

REGULATION OF GLUCOSE METABOLISM IN DAIRY CATTLE

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ABSTRACT

The experiments described herein are designed to elucidate many metabolic changes that occur to regulate the partitioning of nutrients between production and animal maintenance with particular regard to glucose metabolism and insulin sensitivity. The onset of milk production and parturition causes dramatic stress to the cow. The challenge of improving metabolism during this period is being addressed by nutritional management with the possibility of the addition of supplements. The use of Ca-propionate as a supplement did not affect glucose metabolism in transition cows. The data revealed that insulin sensitivity was low during the transition period, but the tissue responses to insulin and the acute insulin response to a glucose infusion increased slightly after calving as compared to late gestation. Glucagon and nonesterified fatty acid concentrations increased throughout the transition period, while glucose, insulin, and thyroxine concentrations decreased. A need for a relatively easy and inexpensive test for evaluating glucose metabolism has been indicated, and it was shown that the Minimal Model computer analysis of the frequently sampled glucose tolerance test was adequate in assessing insulin sensitivity in dairy cattle. The acute hyperinsulinemia induced by the insulin tolerance test or by the hyperinsulinemic euglycemic test did not affect plasma leptin concentrations.

CHAPTER 1

INTRODUCTION

Endocrine regulation of glucose metabolism is of utmost importance in mammalian homeostasis and homeorhesis. Insulin, a key hormone in endocrine regulation, facilitates the movement of glucose across cell membranes, thereby regulating the concentration of blood glucose. Glucose is the principal source of energy for life processes of the mammalian cell. All cells require a constant supply of this indispensable nutrient, and only relatively small changes are tolerated without adverse effects on the health of the animal (Kaneko, 1997).

Transition cow management has a great effect on the subsequent lactation. Accurate prediction of herd health disorders that commonly occur during this period of lactation, such as ketosis, may play a role in reducing disease prevalence. Ketosis is a metabolic disorder which affects approximately 4% of all dairy cows in the United States (de Boer et al., 1985). This period of time can be very critical to the health and production of cows. Therefore, an area of current research emphasis in dairy nutrition is minimizing the metabolic problems that occur during the transition period by adopting better management strategies and improving nutritional strategies. Many substances can be added as supplements to dry cow rations as preventative measures for ketosis. In the first experiment described in this dissertation, the effects of the addition of Ca-propionate as a supplement to alleviate metabolic disorders were studied. During this study frequently sampled glucose tolerance tests with minimal model computer analyses were conducted to determine the effect of Ca-propionate on insulin sensitivity and glucose metabolism prior to and after calving. Many metabolic changes occur to regulate the

partitioning of nutrients between production and animal maintenance, and the relationship between insulin sensitivity and milk production is of particular interest. This concept led to characterizing insulin sensitivity and glucose metabolism in Holstein heifers throughout a lactation cycle in relationship to their milk production. Also, these parameters were compared to previous data collected in the same animals as calves (Stanley et al., 2002) to follow the changes in metabolism as animals transitioned from growing calves to lactating cows.

The minimal model computer program has been used in this laboratory to study insulin sensitivity and glucose metabolism in calves and cows; however, the program had not been evaluated as an acceptable test for measuring these factors in ruminants. The program has been evaluated in many other species and determined to be a good tool to measure these metabolic parameters. In the third experiment, the use of the program was evaluated in Holstein calves and cows by comparing the technique to the hyperinsulinemic clamp test, a method that has been utilized by ruminant researchers. Evaluating the minimal model program in dairy cattle could be important for identifying a tool that would aid in developing nutritional and management strategies for improving glucose utilization in growing and lactating animals.

During recent years it has become apparent that many more factors than insulin sensitivity and glucose metabolism effect metabolic homeostasis and homeorhesis. The metabolic adaptations necessary to accommodate the changes in nutrient partitioning associated with lactogenesis are mediated by the endocrine system. Energy reserves are mobilized in response to an increased nutrient demand to support lactation until the cow can consume enough feed to provide needed nutrients. Insulin and leptin are important

regulators of appetite and energy status of the cell, but little research has investigated the roles of these hormones in regulating metabolism in early lactation dairy cattle. A final experiment was designed to characterize insulin sensitivity and hormone and metabolite profiles at the end of gestation and in early lactation by utilizing several metabolic tests. The physiological responses of leptin and glucagon concentrations to a hyperinsulinemic state at euglycemia and at hypoglycemia were measured by applying the hyperinsulinemic clamp tests and the insulin tolerance tests. The frequently sampled glucose tolerance tests were conducted to gather further data to compare the minimal model computer program to the hyperinsulinemic clamp tests.

CHAPTER 2

REVIEW OF LITERATURE

Methods of Assessing Glucose Metabolism and Insulin Sensitivity

Several methods have been utilized in ruminants for assessing glucose metabolism and insulin sensitivity. One widely used technique is the intravenous glucose tolerance test (IVGTT). This test involves administration of a glucose load followed by serial blood sample collection over a 3 h period. The fractional turnover rate and glucose clearance rate can be calculated in addition to the insulin response curve from the glucose and insulin plasma concentrations. This test is preferred by many researchers because it does not overload the animal with glucose, the infusion can be given easily within the time limits, the blood glucose level is high enough to give a maximal insulin response, and urinary loss of glucose is minimal (Kaneko, 1997).

Another test that may be used is the insulin tolerance test (ITT). In this procedure insulin is injected intravenously or intramuscularly, and blood samples are collected every 30 min for 3 h. The test measures the sensitivity of the blood glucose level to a test dose of insulin and the response of the animal to insulin-induced hypoglycemia. Insulin resistance is identified if the blood glucose level does not decrease by 50% or requires longer than 30 min to reach the maximum hypoglycemic level (Kaneko, 1997).

Andres et al. (1966) introduced clamp techniques for assessing insulin sensitivity and glucose metabolism, and these became widely accepted methods of assessing this physiology. The tests evolved as an alternative to dangerous insulin tolerance tests. The hyperinsulinemic euglycemic clamp (EC) procedure is a widely accepted method of assessing insulin sensitivity. The techniques of measuring insulin sensitivity during an

imposed state of hyperinsulinemia and measuring glucose metabolism during a maintained state of hyperglycemia were published by DeFronzo et al. (1979). Using the glucose clamp techniques, two important physiological variables can be calculated: β -cell response to glucose and the sensitivity of body tissues to insulin.

During the EC plasma insulin is raised by a continuous intravenous insulin infusion, and a variable rate of glucose is infused to maintain a euglycemic level. This physiological response to the test is called the steady state, a period during which the body's uptake of glucose by the tissue is equal to the glucose infused. During the steady state, insulin sensitivity is measured by monitoring the amount of glucose infused to maintain euglycemia under the conditions of hyperinsulinemia (DeFronzo et al., 1979). The EC was originally designed for assessing insulin sensitivity in humans (De Fronzo et al., 1979) and has been used to assess insulin sensitivity in calves (Hostettler-Allen et al., 1994; Hostettler-Allen et al., 1993; Hugi et al., 1998; Sternbauer et al., 1998), heifers (Sternbauer and Luthman, 2002), and cows (Blum et al., 1999; Grinari et al., 1997; Holtenius et al., 2000; Mackle et al., 2000; Mackle et al., 1999; McGuire et al., 1995; Sano et al., 1993; Sano et al., 1991). The EC is considered to be the standard against which other glucose metabolism assessment tests are compared. The test is more difficult and labor-intensive than other procedures. It also requires expensive equipment and well-trained technicians (Coates et al., 1995).

Previous research has utilized the ITT and the IVGTT in ruminants to evaluate glucose metabolism (Bassett, 1989; Hayirli et al., 2001). Although the IVGTT is more practical and easier to perform, information generated from this test is not as easily interpreted as that obtained from the EC. For example, the rate constant for the decline in

blood metabolite concentrations following an intravenous glucose infusion may represent increased utilization or decreased production (Hayirli et al., 2001). The EC provides a safer method of estimating insulin sensitivity than the ITT; the complications and endocrine response to hypoglycemia that might occur during the ITT are avoided in the EC (DeFronzo et al., 1979).

DeFronzo et al. (1979) described a method of assessing the β -cell sensitivity to glucose by utilizing a hyperglycemic clamp test (HC). During the HC plasma glucose concentration is raised 125 mg/dL above basal concentrations and maintained at those concentrations by variable glucose infusion rate. The test also assesses the amount of glucose metabolized under the state of hyperglycemia. During this procedure insulin secretion may be suppressed by administering somatostatin while glycemia is maintained with the variable rate of glucose infusion (Bergman, 1989).

Several alternative methods have been developed to assess insulin sensitivity while minimizing the amount and duration of sampling and gaining the maximum amount of information. During the 1960's the "insulinogenic index" was developed in which the ratio of insulin to glucose was calculated to relate the β -cell function to plasma insulin concentrations. These techniques are simple to employ as a single blood sample is needed. The assumption of this type of sampling is that the relationship of insulin to glucose concentration remains linear at any given concentration and that at any particular moment the patient's insulin concentration reflects the glucose concentration at that same time. The reverse relationship (plasma glucose to insulin concentrations) has been used to estimate insulin sensitivity. The problem with both techniques is that neither insulin nor glucose is held constant (DeFronzo et al., 1979). The homeostasis model assessment

(HOMA) was developed based on the ratio of fasting insulin to fasting glucose (Mathews et al., 1985) and recently a better fitting model, the HOMA2, has been published by Levy et al. (1998). The quantitative insulin sensitivity check index (QUIKI) is a recently developed application (Katz, et al., 2000). Both methods were developed to estimate insulin sensitivity in humans from mathematical equations that utilize fasting glucose and insulin concentrations. The indices derived from the HOMA (Mathews et al., 1985) and QUIKI (Katz et al., 2000) have been successfully compared to the EC in human subjects.

The minimal model computer program (MINMOD) was developed by Bergman et al. (1979) for assessing multiple parameters of glucose and insulin metabolism, and recently a newer version of the MINMOD (millennium edition) has been published (Boston et al., 2003). The frequently sampled intravenous glucose tolerance test (FSIGT) with MINMOD computer analysis provides a simple alternative to the EC procedure (Coates et al., 1995). The FSIGT consists of infusing glucose through a jugular catheter followed 20 min later by infusing bovine insulin. Sequential blood samples are collected through the catheter over a 3 h period relative to glucose administration. The samples are analyzed for glucose and insulin concentrations. These values are entered into the MINMOD computer program to determine glucose effectiveness (S_G), the fractional glucose disappearance at basal insulinemia; insulin sensitivity (S_I) which is the effect of incremental change in plasma insulin to increase the fractional disappearance rate of glucose; and the acute insulin response ($AIR_{Glucose}$) relative to glucose administration (Bergman, 1997).

This MINMOD computer model estimation of S_I has been validated for use in humans, rats, pigs, dogs, and cats (Finegood, 1997). Although this model has been

utilized to determine S_I in ruminants (Bunting et al., 2000; DePew et al., 1998; Majorie et al., 1997; Stanley et al., 2002; Williams et al., 2004), it has not been validated for use in these species. When applying the MINMOD to new species, it is necessary to validate the accuracy of the parameter estimates with a model-independent measure (Finegood, 1997). There have been numerous studies in which the S_I calculated from the MINMOD and EC were correlated including comparisons in humans with normal insulin sensitivity (Beard, et al., 1986; Bergman et al., 1987; Saad et al., 1994) and with non-insulin dependent diabetes mellitus (Coates et al., 1995), in dogs (Finegood et al., 1984) and cats (Petrus et al., 1998) with normal insulin sensitivity.

There have also been some studies in which the MINMOD and EC were not strongly correlated in humans with normal insulin sensitivity (Donner et al., 1985) and in humans with non-insulin dependent diabetes mellitus (Saad et al., 1994). In a previous study Saad et al. (1994) reported that the S_I EC and S_I MINMOD had a strong relationship among humans with a broad range of S_I . However, when the subjects were separated by degrees of S_I into groups of normal glucose tolerance, impaired glucose tolerance, and non-insulin dependent diabetes mellitus, the relationship diminished among the less insulin sensitive subjects. Saad et al. (1994) recommended that even in cases in which the S_I MINMOD did not correlate as well with the S_I EC, the S_I MINMOD was a good index of S_I although the actual measurement of S_I may not have reflected the same values as the S_I EC. The parameter S_G derived from the MINMOD can be compared to the GDR from the HC, but there have been few studies in which this comparison has been made (Finegood, 1997).

Transition Cow Metabolism

Partitioning of Nutrients during the Transition Period. The transition period, which is 3 weeks before and after calving, is a critical period to the health, production, and profitability of the dairy cow (Drackley, 1999). Two types of physiological regulation are instrumental to metabolic coordination during the transition period: homeostasis is the maintenance of physiological equilibrium, and homeorhesis is the orchestrated change in metabolism necessary to support a physiological state. The nutrient demands for pregnant animals at the end of pregnancy are 75% greater than those of non-pregnant animals of a similar body weight. The developing fetus, fetal membranes, gravid uterus, and mammary gland contribute to the increased nutrient demand (Bauman and Currie, 1980).

The majority of the glucose and nitrogen supply for the ruminant fetus is met through placental uptake of glucose and amino acids from maternal circulation (Bell, 1995). Very few short- or long-chain fatty acids or ketones are transported across the placenta. The contribution of acetate to the fetal respiratory fuel is estimated to be 10-15% (Comline and Silver, 1976). The metabolic adaptations in late pregnancy include increased hepatic gluconeogenesis, reduced glucose utilization by the peripheral tissues, increased NEFA mobilization from adipose tissue, and increased peripheral tissue utilization of NEFA and 3-hydroxybutyrate. Muscle proteolysis may increase while there may be decreased protein catabolism in the liver. The metabolic effects of these events increase glucose and amino acid availability to the fetus while the maternal tissues become more reliant on NEFA and ketones (Bell, 1995).

The metabolic adaptations that are necessary to accommodate the changes in nutrient partitioning associated with lactogenesis are mediated by endocrine regulation. Hormone concentrations change to coordinate the metabolic homeostasis and homeorhesis associated with late parturition and the onset of lactation (Bauman and Currie, 1980). Energy reserves are mobilized in response to an increased nutrient demand to support lactation until the cow can consume enough feed to provide needed nutrients. During the transition period there is a balance between the partitioning of nutrients for sustaining metabolic homeostasis and redirecting nutrients to meet the metabolic demands of lactogenesis in homeorhesis (Bauman and Currie, 1980). Three features of homeorhesis during this period have been described by Bell (1995): mobilization of energy reserves, endocrine changes that promote and sustain lactogenesis, and altered sensitivity to insulin.

Many metabolic changes occur to support the demands of lactation, and this complex process of lactation involves not only the mammary gland but many other tissues (Collier et al., 1984). The major metabolic changes that take place to supply the mammary gland with the needed energy, glucose, amino acids, and calcium include increased blood flow to the mammary gland and increased metabolic activity of the mammary gland with the concordant decrease in peripheral tissue utilization (Collier et al., 1984). In the mammary gland there is an increased amount of nutrient usage and uptake to provide substrates for a large amount of milk synthesis (Invartsen and Andersen, 2000). Lipid metabolism in adipose tissue is altered to support this physiological demand resulting in an increase in lipolysis and diminished lipogenesis. Tissue utilization of glucose decreases while there is an increase in use of lipid for energy

(Invartsen and Andersen, 2000). Liver gluconeogenesis and ketogenesis increase. Protein mobilization increases while protein synthesis decreases in muscle and other body tissues to provide substrates for gluconeogenesis. Mineral absorption increases in the gastrointestinal tract while mobilization is increased in the bones. Feed intake increases but not rapidly enough to supply the needed nutrients. There is an increased capacity for nutrient absorption in the digestive tract during early lactation to maximize available nutrient sources (Invartsen and Andersen, 2000).

Decreased insulin concentrations shift glucose availability to tissues such as the mammary gland that do not rely on insulin to facilitate the movement of glucose into the cell and away from the muscle and adipose tissue which respond to insulin facilitated glucose uptake (Collier, 1984). Decreased insulin concentrations also promote the release of NEFA by the adipose tissue (McGuire et al., 1995). During early lactation, glucagon concentrations increase relative to the dry period to stimulate lipolysis and gluconeogenesis to provide the body with needed energy (de Boer et al., 1985). The lower insulin concentrations and higher glucagon concentrations may represent the predominance of gluconeogenesis and glycogenolysis to supply the needed energy to meet nutritional demands (de Boer et al., 1985).

Glucocorticoids are an important component of many physiological functions including stress, metabolism, and immunity (Lefcourt, 1993). Cortisol, the major glucocorticoid in cattle, usually peaks the day of calving (Tucker, 2000). Diminished thyroxine concentrations are associated with decreased metabolic activity of peripheral tissue to allow preferential utilization of substrates by the mammary gland (Collier et al., 1984).

The metabolic adaptations associated with the transition period include increased gluconeogenesis with decreased peripheral tissue utilization of glucose, and increased mobilization of NEFA with increased peripheral tissue utilization of this metabolite (Bell, 1995). Insulin resistance develops in many species during late pregnancy and continues postpartum. This diminished sensitivity with concurrent decreased insulin concentrations alters the body's utilization of glucose by making glucose less available to insulin-responsive cells. The diminished sensitivity also decreases insulin control of lipolysis and NEFA mobilization (Bell, 1995).

A New Frontier. Niswender and Schwartz (2003) reported that many neuropeptides and neurotransmitters modulate feeding behavior by carrying appetite regulatory signals to and within the central nervous system. Some of these factors are released and express their effect in response to a particular nutrient. Other factors are released in response to hormones; furthermore, hormones may stimulate and inhibit the feeding center as well as interact with other substances. Insulin was the first acknowledged signal in the regulation of obesity. It has been demonstrated that intracerebroventricular infusions of insulin decrease food intake and reduce body weight. It is known that serum insulin can enter the cerebrospinal fluid in rodents, humans, and dogs. There are binding sites for insulin in the hypothalamus. Furthermore, insulin receptors have been identified in areas of the brain that control food intake. Hyperphagia is associated with lack of insulin as proven by insulin deficient rodents (Niswender and Schwartz, 2003). It has been demonstrated that insulin and leptin are adiposity signals (Schwartz et al., 2000). Administration of leptin or insulin into the brain causes reduced food intake, and lack of either increases intake. Both hormones circulate in proportion to

the body's fat content and enter the central nervous system in proportion to the circulating blood concentrations (Schwartz et al., 2000).

In humans and rodents that are in positive energy balance, insulin and leptin activate neurons that express proopiomelanocortin and secrete α -melanocyte stimulating hormone, an anorexic neuropeptide (Niswender and Schwartz, 2003). The activation of melanocortin receptors leads to a decrease in food intake. Increased leptin and insulin concentrations also decrease food intake by decreasing neuropeptide Y and agouti related proteins. Conversely, decreased levels of insulin and leptin stimulate neurons that express neuropeptide Y and agouti related proteins which stimulate food intake. Diminished insulin and leptin concentrations also inhibit proopiomelanocortin and diminish α -melanocyte stimulating hormone expression and release, which leads to inactivation of anorexic pathways. (Niswender and Schwartz, 2003).

Leptin is secreted by white adipose tissue in ruminants, and its secretion is decreased when there is an insufficient supply of nutrients (Ahima and Flier, 2000). Increased levels of leptin are associated with decreased feed intake. Low concentrations of leptin increase appetite and prepare the animal to conserve energy (Ahima, 1996). Leptin has been shown to elicit effects in the neurons of the arcuate, ventromedial, and dorsomedial hypothalamus. Following parturition and early lactation when cows are in negative energy balance, plasma leptin levels are low (Block et al., 2001). The decrease in plasma leptin concentrations occur before body fat stores are diminished. The decrease coincides with the onset of negative energy balance which is associated with reduction of plasma insulin concentrations and an increase in growth hormone concentrations. Recent research in lactating dairy cows demonstrates that plasma insulin

has been shown to increase leptin when cows are in positive energy balance (Block et al., 2003). These researchers suggest that reductions in plasma insulin during periods of negative energy balance could be responsible for mediating decreased plasma leptin concentrations. Although the roles of leptin and insulin have been investigated in other species (humans and rodents), little research has been conducted to investigate the role of these hormones in modulating energy intake in lactating dairy cattle (Ingvarlsen, and Andersen, 2000). In humans, reduced leptin and insulin action increase appetite when calories are limited (Schwartz et al., 1992). In early lactation, when dairy cattle experience negative energy balance, it seems reasonable to hypothesize that the decreased secretion of these hormones is a physiological mechanism to regulate energy balance by increasing appetite (Block et al., 2003). Insulin is known to have an important role in nutrient partitioning during the transition period. Research has focused on the roles of insulin and leptin in obesity in humans (Block et al., 2003). Insulin resistance begins before parturition in dairy cows and continues into early lactation (Debras et al., 1989; Prior and Christenson, 1978; Sano et al., 1991). During this transition from gestation to lactation, dairy cows often experience metabolic disorders, including hepatic lipidosis and ketosis. Dietary energy intake is inadequate to meet the energy demands for maintenance and milk production (Collier et al., 1984). As a result of this negative energy balance, catabolic activities are high during this early lactation period. Lactogenesis is accompanied by increased lipolysis and decreased lipogenesis in adipose tissue, increased glycogenolysis and gluconeogenesis in the liver, and increased mobilization of protein reserves from muscle tissue (Collier et al., 1984). This pattern of metabolic activity begins prior to parturition. Therefore, the insulin resistance observed

during the periparturient period may be a factor in the initiation of catabolic activities (Holstenius, 1993).

Diseases of the Transition Period. The occurrence of health problems during the transition period results in not only the acute loss of production, but also a potential for diminished production throughout the entire lactation and decreased reproductive success (Drackley, 1999). The sudden increase in nutrient demand with the lag in dry matter intake is the principle challenge that faces the early lactation dairy cow (Drackley, 1999). A high producing dairy cow experiences metabolic changes after calving. The ability to mobilize body reserves provides the dairy cow with the needed energy until dry matter intake reaches maximum and homeostasis is achieved. Milk fever and ketosis are the most common metabolic complications during this period (Schultz et al., 1993). Other common disorders that occur during this time include displaced abomasum, retained placenta, fat cow syndrome, udder edema, laminitis, and lactic acidosis (Shearer and Horn, 1992). In many cases transition cows suffer from more than one of these diseases and the complications of one become additive to other syndromes. Approximately one-third of the incidence of ketosis is secondary to other diseases such as milk fever, displaced abomasum, or retained placentas (Schultz et al., 1993).

The rapid changes that occur during the transition period are not well understood. The time period has been difficult to study because of the large amount of variation in milk yield, dry matter intake, and response to treatments among cows during the period. With the large degree of variation, the number of cows needed to assess nutritional and management strategies is large (Drackley, 1999).

Transition cow management has a great effect on the subsequent lactation. Accurate prediction of herd health disorders that commonly occur during this period of the lactation, such as ketosis, may play a role in reducing disease prevalence (de Boer et al., 1985). Ketosis is a metabolic disorder which affects approximately 4% of all dairy cows in the United States (de Boer et al., 1985). Ketosis is characterized by elevated blood, milk and urine ketone bodies, low blood glucose, anorexia, decreased milk production, and increased NEFA concentrations. The stress of milk synthesis during early lactation on the metabolic capacity of the cow may lead to primary ketosis in the high-producing dairy. The etiology of the disease is not thoroughly understood, but theories include 1) carbohydrate insufficiency caused by secretion of lactose in milk and 2) excess lipid mobilization caused by the demand for energy for milk synthesis (de Boer et al., 1985). The metabolic drain of early lactation is a big factor in causing metabolic ketosis.

Many high-producing cows experience this metabolic effect during early lactation, and only subclinical ketosis develops if the herd is well-managed (Aiello, 1998). Clinical ketosis develops when all of the ketone bodies are not utilized as an energy source. This results in further lowered blood glucose, increased ketone production, and increased blood NEFA concentration. The signs of clinical ketosis are weight loss, diminished appetite, decrease in milk yield, and dry feces. Cows that exhibit signs of clinical ketosis may also display nervous signs (Aiello, 1998).

During early lactation, cows must use body fat as an energy source as they are unable to meet the energy demands of milk synthesis from dietary intake. In early lactation there is a deficient supply of carbohydrate for the formation of adequate quantities of oxaloacetate (Goff and Horst, 1997). Ketosis occurs when there is a

depletion of carbohydrate sources accompanied by a mobilization of fatty acids (Mayes, 2000). The liver has a limited ability to oxidize fatty acids or to export them as very low-density lipoproteins (VLDL). Triglycerides accumulate in the hepatocytes, and acetyl-coenzyme A is not incorporated into the tricarboxylic acid cycle (TCA). Gluconeogenesis occurs at a reduced rate and hypoglycemia occurs. The acetyl-coenzyme A that is not used in the TCA cycle is converted to acetoacetate, β -hydroxybutyrate, and acetate. These ketone bodies appear in the blood, urine, and milk. The etiology of the deficiency of the liver to completely oxidize fatty acids is not completely understood. Current theories include the following: lack of oxaloacetate to maintain the TCA cycle, the lack of carnitine to transport and oxidize acetyl-coenzyme A, insufficient supply of B vitamins, and endocrine factors (Goff and Horst, 1997). Blood insulin concentrations are lower and glucagon concentrations are higher in ketotic cows than in healthy cows (Sakai et al., 1996). The lower ratio of insulin to glucagon inhibits acetyl-CoA carboxylase in the liver. When insulin concentrations are lower than glucagon concentrations, lipase activity is increased and lipolysis is increased. The blood NEFA concentrations are affected as a result of the altered ratio of the hormones (Mayes, 2000).

Therapy for Treating Ketosis. Intravenous administration of glucose solutions is the most rapid means of temporarily increasing blood glucose concentrations (Schultz et al., 1993). The disadvantages of this therapy are that some glucose may surpass the renal threshold and some may be lost in the urine. Also giving glucose intravenously only restores glucose concentrations for 2 hours (Schultz et al., 1993). This therapy is effective in immediately increasing blood glucose concentrations (Schultz et al., 1993).

Hormonal therapy may be used to treat ketosis. Glucocorticoids (10-30 mg dexamethasone) or adrenocorticotropin hormone (ACTH) are more effective in treating ketosis as their effects last for days (Bobe et al., 2004). Glucocorticoids stimulate the mobilization of amino acids from muscle providing the fuel for gluconeogenesis. ACTH is given to stimulate the release of glucocorticoids from the adrenal cortex, but this treatment is rarely used. Glucagon can also be administered to stimulate gluconeogenesis as a treatment for ketosis. There are disadvantages to the use of these treatments. Hormones may need to be injected multiple times for the complete recovery of ketosis. The over-use of steroids can have negative effects on the immune system. Glucocorticoids and glucagon may cause an increase in NEFA concentration and could potentially aggravate ketosis (Bobe et al., 2004) although the increased rate of gluconeogenesis and the increased availability of gluconeogenic precursors may negate this problem. Glucagon, which is not approved for use in lactating dairy cows, is more liver specific, causing an increase in gluconeogenesis and glycogenolysis, and is less lipolytic. Hormonal therapy is more effective when used in conjunction with other treatments such as providing gluconeogenic precursors (Bobe et al., 2004).

Oral glucose precursors such as sodium propionate (100 g/day), glycerol (1kg/day) and propylene glycol (1L/day) are used to alleviate ketosis (Bobe et al., 2004). Currently propylene glycol is considered to be the most effective of the oral glucose precursors in treating ketosis. Combinations of these oral glucose precursors are also effective in treating ketosis (Bobe et al., 2004). Glycerol increases glucose concentrations for an extended period of time but is slower at initially increasing blood glucose. Sodium propionate increases blood glucose concentrations more rapidly than

glycerol or propylene glycol. Calcium or magnesium propionate is also used as effective treatment for ketosis but is slower in increasing blood glucose concentrations. The use of oral glucose precursors is not without disadvantages (Bobe et al., 2004). They may decrease VFA concentration in the rumen, alter electrolyte balance, and rumen pH. Furthermore, these substances may be toxic when given in excess. The use of oral glucose precursors may be used in conjunction with the other treatments to supply the needed gluconeogenic precursors (Bobe et al., 2004).

Prevention and Supplementation to Reduce Incidence of Ketosis.

Management strategies to prevent ketosis include maximizing dry matter intake after calving and providing gluconeogenic precursors to minimize body tissue mobilization (Hutjens, 2003). Cows should not be over-conditioned at calving. Strategic feeding management during the dry period and transition period is critical to avoiding ketosis. Cows may be separated into a far-off dry cow herd (beginning of dry period to 3 weeks prior to calving), close-up dry herd (3 weeks prior to calving) and a fresh cow herd (2-3 weeks after calving) (Hutjens, 2003).

During the far-off dry period the body condition score can be adjusted. Body condition score should increase only one-half score during the dry period. Fat cows should not lose weight during this time; it is best to prevent them from becoming over-conditioned at the end of lactation. Cows should end lactation and be at a body condition score of 3.5-4.0 based on a scale of 1-5 (Hutjens, 2003). If they are dried off and maintained at this body condition, then energy intake during this phase can be limited to minimize the incidence of transition cow disorders (displaced abomasums, off-feed problems, milk fever, ketosis, retained placenta, udder edema). Feeding goals for this

period include: maintaining optimal fiber, limiting energy intake, avoiding overfeeding of crude protein, and preventing vitamin and mineral deficiencies (Hutjens, 2003).

During the close-up dry period cows should be fed a transition diet to adjust to the lactation diet and prepare the rumen papillae for intense feeding (Hutjens, 2003). During this time the grain concentration of the diet may be increased to stimulate rumen papillae elongation and increase papillae surface area. Dry matter intake may decrease by 15-30% as compared to the previous phase. The fetal calf will be developing and at this time nutritional problems in the cow may begin (Hutjens, 2003).

During the fresh cow period, management of individual cows is critical. The herdsman should watch for abnormalities in dry matter intake, temperature, rumen movements, and uterine discharge. Monitoring milk or urine ketone concentrations may also help prevent clinical ketosis. Adequate hay should be fed to provide for rumen fill to prevent displaced abomasums; however, intake may be lower during this time so the nutrient density of the ration may be increased. The energy needed for milk production will be coming from body stores (Hutjens, 2003).

Many of the treatments for ketosis have been used as supplements to improve performance and avoid clinical ketosis during early lactation (Bobe et al., 2004). Dietary supplementation with monensin and gluconeogenic precursors such as glycerol, propionate, and propylene glycol are possible preventatives for ketosis. The use of top-dressed supplements may be more cost effective than using supplements as oral drenches (Bobe et al., 2004). The use of these products must be applicable to dairy farms. Propylene glycol (1L/day) for the last 20 days of the close-up dry period has been shown to increase plasma glucose and insulin concentrations while lowering NEFA and β -

hydroxybutyrate concentrations (Bobe et al., 2004). This chemical has been more effective in reducing incidence of ketosis when applied in higher doses and as a drench, a problem for practical farm use. Furthermore, higher doses of propylene glycol are toxic to some of the rumen bacteria. Feeding monensin has been shown to prevent ketosis in over-conditioned cows (Duffeld, 2000). By shifting the bacteria population to that which favors propionate production, monensin changes the VFA profile in favor of propionate and increases the glucose supply to the cow (Bobe et al., 2004).

Propionate supplementation (such as calcium propionate) may aid in preventing ketosis by supplying needed substrates for gluconeogenesis (Overton and Waldron, 2004). There have been mixed responses to the use of calcium propionate as a preventative for ketosis. The amount of propionate added to the diet through supplementation may not be adequate to prevent ketosis as this amount may be relatively small when compared to the amount supplied by rumen fermentation (Overton and Waldron, 2004). Goff et al. (1996) also reported that calcium propionate applied in tube form to Jersey cows at calving and 12 h after calving tended to reduce NEFA and β -hydroxybutyrate concentrations. It may have supplied some glucose and may have aided in preventing fatty liver and ketosis. Goff et al. (1996) reported that the supplementation did not affect NEFA or β -hydroxybutyrate concentrations in Holstein dairy cows. Stokes and Goff (2001) investigated the effects of calcium propionate administered to Holstein cows as a drench after calving and 24 h later to supply 0.68 kg of calcium propionate. They reported that this supplementation did not affect blood glucose, NEFA, or β -hydroxybutyrate concentrations. Calcium propionate (NutroCal™, Kemin AgriFoods, Inc., Des Moines IA) can work to alleviate ketosis, displaced abomasums, and parturient

parensis by providing an energy source as propionate. Calcium propionate may help prevent ketosis by correcting the balance of NEFA/VLDL and providing a gluconeogenic precursor as propionate. As the propionate helps to correct the negative energy balance, the NEFA/VLDL ratio will favor the metabolism of fatty acids in the liver. Propionate is metabolized to oxaloacetate in the liver and can be used to provide glucose for the animal through gluconeogenesis or condensed with the acetyl-CoA from the metabolism of fatty acids and provide energy equivalents (Mayes, 2000). Ketosis may be reduced because the fatty acids may be better utilized, ketone body production may be reduced, and the cow is provided with an energy supplement (Bobe et al., 2004). Furthermore, providing a gluconeogenic precursor will have a protein sparing effect by alleviating the need to use body protein as a source of gluconeogenic amino acids (Overton and Waldron, 2004).

During early lactation, dairy cows struggle to meet energy and calcium demands. Hypocalcemia may be a major factor in causing other conditions during the transition period (Stokes and Goff, 2001). Rumen and abomasal motilities are reduced in incidences of induced hypocalcemia due to the role of calcium in smooth muscle contractions (Daniel, 1983). Additionally, calcium plays a critical role in insulin secretion. Following the depolarization of the β -cells of the endocrine pancreas, an influx of calcium through the voltage-sensitive calcium channels causes a release of insulin (Mayes, 2000). Hypocalcemia is often associated with reduced feed intake. This nutrient deprivation can lead to a further negative energy balance which may be followed by ketosis (Stokes and Goff, 2001).

CHAPTER 3

EFFECTS OF PREPARTUM DIETARY ENERGY LEVEL AND CALCIUM PROPIONATE SUPPLEMENTATION ON ENERGY METABOLISM IN DAIRY CATTLE

Introduction

Transition cow management has a great effect on the subsequent lactation. Accurate prediction of herd health disorders that commonly occur during this period of the lactation, such as ketosis, may play a role in reducing disease prevalence. Ketosis is a metabolic disorder which affects approximately 4% of all dairy cows in the United States (de Boer et al., 1985). It is characterized by increased concentrations of blood ketones, decreased concentrations of blood glucose, anorexia, and decreased milk production. The stress of milk synthesis during early lactation on the metabolic capacity of the cow may lead to ketosis in the high-producing dairy cow. The etiology of the disease is not thoroughly understood, but theories include 1) carbohydrate insufficiency caused by secretion of lactose in milk and 2) excess lipid mobilization caused by the demand for energy for milk synthesis (de Boer et al., 1985). During the transition period metabolizable energy requirements greatly increase. As a result the liver must adapt to provide the increased glucose needed for milk production and to process the high quantity of nonesterified fatty acids (NEFA) taken up from extensive mobilization of adipose triglycerides (Drackley et al., 2001). Many substances can be added as supplements to dry cow rations as preventative measures for ketosis. Ca-propionate (CAP) may alleviate metabolic disorders associated with the transition period by providing propionate, an energy source that can be used as a gluconeogenic precursor. Therefore, the objectives of this study were to evaluate the effects of CAP (NutroCal™, Kemin AgriFoods, Inc., Des Moines, IA) to aid in the prevention of ketosis and evaluate

the effects of prepartum dietary energy level on ketosis and glucose metabolism in lactating Holstein cows.

Materials and Methods

Animals and Dietary Treatments. Forty-one Holstein cows were grouped by anticipated parturition date and assigned to one of four treatments that were arranged as a 2 x 2 factorial based on 105 and 145% (NRC, 2001) of prepartum dietary energy requirements with or without addition of CAP (113.5 g/d). The cows were housed at the Dairy Science Research and Teaching Farm in Baton Rouge, Louisiana. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the LSU Agricultural Center. Cows were fed treatment diets from 21 d prior to their anticipated parturition date until parturition. After parturition, all cows were fed a lactation ration with continued supplementation as previously assigned. Individual feed intakes were measured daily using electronic feeding gates (American Calan, Inc., Northwood, NH). Diets and refusals were sampled daily.

Sample Collection and Frequently Sampled Intravenous Glucose Tolerance Tests. Blood samples were collected by venipuncture into 7-mL evacuated tubes containing potassium oxalate and sodium fluoride (Kendall Medical, St. Louis, MO) from a coccygeal vessel during wk -3,-2,-1, 1, 2, and 3 relative to calving. The tubes were centrifuged and plasma was separated and frozen (-20° C) until analyzed for plasma glucose, NEFA, insulin, and thyroxine concentrations.

At wk -1 and +1, frequently sampled intravenous glucose tolerance tests (FSIGT) with minimal model analysis were performed. On the morning of the FSIGT, a catheter was inserted into the jugular vein of the cows (Baxter Healthcare Corporation,

Deerfield, IL). The test consisted of infusing glucose (500 mg/kg BW, 50% w/v in sterile saline) through the jugular catheter followed 19 min later by infusing bovine insulin (0.03 U/kg BW). Blood samples were collected through the catheter at -10, 0, 2, 3, 4, 5, 6, 8, 10, 12, 19, 22, 25, 27, 30, 40, 50, 60, 70, 80, 90, 120, 150, and 180 min relative to glucose administration and placed into 7-mL evacuated tubes containing potassium oxalate and sodium fluoride (Kendall Medical, St. Louis, MO). The tubes were centrifuged and plasma was separated and frozen (-20° C) until analyzed for glucose and insulin concentrations. These values were entered into the minimal model computer program to assess glucose effectiveness (S_G), the fractional glucose disappearance at basal insulinemia; insulin sensitivity (S_I) which is the effect of incremental change in plasma insulin to increase the fractional disappearance rate of glucose; and the acute insulin response ($AIR_{Glucose}$) relative to glucose administration (Bergman, 1997).

Additional blood samples were collected through the jugular catheter at wk -1 and +1 for the measurement of plasma basal cortisol and glucagon concentrations. Samples that were analyzed for cortisol concentrations were placed into 7-mL evacuated tubes containing potassium oxalate and sodium fluoride (Kendall Medical, St. Louis, MO). Samples that were analyzed for glucagon concentrations were placed into 7-mL evacuated tubes containing sodium salts of EDTA (Kendall Medical, St. Louis, MO) and aprotinin was added to deliver 5 trypsin inhibiting units (Sigma Chemical, St. Louis MO). The tubes were centrifuged and plasma was separated and frozen (-20° C) until analyzed for plasma glucagon and cortisol.

Laboratory Methods. Using commercial spectrophotometric kits, plasma was analyzed for glucose (Sigma Tech, Bull. No. 315; Sigma Chemical, St. Louis, MO) and

NEFA concentrations (NEFA-C Kit, ACS-ACOD Method; Wako Chemicals, USA, Richmond, VA) as modified by Drackley et al. (1991). Using commercial radioimmunoassay kits plasma was analyzed for insulin (Coat-A-Count; Diagnostic Products Corporation, Los Angeles, CA), glucagon (Glucagon Kit, ICN Pharmaceuticals, Inc., Orangeburg, New York), cortisol (Cortisol Kit; ICN Pharmaceuticals, Inc., Orangeburg, New York) and thyroxine concentrations (T₄ Mab, ICN Pharmaceuticals, Inc., Orangeburg, New York). The intra-assay CV for insulin, glucagon, thyroxine, and cortisol assays were 4.95, 8.46, 5.73, 6.46 %, respectively. Inter-assay CV for insulin, glucagon, and thyroxine assays were 3.16, 5.4, and 2.7 %, respectively.

Statistical Methods and Calculations. The FSIGT, hormone, and metabolic data were analyzed by ANOVA using the mixed model (Littell et al., 1988). The model included fixed effects of diet, CAP supplementation, week, and the interactions of diet, CAP supplementation, and week. Cow nested within diet and supplementation was included as the random variable and used as the error term for diet, supplementation, and the interaction of diet by supplementation. Week was modeled as the repeated term that was assumed to be correlated within cow with a constant covariance structure. All calculations were completed using SAS (1990).

Results and Discussion

Ca-Propionate and Dietary Energy. The plasma glucose, NEFA, insulin, cortisol, and glucagon concentrations ($P > 0.05$) were not affected by dietary energy level or CAP supplementation. Least squares means for these metabolites and hormones are presented in Table 3.1. There were diet by week by CAP interactions for thyroxine ($P < 0.05$) concentrations as shown in Figures 3.1 and 3.2, respectively. Although there were

diet by week by CAP interactions for thyroxine concentrations, the biological significance of these appears to be of minor importance. The S_I , S_G , and $AIR_{Glucose}$ ($P > 0.05$) were not affected by CAP supplement or dietary energy. The least squares means for these indices derived from the FSIGT are shown in Table 3.2.

During the transition period the primary homeorhetic adaptation of glucose metabolism is increased gluconeogenesis and reduced utilization of glucose by peripheral tissue. The demand for glucose, amino acids, and fatty acids is dramatically increased at the end of gestation and in early lactation. Additionally the demand for calcium increases at the beginning of lactation (Overton and Waldron, 2004). The major gluconeogenic substrates in ruminants include propionate, lactate, amino acids, and glycerol. During the transition period the estimated contribution of propionate to the glucose pool is 50-60% lactate 15-20 %, and glycerol 2-4% (Reynolds et al., 2003). The contribution to gluconeogenic precursors from amino acids may be 20-30% as calculated by difference (Overton and Waldron, 2004). The supplementation with gluconeogenic precursors is used to prevent ketosis. The use of propionate as a top-dressed supplement may be more cost effective than using supplements as oral drenches (Bobe et al., 2004). There have been mixed responses to the use of CAP as a preventative for ketosis. The amount of propionate added to the diet through supplementation may not be adequate to prevent ketosis as this amount may be relatively small when compared to the amount supplied by rumen fermentation (Overton and Waldron, 2004). Goff et al. (1996) reported that CAP when applied in tube form to Jersey cows at calving and 12 h after calving tended to reduce NEFA and β -hydroxybutyrate concentrations. It may have supplied some glucose and may have aided in preventing fatty liver and ketosis. Goff et al. (1996)

Table 3.1. Least squares means for plasma metabolite and hormone data from cows fed a normal or high energy diet with (+) or without (-) Ca-propionate (CAP).

| | Normal energy diet | | High energy diet | | SEM |
|-------------------------|--------------------|--------|------------------|--------|-------|
| | +CAP | -CAP | +CAP | -CAP | |
| NEFA (mEq/L) | 0.60 | 0.46 | 0.50 | 0.57 | 0.07 |
| Glucose (mg/dL) | 54.30 | 53.74 | 54.78 | 55.23 | 1.65 |
| Insulin (μ U/mL) | 3.38 | 2.70 | 2.96 | 3.14 | 0.41 |
| Thyroxine (μ g/dL) | 2.18 | 2.01 | 2.05 | 2.05 | 0.30 |
| Glucagon (pg/mL) | 728.68 | 797.88 | 680.02 | 629.50 | 98.42 |
| Cortisol (μ g/dL) | 0.71 | 0.87 | 0.64 | 0.58 | 0.15 |

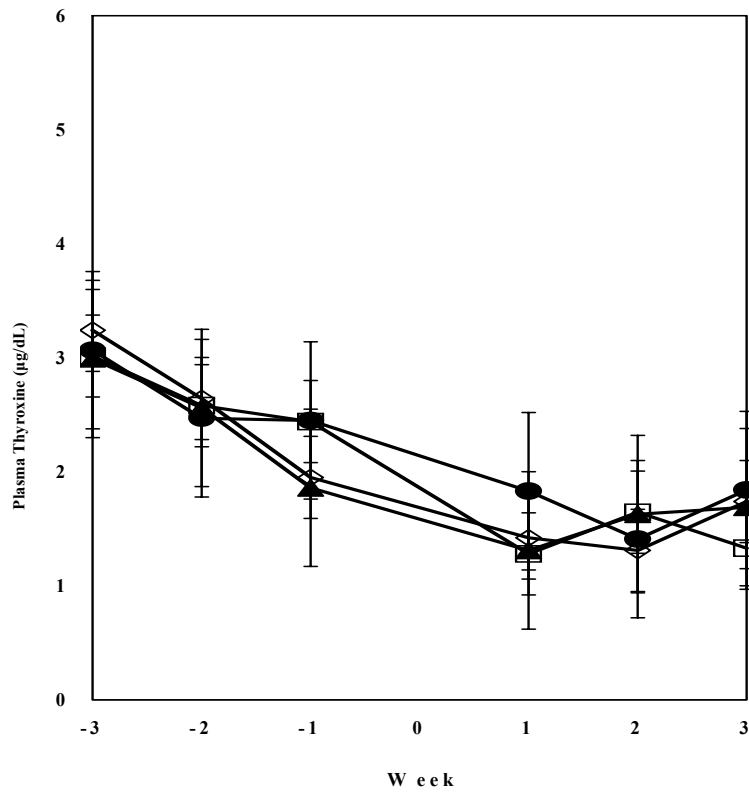


Figure 3.1. Least squares means for thyroxine concentrations for cows fed a high energy diet with (□) and without (◇)Ca-propionate or fed a normal energy diet with (●) and without (▲)Ca-propionate. Interaction of week, diet, and Ca-propionate ($P < 0.05$).

Table 3.2. Least squares means for insulin sensitivity (S_I), glucose effectiveness (S_G), and acute insulin response (AIR_{glucose}) from cows fed a normal or high energy diet with (+) or without (-) Ca-propionate (CAP).

| | Normal energy diet | | High energy diet | | |
|--|--------------------|-------|------------------|-------|------|
| | +CAP | -CAP | +CAP | -CAP | SEM |
| S_I ($\times 10^{-4} \text{ min}^{-1} / \mu\text{U/mL}$) | 3.02 | 1.66 | 2.99 | 2.98 | 0.81 |
| S_G (%/min) | 0.02 | 0.03 | 0.02 | 0.02 | 0.01 |
| AIR_{glucose} ($\mu\text{U/mL}$) | 244.6 | 173.3 | 201.3 | 279.3 | 46.9 |

reported that the supplementation did not affect NEFA or β -hydroxybutyrate concentrations in Holstein dairy cows. Stokes and Goff (2001) investigated the effects of CAP administered to Holstein cows as a drench after calving and 24 h later to supply 0.68 kg of CAP. They reported that this supplementation did not affect blood glucose, NEFA, or β -hydroxybutyrate concentrations.

There was a high incidence of metabolic disorders and disease observed in these cows (Beem, 2003). More than 50% of the cows showed signs of at least one metabolic disorder or disease during the trial, but there was no evidence that treatments were related to the incidence of disease or disorder. The clinical health problems may have affected DMI throughout the trial, and therefore these data may not accurately reflect treatment effects on glucose metabolism.

Week Effects. There was a main effect of week for mean plasma insulin, glucagon, thyroxine, NEFA, and glucose concentrations ($P < 0.05$). These hormones changed to coordinate the metabolic homeostasis and homeorhesis associated with late parturition and the onset of lactation. Least squares means for weekly measurements of plasma NEFA, glucose, insulin, and thyroxine are shown in Table 3.3, and least squares means for plasma glucagon and cortisol are reported in Table 3.4. There was a main effect of week for S_I and $AIR_{Glucose}$ estimates ($P < 0.05$) as indicated in Table 3.5. There was not a main effect of week ($P > 0.05$) in the S_G estimate (Table 3.5).

During the transition period major differences in blood metabolites and hormones can be observed by comparing changes in these concentrations during the prepartum and postpartum periods. The metabolic adaptations necessary to accommodate the changes in nutrient partitioning associated with lactogenesis are mediated by endocrine regulation.

Energy reserves are mobilized in response to an increased nutrient demand to support lactation until the cow can consume enough feed to provide needed nutrients. During the transition period there is a balance between the partitioning of nutrients for sustaining metabolic homeostasis and redirecting nutrients to meet the metabolic demands of lactogenesis in homeorhesis (Bauman and Currie, 1980). Three features of homeorhesis during this period have been described by Bell (1995): mobilization of energy reserves, endocrine changes that promote and sustain lactogenesis, and altered sensitivity to insulin.

Hormones. Insulin concentrations decreased (Table 3.3) in response to the lower glucose concentrations. Decreased insulin concentrations shift glucose availability to tissues such as the mammary gland that do not rely on insulin to facilitate the movement of glucose into the cell and away from the muscle and adipose tissue which respond to insulin facilitated glucose uptake (Collier, 1984). Decreased insulin concentrations also promote the release of NEFA by the adipose tissue (McGuire et al., 1995). Glucagon concentrations increased (Table 3.4) after parturition; this hormone is critical to increasing the body's supply of glucose when energy supply is low relative to the body's demands. During early lactation, glucagon concentrations increased relative to the dry period to stimulate lipolysis and gluconeogenesis to provide the body with needed energy (de Boer et al., 1985). The lower insulin concentrations and higher glucagon concentrations may represent the predominance of gluconeogenesis and glycogenolysis to supply the needed energy to meet nutritional demands (de Boer et al., 1985). Glucocorticoids are an important component of many physiological functions including stress, metabolism, and immunity (Lefcourt, 1993). Cortisol, the major glucocorticoid in

Table 3.3. Least squares means for weekly plasma metabolite and hormone concentrations.

| | Prepartum | | | Postpartum | | | SEM |
|--------------------------------------|-----------|---------|---------|------------|--------|--------|------|
| | Week -3 | Week -2 | Week -1 | Week 1 | Week 2 | Week 3 | |
| NEFA (mEq/L) ^A | 0.40 | 0.31 | 0.45 | 0.74 | 0.70 | 0.59 | 0.05 |
| Glucose (mg/dL) ^A | 58.66 | 58.27 | 57.88 | 53.79 | 49.09 | 49.40 | 1.38 |
| Insulin (μ U/mL) ^A | 4.38 | 3.52 | 2.87 | 2.45 | 2.46 | 2.59 | 0.34 |
| Thyroxine (μ g/dL) ^A | 3.08 | 2.57 | 2.18 | 1.46 | 1.50 | 1.66 | 0.17 |

^A Main effect of week ($P < 0.05$).

Table 3.4. Least squares means for plasma hormone data in cows pre- and postpartum.

| | Pre-partum | Post-partum | SEM |
|-------------------------------|------------|-------------|-------|
| Glucagon (pg/mL) ^A | 609.31 | 808.72 | 67.28 |
| Cortisol (µg/dL) | 0.74 | 0.67 | 0.09 |

^A Main effect of week ($P < 0.05$).

Table 3.5. Least squares means for insulin sensitivity (S_I), glucose effectiveness (S_G), and acute insulin response (AIR_{glucose}) in cows pre- and postpartum.

| | Pre-partum | Post-partum | SEM |
|---|------------|-------------|------|
| S_I ($\times 10^{-4} \text{ min}^{-1} / \mu\text{U/mL}$) ^A | 1.88 | 3.45 | 0.51 |
| S_G (%/min) | 0.02 | 0.02 | 0.01 |
| AIR_{glucose} ($\mu\text{U/mL}$) ^A | 265.4 | 183.8 | 26.4 |

^A Main effect of week ($P < 0.05$).

cattle, usually peaks the day of calving (Tucker, 2000). The cortisol concentrations in the present study may have peaked at this point as well and possibly were declining when measured at 1 wk postpartum. However, cortisol concentrations (Table 3.4) were not significantly different from late gestation to early lactation. Thyroxine concentrations decreased at parturition (Table 3.3). Diminished thyroxine concentrations are associated with decreased metabolic activity of peripheral tissue to allow preferential utilization of substrates by the mammary gland (Collier et al., 1984).

Metabolites. Whole body glucose and NEFA turnover is greater during lactogenesis than during gestation (Reynolds, 2003). The mammary gland demand for glucose, amino acids, and fatty acids are approximately 2.7, 2.0, and 4.5 times that of the gravid uterus during late pregnancy. The energy demand of the mammary gland is about 3 times that of the uterus (Bell, 1995.) Glucose concentrations decreased (Table 3.3) with the progression of the transition period, as cows are in negative energy balance. During this time less glucose is available to the body, and large amounts of glucose are needed for lactation. Reduced insulin binding in adipose tissue during this time increases the availability of glucose to the mammary gland (Collier, 1984). Throughout the progression of the transition period the NEFA concentrations increased (Table 3.3) as the energy reserves were mobilized to meet energy needs. Enhanced NEFA mobilization is usually initiated at the end of pregnancy before the nutrient demand increases at the onset of lactation. The increased NEFA concentration is facilitated by diminished insulin sensitivity during the transition period, as there is a loss of response of insulin to stimulate lipogenesis and oppose lipolysis (Bell, 1995). The metabolic adaptations associated with the transition period include increased gluconeogenesis with decreased

peripheral tissue utilization of glucose, and increased mobilization of NEFA with increased peripheral tissue utilization of this metabolite (Bell, 1995).

FSIGT Parameters. Insulin resistance develops in many species during late pregnancy and continues postpartum (Bell, 1995). This diminished sensitivity with concurrent decreased insulin concentration alters the body's utilization of glucose by making insulin responsive cells less responsive to insulin-stimulated uptake. The diminished sensitivity also decreases insulin control of lipolysis and NEFA mobilization (Bell, 1995). In the present study S_I increased (Table 3.5) in cows from prepartum to postpartum, although indices at both times reflect low insulin sensitivity. Leury et al. (2003) reported that the glucose infused relative to hyperinsulinemia, a reflection of insulin sensitivity, increased during early lactation when compared to late pregnancy. The S_I data in the present study and the data from hyperinsulinemic euglycemic clamp study by Leury et al. (2003) reflect the amount of glucose taken up by insulin-responsive tissues and indicate that cows were more sensitive to insulin in early lactation than during late pregnancy.

The AIR_{glucose} decreased (Table 3.5) after calving, demonstrating that the amount of insulin secreted relative to an acute glucose challenge was diminished in early lactation relative to late pregnancy. There was no statistical difference in the S_G (Table 3.5) in cows during late pregnancy versus early lactation. This data indicated that the glucose uptake of non-insulin responsive tissues was similar during the pre- and postpartum periods. Further work should be performed to characterize the changes in S_I , S_G , and AIR_{glucose} throughout lactation.

Summary and Conclusions

The CAP supplementation or prepartum dietary energy level did not affect glucose metabolism in these transition dairy cows. Although there were diet by week by CAP interactions for thyroxine concentrations, the biological significance appears to be of minor importance. The hormone and metabolite concentrations changed to coordinate the metabolic homeostasis and homeorhesis associated with parturition and the onset of lactation. Clinical health problems not related to dietary treatments of the experimental herd pre- and postpartum may have affected DMI, and therefore these data may not accurately reflect treatment effects on glucose metabolism.

CHAPTER 4

CHARACTERIZATION OF INSULIN SENSITIVITY AND GLUCOSE METABOLISM IN HOLSTEIN DAIRY CATTLE AS NEONATAL CALVES AND AS LACTATING COWS DURING A LACTATION CYCLE: A PRELIMINARY STUDY

Introduction

Glucose is the principal source of energy for life processes of the mammalian cell. All cells require a constant supply of this indispensable nutrient, and only relatively small changes are tolerated without adverse effects on the health of the animal (Kaneko, 1997). Thus, the endocrine regulation of glucose metabolism is of utmost importance in mammalian homeostasis. Insulin, a key hormone in this endocrine regulation, facilitates the movement of glucose across cell membranes, thereby regulating the concentration of blood glucose. In nonruminants and pseudo-nonruminants such as young dairy calves, ingestion of feed and absorption of glucose stimulate the secretion of insulin, which decreases hepatic gluconeogenesis while promoting tissue uptake of glucose. As calves develop into fully functioning ruminants, hepatic gluconeogenesis increases and becomes the primary pathway of hepatic metabolism as the result of an increase in VFA production and absorption (Owens et al., 1986). It has been suggested that calves become insulin resistant as they transition from pre-ruminants to ruminants. (Hugi et al., 1997). It has also been observed that insulin resistance begins before parturition and continues into early lactation (Debras et al., 1989; Prior and Christenson, 1978; Sano et al., 1991). Glucose metabolism and insulin sensitivity have been studied during the transition period, but this area has not been intensely studied throughout lactation particularly not in primiparous cows. The objective of this study was to gather preliminary data to characterize insulin sensitivity and glucose metabolism in Holstein

heifers throughout a lactation cycle. The goal was to measure these metabolic parameters in a group of animals that were born and maintained at the Dairy Science Research and Teaching Farm in Baton Rouge, Louisiana. In previous studies the frequently sampled intravenous glucose tolerance tests (FSIGT) with minimal model analyses were performed at 3 and 6 weeks of life (Stanley et al., 2002) and at 1 week before and after calving (Chapter 3: Effects of Prepartum Dietary Energy Level and Calcium Propionate Supplementation on Energy Metabolism in Dairy Cattle). The original intention was to conduct these tests again at 100, 200, and 300 days in milk in the heifers that were in the original study, but only 3 of the heifers were in the milking herd at the end of their first lactation.

Materials and Methods

Complete data for an entire lactation cycle were only available for 3 of the original 18 calves. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the LSU Agricultural Center.

Sample Collection and Frequently Sampled Intravenous Glucose Tolerance Tests. The FSIGT with minimal model analyses were performed in 3 first calf Holstein heifers (658, 667, and 669) at 1 week before anticipated calving date, 1 week after calving, 100, 200, and 300 days in milk. The FSIGT with minimal model analyses had previously been performed in these animals at 3 and 6 weeks of life (Stanley et al., 2002). On the morning of the FSIGT, catheters were placed in the jugular vein of the cows (Baxter Healthcare Corporation, Deerfield, IL). The test consisted of infusing glucose (500 mg/kg BW, 50% w/v in sterile saline) through the jugular catheter followed 19 min later by infusing bovine insulin (0.03 U/kg BW). Blood samples were collected through

the catheter at -10, 0, 2, 3, 4, 5, 6, 8, 10, 12, 19, 22, 25, 27, 30, 40, 50, 60, 70, 80, 90, 120, 150, and 180 min relative to glucose administration and placed into 7-mL evacuated tubes containing potassium oxalate and sodium fluoride (Kendall Medical, St. Louis, MO). The tubes were centrifuged and plasma was separated and frozen (-20° C) until analyzed for glucose and insulin concentrations. These values were entered into the minimal model computer program to assess glucose effectiveness (S_G), the fractional glucose disappearance at basal insulinemia; insulin sensitivity (S_I) which is the effect of incremental change in plasma insulin to increase the fractional disappearance rate of glucose; and the acute insulin response ($AIR_{Glucose}$) relative to glucose administration (Bergman, 1997).

Laboratory Methods. Using commercial spectrophotometric kits, plasma was analyzed for glucose concentrations (Sigma Tech, Bull. No. 315; Sigma Chemical, St. Louis, MO). Plasma was analyzed for insulin concentrations via commercial radioimmunoassay kits (Coat-A-Count; Diagnostic Products Corporation, Los Angeles, CA). The intra-assay and inter-assay CV for insulin were 4.95 and 3.16%, respectively.

Milk Production Data. Milk production data were obtained from Dairy Herd Improvement Association (DHIA) records maintained at the Dairy Science Research and Teaching Farm. The herd is tested on a monthly basis using official DHIA uniform operation procedures.

Results and Discussion

The milk production data for the first test day and at the test dates closest to 100, 200, and 300 days in milk are reported in Figure 4.1. The days in milk at the test days varied between the animals by 15 days because the herd was only tested once per month.

At the first test day Cow 658 was 18 days in milk, Cow 667 was 33 days in milk, and Cow 669 was 30 days in milk. Generally, peak milk production occurs between 4-8 weeks postpartum, and peak dry matter intake lags behind, occurring at 10-14 weeks postpartum (NRC, 2001). In these growing primiparous cows, peak milk production is not expected to be as high as in mature animals. The management of the dairy farm greatly effected the production of the animals at this time. Interestingly, in two of the cows, milk production increased at the end of this measured period (300 days in milk) rather than declined. This corresponds to the time when better management practices were implemented at the dairy and is probably the result of better nutritional management. Also, these animals were not bred and were kept in the milking herd for a much longer period of time than a normal production cycle. The effect of a subsequent pregnancy, which would normally take place during lactation and affect nutrient partitioning, is therefore not represented in this data set.

A summary of the glucose metabolism and insulin sensitivity parameters and basal glucose and insulin concentrations measured at 3 weeks of life (Model 1), 6 weeks of life (Model 2), 1 week before anticipated calving date (Model 3), 1 week after calving (Model 4), 100 days in milk (Model 5), 200 days in milk (Model 6) and 300 days in milk (Model 7) is reported in Table 4.1. The measurement of S_I is presented in Figure 4.2. At 3 weeks of age (Model 1) these calves were receiving a large portion of their nutrients from milk replacer and were not yet fully functioning ruminants. The S_I was greater at this time point than at any other measured stage in their lives and declined at 6 weeks of age (Model 2) as calves were transitioning from a pre-ruminant to a ruminant state and

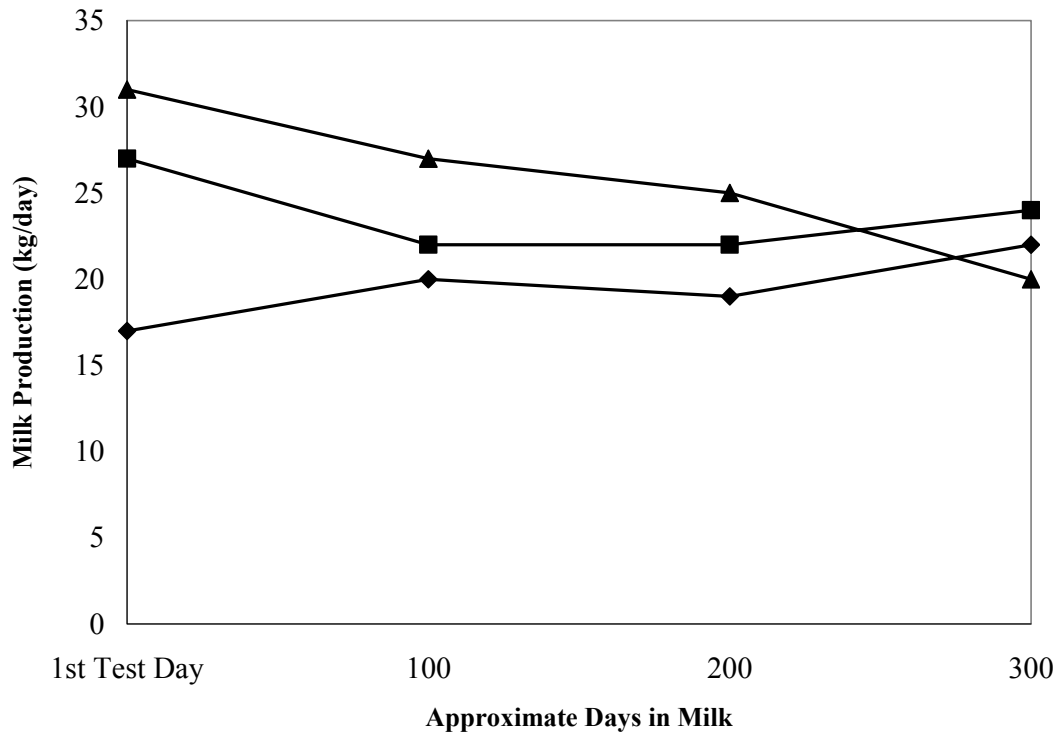


Figure 4.1. Milk production data at the 1st test day, 100, 200, and 300 days in milk. Kilograms of milk produced per day for Cow 658 is indicated by ▲, Cow 667 by ■, and Cow 669 by ◆.

Table 4.1. Insulin sensitivity (S_I), glucose effectiveness (S_G), acute insulin response (AIR_{glucose}), basal glucose and basal insulin concentrations in 3 cows measured at 3 weeks of life (Model 1), 6 weeks of life (Model 2), 1 week before anticipated calving date (Model 3), 1 week after calving (Model 4), 100 days in milk (Model 5), 200 days in milk (Model 6) and 300 days in milk (Model 7).

| Animal | Model | S_I ($\times 10^{-4} \text{min}^{-1} / \mu\text{U/mL}$) | S_G (%/min) | AIR_{glucose} ($\mu\text{U/mL}$) | Glucose (mg/dL) | Insulin ($\mu\text{U/mL}$) |
|--------|-------|--|------------------|--|--------------------|---------------------------------|
| 669 | 1 | 9.14 | 0.016 | 76.8 | 80.22 | 4.81 |
| | 2 | 12.15 | 0.015 | 113.2 | 64.59 | 6.19 |
| | 3 | 0.26 | 0.019 | 423.6 | 69.53 | 15.40 |
| | 4 | 0.91 | 0.016 | 471.8 | 65.44 | 4.58 |
| | 5 | 0.30 | 0.033 | 296.8 | 49.21 | 6.83 |
| | 6 | 2.77 | 0.029 | 359.2 | 57.76 | 3.49 |
| | 7 | 2.05 | 0.019 | 186.4 | 58.49 | 7.67 |
| 667 | 1 | 13.97 | 0.017 | 131.1 | 68.37 | 4.56 |
| | 2 | 10.71 | 0.019 | 145.4 | 66.48 | 5.59 |
| | 3 | 0.14 | 0.020 | 503.5 | 56.87 | 12.56 |
| | 4 | 1.57 | 0.027 | 740.5 | 62.67 | 3.59 |
| | 5 | 1.21 | 0.025 | 494.5 | 53.52 | 10.31 |
| | 6 | 4.24 | 0.031 | 207.0 | 57.71 | 4.27 |
| | 7 | 2.52 | 0.029 | 198.8 | 57.35 | 2.59 |
| 658 | 1 | 22.21 | 0.029 | 120.4 | 59.78 | 7.86 |
| | 2 | 8.97 | 0.028 | 154.6 | 75.98 | 8.13 |
| | 3 | 1.67 | 0.02 | 291.4 | 67.77 | 2.22 |
| | 4 | 2.45 | 0.024 | 363.1 | 61.65 | 6.58 |
| | 5 | 4.11 | 0.019 | 246.1 | 64.78 | 4.19 |
| | 6 | 2.58 | 0.028 | 197.4 | 52.66 | 4.71 |
| | 7 | 1.71 | 0.015 | 307.2 | 55.98 | 3.22 |

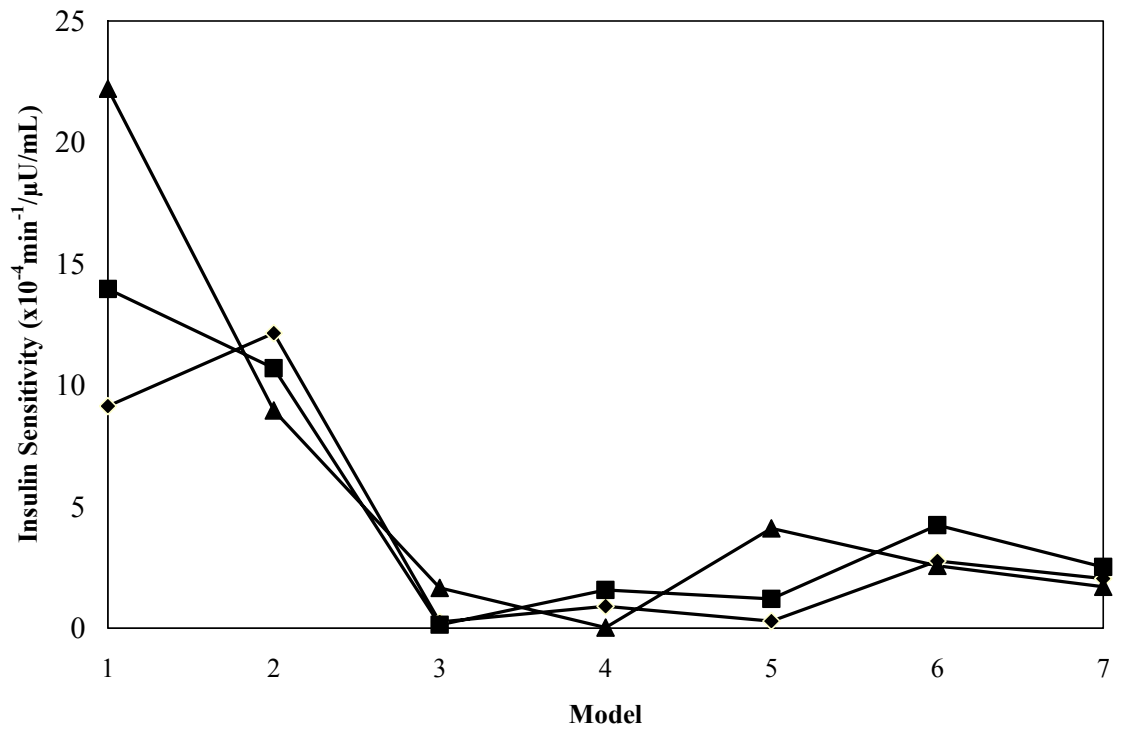


Figure 4.2. Insulin sensitivity in 3 cows measured at 3 weeks of life (Model 1), 6 weeks of life (Model 2), 1 week before anticipated calving date (Model 3), 1 week after calving (Model 4), 100 days in milk (Model 5), 200 days in milk (Model 6) and 300 days in milk (Model 7). Insulin sensitivity for Cow 658 is indicated by ▲, Cow 667 by ■, and Cow 669 by ◆.

becoming more reliant upon microbial fermentation of feedstuffs. During the transition period (3 weeks prior to and after calving) the S_I was low both before parturition (Model 3) and 1 week after calving (Model 4). Insulin resistance develops in many species during late pregnancy. This diminished sensitivity alters the body's utilization of glucose by making insulin responsive cells less responsive to insulin-stimulated uptake, thus shunting glucose to tissues such as the mammary gland and placenta that do not rely upon insulin for glucose transport. The diminished sensitivity also decreases insulin control of lipolysis and NEFA mobilization (Bell, 1995). Future work should incorporate measuring S_I in growing heifers to obtain a more complete description of the changes that occur during growth. Insulin sensitivity appears to begin increasing after 100 days in milk. This period usually follows peak milk production (usually 4-8 weeks postpartum) and is close to peak dry matter intake (10-14 weeks postpartum) (NRC, 2001). An increase in S_I as seen with the progression of lactation corresponds with the time in which milk production normally decreases and cows begin to restore body condition. In this data set it is difficult to distinguish the results of peak milk production and excessive nutritional inadequacies due to mismanagement. Lactating animals are normally in negative energy balance during peak lactation, and therefore the reduction in S_I corresponds to providing nutrients to support milk production. In this study, after improvements in farm management, milk production increased during a time in which it typically would be declining. This may be indicative that these cows were in excessive negative energy balance caused by dietary constraints which may have limited production. The establishment of S_I as measured with the FSIGT with minimal model analyses in a

normal production situation in primiparous cows is necessary to compare the S_I throughout the lactation cycle as milk production decreases.

The $AIR_{Glucose}$ is presented in Figure 4.3. This measurement is not commonly reported in dairy cattle, and therefore there are little data with which to compare this study. There is a marked increase in $AIR_{Glucose}$ 1 week after calving in these 3 heifers; however, in a larger data set (Chapter 3: Effects of Prepartum Dietary Energy Level and Calcium Propionate Supplementation on Energy Metabolism in Transition Dairy Cows) $AIR_{Glucose}$ was greater 1 week prepartum than 1 week postpartum. The transition period is associated with diminished S_I and perhaps the increased insulin secretion in response to a glucose challenge functions to compensate for diminished sensitivity. A larger study might identify times within the transition period where there is an increased $AIR_{Glucose}$ and further distinguish responses of primiparous cows and multiparous cows. The transition period is difficult to study because physiological changes are occurring rapidly, and there is a large amount of variation in response to the metabolic events characteristic of this period (Drackley, 1999).

The assessment of S_G is presented in Figure 4.4. There was no distinguishable pattern of changes in S_G during the periods in which it was measured in these animals. The concept of S_G incorporates the idea of glucose promoting its own disappearance through non-insulin dependant tissues (Bergman, 1997). The non-insulin dependant proteins include the GLUT 1 transporters which are located in the red blood cells, brain, placenta, and fetal tissue and the GLUT 3 transporters, which are in the brain, kidney, placenta, and mammary gland (McGrane, 2000). It is surprising that the onset of milk production did not affect this parameter in these cows.

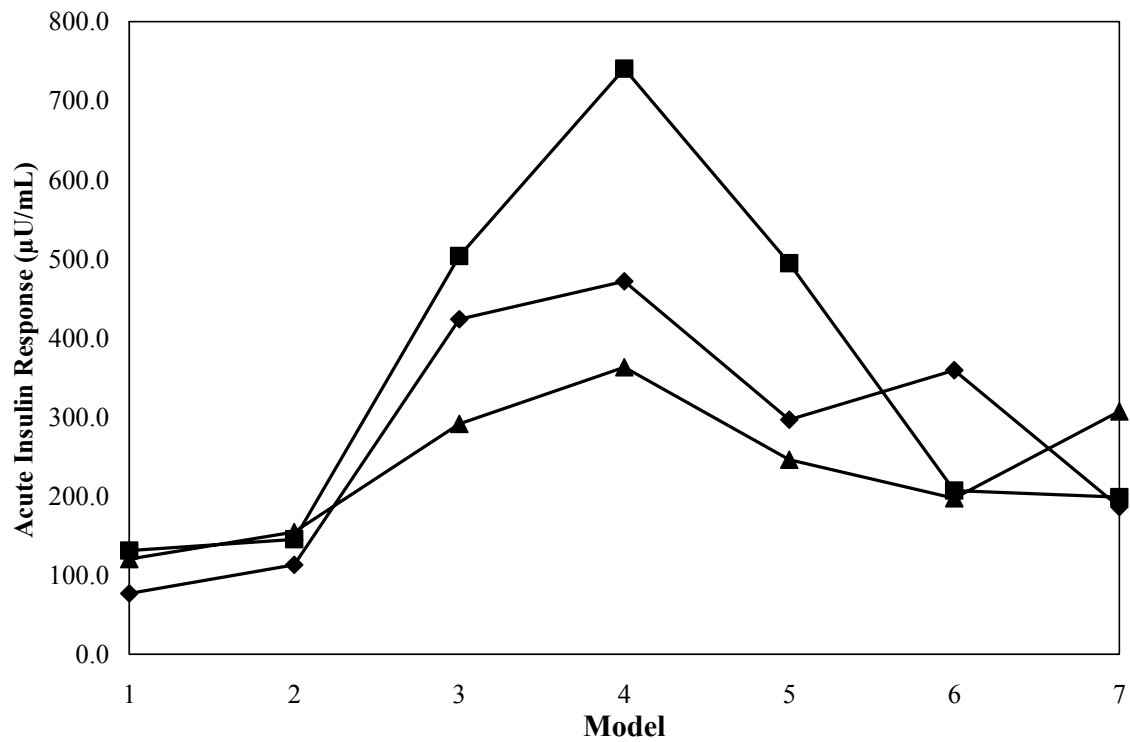


Figure 4.3. Acute insulin response in 3 cows measured at 3 weeks of life (Model 1), 6 weeks of life (Model 2), 1 week before anticipated calving date (Model 3), 1 week after calving (Model 4), 100 days in milk (Model 5), 200 days in milk (Model 6) and 300 days in milk (Model 7). Acute insulin response for Cow 658 is indicated by ▲, Cow 667 by ■, and Cow 669 by ◆.

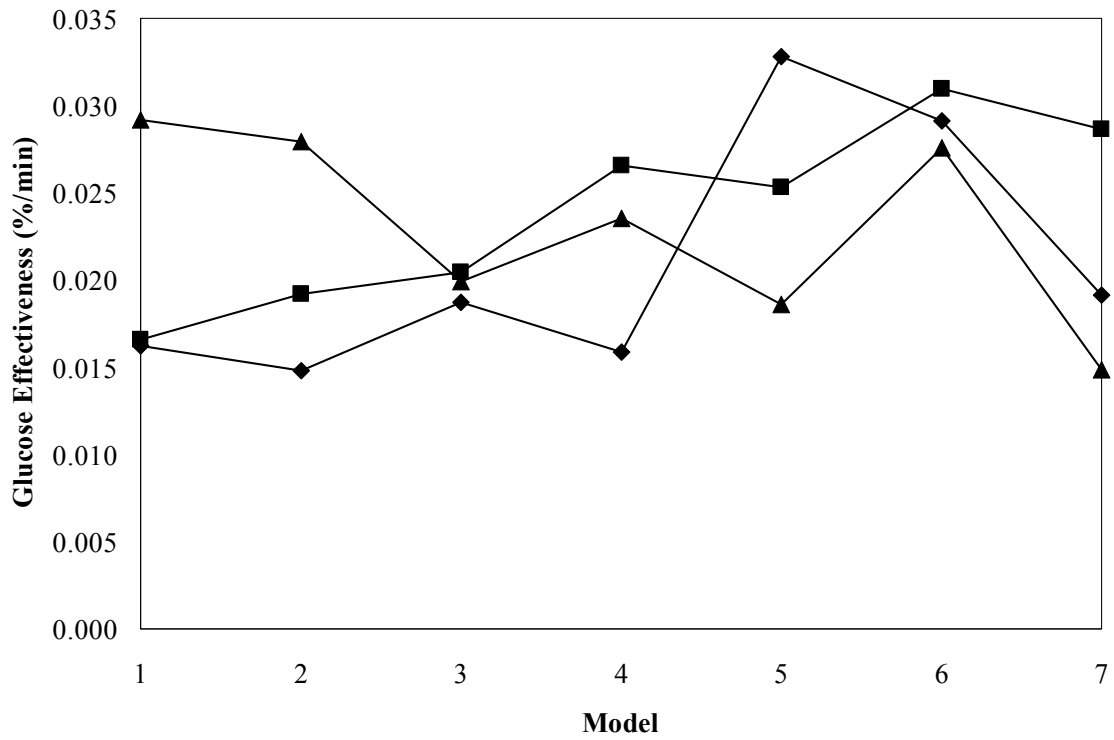


Figure 4.4. Glucose effectiveness in 3 cows measured at 3 weeks of life (Model 1), 6 weeks of life (Model 2), 1 week before anticipated calving date (Model 3), 1 week after calving (Model 4), 100 days in milk (Model 5), 200 days in milk (Model 6) and 300 days in milk (Model 7). Glucose effectiveness for Cow 658 is indicated by ▲, Cow 667 by ■, and Cow 669 by ◆.

The basal glucose and insulin concentrations are presented in Figures 4.5 and 4.6, respectively. Blood glucose concentrations declined as the calves matured as seen in Models 1 and 2. During this time the blood insulin and glucose concentrations were responsive to the type of feed that the calves were digesting and metabolizing. At week 3 (Model 1) the calves were being fed milk replacer and consuming limited amounts of starter, but at 6 weeks of age (Model 2) the calves were weaned and consuming calf starter only.

There was less variation in blood glucose concentrations than insulin concentrations throughout the sampling. The physiological regulation of an animal's body strives to maintain blood glucose concentrations (Bell, 1995). It is when this system becomes unable to compensate for metabolic and dietary changes that metabolic disorders such as diabetes and ketosis occur. Because the animal's system works to maintain constant blood glucose concentrations, there are no profound changes in plasma glucose concentrations in mature ruminants.

In two of the heifers, insulin concentrations were elevated the week before calving. In a larger data set (Chapter 3: Effects of Prepartum Dietary Energy Level and Calcium Propionate Supplementation on Energy Metabolism in Transition Dairy Cows) insulin concentrations decreased the week before calving and remained low for the 3 weeks following calving. Future work should include the measurement of insulin and glucose concentrations in growing bred heifers and continue monitoring the measurements throughout the lactation cycle and the subsequent dry period. Insulin is a hormone that is related to milk yield since low production is associated with increased insulin secretion. Insulin concentrations are also associated with energy balance. During

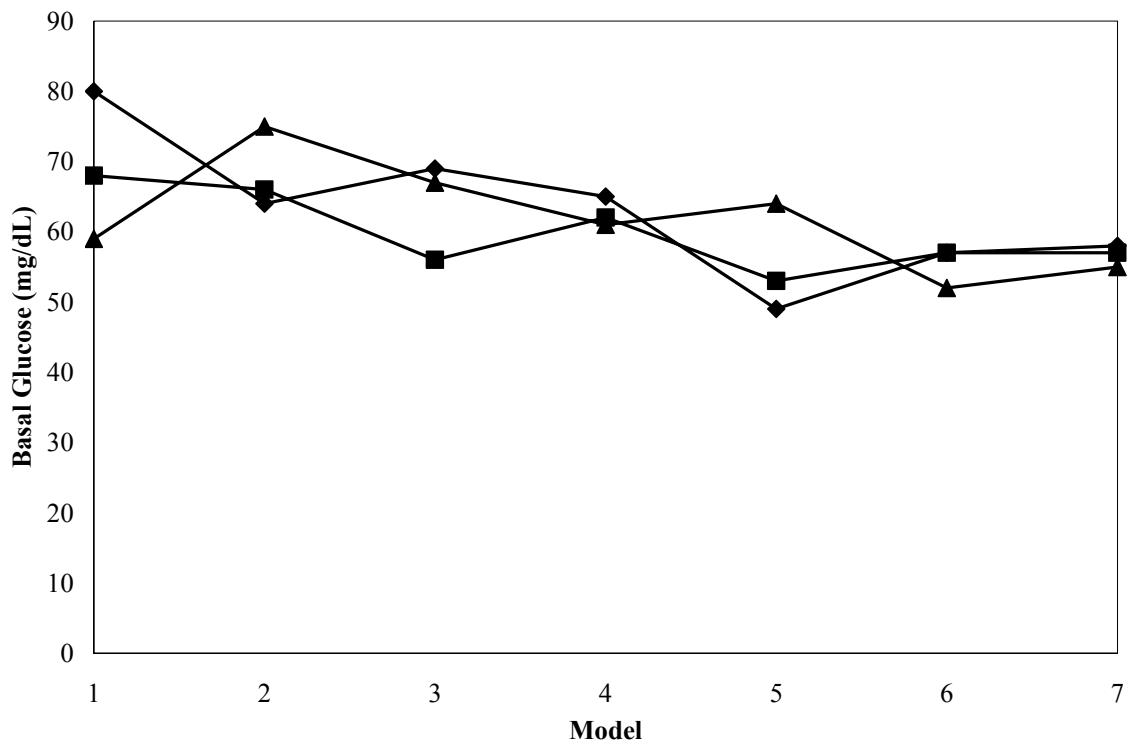


Figure 4.5. Basal glucose concentrations in 3 cows measured at 3 weeks of life (Model 1), 6 weeks of life (Model 2), 1 week before anticipated calving date (Model 3), 1 week after calving (Model 4), 100 days in milk (Model 5), 200 days in milk (Model 6) and 300 days in milk (Model 7). Glucose concentrations for Cow 658 are indicated by ▲, Cow 667 by ■, and Cow 669 by ◆.

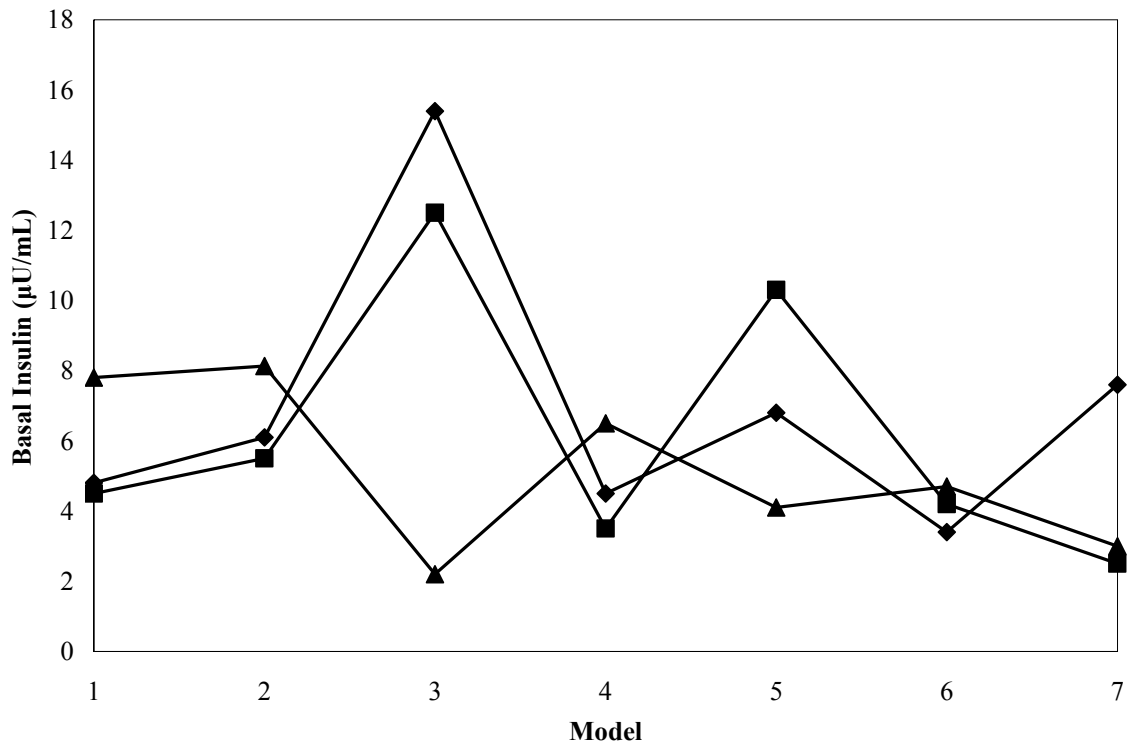


Figure 4.6. Basal insulin concentrations in 3 cows measured at 3 weeks of life (Model 1), 6 weeks of life (Model 2), 1 week before anticipated calving date (Model 3), 1 week after calving (Model 4), 100 days in milk (Model 5), 200 days in milk (Model 6) and 300 days in milk (Model 7). Insulin concentrations for Cow 658 are indicated by ▲, Cow 667 by ■, and Cow 669 by ◆.

times of negative energy balance, such as early lactation, insulin concentrations may reflect the energy status at that point. Low insulin concentrations at this point help allow available glucose to be preferentially shunted to the mammary gland (Collier et al., 1984).

Summary and Conclusions

The heifers included in this study were certainly not a sufficient number to characterize metabolic events that occur from birth through a lactation cycle, but the study has provided insight into an area which needs further investigation. Future work should include conducting FSIGT in calves, growing heifers, transition heifers, lactating primiparous cows, and dry cows. Conducting the FSIGT in the same cows during their second lactation would provide meaningful comparisons to assess the effects of parity on energy metabolism. Further work should include measuring additional hormones such as leptin, glucagon, and cortisol as well as other metabolites such as nonesterified fatty acids to gain a better understanding of the energy metabolism throughout lactation.

CHAPTER 5

INSULIN SENSITIVITY IN LACTATING DAIRY COWS AND NEONATAL CALVES: COMPARISON OF THE MINIMAL MODEL AND THE HYPERINSULINEMIC EUGLYCEMIC CLAMP

Introduction

Glucose is the principal source of energy for life processes of the mammalian cell. All cells require a constant supply of this indispensable nutrient, and only relatively small changes are tolerated without adverse effects on the health of the animal (Kaneko, 1997). Thus, the endocrine regulation of glucose metabolism is of utmost importance in mammalian homeostasis. Insulin, a key hormone in this endocrine regulation, facilitates the movement of glucose across cell membranes, thereby regulating the concentration of blood glucose. In nonruminants and pseudo-nonruminants such as young dairy calves, ingestion of feed and absorption of glucose stimulate the secretion of insulin, which decreases hepatic gluconeogenesis while promoting tissue uptake of glucose. As calves develop into fully functioning ruminants, hepatic gluconeogenesis increases and becomes the primary pathway of hepatic metabolism as the result of an increase in VFA production and absorption (Owens et al., 1986). It has been suggested that calves become insulin resistant as they progress from pre-ruminants to ruminants (Hugi et al., 1997). It has also been observed that insulin resistance begins before parturition and continues into early lactation (Debras et al., 1989; Prior and Christenson, 1978; Sano et al., 1991). During this transition from gestation to lactation, dairy cattle often experience metabolic disorders, including hepatic lipidosis and ketosis. Dietary energy intake is inadequate to meet the energy demands for maintenance and milk production. As a result of this negative energy balance, catabolic activities are high during this early lactation

period. As a result, lactogenesis is accompanied by increased lipolysis and decreased lipogenesis in adipose tissue, increased glycogenolysis and gluconeogenesis in the liver, and increased mobilization of protein reserves from muscle tissue (Collier et al., 1984). This pattern of metabolic activity begins prior to parturition. Therefore, the insulin resistance observed during the periparturient period may be a factor in the initiation of catabolic activities (Holstenius, 1993). As nutritional management strategies are developed to enhance insulin function and, consequently, glucose metabolism in dairy cattle, a reliable method for measuring insulin sensitivity would enhance the capabilities of researchers in this field.

Several methods have been utilized in ruminants for assessing glucose metabolism and insulin sensitivity. Two widely used techniques are the intravenous glucose tolerance test (IVGTT) and the insulin tolerance test (ITT). Andres et al. (1966) introduced clamp techniques for assessing insulin sensitivity and glucose metabolism which became widely accepted methods for studying these parameters. The tests evolved as an alternative to dangerous insulin tolerance tests. The hyperinsulinemic euglycemic clamp (EC) procedure is a widely accepted method of assessing insulin sensitivity. The technique of measuring insulin sensitivity during an imposed state of hyperinsulinemia and measuring glucose metabolism during a maintained state of hyperglycemia was published by DeFronzo et al. (1979). Using the glucose clamp techniques, two important physiological variables can be calculated: β -cell response to glucose and the sensitivity of body tissues to insulin.

During the EC plasma insulin is raised by a continuous intravenous insulin infusion, and a variable rate of glucose is infused to maintain a euglycemic level. This

physiological response to the test is called the steady state, a period during which the body's uptake of glucose by the tissues is equal to the glucose infused. During the steady state insulin sensitivity is measured by monitoring the amount of glucose infused to maintain euglycemia under the conditions of euglycemia (DeFronzo et al., 1979). The EC was originally designed for assessing insulin sensitivity in humans (De Fronzo et al., 1979) and has been used to assess insulin sensitivity in calves (Hostettler-Allen et al., 1994; Hostettler-Allen et al., 1993; Hugi et al., 1998; Sternbauer et al., 1998), heifers (Sternbauer and Luthman, 2002), and cows (Blum et al., 1999; Grinari et al., 1997; Holstenius et al., 2000; Mackle et al., 2000; Mackle et al., 1999; McGuire et al., 1995; Sano et al., 1993; Sano et al., 1991). The EC is considered to be the standard against which other glucose metabolism assessment tests are compared. The test is more difficult and labor-intensive than other procedures. It also requires expensive equipment and well-trained technicians (Coates et al., 1995). Previous research has utilized the ITT and the IVGTT in ruminants to evaluate glucose metabolism (Bassett, 1989; Hayirli et al., 2001). Although the IVGTT is more practical and easier to perform, information generated from this test is not as easily interpreted as that obtained from the EC. For example, the rate constant for the decline in blood metabolite concentrations following an intravenous glucose infusion may represent increased utilization or decreased production (Hayirli et al., 2001). The EC provides a safer method of estimating insulin sensitivity than the ITT; the complications and endocrine response to hypoglycemia that might occur during the ITT are avoided in the EC (DeFronzo et al., 1979).

The minimal model computer program (MINMOD) was developed by Bergman et al. (1979) for assessing multiple parameters of glucose and insulin metabolism, and

recently a newer version of the MINMOD Millennium edition has been published (Boston et al., 2003). The frequently sampled intravenous glucose tolerance test (FSIGT) with MINMOD computer analysis provides a simple alternative to the EC procedure (Coates et al., 1995). The FSIGT consists of infusing glucose through a jugular catheter followed 19 min later by infusing bovine insulin. Sequential blood samples are collected through the catheter over a 6 h period relative to glucose administration. The samples are analyzed for glucose and insulin concentrations. These values are entered into the MINMOD computer program to determine glucose effectiveness (S_G), the fractional glucose disappearance at basal insulinemia; insulin sensitivity (S_I) which is the effect of incremental change in plasma insulin to increase the fractional disappearance rate of glucose; and the acute insulin response ($AIR_{Glucose}$) relative to glucose administration (Bergman, 1997). This computer model has been validated for use in humans, rats, pigs, dogs, and cats (Finegood, 1997). Although this model has been utilized to determine S_I in ruminants (Bunting et al., 2000; DePew et al., 1998; Majorie et al., 1997; Stanley et al., 2002; Williams et al., 2004), it has not been validated for use in these species. When applying the MINMOD to new species, it is necessary to validate the accuracy of the parameter estimates with a model-independent measure (Finegood, 1997). In humans the FSIGT with MINMOD analysis has been validated by correlating the S_I derived from the MINMOD with the EC (Bergman et al., 1987; Coates et al., 1995; Saad et al., 1994).

The objective of this study is to compare the MINMOD Millennium computer analysis of the FSIGT method to the EC in assessing S_I in lactating cows and neonatal calves. The major factor to consider in this potential application is the difference in glucose metabolism between monogastric and ruminant animals. If the MINMOD

Millennium technology is proved to be valid in ruminant animals, it could become an important tool in identifying strategies for improving glucose utilization in growing and lactating animals.

Materials and Methods

Animals. The FSIGT with the MINMOD analysis and the EC were performed in 16 neonatal Holstein calves and 12 lactating Holstein cows in a completely randomized design experiment at the Dairy Science Research and Teaching Farm in Baton Rouge, Louisiana. Prior to all tests, calves were fasted for 12 h (Hostettler-Allen et al., 1994; Hostettler-Allen et al., 1993; Hugi et al., 1998; Sternbauer et al., 1998). Cows were allowed access to feed until 1 h prior to being tethered and catheterized (Holtenius et al., 2000; Sano et al., 1993). Prior to the FSIGT and EC, the animals were fitted with 1 catheter in each jugular vein (Baxter Healthcare Corporation, Deerfield, IL) at least 1 h prior to the test. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the LSU Agricultural Center.

Frequently Sampled Intravenous Glucose Tolerance Tests. The FSIGT consisted of glucose administration (300 mg/kg of BW) followed 20 min later by intravenous administration of bovine insulin (0.03 U/kg BW) through a jugular catheter. Blood samples were collected via another jugular catheter at -10, 0, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 19, 22, 23, 24, 25, 27, 30, 40, 50, 60, 70, 80, 90, 100, 120, 160, 180 and 360 min relative to glucose administration. The 360 min sample was not collected in calves due to their daily feeding schedule. Blood was placed in 7-mL evacuated tubes containing potassium oxalate and sodium fluoride (Kendall Medical, St. Louis MO) and centrifuged. Plasma was separated and frozen at -20°C until analyzed for plasma glucose

and insulin concentrations. The MINMOD Millennium computer program was used to determine the S_I (Bergman, 1997). A low S_I indicates a lower amount of insulin-stimulated glucose clearance.

Hyperinsulinemic Euglycemic Clamp. Prior to the EC, basal glucose concentrations were determined using a glucometer (Accuchek; Roche Diagnostic Corporation, Indianapolis, IN) to establish the target glucose for the animal during the test. The accuracy and precision of commercially available glucometers for use in dairy cattle had previously been assessed by Williams et al. (2004). A mixture of insulin and saline was prepared for each animal to deliver the correct amount of insulin during the EC. Additionally, an aliquot of the animal's blood was dissolved into the insulin-saline mixture at 4% of the total volume to prevent the insulin from adhering to the tubing (Hostettler-Allen et al., 1994). Insulin was administered in amounts of $1 \text{ mU/kg BW} \cdot \text{min}^{-1}$ in calves (Hostettler-Allen et al., 1994) and $6 \text{ mU/kg BW} \cdot \text{min}^{-1}$ in cows (Sano et al., 1993) from 5 min until end of test calves by a volumetric infusion pump (Baxter Travenol Flo Guard 6300 Pump; Diagnostic Products Corporation, Los Angeles, CA) connected to a jugular catheter. An insulin priming dose was used during the first 4 min of the test to stop glycogen breakdown and gluconeogenesis more rapidly. During the first 2 min of the EC insulin was administered at rates 5 times the EC dosage for both cows and calves, and then for 2 min at 2.5 times the EC dosage for cows and calves.

Blood collection was performed through a second jugular catheter every 5 min throughout the procedure and was analyzed for glucose concentration on a handheld glucometer (Accuchek; Roche Diagnostic Corporation, Indianapolis, IN). A 20% glucose solution for calves (Hostettler-Allen et al., 1994) and 50% solution for cows

(Grinari et al., 1997; Mackle et al., 2000; Mackle et al., 1999; McGuire et al., 1995) were infused through a jugular catheter by another volumetric infusion pump at a variable rate to achieve euglycemia (Grinari et al., 1997).

Blood was collected prior to the start of the EC at -25 and -5 minutes, during the EC at 30, 60, and 90 min after the start of the infusions, and during the period of steady state at -45, -25, and at 0 min relative to the start of steady state. Blood was placed in 7-mL evacuated tubes containing potassium oxalate and sodium fluoride (Kendall Medical, St. Louis MO) and centrifuged. Plasma was separated and frozen at -20°C until analyzed for plasma glucose and insulin concentrations.

Steady state was achieved between 90 and 120 min and euglycemia was maintained for a 45 min period during which the glucose disposal rate (GDR) was calculated from the amount of glucose infused per minute per unit of body weight. The GDR, expressed in mg/min/kg BW, is the glucose infusion rate necessary to maintain euglycemia even during hyperinsulinemia. A low GDR indicates lower insulin sensitivity and a higher GDR indicates greater insulin sensitivity. The GDR was converted to a measurement of insulin sensitivity (S_I EC) by dividing the GDR by the change in insulin concentration from the basal state to the hyperinsulinemic state and multiplying by the euglycemic blood glucose concentration. The formula is as follows:
$$\text{GDR} / \Delta \text{ Insulin } (\mu\text{U/mL}) \times \text{euglycemic glucose concentration (mg/dL)} = S_I \text{ EC}$$

(dL/min/kg BW $\cdot \mu\text{U}^{-1} \cdot \text{mL}^{-1}$) (Bergman et al., 1987).

Laboratory Procedures. Glucose concentrations were measured using a commercial spectrophotometric kit (Sigma Tech. Bull. No. 315; Sigma Chemical, St. Louis, MO), and insulin concentrations were measured using a commercial

radioimmunoassay kit (Coat-A-Count; Diagnostic Products Corporation, Los Angeles CA) as validated for cattle by Vicini et al. (1991). The intra-assay and inter-assay CV for the insulin assays were 4.95 % and 3.76 %, respectively.

Statistical Methods and Calculations. The univariate procedure revealed that the data from the EC and the MINDOD analysis of the FSIGT were not normally distributed. Therefore, Spearman correlation coefficients were used to determine the degree of linear association between S_I from the MINMOD and EC.

Results and Discussion

Insulin Sensitivity Indices in Cows and Calves. The median, mean, minimum, and maximum values for S_I MINMOD, GDR, and S_I EC are reported in Table 5.1. In fulfillment of the objective of this study, to verify the agreement of the S_I MINMOD and S_I EC, a broad range of S_I was desired. By using neonatal calves and cattle during all stages of lactation this goal was achieved as cows are generally less sensitive to insulin and calves are more sensitive. The data ranges from the S_I MINMOD, GDR, and S_I EC for calves and cows overlapped. The MINMOD analysis yielded broad range of S_I ($0.04 - 27.70 \times 10^{-4} \text{ min}^{-1} \mu\text{U}^{-1} \cdot \text{mL}^{-1}$) as did the EC analysis of S_I ($1.73 - 197.30 \times 10^{-4} \text{ dL/min/kg} \cdot \mu\text{U}^{-1} \cdot \text{mL}^{-1}$). The increased sample size yielded a data set that was broad and large enough to test the relationships between the parameters. The range for the GDR was 1.43 to 10.88 mg/min/kg.

Spearman rank correlations for the S_I MINMOD, GDR, and S_I EC are reported in Table 5.2. There was a good correlation ($r = 0.60$; $P < 0.05$) between the S_I from the MINMOD and the GDR. Upon normalizing the GDR data from the EC by dividing by the change in insulin concentration from basal to hyperinsulinemia multiplied by the

Table 5.1. Insulin sensitivity (S_i) indices from the minimal model (MINMOD) computer analysis of the frequently sampled glucose tolerance test (FSIGT), the hyperinsulinemic euglycemic glucose clamp (EC), and glucose disposal rate (GDR) in neonatal calves and lactating cows.

| | S_i MINMOD ¹ ($\times 10^{-4} \text{ min}^{-1} \mu\text{U}^{-1} \cdot \text{mL}^{-1}$) | GDR EC ² (mg/min/kg) | S_i EC ³ ($\times 10^{-4} \text{ dL/min/kg} \cdot \mu\text{U}^{-1} \cdot \text{mL}^{-1}$) |
|------------------------|--|------------------------------------|---|
| Cows and calves | | | |
| Median | 7.52 | 4.85 | 41.80 |
| Mean | 8.56 | 5.05 | 55.50 |
| Minimum | 0.04 | 1.43 | 1.73 |
| Maximum | 27.70 | 10.88 | 197.30 |
| Standard Deviation | 7.23 | 2.56 | 58.26 |
| Cows | | | |
| Median | 1.84 | 2.76 | 7.61 |
| Mean | 2.76 | 2.66 | 6.87 |
| Minimum | 0.04 | 1.43 | 1.73 |
| Maximum | 7.65 | 4.01 | 9.19 |
| Standard Deviation | 2.44 | 0.75 | 2.36 |
| Calves | | | |
| Median | 12.40 | 6.46 | 71.30 |
| Mean | 12.90 | 6.85 | 9.19 |
| Minimum | 4.36 | 3.74 | 28.10 |
| Maximum | 27.70 | 10.88 | 197.30 |
| Standard Deviation | 6.80 | 1.96 | 55.00 |

¹ S_i (MINMOD) units = ($\times 10^{-4} \text{ min}^{-1} \mu\text{U}^{-1} \cdot \text{mL}^{-1}$)

²GDR (EC) units = (mg/min/ kg BW)

³ S_i (EC) units = ($\times 10^{-4} \text{ dL/min/kg BW} \cdot \mu\text{U}^{-1} \cdot \text{mL}^{-1}$)

clamped glucose concentration, the relationship between the S_I EC and S_I MINMOD was strong ($r = 0.78$; $P < 0.05$). The relationship of the S_I MINMOD and S_I EC is shown in Figure 5.1. The relationship between the GDR and S_I EC was good ($r = 0.67$, $P < 0.05$).

Both the MINMOD and EC analysis reflect the degree of S_I in dairy cattle by relating the glucose flux to a change in plasma insulin concentration (Finegood et al., 1984). They do not have the same units because the definitions of S_I differ for the tests. In the EC glucose flux is normalized to body weight, whereas it is normalized to glucose distribution volume in the MINMOD (Finegood et al., 1984). In the EC the S_I is defined in terms of the action of insulin to augment glucose utilization. In the MINMOD analysis S_I is defined as the ability to facilitate glucose utilization and inhibit hepatic glucose output (Bergman et al., 1987). The MINMOD was designed to measure the ability of insulin to augment the disappearance of glucose and inhibit endogenous glucose production during a non-steady state period. The MINMOD establishes dynamic relationships between plasma glucose and insulin, whereas the EC dictates that a steady-state be maintained. The MINMOD incorporates insulin secreted from the pancreas in response to infused glucose as well as exogenous insulin infused (Finegood, 1997). The portal insulin in the MINMOD is predicted to have more of a profound effect than the peripheral insulin infused in the MINDOD and EC (Finegood et al., 1984). The duration of hyperinsulinemia is shorter in the MINMOD than the EC.

Hyperinsulinemia is maintained for the period of time until steady state is reached and then throughout steady state in the EC, but the state of hyperinsulinemia is limited in the MINMOD (Saad et al., 1994). In the EC plasma glucose is held constant though the varied infusion of exogenous glucose relative to the constant infusion of insulin. A more

Table 5.2. Spearman rank correlations of insulin sensitivity (S_I) indices from the minimal model (MINMOD) computer analysis of the frequently sampled glucose tolerance test (FSIGT), the hyperinsulinemic euglycemic glucose clamp (EC), and glucose disposal rate (GDR) in neonatal calves and lactating cows.

| | S_I MINMOD | S_I EC | GDR EC |
|-----------------|--------------|----------|--------|
| Cows and calves | | | |
| S_I MINMOD | 1* | | |
| S_I EC | 0.78* | 1* | |
| GDR EC | 0.60* | 0.67* | 1* |
| Cows | | | |
| S_I MINMOD | 1* | | |
| S_I EC | 0.01 | 1* | |
| GDR EC | -0.33 | 0.10 | 1* |
| Calves | | | |
| S_I MINMOD | 1* | | |
| S_I EC | 0.45** | 1* | |
| GDR EC | -0.25 | -0.33 | 1* |

* $P < 0.05$

** $P = 0.08$

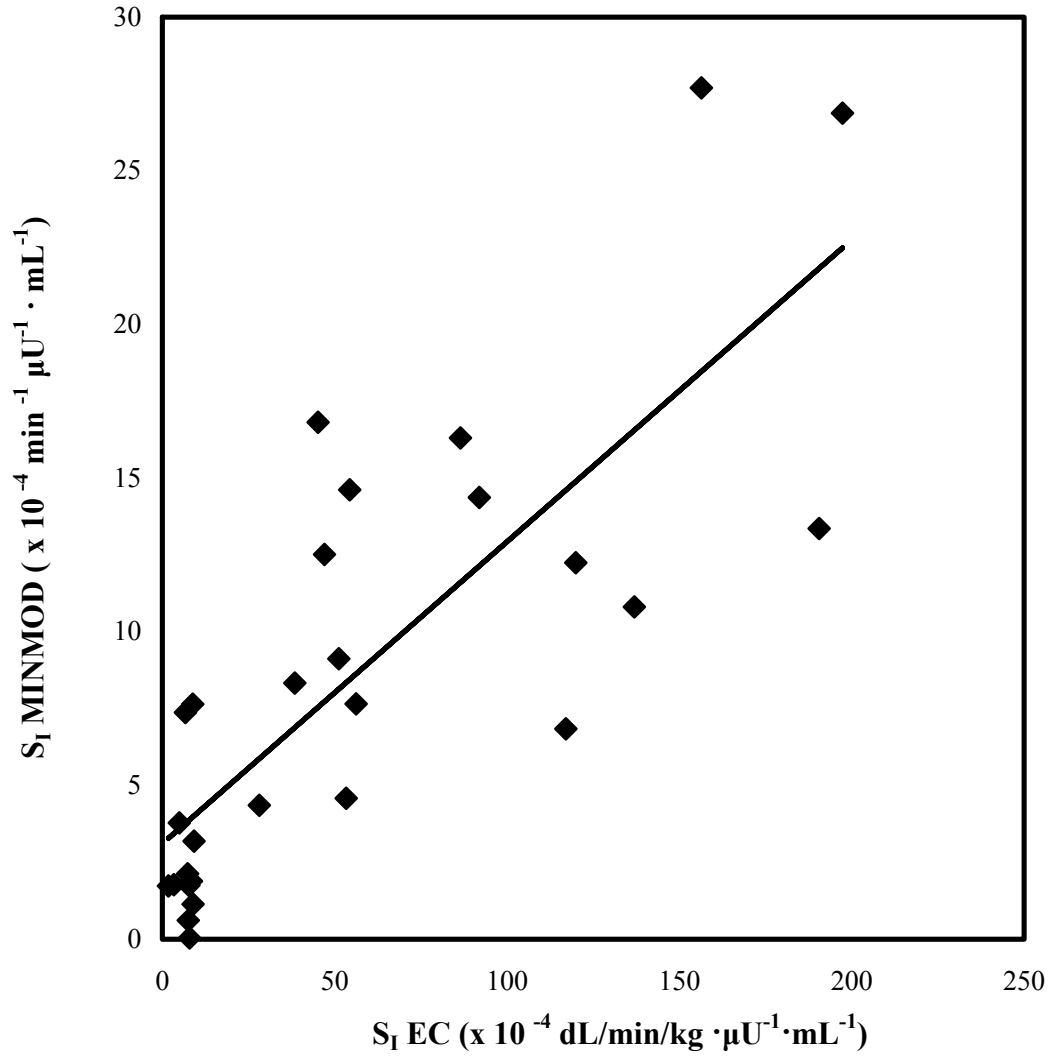


Figure 5.1. Relationship of insulin sensitivity (S_I) indices from the minimal model (MINMOD) computer analysis of the frequently sampled glucose tolerance test (FSIGT) and the hyperinsulinemic euglycemic glucose clamp (EC) in neonatal calves and lactating cows.

* $P < 0.05$; $r = 0.78$

accurate assessment of S_I from the EC would have included conducting the EC at several levels of insulin while clamping glucose rather than choosing an insulin dose and performing the EC at that level of insulinemia (Finegood, 1997). Maintaining an EC in cows or calves for an extended period of time during which steady state would have been established at one insulin dose then another would have been very difficult. Furthermore, maintaining hyperinsulinemia for an extended period of time can increase the GDR even without increasing the insulin dosage as sustained hyperinsulinemia increases insulin-stimulated glucose uptake (Beard et al., 1986).

The day to day variation in glucose metabolism and the stimulation from activity at the farm may have affected the data. Even with this variation the two measurements of S_I appear to be analogous. The MINMOD analysis of the FSIGT has proven to be very useful as a measurement of S_I as the procedure requires no special equipment, utilizes less labor, and can be performed reasonably in a farm environment. Additionally the attainment and maintenance of steady state are not required. Although external stimulation should be limited when performing metabolic tests, complete isolation is not always possible. In such environments the FSIGT was easy to perform, whereas maintaining euglycemia was challenging. The MINMOD analysis of the FSIGT assesses multiple parameters from a single test day, whereas it would be necessary to perform clamps under states of hyperinsulinemia to gather information about S_I and during a maintained state of hyperglycemia to assess a parameter similar to the MINMOD parameter S_G .

Insulin Sensitivity Indices in Cows. The median, mean, minimum, and maximum values for S_I MINMOD, GDR, and S_I EC for cows are reported in Table 5.1.

The median S_I MINMOD for cows was $1.84 \times 10^{-4} \text{ min}^{-1} \mu\text{U}^{-1} \cdot \text{mL}^{-1}$ which was similar to previously reported values (Stanley et al., 2003). The median GDR for cows was 2.76 mg/min/kg which was similar to previous reported values (Sano et al., 1991). The median S_I EC for cows was $7.61 \times 10^{-4} \text{ dL/min/kg} \cdot \mu\text{U}^{-1} \cdot \text{mL}^{-1}$. All indices of insulin function indicated that cows were less sensitive to insulin than calves. Spearman rank correlations for the S_I MINMOD, GDR, and S_I EC are shown in Table 5.2. Upon performing the Spearman correlations on the data from the cows only, there were no significant relationships between any of the parameters measuring insulin action ($P > 0.05$). The relationship of S_I MINMOD and S_I EC in lactating dairy cows is shown in Figure 5.2. The lack of significant correlation among the parameters may be attributed to the reduced sample size or to measuring a lower range of insulin sensitivity in which the magnitude of correlation may be diminished (Saad et al., 1994). The cows utilized in the experiment were in early, mid, and late lactation, a factor which may have contributed to the variation in metabolism within the group of animals.

Insulin Sensitivity Indices in Calves. The median, mean, minimum, and maximum values for S_I MINMOD, GDR, and S_I EC for calves are reported in Table 5.1. The median S_I MINMOD for calves was $12.4 \times 10^{-4} \text{ min}^{-1} \mu\text{U}^{-1} \cdot \text{mL}^{-1}$ which was similar to previously reported values (Stanley et al., 2002). The median GDR for calves was 6.46 mg/min/kg, which was slightly higher than previous reported values in calves (Hostettler-Allen et al., 1994). The median S_I EC for calves was $71.3 \times 10^{-4} \text{ dL/min/kg} \cdot \mu\text{U}^{-1} \cdot \text{mL}^{-1}$. All indices of insulin function indicated that calves were more sensitive to insulin than cows. Spearman rank correlations for the S_I MINMOD, GDR, and S_I EC are shown in Table 5.2. Upon performing the Spearman correlations on the data from the calves only,

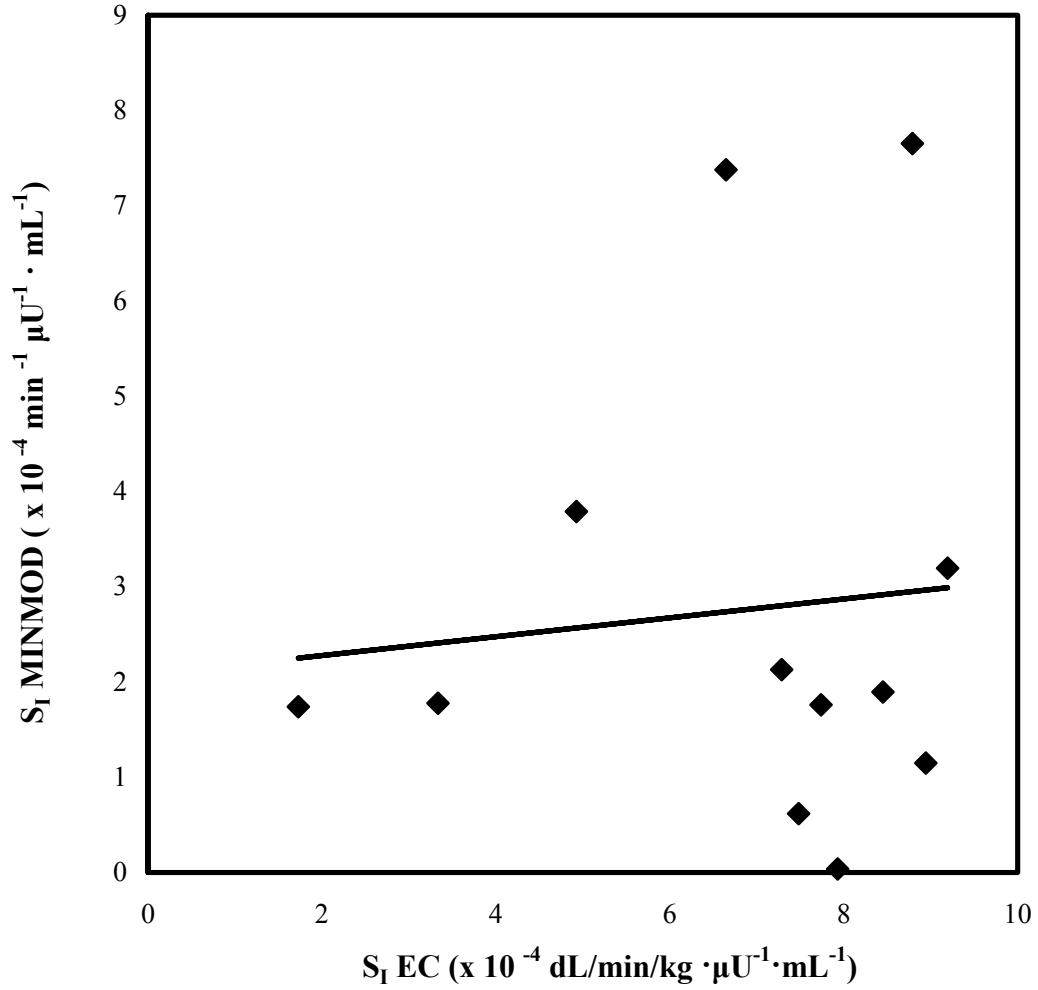


Figure 5.2. Relationship of insulin sensitivity (S_1) indices from the minimal model (MINMOD) computer analysis of the frequently sampled glucose tolerance test (FSIGT) and the hyperinsulinemic euglycemic glucose clamp (EC) in lactating cows.

* $P > 0.05$

there were no significant relationships between the GDR and S_I EC or the S_I MINMOD and the GDR ($P > 0.05$). There was a significant relationship between the S_I EC and the S_I MINMOD ($r = 0.45$, $P = 0.08$). The relationship of S_I MINMOD and S_I EC in calves is shown in Figure 5.3. The decreased sample size may have decreased the magnitude of the correlation in the calves.

In a previous study Saad et al. (1994) reported that the S_I EC and S_I MINMOD had a strong relationship among humans with a broad range of S_I . However, when the subjects were separated by degrees of S_I into groups of normal glucose tolerance, impaired glucose tolerance, and non-insulin dependent diabetes mellitus, the relationship diminished among the less insulin sensitive subjects. Saad et al. (1994) recommended that even in cases in which the S_I MINMOD did not correlate as well with the S_I EC, the S_I MINMOD represented a good index of S_I although the actual measurement of S_I may not reflect the same absolute values as the S_I EC. There have been numerous studies in which the S_I calculated from the MINMOD and EC were correlated including comparisons in humans with normal insulin sensitivity (Bergman et al., 1987; Saad et al., 1994) and with non-insulin dependent diabetes mellitus (Coates et al., 1995), in dogs (Finegood et al., 1984) and cats (Petrus et al., 1998) with normal insulin sensitivity. There have also been some studies in which the MINMOD and EC were not strongly correlated in humans with normal insulin sensitivity (Donner et al., 1985) and in humans with non-insulin dependent diabetes mellitus (Saad et al., 1994).

Summary and Conclusions.

The EC estimation of GDR has been widely accepted in non-ruminant and ruminant animals. The estimation of S_I EC allows the insulin dosage to be accounted for because

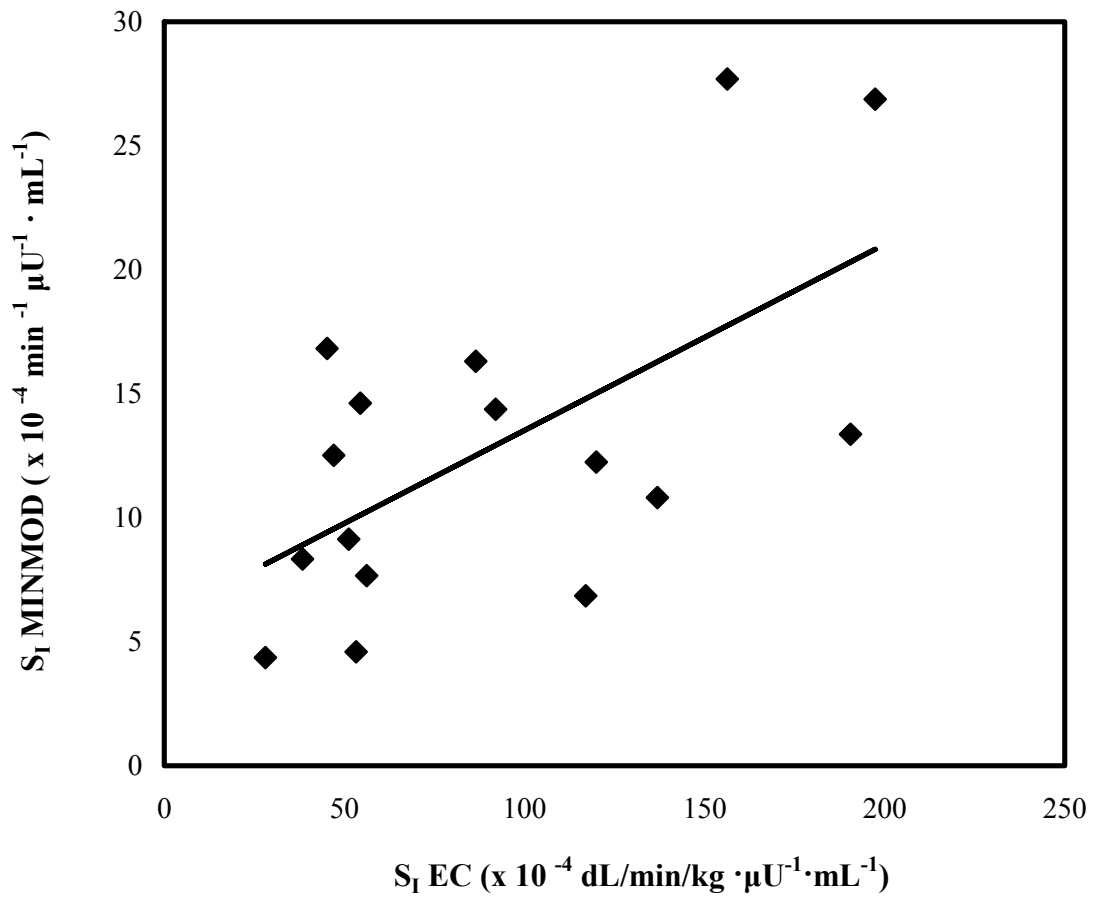


Figure 5.3. Relationship of insulin sensitivity (S_1) indices from the minimal model (MINMOD) computer analysis of the frequently sampled glucose tolerance test (FSIGT) and the hyperinsulinemic euglycemic glucose clamp (EC) in neonatal calves.

* $P = 0.08$; $r = 0.45$

the change in insulin from a basal state to the hyperinsulinemic state is factored into the equation. The S_I estimated from the MINMOD and EC represent the change in plasma insulin to affect a decline in net glucose production and increase glucose uptake (Bergman, 1997). In other species it has previously been determined that the S_I estimated from the MINMOD and EC are equivalent in measuring the same physiological variable. If the MINMOD estimation of S_I was not an accurate estimation of insulin-dependent glucose clearance, it would not be equivalent to the estimation using the EC (Bergman, 1997). There was a strong correlation between the S_I from the MINMOD and EC in dairy cattle, indicating that both the tests are adequate in assessing S_I .

CHAPTER 6

REGULATION OF HORMONES THAT CONTRIBUTE TO THE COORDINATION OF METABOLISM DURING THE TRANSITION PERIOD

Introduction

The transition period, which is 3 weeks before and after calving, is a critical period to the health, production, and profitability of the dairy cow (Drackley, 1999). The metabolic adaptations necessary to accommodate changes in nutrient partitioning associated with lactogenesis are mediated by the endocrine system. Hormone concentrations fluctuate to coordinate metabolic homeostasis and homeorhesis. Energy reserves are mobilized in response to an increased nutrient demand to support lactation until dry matter intake is adequate to meet nutrient requirements. During this transition from gestation to lactation, dairy cattle often experience metabolic disorders, including hepatic lipidosis and ketosis. Dietary energy intake is inadequate to meet the energy demands for maintenance and milk production. As a result of this negative energy balance, catabolic activities are high during this early lactation period. As a result, lactogenesis is accompanied by increased lipolysis and decreased lipogenesis in adipose tissue, increased glycogenolysis and gluconeogenesis in the liver, and increased mobilization of protein reserves from muscle tissue (Collier et al., 1984). It has been observed that insulin resistance begins before parturition in dairy cows and continues into early lactation (Debras et al., 1989; Prior and Christenson, 1978; Sano et al., 1991) and may be a factor in the initiation of catabolic activities (Holstenius, 1993).

The roles of leptin and insulin have been investigated in humans and rodents, but little research has been done in investigating the role of these hormones in modulating

energy intake in lactating dairy cattle (Ingvarsen, and Andersen, 2000). In humans, reduced leptin and insulin action increase the appetite when calories are limited (Schwartz et al., 1992).

In humans and rodents in positive energy balance, insulin and leptin activate neurons that express proopiomelanocortin and secrete α -melanocyte stimulating hormone which is an anorexic neuropeptide (Niswender and Schwartz, 2003). The activation of melanocorticotropin receptors leads to a decrease in food intake. Increased leptin and insulin concentrations also decrease food intake by decreasing neuropeptide Y and agouti related proteins (Niswender and Schwartz, 2003). Conversely, decreased levels of insulin and leptin stimulate neurons that express neuropeptide Y and agouti related proteins which stimulate food intake. Diminished insulin and leptin concentrations also inhibit proopiomelanocortin and diminish α -melanocyte stimulating hormone expression and release leading to inactivation of anorexic pathways (Niswender and Schwartz, 2003).

In ruminants leptin is secreted by white adipose tissue, and its secretion is decreased when there is an insufficient supply of nutrients (Ahima and Flier, 2000). Increased levels of leptin are associated with decreased feed intake. Low concentrations of leptin increase appetite and prepare the animal to conserve energy. Following parturition and early lactation, when cows are in negative energy balance, plasma leptin concentrations are low (Block et al., 2003). The decrease in plasma leptin concentrations occurs before body fat stores are diminished. The decrease coincides with the onset of negative energy balance which is associated with reduction of plasma insulin concentrations and an increase in growth hormone concentrations (Block et al., 2003).

Recent research in lactating dairy cows demonstrates that plasma insulin increases leptin when cows are in positive energy balance (Block et al., 2003). Leury et al. (2003) reported that hyperinsulinemia increased plasma leptin concentrations in late gestation cows but did not have as great an effect on raising plasma leptin concentrations in early lactation. In early lactation, when dairy cattle experience negative energy balance, it seems reasonable to hypothesize that the decreased secretion of these hormones is a physiological mechanism to regulate energy balance by increasing appetite. (Block et al., 2003).

The objective of this study was to characterize insulin sensitivity at the end of gestation and in early lactation by utilizing the frequently sampled glucose tolerance test (FSIGT), insulin tolerance test (ITT), and hyperinsulinemic euglycemic clamp test (EC). The physiological responses of leptin and glucagon concentrations to a hyperinsulinemic state at euglycemia and at hypoglycemia were measured by applying the EC and ITT.

Materials and Methods

Animals. The FSIGT with the minimal model computer analysis (MINMOD), the EC, and the ITT were performed in 5 Holstein cows at 7-10 days prior to anticipated calving date and again at 7-10 days after calving. Each test was performed once in each animal with the order of the test being randomized. The animals were housed and the tests were performed at the Dairy Science Research and Teaching Farm in Baton Rouge, Louisiana. Cows were allowed access to feed until 1 h prior to being tethered and catheterized (Holstenius et al., 2000; Sano et al., 1993). A catheter was inserted (Baxter Healthcare Corporation, Deerfield, IL) in each jugular vein of each cow at least 1 h prior

to the test. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the LSU Agricultural Center.

Frequently Sampled Intravenous Glucose Tolerance Tests. The FSIGT consisted of glucose administration (300 mg/kg of BW) followed 20 min later by intravenous administration of bovine insulin (0.03 U/kg BW) through a jugular catheter. Blood samples were collected via jugular catheter at -10, 0, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 19, 22, 23, 24, 25, 27, 30, 40, 50, 60, 70, 80, 90, 100, 120, 140, 160, 180, and 360 min relative to glucose administration. Blood was placed in 7-mL evacuated tubes containing potassium oxalate and sodium fluoride (Kendall Medical, St. Louis MO) and centrifuged. Plasma was separated and frozen at -20°C until analyzed for plasma glucose and insulin concentrations. These values are entered into the MINMOD Millennium computer program to determine glucose effectiveness (S_G), the fractional glucose disappearance at basal insulinemia; insulin sensitivity (S_I) which is the effect of incremental change in plasma insulin to increase the fractional disappearance rate of glucose; and the acute insulin response ($\text{AIR}_{\text{Glucose}}$) relative to glucose administration (Bergman, 1997).

Hyperinsulinemic Euglycemic Clamp. Prior to the EC, basal glucose concentrations were determined using a glucometer (Accucheck; Roche Diagnostic Corporation, Indianapolis, IN) to establish the target glucose for the animal during the test. The accuracy and precision of commercially available glucometers for use in dairy cattle had previously been assessed by Williams et al. (2004). A mixture of insulin and saline was prepared for each animal to deliver the correct amount of insulin during the EC. Additionally, an aliquot of the animal's blood was dissolved into the insulin-saline

mixture at 4% of the total volume to prevent the insulin from adhering to the tubing (Hostettler-Allen et al., 1994). Insulin was administered at a rate of $6 \text{ mU/ kg BW} \cdot \text{min}^{-1}$ in cows (Sano et al., 1993) from 5 min until end of test by a volumetric infusion pump (Baxter Travenol Flo Guard 6300 Pump, Diagnostic Products Corporation, Los Angeles, CA) connected to a jugular catheter. An insulin priming dose was used during the first 4 min of the test to stop glycogen breakdown and decrease gluconeogenesis. During the first 2 min of the EC insulin was administered at rates 5 times the EC dosage, and then for 2 min at 2.5 times the EC dosage.

Blood collection was performed through a second jugular catheter every 5 min throughout the procedure and was analyzed for glucose concentration on a glucometer (Accucheck; Roche Diagnostic Corporation, Indianapolis, IN). The accuracy and precision of commercially available glucometers for use in dairy cattle had previously been assessed by Williams et al. (2004). A 50% glucose solution (Grinari et al., 1997; Mackle et al., 2000; Mackle et al., 1999; McGuire et al., 1995) was infused through a jugular catheter by another volumetric infusion pump at a variable rate to achieve euglycemia (Grinari et al., 1997).

Blood was collected prior to the start of the EC at -25, -15, and -5 min, during the EC at 30, 60, and 90 min after the start of the infusions, and during the period of steady state at -45, -25, and at 0 min relative to the start of steady state. Blood was placed in 7-mL evacuated tubes containing potassium oxalate and sodium fluoride (Kendall Medical, St. Louis MO) and centrifuged. Plasma was separated and frozen at -20°C until analyzed for glucose and insulin concentrations. Additional samples were collected prior to the start of the tests at -25, -15, and -5 minutes and during steady state at -45, -25, and at 0

min for the measurement of plasma leptin and glucagon concentrations. Samples collected for leptin analyses were placed into 7-mL evacuated tubes containing potassium oxalate and sodium fluoride (Kendall Medical, St. Louis MO). Samples analyzed for glucagon concentrations were placed into 7-mL evacuated tubes containing sodium salts of EDTA (Kendall Medical, St. Louis, MO), and aprotinin was added to deliver 5 trypsin inhibiting units (Sigma Chemical, St. Louis MO). The tubes were centrifuged and plasma was separated and frozen (-20° C) until analyzed for glucagon.

Steady state was achieved between 90 and 120 min, and euglycemia was maintained for a 45 min period during which the glucose disposal rate (GDR) was calculated from the amount of glucose infused per minute per unit of body weight. The GDR, expressed in mg/min/kg BW, is the glucose infusion rate necessary to maintain euglycemia even during hyperinsulinemia. A low GDR indicates lower insulin sensitivity while a higher GDR indicates greater insulin sensitivity. The GDR was converted to a measurement of insulin sensitivity (S_I EC) by dividing the GDR by the change in insulin concentration from the basal state to the hyperinsulinemic state and multiplying by the euglycemic blood glucose concentration. The formula is as follows:

$$\text{GDR}/\Delta \text{ Insulin } (\mu\text{U}/\text{mL}) \times \text{euglycemic glucose concentration (mg/dL)} = S_I \text{ EC} \\ (\text{dL}/\text{min}/\text{kg BW} \cdot \mu\text{U}^{-1} \cdot \text{mL}^{-1}) \text{ (Bergman et al., 1987).}$$

Insulin Tolerance Tests. The ITT consisted of bovine insulin administration (0.1 U/kg of BW) through a jugular catheter. Blood samples were collected via jugular catheter at -30, -15, 0, 15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165, and 180 min relative to insulin administration to measure plasma insulin, leptin, glucagon, and glucose concentrations. Samples collected for plasma leptin, glucose, and insulin analyses were

placed into 7-mL evacuated tubes containing potassium oxalate and sodium fluoride (Kendall Medical, St. Louis MO). Samples collected for glucagon analysis were placed into 7-mL evacuated tubes containing sodium salts of EDTA (Kendall Medical, St. Louis, MO), and aprotinin was added to deliver 5 trypsin inhibiting units (Sigma Chemical, St. Louis MO). The tubes were centrifuged and plasma was separated and frozen (-20° C) until analyzed for hormone and metabolite concentrations. The ITT measures the sensitivity of the blood glucose level to a test dose of insulin and the response of the animal to insulin-induced hypoglycemia. Insulin resistance was identified when the blood glucose level did not fall by 50% or required longer than 30 min to reach the maximum hypoglycemic level (Kaneko, 1997).

Laboratory Procedures. Plasma was analyzed for glucose concentrations using commercial spectrophotometric kits (Sigma Tech, Bull. No. 315; Sigma Chemical, St. Louis, MO). Using commercial radioimmunoassay kits, plasma was analyzed for insulin (Coat-A-Count; Diagnostic Products Corporation, Los Angeles, CA) and glucagon concentrations (Linco Glucagon Radioimmunoassay Kit; Linco Research, Inc., St. Charles, MO). Plasma was analyzed for leptin concentrations by radioimmunoassay (Cartmill et al., 2003). The intra-assay CV for insulin, glucagon, and leptin assays were 4.55, 7.57, 6.96 %, respectively. Inter-assay CV for insulin, glucagon, and leptin assays were 4.16, 6.46, and 4.66 %, respectively.

Statistical Methods and Calculations. The EC, ITT, FSIGT, hormone, and metabolic data were analyzed by ANOVA using the mixed model (Littell et al., 1988) in SAS (1990). The basal concentrations for plasma leptin, glucose, insulin, and glucagon were averaged from the samples taken prior to infusions on the days of the ITT, EC, and

FSIGT for the pre- and post-calving periods for each cow. For the analysis of the GDR, S_I EC, S_I MM, S_G , and $AIR_{Glucose}$ data and for basal insulin, glucose, glucagon and leptin concentrations week was modeled as the repeated term and was assumed to be correlated within cow using a compound symmetry covariance structure. The model included fixed effects of week. The percent differences in concentrations from basal to hyperinsulinemia were calculated for leptin, insulin, glucose, and glucagon. In the analysis of the ITT data, time and week were modeled as the repeated terms for analyzing the concentrations of metabolites, hormones and percent differences. For the analysis of the hormone and metabolite concentrations in the EC, phase (basal and steady state) and week were modeled as the repeated terms.

Results and Discussion

Least squares means for plasma metabolite and hormone concentrations are presented Table 6.1. There was a main effect of week for mean plasma glucagon concentrations ($P < 0.05$) with an increase in basal glucagon concentrations after calving. There were no main effects of week for mean basal plasma insulin, glucose, or leptin concentrations, although numerically there were decreases in all of these metabolite and hormone concentrations after calving. The least squares means for the GDR, S_I EC, S_I MM, S_G , $AIR_{glucose}$ are presented in Table 6.2. There were no effects of week for any of these parameters measured. The glucose disappearance in the ITT, GDR and S_I from the EC, and the S_I from the MM, reflected a numerical increase in insulin sensitivity after calving although the responses indicated low insulin sensitivity both pre- and postpartum.

The increased glucagon concentrations after parturition were critical in increasing the body's supply of glucose when energy supply was low relative to the demands.

Table 6.1. Least squares means for basal plasma metabolite and hormone concentrations in cows before and after parturition.

| | Prepartum | Postpartum | SEM |
|---|-----------|------------|-------|
| Basal Glucose (mg/dL) | 49.21 | 43.1 | 3.34 |
| Basal Insulin ($\mu\text{U}/\text{mL}$) | 6.15 | 4.92 | 1.78 |
| Basal Leptin (pg/mL) | 4.14 | 2.77 | 1.39 |
| Basal Glucagon (pg/mL) ^A | 44.46 | 65.62 | 12.92 |

^AMain effect of week ($P < 0.05$).

Table 6.2. Least squares means for glucose disposal rate (GDR), insulin sensitivity measured by the hyperinsulinemic euglycemic clamp test (S_I EC), measurement of insulin sensitivity from the minimal model analysis (S_I MM), glucose effectiveness (SG), and acute insulin response ($\text{AIR}_{\text{glucose}}$) before and after calving.

| | Prepartum | Postpartum | SEM |
|---|-----------|------------|-------|
| GDR EC (mg/min/kg) | 2.00 | 2.96 | 0.51 |
| S_I EC ($\times 10^{-4}$ dL/min/kg $\cdot \mu\text{U}^{-1} \cdot \text{mL}^{-1}$) | 0.23 | 0.49 | 0.20 |
| S_I MM ($\times 10^{-4}$ min ⁻¹ / $\mu\text{U}/\text{mL}$) | 1.72 | 2.11 | 0.71 |
| SG (%/min) | 0.02 | 0.02 | 0.002 |
| $\text{AIR}_{\text{glucose}}$ ($\mu\text{U}/\text{mL}$) ^A | 488.1 | 276.3 | 95.5 |

^AMain effect of week ($P = 0.098$).

During early lactation, glucagon concentrations increased relative to the dry period to stimulate lipolysis and gluconeogenesis to provide the body with needed energy (de Boer et al., 1985). Increased glucagon concentrations were previously observed postpartum (Chapter 3: Effects of Prepartum Dietary Energy Level and Calcium Propionate Supplementation on Energy Metabolism in Transition Dairy Cows).

Block et al. (2001) reported decreased leptin concentrations after parturition as the result of negative energy balance associated with lactation. Leptin has also been shown to be regulated by insulin concentrations. Long-term increases in insulin increase leptin concentrations, and it is believed that a decrease in insulin concentrations around the periparturient period partially initiates the decreased blood leptin concentrations. It is hypothesized that in dairy cattle leptin concentrations will decrease as the result of lower insulin concentrations and the mobilization of body fat (Block et al., 2003). Decreased insulin concentrations shift glucose availability to tissues such as the mammary gland that do not rely on insulin to facilitate the movement of glucose into the cell and away from the muscle and adipose tissue which respond to insulin facilitated glucose uptake (Collier, 1984). As cows began lactating, there was a numeric decrease in basal glucose concentrations which coincided with the onset of negative energy balance.

There are several possible reasons for the lack of statistical differences found in the basal plasma insulin, glucose, or leptin concentrations or in these metabolic tests from pre-calving to post-calving. The transition period is difficult to study because physiological changes are occurring rapidly, and there is a large amount of variation in response to the metabolic events characteristic of the period (Drackley, 1999). By measuring the metabolic differences from prepartum to postpartum in only 5 cows,

individual variation may have affected the results; therefore, utilizing more animals may have minimized the variation contributed by the individuals. The demand for glucose, amino acids, and fatty acids is dramatically increased in the transition period (Overton and Waldron, 2004). Measuring these parameters earlier in the dry period before the onset of metabolic stresses such as impending calving, colostrogenesis, and milk production may have revealed a more dramatic metabolic change.

The least squares means for plasma glucagon, leptin, insulin, and glucose concentrations during two phases (basal and steady states) of an EC are presented in Table 6.3. The percent difference in leptin concentrations relative to basal after insulin infusion in an ITT in cows approximately 1 week prior to calving and 1 week after calving is presented in Figure 6.1. There was a significant interaction of week by time ($P = 0.085$) for the percent change in plasma leptin concentrations. Plasma leptin concentrations during an ITT in cows approximately 1 week prior to calving and 1 week after calving are presented in Figure 6.2.

It has been determined that body fatness alone does not control leptin secretions, as it may be effected by both insulin and glucose concentrations. It has been reported that plasma leptin concentrations are positively correlated with glucose and insulin concentrations in humans (Boden et al., 1996). The objective of measuring leptin during an ITT and EC was to isolate the changes in plasma leptin as a result of hyperinsulinemia with euglycemia and hyperinsulinemia with hypoglycemia.

Plasma leptin concentrations were not affected by the hyperinsulinemia induced by the ITT or by the EC. The duration of the EC was not long enough to induce an insulin

Table 6.3. Least squares means for plasma glucagon, leptin, insulin, and glucose concentrations during two phases (basal and steady states) of a hyperinsulinemic euglycemic clamp tests.

| | Prepartum | | Postpartum | | SEM |
|---------------------------------|-----------|--------------|------------|--------------|-------|
| | Basal | Steady state | Basal | Steady state | |
| Glucagon (pg/mL) ^{A,B} | 42.78 | 30.7 | 71.58 | 47.57 | 15.15 |
| Leptin (pg/mL) | 4.09 | 3.24 | 2.90 | 3.48 | 1.56 |
| Insulin (μU/mL) ^B | 1.62 | 210.84 | 2.24 | 384.1 | 84.2 |
| Glucose (mg/dL) ^{1 B} | 41.37 | 46.72 | 42.29 | 44.01 | 4.38 |
| Glucose (mg/dL) ² | 42.64 | 43.53 | 42.75 | 43.75 | 5.42 |

¹Glucose measured by commercial spectrophotometric kit (Sigma Tech. Bull. No. 315; Sigma Chemical, St. Louis, MO).

²Glucose measured by glucometer (Accucheck, Roche Diagnostic Corporation, Indianapolis, IN).

^A Main effect of week ($P < 0.05$).

^B Main effect of phase ($P < 0.05$).

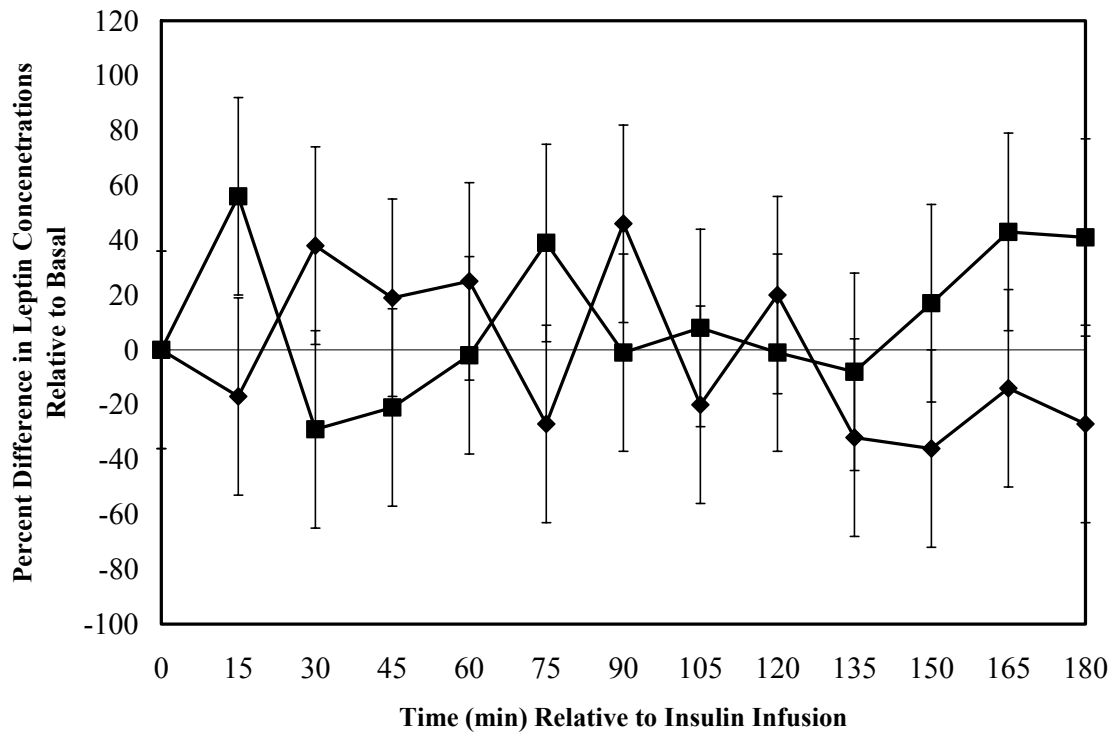


Figure 6.1. Percent difference in leptin concentrations relative to basal after insulin infusion in an insulin tolerance test in cows approximately 1 week prior to calving (♦) and 1 week after calving (■). Interaction of week by time ($P = 0.085$).

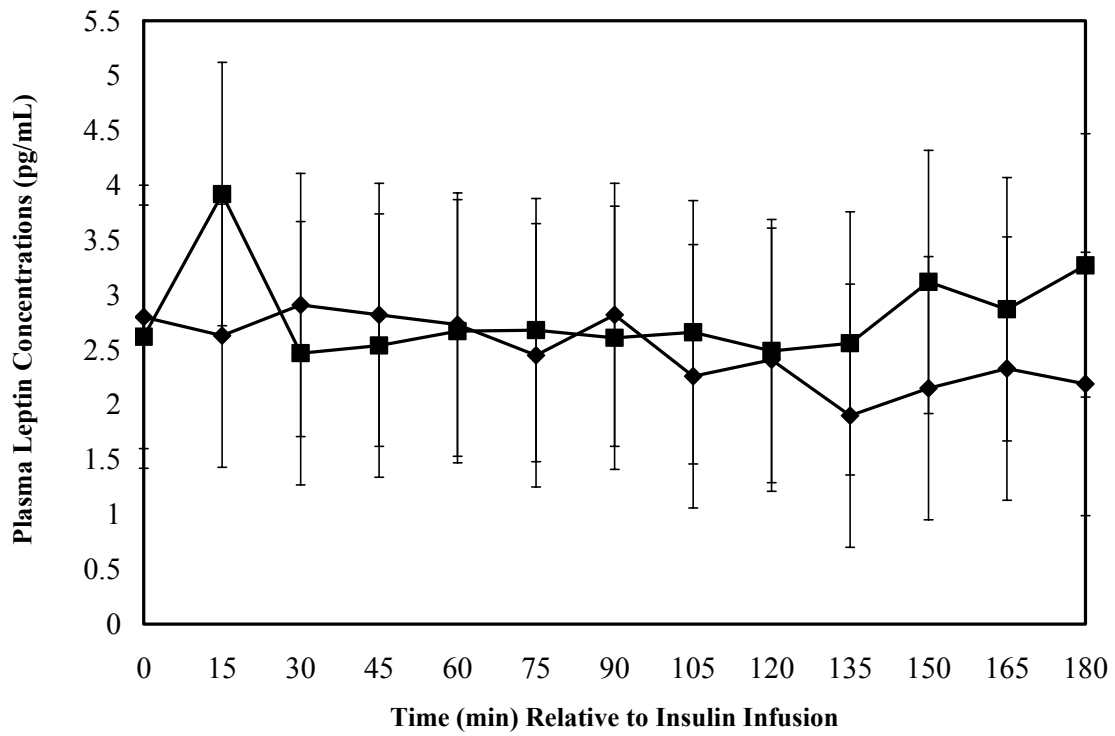


Figure 6.2. Plasma leptin concentrations during an insulin tolerance test in cows approximately 1 week prior to calving (♦) and 1 week after calving (■).

stimulated change in plasma leptin concentrations. Block et al. (2003) summarized that chronic hyperinsulinemia may be required to induce a leptin response in dairy cattle; therefore inducing hyperinsulinemia for a short period of time as in the ITT and the EC performed in these transition cattle may not have been sufficient to cause a change in leptin concentrations. Leury et al. (2003) reported that hyperinsulinemia during an EC conducted in late pregnancy and early lactation increased leptin concentrations in dairy cattle. These researchers reported that plasma leptin concentrations in both late pregnancy and early lactation cows began to rise 4-8 hours after beginning the EC and reached a plateau at 24 hours after initiation. There was a significant interaction of week by time ($P = 0.085$) for the percent difference in leptin concentrations relative to basal after insulin infusion in an ITT. Plasma leptin concentrations increased after the insulin infusion during the ITT in cows 1 week after calving but decreased at the same time point during the week before calving. The change in response led to a significant interaction of week by time for the percent change in plasma leptin concentrations. Previous research has reported that increased insulin concentrations cause an increase in plasma leptin after a greater period of time than the short-term hyperinsulinemia of an ITT. Perhaps during the postpartum period the animals were more responsive to the changes in insulin concentrations thus yielding a significant change from basal concentrations during the test.

The percent difference in glucagon concentrations relative to basal after insulin infusion in an ITT in cows approximately 1 week prior to calving and 1 week after calving is presented in Figure 6.3. The plasma glucagon concentrations during an ITT in

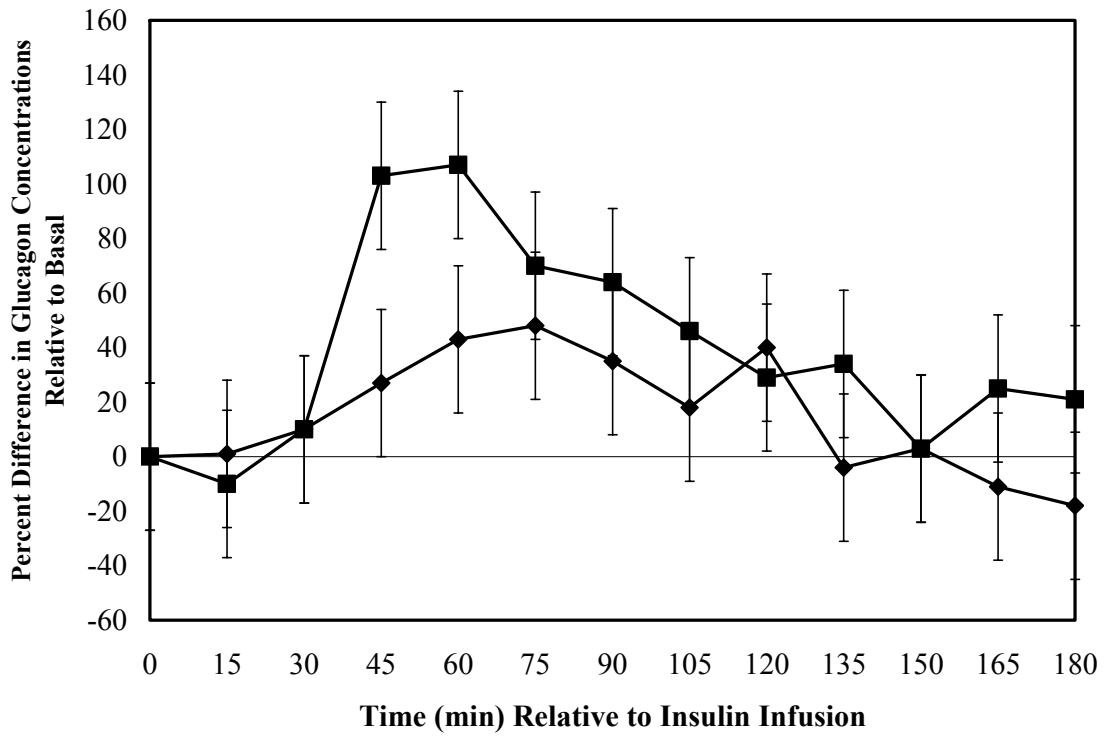


Figure 6.3. Percent difference in glucagon concentrations relative to basal after insulin infusion in an insulin tolerance test in cows approximately 1 week prior to calving (♦) and 1 week after calving (■). Main effects of time ($P < 0.05$) and week ($P < 0.05$).

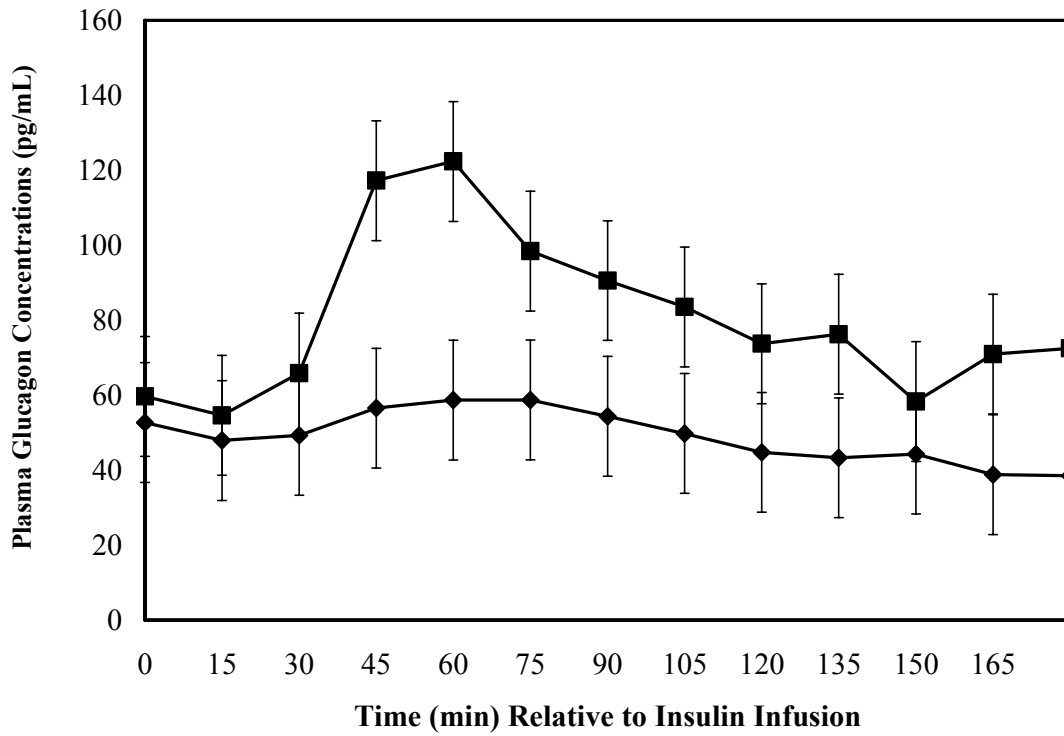


Figure 6.4. Plasma glucagon concentrations during an insulin tolerance test in cows approximately 1 week prior to calving (♦) and 1 week after calving (■). Main effects of time ($P < 0.05$) and week ($P < 0.05$).

cows approximately 1 week prior to calving and 1 week after calving are presented in Figure 6.4. There were main effects of time ($P < 0.05$) and week ($P < 0.05$) for the percent change in plasma glucagon concentrations and for the plasma glucagon concentrations. During the EC there were main effects of week ($P < 0.05$) and phase ($P < 0.05$). Basal plasma glucagon concentrations increased after calving, and glucagon appeared to be more responsive to the hypoglycemia induced in the ITT. The basal phase of the EC demonstrates the increased concentrations of glucagon after calving. The secretion of glucagon was effective in restoring glucose concentrations to basal concentrations in the ITT. During the EC, as insulin concentrations were increased and glucose was maintained close to basal ranges, glucagon concentrations decreased. This effect revealed that the exogenous glucose supplied during the EC was the primary energy source for the cells.

The percent difference in glucose concentrations relative to basal after insulin infusion in an ITT in cows approximately 1 week prior to calving and 1 week after calving is presented in Figure 6.5. Glucose concentrations during the ITT in cows approximately 1 week prior to calving and 1 week after calving are presented in Figure 6.6. There was a main effect of time ($P < 0.05$) for the percent change in basal glucose concentrations, and there were main effects of time ($P < 0.05$) and week ($P < 0.05$) for plasma glucose concentrations during the ITT. As presented in Table 6.1 and demonstrated by the effect of week, plasma glucose concentrations were lower after calving as cows entered the phase of milk production. Although there was not a significant interaction for week by time for the percent change in glucose or concentration, the numerical percent difference at the nadir of the glucose concentrations

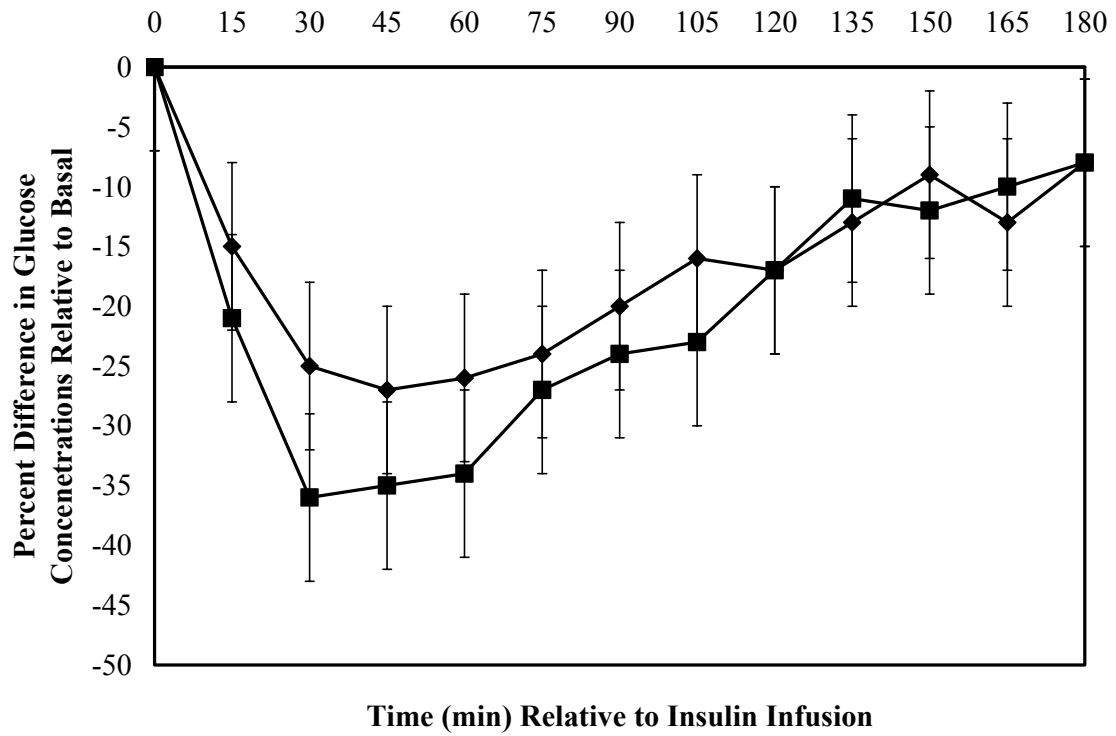


Figure 6.5. Percent difference in glucose concentrations relative to basal after insulin infusion in an insulin tolerance test in cows approximately 1 week prior to calving (◆) and 1 week after calving (■). Main effect of time ($P < 0.05$).

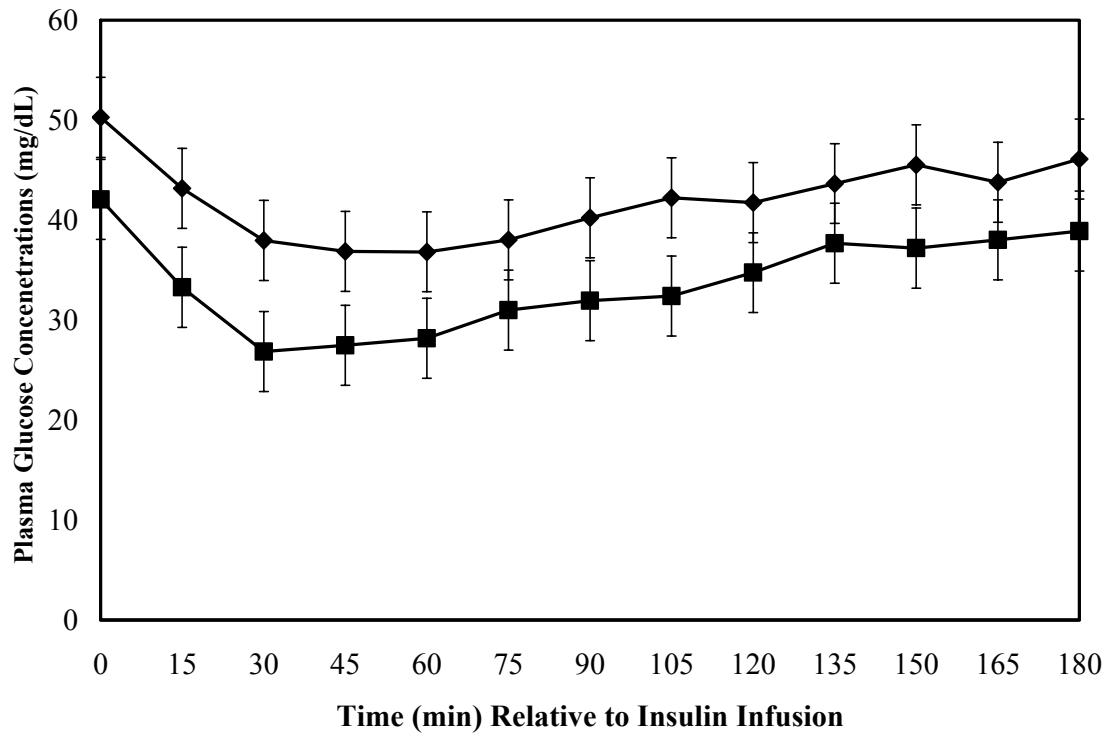


Figure 6.6. Plasma glucose concentrations during an insulin tolerance test in cows approximately 1 week prior to calving (♦) and 1 week after calving (■). Main effects of time ($P < 0.05$) and week ($P < 0.05$).

during the ITT was greater after calving, implying that they may have been slightly more responsive to insulin after calving than in late pregnancy. Insulin resistance was identified in both physiological stages as the blood glucose level did not decrease by 50% during the ITT (Kaneko, 1997). These data are consistent with the GDR, S_I from the EC and the S_I from the MM. During the EC the goal is to maintain plasma glucose within 10% of basal glucose while elevating insulin; therefore, basal glucose and steady state glucose should be equal. There was an effect of phase during the EC when glucose was measured via laboratory assay; however, no effect was measured when using the glucometer. At the farm, adjustments to glucose infusion rate by supplying exogenous glucose were based on concentrations obtained with the glucometer. Additionally, no effect of week was measured for plasma glucose concentrations during the EC.

The percent difference in insulin concentrations relative to basal during an ITT in cows approximately 1 week prior to calving and 1 week after calving is presented in Figure 6.7. Insulin concentrations during an ITT in cows approximately 1 week prior to calving and 1 week after calving are presented in Figure 6.8. There was a main effect of time ($P < 0.05$) for plasma insulin concentrations and for the percent change in plasma insulin in response to the infusion of insulin. During the EC there was a main effect of phase ($P < 0.05$) as insulin was increased from a basal state to the hyperinsulinemia.

Summary and Conclusions

There was a significant increase in basal glucagon concentrations after calving; however, there were no significant changes in plasma insulin, glucose, or leptin concentrations from prepartum to postpartum. Numerically they did decline in a manner that would be consistent with the onset of lactation. All of the tests that measured tissue

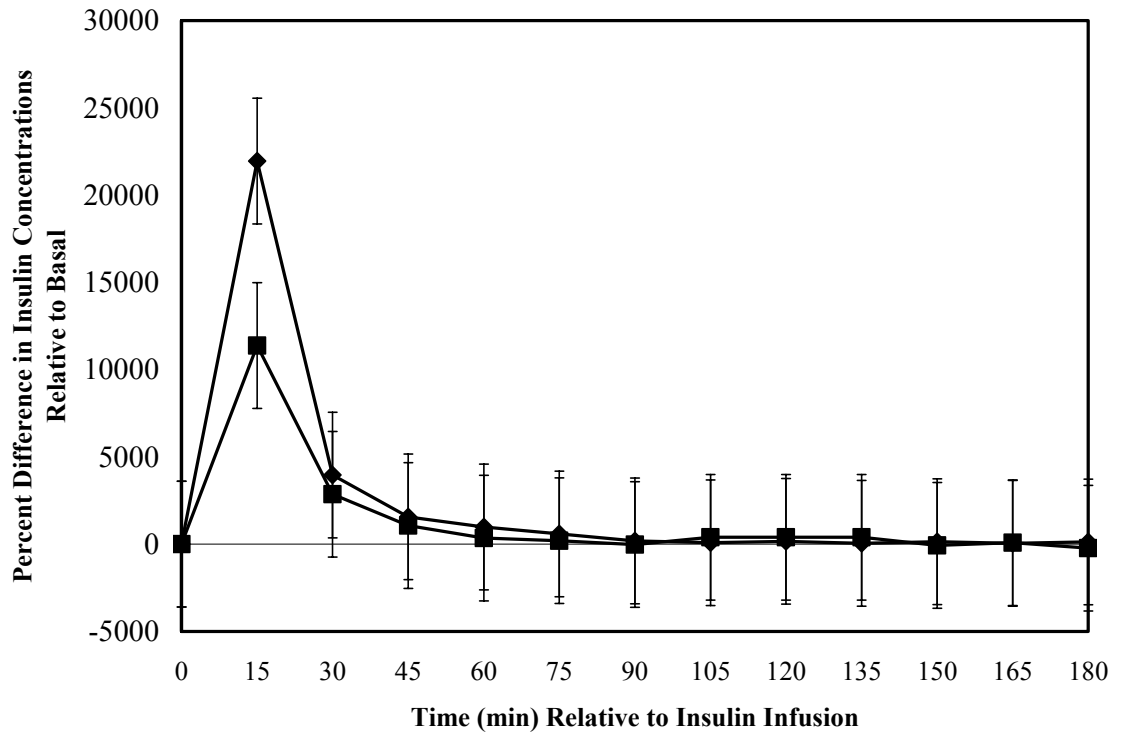


Figure 6.7. Percent difference in insulin concentrations relative to basal after insulin infusion in an insulin tolerance test in cows approximately 1 week prior to calving (♦) and 1 week after calving (■). Main effect of time ($P < 0.05$).

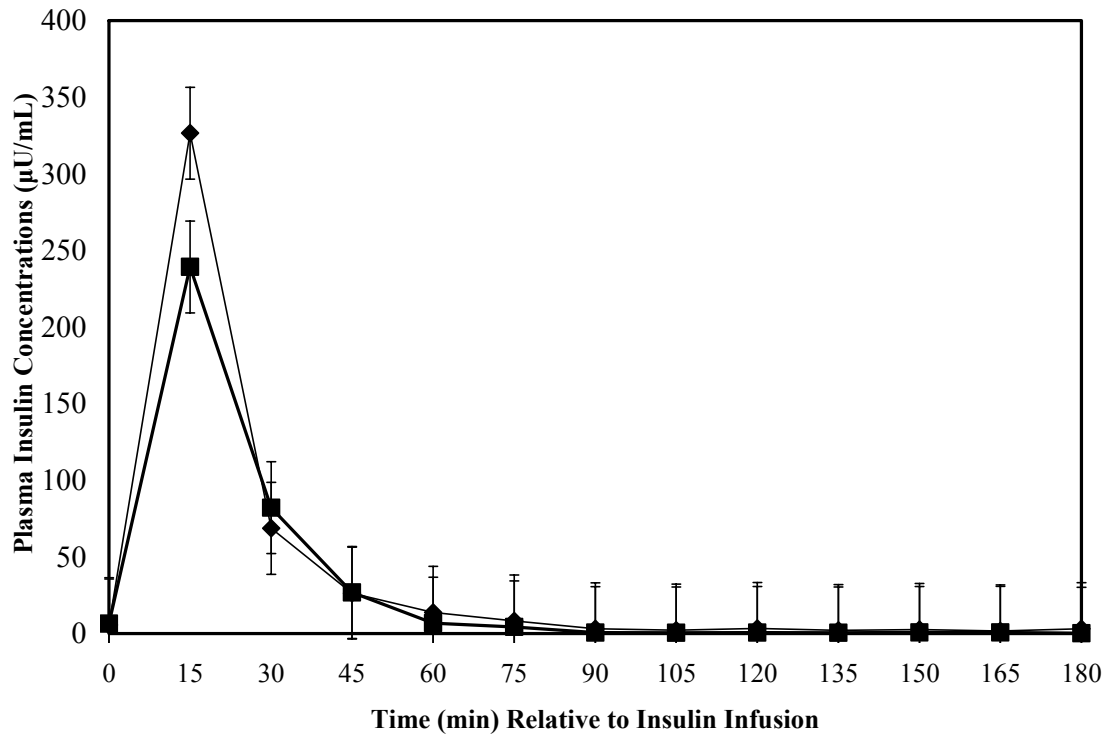


Figure 6.8. Plasma insulin concentrations during an insulin tolerance test in cows approximately 1 week prior to calving (◆) and 1 week after calving (■). Main effect of time ($P < 0.05$).

responses to insulin reflected a numerical increase in insulin after calving although insulin sensitivity was low both pre- and postpartum. These data provided further confirmation that the MM and EC are appropriate in measuring insulin sensitivity in dairy cattle as previously reported (Chapter: 5 Insulin sensitivity in lactating dairy cows and neonatal calves: Comparison of the Minimal Model and the Hyperinsulinemic Euglycemic Clamp). The acute hyperinsulinemia induced by the ITT or by the EC did not affect plasma leptin concentrations. Inducing hyperinsulinemia for a longer period of time may have increased plasma leptin concentrations; however, distinguishing the effects of hyperinsulinemia at euglycemia from hyperinsulinemia at hypoglycemia on leptin concentrations may not be possible because of the potential negative effects of inducing hypoglycemia for an extended period of time.

CHAPTER 7

CONCLUSIONS

The emphasis of this dissertation has been the hormonal regulation and utilization of plasma glucose in dairy cattle. The area of transition cow management as well as the regulation of metabolism throughout lactation has been studied. Drackley (1999) referred to the transition period as “The Final Frontier” because it is an area of metabolism that dairy cattle researchers understand the least. This period affects the entire lactation, and events that occur throughout a lactation cycle affect the health of cows during the transition period. There is a need for more information about the metabolism in transition cows as well as lactating cows.

The development of better nutritional plans as well as the incorporation of supplements to improve metabolism are important strategies that are being developed to improve performance during lactation. Chapter 3 demonstrated that Ca-propionate supplementation or prepartum dietary energy level did not affect glucose metabolism in the transition dairy cows studied. Valuable data were collected to demonstrate how hormone and metabolite concentrations changed to coordinate the metabolic homeostasis and homeorhesis associated with parturition and the onset of lactation.

The objective in Chapter 4 was to characterize metabolic events that occur from a young age to adults and throughout a lactation cycle. The study provided insight into areas which need further investigation such as conducting glucose metabolism tests in calves, growing heifers, transition heifers, lactating primiparous cows, and dry cows. A need for a relatively easy test for evaluating glucose metabolism has been demonstrated.

The Minimal Model computer program provides an estimate of many factors that effect glucose metabolism.

The study in Chapter 5 demonstrated that this evaluation was adequate in assessing insulin sensitivity in dairy cattle. The data in Chapter 6 provided further confirmation that the Minimal Model computer program is appropriate in measuring the tissue responses to insulin in dairy cattle. The objective of Chapter 6 was to measure the response of leptin concentrations to the acute hyperinsulinemia induced by an insulin tolerance test or by the hyperinsulinemic euglycemic clamp test. The short periods of hyperinsulinemia did not alter leptin concentrations. Inducing hyperinsulinemia for a longer duration of time may have increased plasma leptin concentrations; however, distinguishing the effects of hyperinsulinemia at euglycemia from hyperinsulinemia at hypoglycemia on leptin concentrations may not be possible because of the potential negative effects of inducing hypoglycemia for an extended period of time. The completion of the experiments in this dissertation has provided insight into the glucose metabolism of dairy cattle and has revealed areas that should be studied further.

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APPENDIX A

NONESTERIFIED FATTY ACIDS ASSAY

(REF: Wako Chemicals USA, Inc. NEFA C Kit, Wako Chemicals USA, Inc. 1600 Bellwood Road, Richmond VA 23237, U.S.A., modified according to Drackley et al., 1991)

Principle

Non-esterified fatty acids (NEFA) in serum, in the presence of adenosine triphosphate, magnesium cations, and CoA form the thiol esters of CoA known as acyl-CoA as well as the byproducts adenosine monophosphate and pyrophosphate. During the second part of the procedure, the acyl-CoA is oxidized by added acyl-CoA oxidase to produce hydrogen peroxide which in the presence of added peroxidase allows the oxidative condensation of 3-methyl-N-ethyl-N-(β -hydroxyethyl)-aniline with 4-aminoantiprine to form a purple colored adduct with an absorption maximum at 550 nm. From the optical density measured at 550 nm the amount of NEFA in the standard can be measured. Ascorbate oxidase is added to the reaction mixture to remove ascorbic acid from the sample.

Preparation of Reagent Solutions

A. Color Reagent A Solution

1. Add 10 mL of Diluent to one vial of dry Color Reagent A.
2. Mix gently by inverting the vial until the contents are completely dissolved.
3. Add 16 mL distilled water to reagent A and their diluent.
4. Mix gently by inverting the vial until the contents are completely dissolved. Store solution in the refrigerator (2-8° C) for up to 5 days.

B. Color Reagent B Solution

1. Add 20 mL of Diluent to one vial of dry Color Reagent B.
2. Mix gently by inverting the vial until the contents are completely dissolved.
3. Add 33 mL distilled water to reagent B and their diluent.
4. Mix gently by inverting the vial until the contents are completely dissolved. Store solution in the refrigerator (2-8° C) for up to 5 days.

Assay Procedure

1. Pipette
 - Sample tubes: 25µl plasma/serum
 - Blank: 25 µl distilled water
 - Standard: 25 µl of the standard solution
2. Pipette 0.4 mL of Color Reagent A Solution into all tubes.
3. Mix tubes well.
4. Incubate in 37 °C water bath for 10 minutes
5. Pipette 0.8 mL of Color Reagent B Solution into all tubes.
6. If using blanks to account for hemolysis, pipette 25 µl of plasma/serum into Serum Blank Tube.
7. Mix tubes well.
8. Incubate in 37 °C water bath for 10 minutes.
9. Remove from water bath and allow tubes to equilibrate with room temperature (5 min).
10. Read the optical density of all tubes at 550 nm versus the Reagent Blank.
11. Record the optical density of the Reagent Blank at 550 nm.

A.A.1. Nonesterified Fatty Acid Assay Standard Curve

| Level | NEFA STD (µl) | Deionized Water(µl) | Reagent A (mL) | Reagent B (mL) |
|-------|---------------|----------------------|-------------------|----------------|
| Blank | 0 | 25 | 0.4 | 0.8 |
| Std 1 | 12.5 | 12.5 | 0.4 | 0.8 |
| Std 2 | 25 | 0 | 0.4 | 0.8 |
| Std 3 | 37.5 | 12.5 | 0.4 | 0.8 |
| Std 4 | 50 | 0 | 0.4 | 0.8 |

Calculation

$$\text{Plasma NEFA concentration} = \frac{\text{A sample} \times \text{C standard}}{\text{A standard}} = \text{C sample (mEq/L)}$$

Where: A = Absorbance at 550m
 C= NEFA Concentration (mEq/L)

APPENDIX B

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 = $\frac{\text{Abs sample}}{\text{Abs standard}} \times C \text{ standard} = C \text{ sample (mg/dl)}$

APPENDIX C

PLASMA INSULIN RADIOIMMUNOASSAY

(REF: Diagnostic Products Corporation, Coat-A-Count Insulin Kit, Catalog Number TKIN5, 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 non-labeled insulin in the patient sample for 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 additional antibody-coated tubes, also in duplicate, for controls and test samples.
2. Pipet 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. Pipet directly to the bottom.
3. Add 1.0 mL of ¹²⁵I Insulin to every tube. Vortex.
4. Incubate for 18-24 hours at room temperature (15-28°C)
5. Decant thoroughly.
6. Count for one minute in a gamma counter.

A. C.1. Plasma Insulin Radioimmunoassay Standard Curve

| <i>Calibrator</i> | <i>Approximate $\mu\text{U}/\text{mL}$</i> |
|-------------------|---|
| A (MB) | 0 |
| B | 5 |
| C | 15 |
| D | 50 |
| E | 100 |
| F | 200 |
| G | 400 |

Preparation of Reagent Solutions

1. ^{125}I Insulin: Add 100 mL 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 6.0 mL of distilled or deionized water. Reconstitute the remaining calibrators B through G with 3.0 mL each of distilled or deionized water. Use volumetric pipets and mix by gentle 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 tubes and remaining tubes.
2. Subtract the average NSB counts from each average count.
3. Calculate the percentage of tracer bound [(Maximum Binding Counts / Total Counts) X 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 $\mu\text{U}/\text{mL}$ of insulin in the unknown samples by interpolation of the reference curve.

APPENDIX D

PLASMA GLUCAGON RADIOIMMUNOASSAY (LINCO RESEARCH, INC)

(REF: Linco Research, Inc., Linco Glucagon Radioimmunoassay Kit Catalog #GL-32K, 14 Research Park Drive, St. Charles , Missouri 63304, USA)

Principle

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

Procedure

Assay Set-up

Pipette 300 μl of assay buffer to the non-specific binding (NSB) tubes (3-4), 200 μl to the Maximum Binding Tubes (5-6), and 100 μl to tubes 7 through the end of the assay.

1. Pipette 100 μl of standards and quality control in duplicate.
2. Pipette 100 μl of each sample in duplicate.
3. Pipette 100 μl of glucagon antibody to all tubes except total count tubes (1-2) and NSB tubes (3-4).
4. Vortex, cover, and incubate overnight (20-24 hours) at 4 °C.

Next Day

1. Pipette 100 μl of ^{125}I -glucagon to all tubes.
2. Vortex, cover, and incubate overnight (20-24 hours) at 4 °C.

Second Day

1. Add 1.0 mL of cold (4 °C) precipitating reagent to all tubes except total count tubes (1-2).
2. Vortex and incubate 20 minutes at 4°C.
3. Centrifuge, 4°C, all tubes (except for total count tubes) for 20 minutes at 2,000-3,000 x g.
4. Immediately decant the supernatant from all tubes except the total count tubes.
5. Drain tubes for 15-60 seconds and blot excess liquid from lip of tubes.
6. Count all tubes in a gamma counter for 1 minute.

**A.D.1. Plasma Glucagon Radioimmunoassay (Linco Research, INC)
Standard Curve**

| Calibrator | Approximate pg/mL |
|------------|-------------------|
| A | 20 |
| B | 50 |
| C | 100 |
| D | 200 |
| E | 400 |

Calculations

1. Average duplicate counts for Total Count tubes, NRS tubes, Maximum Binding tubes and remaining tubes.
2. Subtract the average NRS counts from each average count.
3. Calculate the percentage of tracer bound [(Maximum Binding Counts/ Total Counts) X 100]. This should be 35-50%.
4. Calculate the percentage of maximum binding [%B/ B₀ = (Sample or Standard / Maximum Binding) X 100].
5. Plot the %B/ B₀ 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 glucagon in the unknown samples by interpolation of the reference curve.

APPENDIX E

PLASMA GLUCAGON RADIOIMMUNOASSAY (ICN PHARMACEUTICALS, INC)

(REF: Glucagon Kit, catalog Number 07-153101, ICN Pharmaceuticals, Inc., Diagnostics Division, 13 Mountain View Avenue, Orangeburg, New York, USA 10962-1294)

Principle

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

Procedure

1. Reconstitute reagents with the provided ultra pure water. Keep reagents between 0-4°C.
2. Set up assay in duplicate in glass test tube in an ice water bath.
3. Pipette 200 μL of ultra pure water into all tubes except total count tubes and NRS tubes.
4. Pipette 200 μL of sample, quality controls, or standard into the appropriate tubes.
5. Add 200 μL of anti-glucagon to all tubes except total count tubes.
6. Vortex tubes and incubate for 6 hours at 4°C.
7. Reconstitute Glucagon ^{125}I and add 100 μL to all tubes.
8. Vortex tubes and incubate for 16 hours at 4°C.
9. Add second antibody to all tubes except total count tubes.
10. Vortex and add 1 mL of cold distilled water to all tubes except total count tubes.
11. Centrifuge, 4°C, all tubes (except for total count tubes) for 15 minutes at 2,000-3,000 x g.
12. Immediately decant the supernatant from all tubes except the total count tubes.
13. Drain tubes for 15-60 seconds and blot excess liquid from lip of tubes.
14. Count all tubes in a gamma counter for 2 minutes.

A. E. 1. Plasma Glucagon Radioimmunoassay (ICN Pharmaceuticals, INC)

Standard Curve

| Calibrator | Approximate pg/mL |
|------------|-------------------|
| A | 25 |
| B | 50 |
| C | 100 |
| D | 250 |
| E | 500 |
| F | 1000 |
| G | 2000 |

Calculations

1. Average duplicate counts for Total Count tubes, NRS tubes, Maximum Binding tubes and remaining tubes.
2. Subtract the average NRS counts from each average count.
3. Calculate the percentage of tracer bound $[(\text{Maximum Binding Counts} / \text{Total Counts}) \times 100]$. 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 glucagon in the unknown samples by interpolation of the reference curve.

APPENDIX F

PLASMA THYROXINE RADIOIMMUNOASSAY

(REF: T₄ Monoclonal Solid Phase Radioimmunoassay; Catalog Number 06B56100-R6, ICN Pharmaceuticals, Inc., Diagnostics Division, 13 Mountain View Avenue, Orangeburg, New York, USA 10962-1294)

Principle

In radioimmunoassay (RIA), the antibody used should have an equal affinity for the standard and the analyte which is present in the serum. The unlabeled analyte competes with labeled analyte for the limited number of available antibody sites thereby reducing the amount of labeled analyte bound to antibody. The level of radioactivity bound is, therefore, inversely related to the concentration of the analyte in the sample or standard. After an adequate incubation period, the bound and free fractions are separated and the radioactivity quantitated. The ICN Pharmaceuticals [¹²⁵I] T₄ (Thyroxine) Solid Phase Radioimmunoassay used tubes coated with Monoclonal T₄ antibody.

Radioimmunoassay Procedure

1. Number an appropriate number of antibody coated tubes for the standard curve and clinical samples.
2. Add 25µL of the T₄ Standards and samples to the appropriate tubes.
3. Add 1.0 mL of ¹²⁵I T₄ Tracer to each tube. Vortex.
4. Incubate for 1 hour at room temperature (15-28°C).
5. Decant thoroughly. Allow the tubes to drain from each tube for at least one minute. Tap and blot any residual liquid from the tube rims.
6. Count the radioactivity bound to the coated tubes in sequence for one minute with a gamma counter.

A. F.1. Plasma Thyroxine Radioimmunoassay Standard Curve

| Standard | Concentration (T ₄ µg/dL) |
|----------|--------------------------------------|
| A | 0 |
| B | 2 |
| C | 4 |
| D | 8 |
| E | 12 |
| F | 20 |

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 tracer bound [(Maximum Binding Counts /Total) X100]. 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 µg/dL of T₄ in the unknown samples by interpolation of the reference curve.

APPENDIX G

PLASMA CORTISOL RADIOIMMUNOASSAY

(REF: ImmuChem™ Cortisol ; Catalog Number # 07-221105,
ICN Pharmaceuticals, Inc., Diagnostics Division, 13 Mountain View Avenue,
Orangeburg, New York, USA 10962-1294)

Principle

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

ASSAY PROCEDURE

A. ASSAY PREPARATIONS

1. Bring all standards, samples, controls, coated tubes, and CORTISOL- ^{125}I to room temperature prior to use.
2. Place the required number of anti-CORTISOL tubes in a test tube rack. Reseal the unused tubes in the plastic bag along with the desiccant and refrigerate.
3. Add all solutions in the quantities specified directly from the reagent vials.

B. ASSAY STEPS

1. Pipette 25 μL of each standard, control and patient sample into its respective coated tube.
2. Add 1.0 mL of the CORTISOL- ^{125}I to all tubes and vortex all the tubes.
3. Incubate for 45 minutes at $37 \pm 1^\circ\text{C}$.
4. Aspirate or decant the contents of the tubes. (If decanting, touch the rim of the tubes on absorbent paper before turning upright.)
5. Count the tubes in a gamma counter calibrated for ^{125}I .

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 tracer bound [(Maximum Binding Counts /Total) X100]. 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 $\mu\text{g/dL}$ of cortisol in the unknown samples by interpolation of the reference curve.

A. G. 1. Plasma Cortisol Radioimmunoassay Standard Curve

| Calibrator | Cortisol Concentration ($\mu\text{g/dL}$) |
|------------|---|
| A | 0 |
| B | 1.0 |
| C | 3.0 |
| D | 10.0 |
| E | 30.0 |
| F | 100 |

APPENDIX H

PLASMA LEPTIN RADIOIMMUNOASSAY

Principle

This assay is composed of a solid-phase radioimmunoassay with ^{125}I -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 μL of PBS-gel/0.05% TritonX-100 (Leptin Assay buffer) to the NRS, and BC tubes; add 100 μL to all other tubes except TC.
3. Pipette samples at 100 μL 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 μL of PBS-gel in tubes 1-7 and 600 μL of the standard in tube #8.
 - c. Transfer 300 μL of the standard from tube 8 to tube 7 and mix thoroughly.
 - d. Transfer 300 μL of the mixture in tube 7 to tube 6 and mix thoroughly.
 - e. Repeat the process through tube #1 which will have 300 μL because no other dilutions are made.
 - f. Pipette 100 μL 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 μL).
6. Pipette 200 μL of NRS #3 1:105 into NRS tubes.
7. To all other tubes except TC and NRS add 200 μL antibody solution (KLH-Red, 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.

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.

Day 5: ARGG precipitation

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

Day 7: Pour-off

11. Routine pour-off is two spins. Centrifuge, 4°C, all tubes (except for total count tubes) for 30 minutes at 2,000-3,000 x 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 $[(BC / TC) \times 100]$. This should be 35-50%.
4. Calculate the percentage of maximum binding $\%B / B_0 = (\text{Sample or Standard} / BC) \times 100]$.
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 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 |

VITA

Christie Cheatham Stanley was born on February 27, 1975, in Ruston, Louisiana, to Robert and Mary Cheatham. Christie graduated from Ruston High School in 1993 and began her studies at Louisiana Tech University. In 1997, Christie received her bachelor of science in animal science from Louisiana Tech University and in the spring of 1999 began graduate school in the area of dairy calf nutritional physiology. On April 21, 2001, she was married to Mike Stanley, and in May of 2001, she received the Master of Science degree in the Interdepartmental Program in Animal, Dairy, and Poultry Science. Upon completion, she entered a doctoral program at Louisiana State University and Agricultural and Mechanical College under the direction of Dr. Cathleen Williams in the Department of Dairy Science. During her doctoral program she assessed the use of the Minimal Model computer program in dairy calves and cows, characterized hormonal regulation of metabolic changes during the transition period, and assessed the use of calcium propionate as a supplement in transition period.