0.05$)

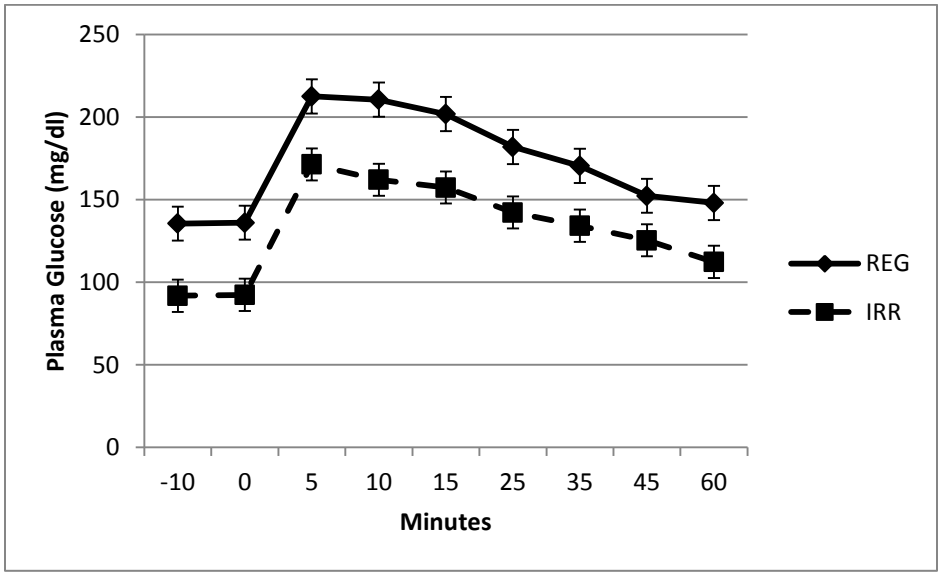

Figure 9. Least squares means of glucose concentrations by minute during the IVGTT in calves fed MR at regular (REG) versus irregular (IRR) feeding times. There was a main effect of time ($P<0.05$).

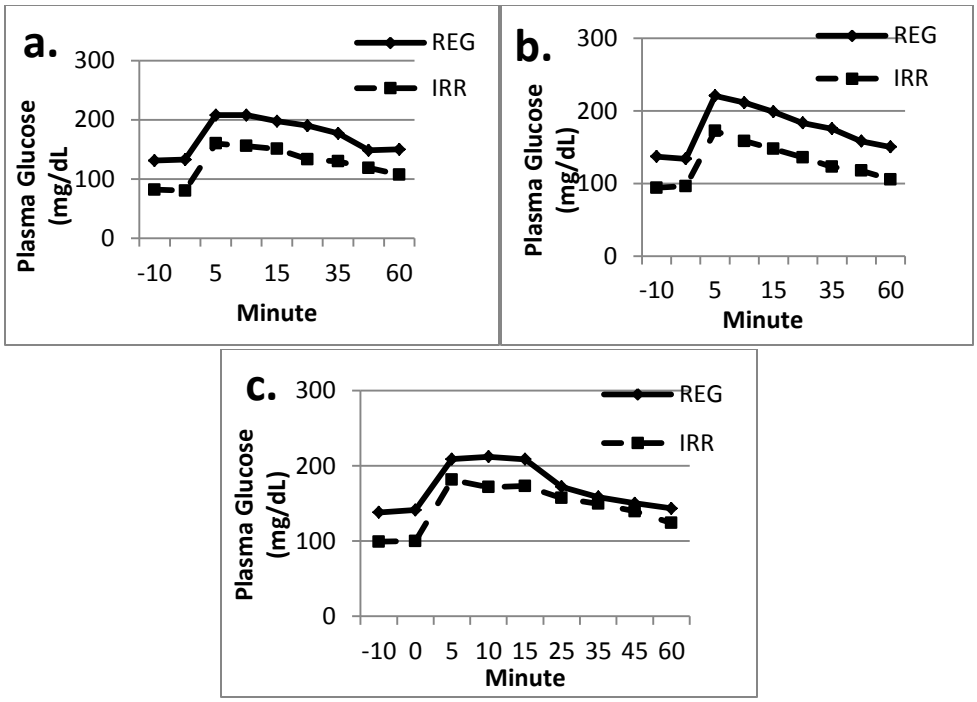

Figure 10. Least squares means of glucose concentrations by minute during the IVGTT in calves fed MR at regular (REG) versus irregular (IRR) feeding times. a. 3 weeks b. 6 weeks c. 9 weeks There was a main effect of week ($P<0.05$), and a treatment by week interaction ($P<0.0001$).

Least squares means for insulin concentrations over time during the IVGTT are displayed in figure 11. Figure 12 shows peak insulin concentrations after glucose infusion at each week for calves fed MR regularly versus irregularly. There was a main effect of week on insulin concentrations ($P<0.05$) during the IVGTT, with insulin concentrations increasing as calves aged. Although no significance was observed between treatments, calves fed MR at regular times had numerically higher glucose concentrations over all weeks, while irregularly fed calves displayed higher insulin concentrations.

Least squares means of AUC for glucose and insulin during the IVGTT are displayed in figure 14 and 15, respectively. A treatment by week interaction was not observed for AUC of glucose, nor were main effects of treatment or of week observed ($P>0.10$). Similarly, there was no treatment by week interaction or main effect of

treatment for insulin AUC (P>0.10). However, a main effect of week (P<0.10) was observed for AUC of insulin. Peak insulin concentrations and AUC for insulin in response to the IVGTT did increase with age of the calves in both treatment groups, while glucose clearance rate and glucose half-life did not differ. This implies that calves become less sensitive to insulin as they transition from a monogastric type gastrointestinal system to that of a functioning ruminant. Stanley et al. (2002) also reported a decrease in insulin sensitivity with age in young dairy calves. In contrast, similar research by Simon (2010) observed peak insulin concentrations to be greater for calves fed MR at irregular times versus regular times, but showed no effect of age. Simon (2010) also observed no difference in glucose concentrations between calves fed at regular versus irregular feeding times.

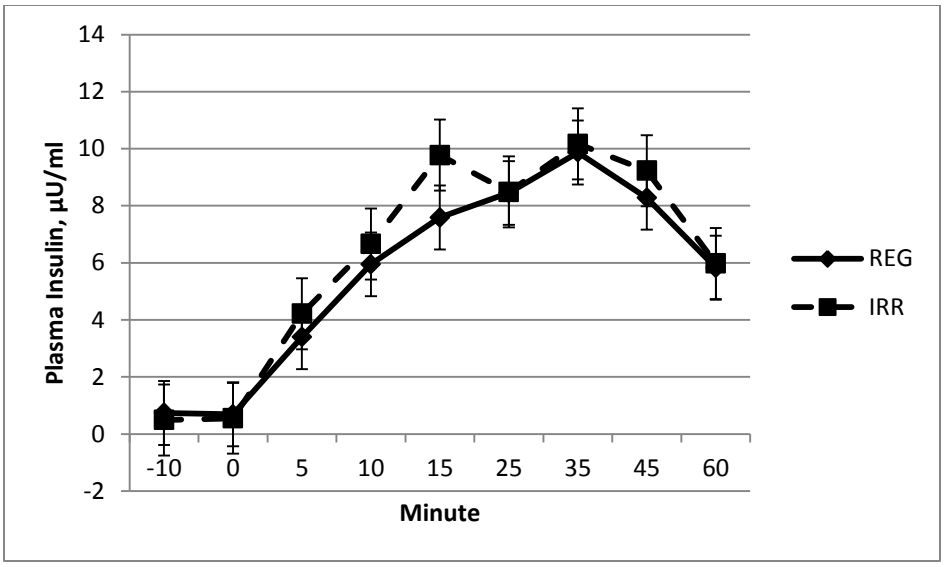

Figure 11. Least squares means of peak insulin concentrations by minute during the IVGTT in calves fed MR at regular (REG) versus irregular (IRR) feeding times. There was a main effect of time (P<0.05).

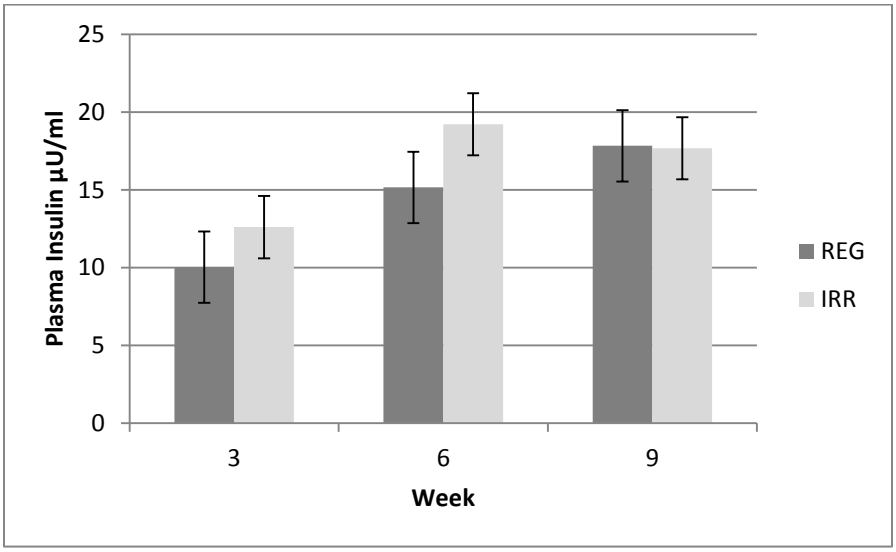

Figure 12. Least squares means of peak insulin concentrations by week in calves fed MR at regular (REG versus irregular (IRR) feeding times. There was a main effect of week (P<0.05).

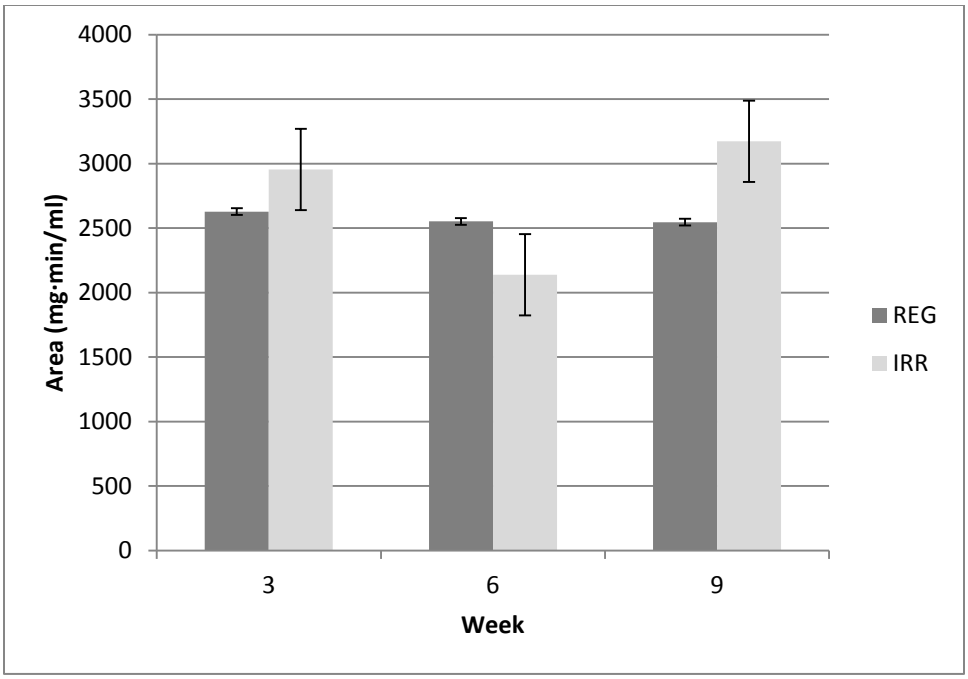

Figure 13. Least squares means of AUC for glucose concentration by week in calves in response to insulin during the IVGTT in calves fed MR at regular (REG) versus irregular (IRR) feeding times.

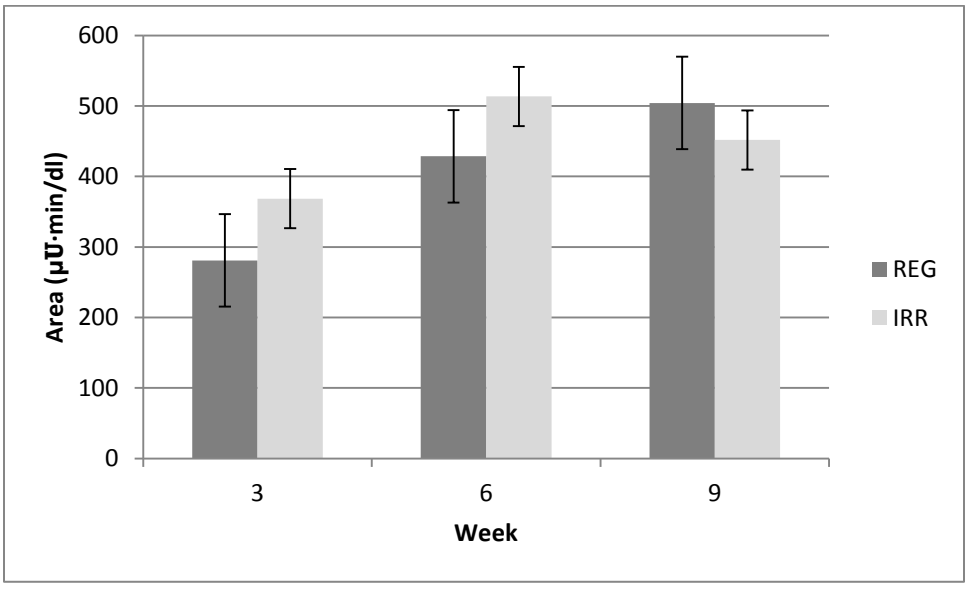

Figure 14. Least squares means of AUC for insulin concentration by week in calves in response to an IVGTT in calves fed MR at regular (REG) versus irregular (IRR) feeding times. A main effect of week was observed ($P<0.10$)

CHAPTER V
SUMMARY AND CONCLUSION

Summary

The purpose of this study was to determine if irregular feeding times can affect the secretion of ghrelin and growth hormone in pre and post weaned dairy calves. An intravenous glucose tolerance test was performed pre and post weaning to evaluate the effects of varied feeding times on glucose metabolism and insulin secretion. Eighteen neonatal Holstein bulls were utilized in this 9 week study. Calves were divided into two treatment groups consisting of regularly fed MR and irregularly fed MR. Regularly fed calves were offered MR once daily at 630 h. Irregularly fed calves were offered MR at 1030, 0800, 0630, 0830, 0530, 0930, and 0730 h on Monday, Tuesday, Wednesday, Thursday, Friday, Saturday, and Sunday, respectively.

Body weights were collected weekly from birth to wk 9, water intake was measured daily from wk 1 to wk 9, and starter intake was measured daily from wk 4 to wk 9. Serial blood collections were performed at weeks 2, 4, 6, and 8, beginning at 0530 h and continuing for 120 minutes. Plasma was analyzed for ghrelin and growth hormone. An IVGTT was performed at weeks 3, 6, and 9. Plasma was analyzed for insulin and glucose.

Overall the results showed no effect of an irregular feeding pattern on body weight or starter intake ($P>0.10$). There was a main effect of week on body weight and starter intake ($P<0.05$). An irregular feeding pattern did have an effect on water intake ($P<0.10$), with significant increases at 1 wk and 3 wk ($P<0.05$).

A treatment by week interaction and a main effect of week were exhibited for mean ghrelin concentrations ($P<0.05$), and regularly fed calves exhibited increased ghrelin concentrations at week 4 ($P<0.10$). Plasma ghrelin concentrations increased with age until weaning, at week 6, and then decreased t week 8. No treatment by time interaction or main effect of time on ghrelin concentration were observed ($P>0.10$) in relation to feeding time. Regularly fed calves displayed a significant increase in GH concentration at t= 75 ($P<0.05$), 90 ($P<0.10$), and 120 ($P<0.10$) min. A treatment by week interaction and a main effect of week were observed for mean GH concentrations ($P<0.0001$). Regularly fed calves had higher mean GH concentrations at weeks 2 and 4 ($P<0.05$).

No differences were reported between treatments or with age for fractional turnover rate and half-life of glucose. Peak insulin concentrations ($P<0.05$) and AUC for insulin ($P<0.10$) increased as calves aged, suggesting that calves become less sensitive to insulin as they develop.

Conclusion

Feeding MR to neonatal calves at regular or irregular feeding times does not affect the growth of the animal. There does appear to be an effect on anabolic hormone status, with differences in ghrelin and GH concentrations. No effects of feeding time were observed for glucose metabolism and insulin sensitivity. However, age related differences were observed in ghrelin, GH, and glucose metabolism, thus indicating the developmental changes that occur in energy metabolism in young ruminant animals.

REFERENCES

Alba-Roth, J., O.A., Muller, J, Schopohl, and K, Von Werder. 1988. Arginine stimulates growth Hormone secretion by suppressing endogenous stomach secretion. J. Clin. Endocrinol. Metab. 67 (6): 1186-9.

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

Bovine Alliance on Management and Nutrition. 1995. A guide to colostrum and colostrum management for dairy calves. Arlington, VA: American Feed Industry Association.

Bradford, B. J., and M. S. Allen. 2007. Negative energy balance increases periprandial ghrelin and growth hormone concentrations in lactating dairy cows. Domest. Anim. Endocrinol. 34:196-203.

Briatore, L., G. Andraghetti, and R. Cordera. 2003. Acute plasma glucose increase, but not early insulin response, regulates plasma ghrelin. Eur. J. Endocrinol. 149:403 406.

Broglio, F., E. Arvat, A. Benso, C. Gottero, G. Muccioli, M. Papotti, A.J. Van der Lely, R. Deghenghi, and E. Ghigo. 2001. Ghrelin, a natural GH secretagogue produced by the stomach, induces hyperglycemia and reduces insulin secretion in humans. J. Clin. Endocrinol. Metab. 86:5083–5086.

Bryant, M. P., N. Small, C. Bouma, and J,Robinson. 1958. Studies on the composition of the ruminal flora and fauna of the young calf. J. Dairy Sci. 41:654-667.

Cummings D.E., J.Q. Purnell, R.S. Frayo, K. Schmidova, B.E. Wisse, and D.S. Weigle. 2001. A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans. Diabetes. 50:1714–1719.

Date, Y., M. Nakazato, S. Hashiguchi, K. Dezaki, M.S. Mondal, H. Hosoda, M. Kojima, K. Kangawa, T. Arima, H. Matsuo, T. Yada, and S. Matsukura. 2002. Ghrelin is present in pancreatic α-cells of humans and rats and stimulates insulin secretion. Diabetes. 51:124–129.

Dickin, J.C., T.D. Thue, and F.C. Buchanan. 2004. An alternative splice site in ghrelin is missing in ruminants. Anim. Gen. 35:411–412.

Drazen, D., T. Vahl, D. D'Alessio, R. Seeley, and S. Woods. 2006. Effects of a Fixed Meal Pattern on Ghrelin Secretion: Evidence for a Learned Response Independent of Nutrient Status. Endocrinology. 147(1):23.

Flatt, W. P., R. G. Warner, and J. K. Loosli. 1958. Influence of purified materials on the development of the ruminant stomach. J. Dairy Sci. 41:1593-1600.

Frohman, L. A., R. D. Kineman, J. Kamegai, S. Park, L. T. Teixeira, K. T. Coschigano, and J. Kopchic. 2000. Secretagogues and the somatotrope: Signaling and proliferation. Rec. Prog. Horm. Res. 55: 269-290.

Fukumori, R., T. Sugino, Y. Hasegawa, M. Kojima, K. Kangawa, T.,Obitsu, and K. Taniguchi. 2011. Plasma ghrelin concentration is decreased by short chain fatty acids in wethers. Domest. Anim. Endocrinol. 41:50–55.

Fukumori, R., T. Mita, T. Sugino, Y. Hasegawa, M. Kojima, K. Kangawa, T. Obitsu, and K. Taniguchi. 2012. Effects of glucose and volatile fatty acids on blood ghrelin concentrations in calves before and after weaning. J. Anim. Sci. 90:4839-4845.

Fukumori, R., T. Mita, T. Sugino, Y. Hasegawa, M. Kojima, K. Kangawa, T. Obitsu, and K. Taniguchi. 2013. Effects of intravenous ghrelin injection on plasma growth hormone, insulin, and glucose concentrations in calves at weaning. Anim. Sci. J. 84:310-15.

Gauna, C., P.J. Delhanty, and L.J. Hofland. 2004. Ghrelin stimulates, whereas des octanoyl ghrelin inhibits, glucose output by primary hepatocytes. J. Clin. Endocrinol. Metab. 90:1055-1060.

Geary, N. 2004. Endocrine controls of eating: CCK, leptin, and ghrelin. Physiol. Behav. 81:719-733.

Gentry, P. C., J. P. Willey, and R. J. Collier. 2003. Ghrelin, a growth hormone secretagogue, is expressed by bovine rumen. J. Anim. Sci. 81(Suppl.1):123.

Granger, A. L., W. E. Wyatt, W. M. Craig, D. L. Thompson, Jr., and F. G. Henbry. 1989. Effects of breed and wintering diet on growth, puberty, and plasma concentration of growth hormone, and insulin-like growth hormone in heifers. Dom. Anim. Endocrinol. 6:253-262.

Grouselle D., E. Chaillou, A. Caraty, M.T. Bluet-Pajot, P. Zizzari, Y. Tillet, and J. Epelbaum. 2008. Pulsatile cerebrospinal fluid and plasma ghrelin in relation to growth hormone secretion and food intake in the sheep. J. Neuroendocrinol. 20:1138–1146.

Guilloteau P, R. Zabielski, J.C. David, J.W. Blum, J.A. Morisset, M. Biernat, J. Wolinski, D. Laubitz, and Y. Hamon. 2009. Sodium-butyrate as a growth promoter in milk replacer formula for young calves. J. Dairy Sci. 92:1038–1049.

Hayashida, T., K. Murakami, K. Mogi, M. Nishihara, M. Nakazato, M. S. Mondal, Y. Horii, M. Kojima, K. Kangawa, and N. Murakami. 2001. Ghrelin in domestic animals: distribution in the stomach and its possible role. Domest. Anim. Endocrinol. 21:17-24.

Hosoda, H., M. Kojima, H. Matsuo, K. Kangawa. 2000. Ghrelin and des-acyl ghrelin: two major forms of rat ghrelin peptide in gastrointestinal tissue. Biochem. Biophys. Res. Commun. 279:909-913.

Hosoda, H., M. Kojima, T. Mizushima, S. Shimizu, and K. Kangawa. 2003. Structural divergence of human ghrelin. Identification of multiple ghrelin-derived molecules produced by post translational processing. J. Biol. Chem. 278:64-70.

Itoh, F., T. Komatsu, M. Yonai, T. Sugino, M. Kojima, K. Kangawa, Y. Hasegawa, Y. Terashima, and K. Hodate. 2005. GH secretory responses to ghrelin and GHRH in growing and lactating dairy cattle. Domest. Anim. Endocrinol. 28:34–45.

Itoh, K., T. Komatsu, S. Kushibiki, and K. Hodate. 2006. Effects of ghrelin injection on plasma concentrations of glucose, pancreatic hormones and cortisol in Holstein dairy cattle. Comp. Biochem. and Phys. -Part A: Molecular & Integrative Physiology. 143(1):97-102.

Kalra S.P., M. Bagnasco, E.E. Otukonyong, M.G. Dube, and P.S. Kalra. 2003. Rhythmic, reciprocal ghrelin and leptin signaling: new insight in the development of obesity. Regul. Pept. 111:1–11.

Kaneko, J. J. 1997. Carbohydrate Metabolism and its Diseases. IN: Clinical Biochemistry of Domestic Animals. Academic Press. San Diego, CA. p. 66.

Katoh, K., G. Furukawa, K. Kitade, N. Katsumata, Y. Kobayashi and Y. Obara. 2004. Postprandial changes in plasma GH and insulin concentrations, and responses to stimulation with GH releasing hormone (GHRH) and GHRP-6 in calves around weaning. J. Endocrinol. 183:497-505.

Kobayashi, Y., M. Onodera, M. Yoshida, T. Sugino, K. Katoh, and Y. Obara. 2006. Postprandial changes in plasma concentrations of growth hormone and ghrelin around weaning in the goat. Gen. Comp. Endocrinol. 148:368–374.

Kojima M., H. Hosoda, and Y. Date. 1999. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature. 402:656-660.

Krueger, T., and P. Melendez. 2012. Effect of ghrelin on feed intake and metabolites in lambs. Appetite. 58:758-759.

Littell, R. C., P. R. Henry, and C. B. Ammeran. 1998. Statistical analysis of repeated measures data using SAS procedures. J. Anim. Sci. 76: 1216-1231.

Lyford, Sidney J. 1988. Growth and Development of the Ruminant Digestive System. IN: The Ruminant Animal Digestive Physiology and Nutrition. Waveland Press Inc. Long Grove, IL. 46-49.

Masuda, Y., T. Tanaka, N. Inomata, N. Ohnuma, S. Tanaka, Z. Itoh, H. Hosoda, M. Kojima, and K. Kangaw. 2000. Ghrelin stimulates gastric acid secretion and motility in rats. Biochem. Biophys. Res. Commun. 276:905–908.

McCowen, K.C., J.A. Maykel, B.R. Bistrian, and P.R. Ling. 2002. Circulating ghrelin concentrations are lowered by intravenous glucose or hyperinsulinemic euglycemic conditions in rodents. J. of Endocrinol. 175:R7–R11.

Nakagawa, E., N. Nagaya, H. Okumura, M. Enomoto, H. Oya, F. Ono, H. Hosoda, M. Kojima, and K. Kangawa. 2002. Hyperglycaemia suppresses the secretion of ghrelin, a novel growth hormone-releasing peptide: responses to the intravenous and oral administration of glucose. Clin. Sci. (Lond). 103:325–328.

Nakazato, M., N. Murakami, Y. Date, M. Kojima, H. Matsuo, K. Kangawa, and S. Matsukura. 2001. A role for ghrelin in the central regulation of feeding. Nature. 409:194–198.

Preston, T. R., 1963. The nutrition of the early weaned calf. World Rev. Nutr. Diet. 4:117-139.

Reimer, M.K., G. Pacini, and B. Ahren. 2003. Dose-dependent inhibition by ghrelin of insulin secretion in the mouse. Endocrinol. 144:916–921.

Rindi, G., V. Necchi, A. Savio, A. Torsello, M. Zoli, V. Locatelli, F. Raimondo, Cocchi, and E. Solcia. 2002. Characterization of gastric ghrelin cells in man and other mammals: studies in adult and fetal tissues. Histochem. Cell Biol. 117:511-519.

Salfen, B.E., J.A. Carroll, D.H. Keisler, and T.A. Strauch. 2004. Effects of exogenous ghrelin on feed intake, weight gain, behavior, and endocrine responses in weanling pigs. Journal of Animal Science. 82:1957-1966.

Sander, E.G., R.G. Warner, H.N. Harrison, and J.K. Loosli. 1959. The stimulatory effect of sodium butyrate and sodium propionate on the development of rumen mucosa in the young calf. J. Dairy Sci. 42:1600±1605.

Shiiya, T., M. Nakazato, M. Mizuta, Y. Date, M. S. Mondal, M. Tanaka, S. Nozoe, H. Hosoda, K. Kangawa, and S. Matsukura. 2002. Plasma ghrelin levels in lean and obese humans and the effect of glucose on ghrelin secretion. J. Clin. Endocrinol. Metab. 87:240–244.

Simon, K.C. 2010. Effects of meal timing on anabolic hormone status and energy metabolism in neonatal dairy cattle. M.S. Thesis. Louisiana State University, Baton Rouge.

Stanley, C.C., C. C. Williams, B. F. Jenny, J. M. Fernandez, H. G. Bateman, II, W. A. Nipper, J. C. Lovejoy, D. T. Gantt, and G. E. Goodier. 2002. Effects of feeding milk replacer once versus twice daily on glucose metabolism in Holstein and Jersey calves. J. Dairy Sci. 85:2335- 2343.

Sugino T, Y. Hasegawa, Y. Kikkawa, J. Yamaura, M. Yamagishi, Y. Kurose, M. Kojima, K. Kangawa, and Y. Terashima. 2002. A transient surge of ghrelin secretion before feeding is modified by different feeding regimens in sheep. Biochemical and Biophysical Research Communications. 298:785–788.

Sugino T, Y. Hasegawa, Y. Kurose, M. Kojima, K. Kangawa, and Y. Terashima. 2004 Effects of ghrelin on food intake and neuroendocrine function in sheep. Anim.Repro. Sci. 82–83: 183–194.

Takahashi T, T. Ida, T. Sato, Y. Nakashima, Y. Nakamura, A. Tsuji, M. Kojima. 2009. Production of n-octanoyl-modified ghrelin in cultured cells requires prohormone processing protease and ghrelin O-acyltransferase, as well as n-octanoic acid. J Biochem.146(5):675-82.

Takaya, K., H. Ariyasu, N. Kanamoto, H. Iwakura, A. Yoshimoto, M. Harada, K. Mori, Y. Komatsu, T. Usui, A. Shimatsu, Y. Ogawa, K. Hosoda, T. Akamizu, M. Kojima, K. Kangawa, K. Nakao. 2000. Ghrelin strongly stimulates growth hormone release in humans. J. Clin. Endocrinol. Metab. 85:4908–4911.

Tannenbaum, G.S., M. Lapointe, A. Beaudet. 1998. Expression of growth hormone secretagogue-receptors by growth hormone-releasing hormone neurons in the mediobasal hypothalamus. Endocrinol. 139:4420-3.

ThidarMyint, H., H. Yoshida, T. Ito, and H. Kuwayama. 2006. Dose-dependent response of plasma ghrelin and growth hormone concentrations to bovine ghrelin in Holstein heifers. J. Endocrinol. 189:655–664.

ThidarMyint, H., H. Yoshida, T. Ito, M. He, H. Inoue, H. Kuwayama. 2008. Combined administration of ghrelin and GHRH synergistically stimulated GH release in Holstein preweaning calves. Domest. Anim. Endocrinol. 34:118-123.

Thomas, G. B., J. T. Cummins, H. Fransis, A. W. Sudbury, P. I. McCloud, and I. J. Clarke. 1991. Effects of restricted feeding on the relationship between hypophysial portal concentrations of growth hormone (GH)-releasing factor and somatostatin, and jugular concentrations of GH in ovariectomized ewes. Endocrinol. 128:1151–1158.

Toshinai, K., H. Yamaguchi, and Y. Sun. 2006. Des-acyl ghrelin induces food intake by a mechanism independent of the growth hormone secretagogue receptor. Endocrinol. 147(5):2306–2314.

Tschop, M., D.L. Smiley, and M.L. Heiman. 2000. Ghrelin induces adiposity in rodents. Nature.407:908–913.

Veldhuis, J.D. 2003. A tripeptidyl ensemble perspective of interactive control of growth hormone secretion. Horm. Res. 60:86–101.

Wertz-Lutz, A. E., T. J. Knight, R. H. Pritchard, J. A. Daniel, J. A. Clapper, A. J. Smart, A. Trenkle, and D. C. Beitz. 2006. Bovine ghrelin fluctuates relative to nutritional status and influences feeding behavior. J. Anim. Sci. 84:3285-3300.

Wertz-Lutz, A.E., J.A. Daniel, J.A. Clapper, A. Trenkle, and D.C. Beitz. 2008. Prolonged, moderate nutrient restriction in beef cattle results in persistently elevated circulating ghrelin concentrations. J. Anim. Sci. 86 (3):564-575.

Wertz-Lutz**,** A. E., J.S. Jennings, and J.A. Clapper. 2010. Plasma ghrelin concentrations of beef cattle consuming a similar amount of dietary energy supplied by different ingredients. J. Anim. Sci. 88(7):2289-2299.

Wilcox, G. 2005. Insulin and Insulin Resistance. Clin Biochem Rev. 26(2):19–39.

Zhang, W., B. Chai, J.-Y. Li, H. Wang, and M. W. Mulholland. 2008. Effect of des-acyl ghrelin on adiposity and glucose metabolism. Endocrinol. 149 (9):4710–4716.

Zizzari, P. and M.T. Bluet-Pajot. 2004. Ghrelin, from GH secretagogue to orexigenic peptide. Correspondances en Metabolismes, Hormones, Diabetes et Nutrition. 8:58–65.

APPENDIX A
MBC QT II™ BOVINE SERUM IgG

Assay Procedure

1. Allow the sample and reagent vial to warm to room temperature (approximately 20 minutes).

2. Insert the zero vial in the MBC QTII®. Align the ▼on the vial with the ▲ on the adapter to obtain a continuous beeping and view ******* across the display. If ******* an beeping is not observed, the vial may need to be rotated left or right to initiate the zero feature. Cover the vial. When completed “0.000”, “ZERO”, and “0 Program” will be displayed.

3. Ad 10 µL of sample to the reagent vial. Re-cap and mix the contents by inverting several times. Wipe off any liquid or smudges from the exterior of the vial.

4. Incubate the reagent vial containing the sample for 5 minutes at room temperature.

5. Mix the contents again by inverting several times. Insert the vial into the MBC QTII®. Align the ▼on the vial with the ▲ on the adapter. Rotate the vial left or right to initiate measurement if necessary. Cover the vial.

6. The MBC QTII® will display the result in mg/dL. Record the value.

Limitations:

This method is linear to a Bovine IgG level of 1600 mg/dL. The MBC QTII® will indicate if a sample is out of range with the message “OVERRNG” or “UNDRRNG”. Sample higher than 1600 mg/dL should be re-assayed after dilution with 0.9% saline. The results should then be multiplied by the dilution factor.

Calculations:

Diluted Concentration X Dilution Factor = Concentration

APPENDIX B
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 4oC.

Day Two

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

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 x 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 = (Sample or Standard/Total Binding) X 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 µl, an appropriate mathematical adjustment must be made to accommodate for the dilution factor (e.g., if 50 µl of sample is used, then calculated data must be multiplied by 2).]

APPENDIX C
PLASMA GROWTH HORMONE RADIOIMMUNOASSAY

Volumes of reagents to pipette:

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

What Goes in Each Tube

1. TC 125I-bGH (HH)
2. NRS PBSG (200µL) + #3 1:105 NRS + HH + 1:45 (4X)pARGG
3. BC PBSG (200µL) + 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 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 quinoeimene 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 E
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 µIU/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 though 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 tractor bound [(Maximum Binding Counts /Total) X 100]. This should be 35-50%.
4. Calculate the percentage of maximum binding %B/Bo = (Sample or Standard / Maximum Binding) X 100.
5. Plot the %B/Bo 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.

VITA

Erica Chartier is the daughter of Michael and Susan Chartier of Kenner, LA. She graduated from Grace King High School in May of 2005, and began her bachelor's degree in August of 2005 at University of New Orleans. Transferring to Louisiana State University in August, 2008, it was here that she discovered her passion for dairy cows. In December of 2010, she earned her Bachelor of Science degree in Animal Science. After graduating, Erica began her graduate studies in August, 2011 in dairy calf nutrition physiology. She will receive her masters in December, 2013.