AN ABSTRACT OF THE DISSERTATION OF

Steven B. Puntenney for the degree of Doctor of Philosophy in Animal Science presented on May 9, 2006.

Title: The Effect of Prepartum Anionic Diets on Cortisol, Adiponectin, and Tumour Necrosis Factor-α Expression at Varying Levels of Body Mass Index in Preparturient Dairy Cows; Implications for Insulin Resistance

Twenty-six Holstein and 18 Jersey multiparous cows in a randomized block design were assigned by calving date and breed to a non-anionic prepartum diet (CTRL) or a treatment diet containing a commercial anionic salt supplement (Animate®). Control and treatment diets were formulated to a dietary anion-cation difference (DCAD) of 21.3 and -14.3 meq/100 grams.

The hypothesis that prepartum anionic diets and level of adiposity, measured by body condition score (BCS) and body weight, positively affect pre and postpartum dry matter intake (DMI) and energy balance were tested. We evaluated several markers of the mobilization of adipose tissue, including plasma β-hydroxybuterate (BHB), non-esterified fatty acid (NEFA), and glucose, and the serial expression of cortisol, adiponectin, tumour necrosis factor-alpha (TNF-α), and insulin, and their effect on energy balance. The effect of anionic prepartum diets on post-parturient metabolic disorders, body weight loss, and subsequent milk production were also investigated.

Cows were BCS scored prior to parturition and assigned to five prepartum groups. The effects of prepartum dietary treatment by BCS were evaluated for serum concentrations of cortisol, adiponectin, insulin, TNF-α, calcium and magnesium from day -21 prepartartum through d 21 days postpartum. No difference in prepartum or postpartum energy balance was observed based on prepartum diet. Cows receiving the anionic diet returned to positive energy balance faster than CTRL. DMI by day was 3.71 kg higher for Holsteins fed the anionic diet. Milk yield for Holsteins fed the anionic diet was 3.22 kg. more than CTRL. Holsteins fed the anionic prepartum diet produced 46.2 kg. ECM compared to 39.5 kg. ECM for the control diet. Milk production of Jersey cows did not differ based on prepartum diet. Plasma NEFA, cortisol, and adiponectin concentrations were unaffected by dietary treatment, nor by BCS. BHB concentrations were not directly affected by dietary treatment. Interactions for BCS by treatment were observed for BHB and TNF-α, with a crossover points at BCS 3.5. An interaction for plasma insulin by BCS was observed. No differences based on prepartum diet were observed in the incidence of milk fever, retained placenta, nor clinical mastitis in the first 21 days of lactation.

The Effect of Prepartum Anionic Diets on Cortisol, Adiponectin, and Tumour Necrosis Factor-α Expression at Varying Levels of Body Mass Index in Preparturient Dairy Cows: Implications for Insulin Resistance

by
Steven B. Puntenney

A DISSERTATION

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Presented May 9, 2006
Commencement June 2006

ACKNOWLEDGEMENTS

I would like to sincerely thank all of those who have supported me in this project. I would especially like to thank Dr. Patrick French, whose support over the past several years has been invaluable, not only with this project, but in my consulting business as well. I would also like to thank my committee for their direction and guidance through this journey, including Dr. Neil Forsberg, Dr. David Froman, Mr. Mike Gamroth, Dr. Bart Eleveld, and Dr. Luis Bermudez. In trying to maintain a balance between running a business and the time commitment of graduate school, I have had to accomplish most of the analytical work on weekends. The graduate students mentioned below went the extra mile in giving up their free time to assist me with this project, largely on weekends as well, and for this I am eternally grateful. I would like to especially thank a special friend, Dr. Yongqiang Wang, for all of his help and encouragement in the lab, and for not letting me give up on developing the adiponectin assay. There were a number of graduate students involved in the feeding and taking of blood samples, without whose help this project could not have been accomplished, including Dr. Michael DeGroot, Kathyrn Higgs, Jennifer Sumner, and Annette Buyserie. Lastly, I would like to thank Mark Keller for his guidance in the set up of the assays and for running down to the lab at odd hours on weekends to ensure that equipment was available and operating.

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

Page

LIST OF FIGURES

LIST OF FIGURES (continued)

LIST OF TABLES

The Effect of Prepartum Anionic Diets on Cortisol, Adiponectin, and TNF-α Expression at Varying Levels of Body Mass Index in Preparturient Dairy Cows: Implications for Insulin Resistance

INTRODUCTION

Milk fever or hypocalcemia in dairy cows, occurs when homeostatic mechanisms fail to maintain extracellular plasma calcium levels in a normal range of 9 - 12 mg/dl, resulting in symptoms of impaired nerve and muscle function. Mixtures of anionic salts including calcium chloride, ammonium chloride, ammonium sulfate, magnesium sulfate, calcium sulfate, magnesium chloride, and hydrochloric acid have been successfully used to improve calcium homeostasis and reduce susceptibility of periparturient cows to hypocalcemia. (Moore, *et al.* 2000, Goff, *et al.* 2003, Goff, *et al.* 1998). Decreases in dry matter intake (DMI) have often been exacerbated on anionic salt based diets, especially for primiparous cows are well documented in the literature (Moore, *et al.* 2000, Vagnoni, 1998, Joyce, *et al.* 1997, Horst, *et al.* 1994, Oetzel, *et al.* 1993).

Dry matter intake (DMI) reductions of 20 to 30% are characteristically observed in Holstein cows during the 48 hours immediately preceding parturition, even without the use of anionic salts. As a result, following parturition, animals often exhibit elevated plasma concentrations of non-esterified fatty acid (NEFA), liver triglycerides, and plasma β-hydroxybutyrate (BHB), as well as decreased liver glycogen, lowered insulin levels, and hypoglycemia (Grummer, *et al.* 1993, Baird, *et al.* 1982). Animals with acutely depressed intakes during the prepartum period may be more susceptible to immunosuppression, resulting in an increased incidence

of metritis and mastitis, as well as an incidence of post-calving disorders including dystocia, retained placenta, ketosis, and lameness, resulting in considerable cost to the dairy industry (Goff, *et al*. 2006). In a study of 8,070 multiparous Holstein cows, Grohn, et al. (1995) reported the incidence of milk fever at 6.0%, retained placenta at 8.0%, metritis at 8.0% ketosis at 5.0%, and displaced abomasums at 3.0%. Guard et al. (1996) estimated the cost of postpartum disorders at $344 per incidence for milk fever, retained placenta at $285.00, ketosis at $145, and displaced abomasums at $340. With a national dairy herd of 9 million cows, the potential losses to the U.S. dairy industry for postpartum disorders is well over $600 million per year.

Post-parturition DMI increases of 6 or more pounds have been reported in a number of field trials using anionic salt based prepartum diets, as opposed to non-anionic diets for multiparous cows. Intake increases for first calf heifers appear to be similar vs. their contemporaries on other close-up dry cow programs. A mode of action for anionic transition cow diets to alter DMI has not yet been biologically tested, however, I hypothesize that feeding a diet with a negative dietary anionic difference (DCAD) may alter the endocrine response related to the stimulation of appetite. While there have been reports of substantially elevations in prepartum DMI by reducing neutral detergent fiber (NDF) thereby increasing energy density of rations in field trials, it is not likely that intake is solely related to ration energy density. It is entirely possible that the observed responses are related to improved lipid metabolism and that the hormones involved in the mobilization of body lipid may play a key role in the observed intake response. Significant reductions in

retained placenta, metritis, and mastitis have been observed related to the feeding of prepartum anionic diets, possibly the result of an enhanced innate immune response to lowered blood NEFA.

Adiponectin is a 244 amino acid protein, secreted by fully differentiated adipocytes. High levels of adiponectin are found in human plasma (2.0 to 17 micrograms/ml)(Scherer, *et al.* 1995). Adiponectin has a modulatory effect on the expression of tumour necrosis factor-alpha (TNF-α) induced vascular cell adhesion via suppression of VCAM-1, E-selectin, and ICAM-1 (Ouchi, *et al.* 1999). Adiponectin has an inhibitory effect of on macrophage phagocytic activity, via suppression of TNF-α by blocking the c1qRp receptor. Adiponectin has a down-regulatory effect on macrophage TNF-α production in response to bacterial lipopolysaccharide (LPS) by attenuating TNF-α gene transcription, and is thought to be a negative regulator of the inflammatory response. (Yokota, 2000).

In murine models, Freubis, *et al.* (2001) showed that the treatment of mice with the adiponectin homolog Acrp30, resulted in significantly lowered plasma concentrations of NEFA, glucose, and triacylglycerol, via stimulation of increased fatty acid oxidation by muscle tissue, suggesting that adiponectin could play a role in the treatment of ketosis.

Insulin is the primary regulator of glucose uptake in skeletal muscle and adipose tissue, and also functions to inhibit hepatic glucose output. Approximately 75% of insulin-dependent glucose disposal occurs in skeletal muscle, with adipose tissue accounting for only a small percentage of the total. Fasting glucose levels are

regulated by insulin's blocking effect on hepatic gluconeogenesis and glycogenolysis, and by its stimulatory effect on glycogen synthesis (Salteil, *et al*. 2001). Release of insulin decreases rapidly in response to low blood glucose levels, with a concurrent increase in the amount of glucagon secreted, both by the pancreas. During prolonged periods of low blood glucose concentrations, receptors in the hypothalamus are stimulated to signal the adrenal glands to secrete epinephrine. The increase in epinephrine and plasma glucagon concentrations, along with the decrease in plasma insulin results in glycogenolysis and lipolysis of adipose tissue (Fahey, *et al.* 1988).

The placenta secretes increasing amounts of TNF-α to asymmetrically to the maternal circulation during late pregnancy to regulate energy partitioning to the developing fetus. TNF-α levels fall rapidly after parturition due to cessation of placental secretion (Kirwan, *et al.* 2002). TNF-α down-regulates insulin stimulated glucose uptake by several mechanisms. These include inhibiting tyrosine phosphorylation of the insulin receptor substrate (IRS-1), thereby inhibiting insulin receptor activity (Fruhbeck, *et al.* 2002), as well as by downregulating mRNA transcription of GLUT4 by up to 50% and the insulin receptor substrate (IRS-1) by up to 80% (Hirosimi, *et.al*, 2002, Stephens, *et al.* 1996). Insulin resistance often reverses following parturition due to the decreased TNF-α contribution from the placenta. Increased expression of TNF-α by the placenta occurs in late gestation and is correlated with an increased incidence of insulin resistance, especially in obese subjects (Kirwan, et al. 2002). This may partially explain the degree of dry matter

intake depression commonly observed in the week prior to parturition and initiation of excessive body weight loss (Grummer, *et al.* 1993, Baird, *et al.* 1982).

Glucose demand at the onset of lactation rises rapidly and outpaces the liver's ability to synthesize it, generally to 21 d post calving. Glucose synthesis is usually adequate from 21 d up to 3 d prior to calving (Piepenbrink, *et al.* 2000, Overton, *et al.* 2000). Glucose is synthesized by the liver from gluconeogenic precursors entering the TCA cycle, including propionic acid, glucogenic amino acids, lactate, and glycerol in ruminants. Excessive body reserves have been consistently associated with significant reductions in preparturient voluntary DMI in dairy cows (Hayirli, *et al.* 2002, Bareille, *et al.* 1996), thereby reducing the availability of gluconeogenic precursors. Alteration of metabolism to meet the increased glucose demand during negative energy balance, results in mobilization of large amounts of body fat and the release of NEFA into the blood. Up to 40% of milk fat composition during the first day of lactation originates from NEFA in the form of VLDL (very low density lipids). NEFA are utilized as a fuel for muscle tissue during periods of negative energy balance. The liver takes up NEFA proportionally to the supply, however the ability of the liver to utilize NEFA as fuel or export it to the mammary gland is limited, resulting in excessive fatty acid accumulations in the liver deposited in the form of triglycerides, a condition referred to as fatty liver. In cows with fatty liver, plasma lipoprotein concentrations decrease. Additionally, concentrations of apolipoproteins are significantly reduced, resulting in decreased transport of triglycerides out of the liver to mammary and steroidogenic tissues (i.e. the adrenal

cortex and corpus luteum). Fat accumulation in the liver impairs synthesis of glucose (Oikawa, *et al.* 1997). Insulin promotes uptake of glucose by cells of peripheral tissues and increases the activities of liver enzymes and carbohydrate utilization. When the supply of dietary carbohydrate is sufficient, fatty acids transported to the liver are esterified and exported to the tissues and mammary system (Sakai, *et al.* 1993). Susceptibility to fatty liver and ketosis has been associated with overfeeding and overconditioning in late lactation, and is especially evident where reproductive problems lead to extended lactations in dairy cows (Overton, *et al.* 2000).

Cortisol levels progressively increase beginning around the second trimester of pregnancy, via the effect of corticotrophin-releasing hormone of the hypothalmo-pituitary-adrenal axis (HPA). Glucocorticoids are involved in fetal organ development and in the process of initiation of labor at parturition (Trainer, *et al.* 2002). Burton et al also showed that parturition is initiated by a significant increase in blood cortisol levels between day 0 and day +1, but that this event was highly correlated (p-value = .003) with pre-translational down-regulation of neutrophil CD62L expression. CD62L is a type 1 transmembrane protein expressed on the surface of neutrophils allowing margination and trans-endothelial migration to infection sites in underlying tissue.

In this study, I tested the hypothesis that prepartum anionic diets may induce a carry-over affect on post-partum DMI and that increasing levels of adiposity, as measured by body condition score and body weight, may negatively impact

postpartum DMI, as well as calculated the effect of both pre and postpartum DMI on energy balance in Holstein and Jersey cows. I evaluated several markers for the mobilization of adipose tissue, including plasma BHB, NEFA, and glucose, as well as the serial expression of cortisol, adiponectin, TNF-α, and insulin, and their potential effect on energy balance. Lastly, I investigated the effect of anionic prepartum diets on post-parturient metabolic disorders, body weight loss, and subsequent milk production.

LITERATURE REVIEW

Ruminant Energy Metabolism

The primary source of glucose in ruminants is from hepatic gluconeogenesis. Glucose synthesis is increased in late pregnancy to supply the increasing energy demand of the rapidly developing fetus and subsequently to supply energy for the initiation of lactation. Glucose is the primary nutrient for the synthesis of lactose, the main precursor of milk production.

Ruminants derive the majority of their required protein from microbial protein. A large proportion of ruminant energy requirements are derived from the fermentation of plant fiber sources, primarily cellulose and hemi-cellulose. Non-structural dietary carbohydrate (NSCHO) sources of energy include sugars, starches, and pectins. NSCHO's undergo amylolytic and microbial fermentation in the rumen and are absorbed through the rumen epithelium as volatile fatty acids (VFA's), primarily as acetate, butyrate and propionate.

Glucose from starches, sugars, and pectins escaping ruminal fermentation pass to the lower gut, where active transport systems are required to move glucose into intestinal and renal cells. Due to the impermeable nature of the lipid bi-layer of cell membranes to carbohydrates, glucose transport systems utilizing energy derived from sodium co-transport are required to actively transport glucose into intestinal cells against a glucose concentration gradient (Shepard, *et al.*1999, Wright, *et al.* 1991).

In other cell types, glucose is transported via five homologous GLUT proteins, numbered 1 through 5, all having distinctly different substrate specificities, kinetics, and cell type distributions. GLUT-1 is a constitutive glucose transporter highly concentrated in brain tissue, erythrocytes, and epithelial cells. GLUT-2 primarily functions as a glucose sensor in pancreatic beta cells and also functions as a low affinity glucose transporter for the epithelial cells of the liver, kidney, and small intestine. GLUT-3 AND GLUT-4 are high affinity glucose transporters for skeletal and cardiac muscle cells, as well as for adipose tissue. GLUT-4 is insulin dependent. GLUT-5 has a very low affinity for glucose, and functions primarily as a fructose transporter for muscle, brain, kidney, sperm, and small intestinal cells.

In ruminants, propionate, from microbial degradation of fermentable fiber and non-structural carbohydrate, is the primary substrate for hepatic gluconeogenesis. Propionate absorbed by epithelial cells in the ruminal wall, is released into the portal venous blood supply, to be subsequently taken up in the liver (Block, *et al.* 1986, Curtis, *et al.* 1985). The supply of propionate available for

gluconeogenesis is highly correlated to the intake of fermentable substrate, especially fermentable non-structural carbohydrate. When propionate supplies are inadequate to meet energy demands, amino acids, including glutamine and alanine, lactate and glycerol can also be utilized as glucogenic precursors (Curtis, *et al.* 1985). Glucagon and insulin are the principal regulators of hepatic utilization of these glucogenic precursors. With the onset of lactogenesis, the mammary demand for glucose increases to 2.7 times that of the gravid uterus during late pregnancy. Demands for amino acids and fatty acids also increase by 2.0 and 4.5 times respectively (Bell, *et al.* 1995). Amino acids are derived primarily from the catabolism of skeletal muscle, indicated by a 25% reduction in skeletal muscle fiber diameter in dairy cows immediately after parturition (Reid, *et al.*1980).

Lipid Metabolism

Dietary fats in ruminant diets are primarily found in the esterified form. Fatty acids undergo lipolysis in the rumen, where they are enzymatically hydrolyzed into glycerol and free fatty acids (Jenkins, *et al.* 1993). Biohydrogentation of unsaturated fatty acids subsequently occurs via ruminal microbial activity, providing a limited hydrogen sink (1 -2% of metabolic hydrogen) for rumen microbes to dispose of excess hydrogen ions (Jenkins, *et al.* 1993). Fatty acids enter the small intestine as NEFAs in a predominantly saturated form, and are primarily converted to stearic acid (C18:0) (Baumann, *et al.* 2005). Fats bind with minerals and other particulate matter in the ruminal ingesta, becoming protonated and highly soluble after

encountering the low pH of the abomasum. Due to exposure to low abomasal pH's and coupled with the limited buffering capacity of the small intestine, complexed minerals and free fatty acids are released for absorption in the small intestine, as are the fatty acids themselves. As opposed to non-ruminants, lipid enters the ruminant small intestine primarily as highly saturated NEFA, rather than triacylglycerol. Ingested fatty acids are emulsified by bile salts in the lumen of the small intestine to form aggregated micelles (Baumann, *et al.* 2005). Pancreatic lipase secreted into the small intestine via the bile duct hydrolyzes any triacylglycerol escaping ruminal breakdown, yielding a mixture of free fatty acids, mono- and di-acylglycerols, and glycerol. Pancreatic lipase is responsible for the hydrolysis of dietary fats in ruminants. The action of pancreatic lipase digestion on large micelles of fatty acids results in association of fatty acids into smaller micelles capable of being absorbed across the cell walls of the epithelial cells of the villus of the jejenum. Absorption by the villus cells occurs via passive diffusion (Baumann, *et al.* 2005).

Intestinal cells, or enterocytes, resynthesize free fatty acids into triacylglycerols in the smooth endoplasmic reticulum utilizing the products of pancreatic lipase hydrolysis. Triacylglycerol is further complexed with transport proteins forming apolipoproteins, known as chylomicrons and various forms of cholesterols in the rough endoplasmic reticulum of enterocytes. Final processing of chylomicrons occurs in the Golgi apparatus of the enterocyte followed by release into the intercellular space via exocytosis. In ruminants, apolipoproteins are produced primarily in the mucosal cells of the small intestine, with minor levels of

these compounds being synthesized in the liver. Very low density lipoproteins (VLDL's) comprise the dominant form of transport apolipoproteins in ruminants, however several forms of phospholipids and cholesterol also play a significant role in metabolism (Byers, *et al.* 1988).

Following exocytosis and release into the intercellular space, lipoproteins enter the lamina propria through gaps in the basement membrane of the intestinal mucosa, subsequently passing into the lymph lacteal. Once they have entered the lymphatic system they are transported into the circulatory system via the thoracic duct.

The primary form of transport lipoprotein in ruminants is cholesterol, specifically VLDL, as opposed to non-ruminants where the chylomicron is the predominant form. Chylomicrons are large lipid droplets surrounded by a phospholipids monolayer, resulting from the absorption of unsaturated fatty acids, whereas the absorption of saturated fatty acids results in the formation of VLDL. Ruminants also differ from non-ruminants in the rate of lipid transport. In the case of ruminants the rate is quite slow and steady, as compared to non-ruminants where post-prandial surges in transport of lipid occurs.

Apoproteins complex with lipoproteins to transport triacylglycerol to the surface of various cells. Apo C-II prevents hydrolysis VLDL by liver cells, and also functions to activate lipoprotein lipase, a cell surface enzyme that initiates hydrolysis of transported triacylglycerol at the cell surface. Following this hydrolysis, fatty acids and glycerol are available to cross cell membranes of adipocytes, muscle, and

mammary cells either to be catabolized immediately for energy, stored in adipocytes, or conserved as essential fatty acids through preferential absorption and esterification as phospholipids (Byers, *et al.* 1988).

The role of the liver in the synthesis of fatty acids is insignificant in ruminants. NEFAs are converted to VLDL in the liver depending on the concentration in circulation. Depot fat in the form of triacylglycerol is stored in the adipocytes, with copious quantities accumulating in the case of obesity. Fat mobilization from adipose tissue involves the hydrolysis of triacylglycerol, yielding one molecule of glycerol and three molecules of NEFAs per molecule of triacylglycerol. The catabolism of triacylglycerol is hormonally initiated by β-corticotropin and epinephrine during stress and glucagon during fasting. These hormones bind to the adipocyte's plasma membrane receptor resulting in activation of adenosine 3',5'-monophosphate or cAMP. Cyclic AMP activates protein kinase A in turn phosphorylating triacylglycerol lipase, also known as hormone sensitive lipase. This is followed by the activation of diacylglycerol and monoacylglycerol lipases, completing the breakdown of triacylglycerol to free fatty acids and glycerol. The products of hydrolysis cross the adipocytes cell wall, where they complex with albumin for transport to other cells to be utilized as an energy substrate. Fatty acids diffuse across target cell walls in a concentration dependent manner. Once inside the cell, fatty acids are converted to fatty acyl CoA and transported to the mitochondrial membrane, where they pass through bound to the transport protein carnitine. They subsequently undergo β-oxidation to generate ATP and NADH. Glycerol released

into the circulation is removed by the liver for use as a gluconeogenic substrate (Byers, *et al.* 1988).

During periods of negative energy balance or fasting, such as in lactogenesis, a state of flux occurs in the citric acid cycle. As substrate in the form of carbohydrate or energy in the form of NAD^+ becomes limited, the generation of oxaloacetate through the citric acid cycle and subsequent generation of citrate synthase is inadequate for the oxidation of acetyl-CoA in mitochondrial cells, resulting in the accumulation of acetyl-CoA. In this case, an alternative pathway known as ketogenesis is utilized to generate energy for certain cell types including heart muscle, brain, and kidney. Ketogenesis results in the production of ketone bodies through a reversal of a thiolase reaction to convert two molecules of acetyl-CoA to acetoacetyl-CoA. This compound is further reduced by NADH to yield D-β-hydroxybutyrate and can be decarboxylated to form acetone (Byers, *et al.* 1988). A buildup of these compounds in the blood is associated with a reduction of dietary intake and the transition cow disorder known as ketosis.

Ketosis

Post–calving metabolic disorders, other than mastitis, have been identified as a major cost to the dairy industry. Ketosis is often secondary to retained placentas, metritis, mastitis, displaced abomasums, dystocia, and milk fever. Ketosis in dairy cows and other species is characterized by elevated levels of ketones in blood, urine,

and milk, measured as plasma BHB. Concurrent decreases in blood glucose and liver glycogen are also characteristic of this disorder. Depression of appetite is observed in clinically ketotic animals and associated BW losses of 2 or more body condition scores are not uncommon. First test day milk butterfat concentrations in excess of 5% butterfat (Holsteins), are generally indicative of ketosis. Animals affected by ketosis often exhibit noticeable rapid loss of BW, decreased DMI, lethargy or hyperexcitability. Metabolically, animals often have increased plasma NEFA concentrations, increased liver triglycerides, decreased liver glycogen, lowered insulin levels, hypoglycemia, and increased blood ketone production (Baird, *et al.* 1982). Milk production of dairy cows with acute ketosis will usually be markedly reduced, however subclinical cases often go unnoticed and untreated, and can be a significant hidden cost of production.

Through ketogenesis, ketone bodies are produced by liver mitochondria from fatty acids via the action of hormone-sensitive lipase on triacylglycerols stored in adipose tissue. Fatty acids undergoing β-oxidation yield acetyl-CoA. Acetyl-CoA may enter the TCA cycle for citrate synthesis may be directed away from the mitochondria to the cytosol and subsequently to the liver for gluconeogenesis or in the case of periods of negative energy balance, it can react with acetyl-CoA thiolase to forming acetoacetyl-CoA. Lyase reacts with acatoacetyl-CoA to form acetoacetate and recyclable acetyl-CoA. Acetoacetate is decarboxylated by D-3-hydroxybuterate to form the ketone body, D-β-hydroxybutyrate, and in the process regenerates NAD. Small amounts of acetone are also produced from acetoacetate through a

decarboxylation reaction. Some amino acids can also be used for ketogenesis entering as acetyl-CoA, 3-hydroxy-3-methylglutaryl-CoA (HMGCoA), acetoacetate, or acetoacetyl-CoA (Salway, *et al.* 1999).

With negative energy balance, as glycogen reserves are depleted, ketone body production from fatty acids by the liver increases. The brain cannot directly use fatty acids as an energy source, however ketone bodies can be used to generate ATP. This provides a sparing effect to the further breakdown of muscle tissue for glycogenolysis to produce glucose. D-β-hydroxybuterate yields the equivalent of 21.25 ATP and acetoacetate generates 18.75 molecules of ATP from oxidation via the TCA cycle. The rapid production of D-β-hydroxybuterate and acetoacetate results in high concentrations of protons (H+) which overwhelm the buffering capacity of the blood resulting in severe decrease in blood pH (metabolic acidosis)(Salway, *et al.* 1999).

Significant reductions in DMI and negative energy balance in early lactation of dairy cows have been associated with metabolic disorders, fatty liver syndrome, and ketosis. Glucose demand at the onset of lactation rises rapidly and frequently outpaces the liver's ability to synthesize it, generally beyond 21 d post calving. Glucose synthesis is normally adequate from 21 d through 3 d prior prepartum (Piepenbrink, *et al.* 2000). DMI typically drops by as much as 30% during the three d prior to calving.

Glucose is normally synthesized by the liver from precursors, including propionic acid, glucogenic amino acids, lactate, and glycerol. Alteration of metabolism to meet the inadequate glucose demand during negative energy balance, results in mobilization of large amounts of body fat and release of NEFA into the

blood. NEFAs make up about one third of the milk fat, with the balance originating from lipoprotein triglyceride during the first d of lactation. The liver takes up NEFA proportionally to the supply, however the ability of the liver to dispose of it as fuel or export it to the mammary gland is limited, consequently NEFAs accumulate in the liver as fat in the form of triglyceride during early lactation. In cows with fatty liver, plasma lipoprotein concentrations decrease. In addition, concentrations of apoproteins are also reduced, which are essential for the transport of triglycerides out of the liver to mammary and steroidogenic tissues, such as the adrenal cortex and corpus luteum. The net result is hepatic fat accumulation and the impairment of liver function to synthesize glucose (Overton, *et al.* 2000). Insulin promotes the uptake of glucose by cells in peripheral tissues and increases the activitation of liver enzymes for carbohydrate utilization. If the supply of carbohydrate is sufficient, fatty acids transported to the liver are esterified and are exported to the tissues and mammary system (Sakai, *et al.* 1999).

Drackley et al. (1991) hypothesized that normally neither feed restriction nor butanediol production, an important precursor to ketone bodies, induced fatty liver or ketosis, and further suggested that ketosis is secondary to other metabolic stressors. Curtis et al. (1985) calculated odds ratios for the occurrence of metabolic disorders and their relation to ketosis and reported that cows with retained placentas were 5.7 times more likely than their cohorts to develop metritis and 16.7 times more likely to develop ketosis. Cows with parturient paresis (milk fever) were 4 times more likely to have retained placentas, and 23.6 times more likely to be predisposed to ketosis. The

relationship between immune cell function and ketosis has been investigated. Klucinski et al. (1988) reported that β-hydroxybutyrate had an inhibitory effect on in vitro phagocytic activity of blood and milk macrophages and polymorphonuclear leukocytes. Kremer et al. (1993) found that severity of coliform mastitis increased in experimentally infected ketonemic cows.

Monitoring transition cow feeding programs and milk yields during early lactation, between the periods three wks prepartum and the three wks postpartum, has been beneficial in reducing the incidence of ketosis. Early intervention and treatment greatly minimizes the impact of the ketosis. Overton et al. (2000) observed that rodents and birds fed choline deficient diets developed fatty liver because critical phospholipids needed for VLDL synthesis are directly derived from choline. Ruminants synthesize choline in the rumen, however, it is speculated that with reduced feed intake prepartum, choline is not synthesized in sufficient amounts for adequate liver conversion of VLDL from NEFA. Supplemental rumen protected choline in transition cow diets has been shown to increase liver disposal of NEFA and reduce triglyceride accumulation in the liver (Table 1). Increasing amounts of rumen-protected choline (0, 45, 60, and 75 grams per day) were fed to transition dry cows from 21 d prior to calving to 21 d post-calving to determine the rate of utilization of NEFA and the rate of accumulation of fat in the liver. Rate of NEFA utilization for fuel, was not affected by the treatment, however accumulation of NEFA and triglyceride in the liver decreased with increasing level of choline supplementation. Triglyceride to glycogen ratios are typically used as a marker of ketosis susceptibility

(Drackley, *et al.* 1991). Triglyceride to glycogen ratios were reduced in the aforementioned trial, indicating reduced susceptibility to ketosis (Table 1). The conclusions from this trial would indicate that supplemental choline may modulate the capacity of the liver to export NEFAs as triglycerides in the form of VLDL (Overton, *et al.* 2000).

Table 1. Composition of liver samples of cows fed supplemental rumen-protected choline from 21d prepartum to 63d postpartum.

	Rumen-protected choline, g/d				
Level Fed	0	45	60	75	SE
Triglyceride% (wet wt.)	15.60	14.9	13.20	11.40	2.21
Glycogen(wet wt.)	0.79	0.81	1.12	1.40	0.21
Triglyceride:Glycogen	19.70	18.40	11.80	8.20	

(Piepenbrink and Overton, 2000)

Susceptibility to fatty liver and ketosis has been associated with overfeeding and overconditioning in late lactation (Overton, *et al.* 2000). Typically, this situation results from cases where cows are not moved to lower energy diets soon enough in late lactation to prevent overconditioning (Moore, *et al.* 1997). Adjusting ration energy and protein levels in late lactation and monitoring body condition scores to a target of 3.5 at dry off, so that consumption of energy and protein is not in excess of that required for the reduced milk production associated with late lactation, is the suggested protocol for preventing overconditioning (Maruksfeld, *et al.* 1985). The use of bovine somatotropin (BST) can facilitate control of excessive BW gain in late lactation.

In an unpublished trial by Sanchez in 2000, feeding protected fat in the transition period at ¼ lb per head per day reduced the incidence of ketosis and reduced the number of d to 1st breeding. In the first trial, 600 Holstein cows were fed ¼ lb. of rumen protected for the 21 d prior to calving. Peak milk increased by 4.7 lbs. per day and ketosis incidence was reduced from 12.5% in the control group to 4.5% in the rumen protected fat group. In a second trial, 900 Holstein cows fed ¼ lb. of rumen protected fat for the 21 d prior to calving, showed a peak milk increase of 1.4 lbs. per head per day versus the control group, a reduction in the incidence of ketosis from 5.2% in the controls vs. 4.5% in the rumen protected fat fed group, and a reduction in d to first breeding from 74 d in the control group to 72.5 d in the treatment group.

The use of anionic salts in the transition ration, has at times been beneficial in reducing the incidence of milk fever, and is well documented in the literature (Block, *et al.* 1986, Goff, *et al.* 1998, Sanchez, *et al.* 2000). Retained placenta, dystocia, metritis, prolapsed uterus, and displaced abomasums have also been effectively reduced with the use of anionic salts, particularly with the improved palatability of the new generation of anionic supplements. Secondary disorders may be due to poor muscle contractions and poor appetite associated with insufficient blood calcium (Curtis, *et al.* 1985).

Propylene glycol has been traditionally used as the standard prevention and treatment for ketosis. Propylene glycol escapes destruction in the rumen and serves as a precursor to glucose. Niacin fed to pre and post-partum cows at the respective levels of 6 and 12 grams per day has been shown to increase blood glucose levels and lower

free fatty acids (Dufva, *et al.* 1983). In a study by Shpigel et al.(1996), clinically ketotic cows treated with 40 mg of dexamethasone and 500 ml of a 50% glucose solution, had increased blood glucose concentrations of 150% of pretreatment and reductions of β-hydroxybuterate and acetoacetate to 83 and 48% of pretreatment levels (Shpigel, *et al.* 1996). Sakai et al. (1993), showed that simultaneous treatment of ketosis with insulin and glucose for six days was also an effective means of increasing blood glucose levels.

Practical implications in addressing the ketosis problem in dairy cows involve controlling the supply of NEFA to the liver and optimizing the capacity of the liver to dispose of, rather than accumulate NEFA as fat in the liver. Most strategies will be related to maintaining proper ration protein and energy levels, and appropriately monitoring and addressing BW status of late lactation and dry cows. Maximization of DMI in transition cows, especially in the 72 hours prior to calving, has been beneficial in preventing ketosis and other post-calving disorders (Hammon, *et al.* 2006). Overcrowding in the prefresh and transition pens can reduce DMI as well. Poor reproductive performance can lead to extended lactations and will add to the overconditioning problem. As stated earlier, metritis predisposes an animal to ketosis, and dirty calving areas, dystocia, and calcium status of the animal can all be correlated to the incidence of uterine infections. Placing cows on low energy rations for extended periods post parturition can exacerbate negative energy balance and predispose animals to ketosis.

. In a study of New York State dairies, Grohn (1997) estimated the incidence of ketosis to be 5% of cows freshening with an associated 7.6% loss in milk yield. Guard (1996) estimated the cost per incidence of ketosis at $145, including the cost of labor, culling, veterinary assistance and drugs. Controlling BW loss and the maximization of the DMI of transition cows are paramount to minimizing losses of profitability from ketosis

Milk Fever

Milk fever or hypocalcemia affects approximately 6 to 9% of the cows in the United States (Joyce, *et al.* 1997, NAHMS, 1996) and occurs when homeostatic mechanisms fail to maintain extracellular plasma calcium levels in the normal range of 9 to 12 mg/dl. Colostrum contains 2.3 grams of calcium per liter and a cow producing 10 liters would draw down the serum calcium pool by 23 grams, which could rapidly deplete the plasma pool should the homeostatic mechanisms of intestinal absorbtion of dietary calcium, calcium release from bone, and decreased urinary calcium turnover be inadequate to maintain plasma calcium levels (Joyce, *et al.* 1997). The sudden onset of lactation and the production of colostrum at parturition often results in significant decreases in plasma calcium, typically in the to 2 - 6 mg/dl range, resulting in symptoms of impaired nerve and muscle function. With the high calcium demand for the production of colostrum, the animal's ability to increase the rate of dietary calcium absorption and to concurrently increase mobilization of skeletal reserves of calcium is significantly outpaced by the

efficiency of the mammary gland to remove calcium from the plasma pool. Hypocalcemia generally occurs within 72 hours of parturition and is simply the result of an inability to replace the large losses of extracellular calcium at the onset of lactation, resulting in clinical symptoms of lateral recumbancy, inappetance, an inability to urinate or deficate, tetany, coma, and death if left untreated (Goff & Horst, 1997).

Parathyroid hormone (PTH) is released in response to a drop in plasma calcium concentrations, resulting in decreased urinary excretion and increased renal absorption of calcium. Depending on the magnitude of the calcium deficit in the extracellular plasma pool, continued PTH secretion will result in mobilization of skeletal calcium. Bone reserves of calcium exist as soluble calcium in solution surrounding osteoclasts. The majority of skeletal calcium is tightly bound in the bone collagen matrix. Secretion of PTH signals osteoblasts to release enzymes and acids onto the bone matrix resulting in the digestion, release, and transport of calcium into the extracellular fluid. (Goff, *et al.* 1991).

PTH also stimulates the activation 1,25-dihydroxycholecalciferol to vitamin D_3 and its release by renal tissues, thereby increasing active intestinal transport of calcium. Intestinal absorption of calcium by epithelial cells generally occurs via passive diffusion, however when calcium demand is high can switch over to active transport. This is accomplished through activation of vitamin D_3 dependent calcium binding protein expressed on the epithelial cell surface, and Ca:Mg ATPase

dependent pumps capable of moving calcium against a 1000 fold concentration gradient (Goff, *et al.* 1991).

Hypercalcemia is regulated by the hormone calcitonin, which has a negative effect on the release of calcium by osteoclasts. Similarly, excess plasma vitamin $1,25(OH)_2D_3$ concentrations result in renal stimulation of $23(OH)_2D_3$ and $24(OH)_2D_3$ hydroxylases, which catabolize and inactivate vitamin 1, 25 $(OH)_2D_3$. Expression of vitamin D_3 receptors decreases significantly around parturition, however does not appear to differ between cows with or without hypocalcemia. Goff et al. reported that receptor recovery to preparturient levels was slower in hypocalcemic cows (Goff, *et al.* 1991).

Metabolic alkalosis induced by an abundance of dietary cations, particularly potassium and sodium, impairs the cells ability to respond to PTH. PTH levels are normally increased in response to declining plasma calcium levels, however the responsiveness of cells to PTH is significantly impaired during periods of metabolic alkalosis due to priority given to maintenance of an arterial pH of 7.4. The feeding of diets high in cations may impair the animal's ability to maintain calcium homeostasis (Goff 1991).

Anionic Diets

Transition cow diets tend to be forage based and therefore typically can be high in cation content, particularly K^+, Ca^{2+} and Na^+, resulting in a state of mild alkalosis. Metabolic alkalosis reduces tissue responsiveness to PTH, resulting in reduced activation of 1α-hydroxylase necessary for the conversion of 25-(OH)D_3 to the active form 1,25-dihydroxycholecalciferol or vitamin D_3. It has been suggested by Goff and Horst in 1997 that the underlying cause of milk fever is decreased responsiveness to PTH in both skeletal and renal tissue during metabolic alkalosis, significantly disrupting calcium homeostasis.

Dietary cation-anion difference (DCAD) is calculated by difference in the milliequivalents of [(Na + K) – (Cl + S)] / 100 g of DM. Meta-analysis for dietary factors influencing the risk of milk fever showed a negative correlation for sulfur and a positive correlation for sodium, suggesting that individually these elements could impact the strong ion difference thereby increasing the risk of milk fever. Surprisingly, this analysis also showed that extremes in dietary calcium (<0.4% or >2.0%) resulted in the reduction in the risk for milk fever, and that dietary phosphorus and calcium:phosphorus ratio had no effect on the incidence of milk fever (presumably when fed at moderate levels) (Oetzel, *et al.* 1991).

Decreasing the DCAD during the last 3 wk prior to parturition alters acid-base status improving Ca homeostasis by increasing tissue PTH responsiveness and markedly reducing hypocalcemia (Moore, *et al.* 2000, Goff, *et al.* 2003, Joyce, *et al.* 1997). Anionic salts have been widely used to decrease DCAD in preparturient

diets. Anionic salts including calcium chloride, ammonium chloride, ammonium sulfate, magnesium sulfate, calcium sulfate, magnesium chloride, and hydrochloric acid have been successfully used for this purpose (Moore, *et al.* 2000, Goff, *et al.* 2003, Goff, *et al.* 1998). Decreases in DMI on some anionic diets, especially for primiparous cows are well documented in the literature (Moore, *et al.* 2000, Vagnoni, 1998, Joyce, *et al.* 1997, Horst, *et al.* 1994, Oetzel, *et al.* 1993). Experiments quantifying the relative acidifying capacity of various sources of dietary anions revealed that chloride sources are superior to sulfate sources when measured by their ability to reduce urine pH and blood standard base excess (Goff, *et al.* 2004). In an experiment using HCl as the acidifier, Goff and Horst (1998) showed increased DMI. Commercial mixtures of anion sources have been developed to address the safety issues surrounding feeding HCL, as well as to address the palatability issues related to crude anion sources.

Moore (2000) also reported increased serum NEFA concentrations, increased liver tryglycerides and no improvement in calcium metabolism in primiparous cows fed anionic diets. Additionally, reduced prepartum DMI has been shown to be a risk factor for subclinical ketosis and displaced abomasum (LDA), though no association of clinical milk fever to LDA has been observed (LeBlanc, *et al.* 2004). Most studies have utilized diets between -10 meq/100g and -15 Meq/100 g in treatment diets to induce an adequate metabolic acidosis to significantly reduce hypocalcemia (Overton, *et al.* 2004, Moore, *et al.*2000, Joyce, *et al.* 1997, Goff, *et al.* 1994,).

When anionic diets are fed, there is a measurable and predictable decrease in urine pH. Moore et al. (2000) observed that a DCAD of -15 Meq/100 g was effective in preventing most cases of hypocalcemia and was associated with a urine pH of 6.0. When a DCAD of 0 Meq/100g was fed urine pH dropped from 8.0 to 7.3 and was less effective in reducing the incidence of milk fever than a DCAD of -15 Meq/100g. Most researchers suggest feeding an adequate amount of anionic salts to reduce urine pH to 6.5. Lennon and Piering (1970) found that urine organic acid excretion was significantly increased following ammonium chloride induced metabolic acidosis and glucose administration in human subjects. Decreased urinary reabsorption of calcium and magnesium were also observed as well as a subsequent increase in their excretion. During induced acidosis utilizing oral doses of ammonium chloride only, the researchers observed increased urinary calcium, but not magnesium excretion. Oral glucose administration in the absence of NH_4Cl also yielded an increase in urinary net acid excretion, and increased urinary calcium and magnesium excretion. With a glucose load administered during NH_4Cl induced acidosis, an additive effect on calcium excretion was observed, however magnesium excretion did not increase beyond what was observed with NH_4Cl treatment alone. Urinary sodium excretion tended to decrease at 4 hours following NH_4Cl dosing, indicating increased renal reabsorption. Vagnoni (1998) observed decreased urine pH values and bicarbonate excretion, as well as increased urine ammonia concentrations by 2.2 mM when anionic diets were fed to Holstein cows. Blood pH in this trial did not differ from control diets. This trial also showed reduced ruminal

VFA concentrations for cows consuming an ammonium chloride based anionic diet. Both ammonium chloride induced acidosis and glucose ingestion during metabolic acidosis have both been shown to increase urinary calcium and magnesium excretion in bovine models. Hypercalcuria was also demonstrated in trials by Joyce and Sanchez in 1997, and it was concluded to be the result of increased calcium flow through the exchangeable calcium pool. When anionic diets are fed to prepartum lactating cows, dietary calcium concentrations should be increased to between 120 and 150 grams per d to account for the increased urinary calcium loss.

In contrast to the use of anionic diets, removal of cations from the diet has been shown to be partially successful in reducing the incidence of hypocalcemia. Horst et al. (1997) suggest that reducing dietary potassium levels to below 1.1% in the diet would preclude the necessity of using anionic salts in the diet to prevent hypocalcemia. Other strategies that have been used to limited success for the reduction of milk fever have been to reduce dietary calcium intake to below 20 grams per day, which is significantly below the calcium requirement of the animal. The negative calcium balance stimulates the release of PTH and activation of $1,25(OH)_2D_3$ (Horst, *et al.* 1997). Feeding diets high in dietary phosphorus (>80g) has also been found to increase the incidence of milk fever by inhibiting the enzyme 1α-hydroxylase from activating vitamin D_3, in turn compromising intestinal absorption of calcium (Tanaka, *et al.* 1973).

TNF-α and the Endocrine System

TNF-α is a cytokine produced by activated macrophages and neutrophils, as well as a variety of other cell types including T-cells and fibroblasts. A large proportion of TNF- α is also produced by the stromovascular cells making up the protein fraction of the adipose tissue matrix (Fain, *et al.* 2004). Circulating levels of this protein tend to be significantly elevated in obesity and non-insulin dependant diabetes mellitus (NIDDM). This cytokine has direct effects on adipose tissue including impaired preadipocyte differentiation, induction of apoptosis, suppression of lipogenesis, and the stimulation of lipolysis (Prins *et al.*, 1997, Warne *et al.*, 2003).

In the case of macrophages, TNF-α is cleaved from a 25 kDa transmembrane protein to a shorter fragment and released into circulation in response to the presence of the lipopolysaccharide (LPS) cell wall component of gram negative bacteria. TNF-α activates macrophages to secrete inflammatory cytokines in response to pathogens and is responsible for signaling the upregulation of endothelial adhesion molecules (i.e. L-selectin, E-selectin and P-selectin), allowing the extravasation of neutrophils and T-cells through the vasculature into infected tissues. Aside from its role in immune response, at high serum concentrations TNF-α may act in a paracrine manner directly affecting energy metabolism. It has been associated with insulin resistance in conditions such as aging, gestational diabetes, obesity, sepsis, and muscle damage. In cultured adipocytes (Hotamisligil, *et al.* 1996), hepatocytes (Feinstein, *et al.* 1993), and skeletal muscle (Del Aguiila, *et al.* 1999), TNF-α has been shown to down-regulate insulin receptor signaling by inducing phosphorylation

of the serine residues on the insulin receptor substrate (IRS-1), thereby inhibiting insulin receptor activity. Increases in TNF-α secretion in late gestation are the result of placental secretion delivered to the maternal side (Kerwin, *et al.* 2002). TNF-α is also secreted by the adipose tissue matrix, particularly in obese individuals, however Kerwin, et al. (2002) found insulin resistance to be independently correlated with serum leptin, cortisol, estradiol, or progesterone levels associated with late gestation. In murine models, Hotamisligil, *et al.* (1996) reported a close association with increased adiposity and TNF-α expression, blocking insulin receptor activity and precluding glucose from entering the cell, the result of phosphorylation of the serine residues on IRS-1. Body Mass Index (BMI) is a function of body weight and height and is used to access the relative level of obesity in humans, with subjects with an index of <18.5 considered underweight, 18.5 to 24.9 considered normal, 25.0 to 29.9 considered overweight and >29.9 considered obese. In a study of the relationship of BMI to adipokine release in humans, circulating TNF-α levels increased by 300% in individuals with a BMI of 45, considered to be extremely obese, versus a BMI of 32, considered to be slightly obese. Additionally, this study found a 246% increase in TNF- α released from subcutaneous adipose cells incubated in primary culture taken from individuals with a BMI of 45 versus a BMI of 32. Approximately 70% of adipocytes are from subcutaneous origin and 30% originate from visceral adipose tissue (Fain, *et al.* 2004).

In studies with cultured human preadipocytes and differentiated adipocytes in vitro treated with TNF- α in serum–free medium for 4 hours, Prins, *et al.* (1997)

observed structural changes in adipocytes consistent with apoptosis, including nuclear shrinkage, increased density of the cytoplasm, condensation of chromatin, and nuclear fragmentation. While this study was conducted with very high levels of TNF-α administration, considered to be beyond physiological relevance (425 ng/ml), the data does provide evidence that TNF- α has a role in the regulation of adipose cell number, and thus adipose tissue mass and lipolysis. Qian, et al., (2001) also showed that TNF- α increased adipocyte apoptosis. In this study with rat adipocyte cell cultures, a maximal level of apoptosis was achieved with the addition of 15 ng/ml of TNF- α. They also observed cellular changes initiated by the upregulation of interleukin 1-β converting enzyme (ICE), followed by an insulin dependent increase in caspase-3 activity of up to 3 fold. Caspase-3 subsequently initiates a proteolytic cascade leading to apoptosis of adipocytes.

Pancreatic β-cell apoptosis can be result of an auto immune assault on the pancreas by macrophages and T cells, a common feature of type 1 diabetes mellitus (T1DM). Additionally, β-cell apoptosis occurs with T2DM, however in this case is initiated as a response to increased circulating FFA levels and hyperglycemia. Intracellular calcium stores regulate apoptosis in a variety of cell types, including pancreatic β-cells. Three types of sensitive Ca^{2+} stores exist in pancreatic β-cells, NADP, inositol triphosphate (IP_3)/thapsagarin, and cyclic ADP ribose/ryanodine. The ryanodine receptor (RyR) transmits Ca^{2+} signals to the mitochondria, thereby regulating ATP production. Two subtypes of RyR are found in pancreatic β-cells, RyR1 and RyR2. With excessive Ca^{2+} influx, as occurs with hypoglycemia and

exposure to high levels of FFA's, RyR signaling is blocked, and an increase in the rate of apoptosis of pancreatic β-cells occurs (Johnson, 2004). Increased adiposity is associated with increased release of FFA's into the circulation and also the increased release of TNF-α. Johnson, et al. 2004 identified two distinctly differing pathways of apoptosis for pancreatic β-cells, one involving a pathway induced by low blood glucose, palmitate exposure, or ryanodine exposure activating a calpain-10 pathway and one involving a caspase-3 dependent pathway, more closely associated with hyperglycemia. Glucagon-like peptide was found to reduce the effect of ryanodine induced activation of the calpain-10 pathway. Interestingly, in this study, β-cell apoptosis was increased by chronic hyperglycemia, which was found to activate the caspase-3 apoptosis pathway. In a subsequent study, Kharroubi, et. al. (2004) also concluded that the rate of β-cell apoptosis involves two distinctly different pathways, identifying TNF-α induced activation of NF-kβ via the caspase-3 pathway, the result of increased secretion of TNF-α associated with increased adiposity, and the other as FFA-induced apoptosis, the result of the activation of the calpain-10 pathway.

TNF-α levels are known to be elevated in obesity and NIDDM, in both adipose tissue and in circulation (Prins, *et al.* 1997). Plasma leptin levels are also positively correlated with adipose tissue mass, and high plasma leptin levels are associated with obesity in humans and other animal models. The hormone leptin is also produced by adipose tissue and has been shown to be a potent regulator of food intake. Treatment of calorically restricted hamsters with 17 μg/100 g BW of TNF- α was shown to increase leptin mRNA levels in adipose tissue and leptin protein in

circulation with a concurrent decrease in food intake of approximately 30% observed (Grunfeld, *et al.* 1996). Early experiments using homozygous mice for the obesity gene (ob/ob) reported that infusion of leptin caused a significant depression of appetite (Campfield, *et al.* 1997, Pelleymounter, *et al.* 1995, Halaas, *et al.* 1995). Conversely, decreased leptin levels typically seen in fasted animals, compared to fed animals, have been correlated with a stimulatory effect on appetite. Another early study showed that leptin injections induced mobilization of adipose lipid content and reduced total adipose tissue mass in excess of 30% in genetically normal murine models (Halaas, *et al.* 1995).

There are two determinants of adipose tissue mass, adipose cell number and adipocyte volume or triglyceride content. Adipose cell number is determined by the rate of preadipocyte proliferation and differentiation, and the rate of adipocyte apoptosis (Prins, *et al.* 1997).

There are two principle receptors for TNF-α, TNFR-1 and TNFR-2. The role of TNFR-1 is to signal apoptosis, effectively reducing the number of lymphocytes at the termination of an infection. TNFR-2 plays a minor role in the apoptosis function, however additionally functions in the translocation of NF-kB from the cytoplasm to the nucleus, where it is involved in the transcription of a large and diverse group of inflammatory and immune response mediators. The binding of TNF-α to the TNFR-2 receptor is involved in signaling differentiation and maturation of thymocytes (Prins, *et al.* 1997) .

The signaling pathway for TNFR-1 for the induction of apoptosis has an initial step of cytoplasmic complexing of a number of recruited proteins following the extracellular binding of TNF-α to the TNFR-1 receptor. The complex includes TRADD (TNFR-1-associated death domain), (RIP) receptor interacting protein, RAIDD (RIP-associated ICH1/CED3-homologous protein with a death domain), MADD (MAP kinase-activating death domain protein), FADD (Fas-associated death domain), and TRAF2 (TNFR-associated factor 2) The receptor activated complex subsequently activates caspase-8 at the plasma membrane, initiating a cascade of other members of the caspase family, incuding caspase-3, caspase-6, and caspase-7. The activated caspases then cleave various cellular substrates to cause apoptotic DNA fragmentation and ubiquitination. TNFR-2 may be involved in cytotoxicity due to its rapid association/dissociation kinetics with TNF-α, allowing it to bind TNF-α and present it to TNFR-1 thereby serving as a ligand source to TNFR-1.

TNFR-2's role is to activate NF-kappaB resulting in induction of genes involved in the chronic and acute inflammatory process and an interruption of the cell cycle resulting in cell survival. This pathway involves the complexing of ITRAF (TRAF-interacting protein), (RIP) receptor interacting protein, and TRAF proteins (tumor necrosis factor receptor-associated factor), when the TNFR-2 receptor binds to TNF-α. This induces the phosphorylation of NF-kappaB inducing kinase (NIK), and subsequent phosphorylation of I-kappa-B kinase (IKK), which activates NF-kappaB. Through actions of the TNFR-1 receptor on activation of MADD and TRAF-2, phosphorylation of c-Jun amino-terminal kinases (JNK)

occurs, activating Adapter Protein Complex (AP-1). Acting together in the nucleus, AP-1 and NF-kB initiate TNF gene expression of additional inflammatory cytokines and suppression of apoptosis (Prins, *et al.* 1997).

TNF-α and Lipolysis

The flux of FFA's is largely dependent on the rate of lipolysis. In obese and type 2 diabetes, levels of circulating FFA's are typically elevated, which is thought to be associated with insulin resistance (Boden, *et al.* 1997). TNF-α has been shown to increase lipolysis in human and rodent models (Zhang, *et al.* 2002, Hauner, *et al.* 1995, Hotamisligil, *et al.* 1995).

Adenosine is endogenously released from the adipose tissue matrix primarily from stromovascular cells, comprising approximately 75% of the total cell population in adipose tissue, due to their relatively small size compared with differentiated adipose cells (Vernon, *et al.* 1991, Gassic, *et al.* 1999). Adenosine has an inhibitory effect on lipolysis through its affinity to bind to the adenosine receptors, R_A or R_i, resident in the plasma membrane of adipose cells. The rate of lipolysis is regulated by a signal transduction system of GTP-binding proteins, known as G proteins. G proteins are categorized as either stimulatory G proteins (G_s) or inhibitory G proteins (G_i) (Lanna, *et al.* 1999). The binding of adenosine to its receptor causes disassociation and activation of guanosine-triphosphate (GTP). The rate of cyclic AMP production is differentially regulated by the binding of G_i and G_s proteins to adenylate cyclase (Vernon, *et al.* 1991, Gasic, *et al.* 1999), which subsequently activates protein kinase A. Protein kinase A is enzymatically

phosphorylated , in turn activating hormone-sensitive lipase, resulting in hydrolysis of carbon #1 or carbon #3 of the glycerol moiety forming triacylglycerol (Matthews, *et al.* 1999).

Adenosine production is substrate dependent based on the concentration of intracellular cAMP (Zhang, *et al.* 2002) and TNF-α has been shown to block endogenous adenosine release via this mechanism of action (Gasic, *et al.* 1999). The reduction of cAMP is enzymatically initiated by 5'-nucleotidase or by the hydrolysis of S-adenosyl-homocysteine.

Adenosine Pathway for Lipolysis

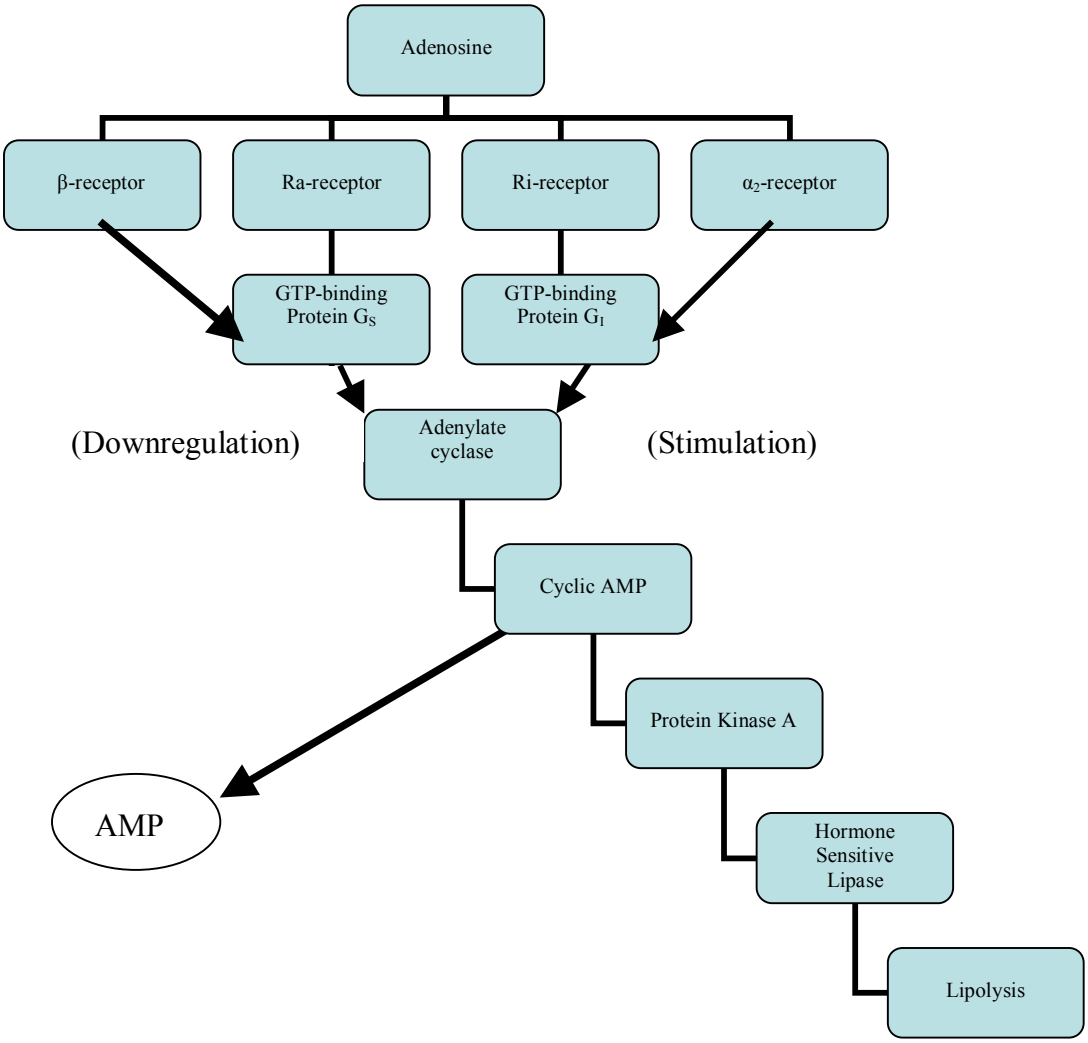

Figure 1. The binding of adenosine to its receptor causes either disassociation or activation GTP-binding protein (inhibitory or stimulatory), which is rate limiting for adenylate cyclase, thereby controlling the rate of cyclic AMP production. The cascade through protein kinase A causes activation of hormone sensitive lipase initiating lipolysis (Matthews 1999).

In a slightly different model, Zhang, et. al. identified a pathway for TNF-α stimulated lipolysis involving the activation of the MEK1/2-ERK1/2 pathway, resulting in a 50% downregulation of cyclic-neucleotide phosphodiesterase 3B (PDE3B), causing a two-fold increase in cAMP and activation of PKA. In this model, PKA hyperphosphorylates the perilipin coating of lipid droplets, allowing increased contact with lipases at the lipid droplet surface, resulting in increased lipolysis. This study, utilizing fully differentiated human adipocyte cell cultures, identified an alternative pathway for the intracellular elevation of cAMP. Zhang et al. (2002) reported that lipolysis involved TNF-α activation of extracellular signal-related kinase (ERK)-1 and -2 to stimulate PKA, rather than increase G_i protein concentrations. In contrast, other researchers have observed decreases in G_i proteins, followed by a marked increase in adenyl cyclase and cAMP expression, leading to the activation of PKA, resulting in an increase in lipolysis (Figure 1)(Gasic, *et al.* 1999).

In yet another experiment, Gasic et al. showed that in rat 3T3 adipose cells treated with TNF-α, there was a complete loss of the inhibitory effect of both nicotinic acid and adenosine on lipolysis, primarily due to decreased expression of G_i proteins, allowing adenyl cyclase activity to increase. The conclusions from this experiment were that the mode of action for nicotinic acid's inhibitory effect on adenyl cyclase activation is GTP-dependent, with the absence of TNF-α causing up-

regulation G_i protein expression. Prolonged treatment with growth hormone has similarly been shown to decrease the expression of G_i proteins and to increase lipolysis, thus having a similar effect to TNF-α (Doris, *et al.* 1994, Stich, *et al.* 2004). In another experiment with bST, decreased sensitivity to adenosine in bovine adipocyte cell cultures was reported in an isoproterenol stimulated lipolysis model. In this study, a β-adrogenic receptor agonist was utilized to reduce the endogenous production of adenosine and controlled amounts of PIA, (-) – N^6 – (2-phenylisopropyl)-adenosine, an adenosine analog that is resistant to adenosine deaminase hydrolysis, were utilized to control the amounts of adenosine present in the culture medium. The results showed a 39% increase in the rate of lipolysis when both bST and adenosine were included in the culture, but no effect of bST in the absence of adenosine. Lanna and Bauman (1999) concluded that bST's ability to reduce the antilipolytic response of adenosine was related to a decreased ability of the α subunit of the G_i proteins to interact with and reduce activation of adenyl cyclase, thereby increasing the subsequent breakdown of triacylglycerol and release of NEFA and glycerol.

Fatty Liver Syndrome

For a number of reasons, including negative energy balance, hormonal changes occurring around parturition, and infections, plasma NEFA mobilized from adipose tissue is either taken up by muscle and other tissues to be oxidized for energy, diverted to mammary tissue to be used as a source to synthesize milk fat, or

synthesized into triacylglycerol in the liver (Bremmer, *et al.* 2000, Blaak, *et al.* 2003, Bobe, *et al.* 2004). The ability of muscle to utilize NEFA has been shown to be impaired in obese human subjects and subjects with type 2 diabetes mellitus (Blaak, *et al.* 2003). Fatty liver in dairy cows occurs when the rate of hepatic triglyceride (TG) synthesis exceeds the ability of the animal to hydrolyze TG and assemble very low density lipoproteins (VLDL), a lipoprotein complex that facilitates transport of TG from the liver. The rate of mobilization of hepatic TG is dependent on the rate of synthesis of VLDL (Bremmer, *et al.* 2000). NEFA are mobilized in amounts in excess of tissue removal rates and in excess of the hepatic disposal abilities during hormonal changes around parturition, infection, and periods of negative energy balance (McNamara, *et al.* 2000, Goff and Horst 1997). Correspondingly, catecholamines and glucocorticoids may stimulate excessive lipolysis through activation and upregulation of the number of β1 and β2 adrenoreceptors (Stich and Berlan, 2004, Lanna and Bauman, 1999), contributing to NEFA overload.

Due to the accumulation of TG in animals with fatty liver, gross pathology shows liver enlargement and a pale to yellowed appearance. Hepatocytes display increased volume, mitochondrial damage,and compression and decreased volume of nuclei, rough endoplasmic reticulum, sinusoids, and other organelles. A decrease in the number of organelles in hepatic cells and fatty cysts in the liver parenchyma have been observed (Bobe, *et al.* 2004).

Exacerbating the disposal of liver TG is the reduction of proteins necessary for the formation of VLDL, including apoprotein B, protein kinase C, and carnitine

palmitoyltransferase (Bobe, *et al.* 2004), however microsomal triacylglyceride transfer protein does not appear to be affected by TG accumulation (Bremmer, *et al.* 2000). Fatty liver is associated with the decreased release of energy regulatory hormones including glucagons, glucocorticoids, growth hormone, IGF-1, insulin, thyroxin, and triiodothyronine and also tissue sensitivity to these hormones. Fatty liver is associated with increased plasma concentrations of BHBA and acetoacetate, both of which have metabolic and toxic effects on the animal at high concentrations (Bobe, *et al.* 2004). Cows fed high energy diets in the pre-parturient period, designed to induce fatty liver, and allowed to gain excessive amounts of BW (>80 kg.) were shown to lipolize more adipose tissue, as indicated by significantly higher plasma NEFA levels, had significantly higher liver triglyceride levels in the post-parturient period, and significantly lower liver glycogen concentrations at 1 wk post-parturition (Rukkwamsuk, *et al.* 1998). In a second similarly designed trial, the proportional makeup of liver fatty acids was significantly different in obese cows versus cows of normal body mass. There were significantly higher liver concentrations of palmitic acid and oleic acid at 2 wk post-parturition, indicating intense lipolysis, and significantly lower liver concentrations of stearic and linoleic acids. Because linoleic acid is a precursor to prostaglandin synthesis, specifically $PGF_{2\alpha}$, an effect of fatty liver on reproduction and immune function has been implicated (Rukkwamsuk, *et al.* 1999, Bobe, *et al.* 2004). A number of treatments have been designed to treat fatty liver, however most of these have been met with limited success (Bobe, *et al.* 2004).

TNF- α and Insulin Resistance

Insulin resistance has been defined as an inability of insulin to regulate glucose homeostasis in various cells and target tissues. It has been characteristically associated with type 2 diabetes mellitus and obesity (Nguyen, *et al.* 2005, Uysal, *et al.* 1997).

Insulin is the primary regulator of glucose uptake in skeletal muscle and adipose tissue, and also functions to inhibit hepatic glucose output. Approximately 75% of insulin-dependent glucose disposal occurs in skeletal muscle, with adipose tissue accounting for only a small percentage of the total. Fasting glucose levels are regulated by insulin's blocking effect on gluconeogenesis and glycogenolysis, and by its stimulatory effect on glycogen synthesis, however insulin is not directly involved in the uptake of glucose by the liver (Saltiel, *et al.* 2001).

The primary insulin signaling pathway involves insulin binding to its receptor on the cell surface. The insulin receptor is composed of tetrameric proteins consisting as two paired subunits, alpha and beta. Tyrosine kinase activity prevents transphosphorylation of the beta subunit, until binding of insulin to the alpha subunit occurs. Insulin binding at the alpha subunit blocks tyrosine kinase inhibition of transphosphorylation leading to a conformational change in the beta subunit and signaling to the insulin receptor substrate (IRS). A number of insulin receptor kinases, as well as other substrates including Gab-1, $p60^{dok}$, Cbl, APS, and isoforma

of Shc, have been identified as belonging to the family of substrates included in the IRS. Proteins of the IRS containing SH2 domains (Src-homology-2) function as adapter molecules (including the p85/p110-type phosphatidyl-inositol-3-OH kinase subunit known as PI(3)K and Grb2, and CrkII). PI(3)K phosphorylates the 3' position of the inositol head of phosphatidylinositol lipids resulting in the formation of phosphatidyl-inositol-3,4,5-triphosphate (PIP3), leading to the activation of G proteins, which signal inactive GLUT4 in the golgi apparatus to translocate to the cell surface via a series of microtubules. (Czech, *et al.* 2000, Salteil, *et al.* 2001). Other SH2 proteins binding to IRS, including phosphotyrosine phosphatase (SHP2) and cytoplasmic tyrosine kinase Fyn, have an inhibitory effect on glucose transport (Salteil, *et al.* 2001). Both the insulin receptor and IRS proteins can undergo serine phosphorylation thereby decreasing tyrosine phosphorylation, resulting in attenuation of insulin signaling and insulin resistance. This situation may occur with obesity and type II diabetes, where attenuation of insulin signaling may result from the sequential activation of protein kinase C (PKC) and nuclear factor-kβ (Yuan, *et al.* 2001). Lipid infusion in rats has been shown to cause insulin resistance in skeletal muscle cells by activation of IKK-β, with subsequent increased serine phosphorylation of IRS-1. Salicylates have been shown to supress this process (Kim, *et al.* 2001). Salicylate has also been demonstrated to effectively reduce TNF-α stimulated IKK-β activity, by competitively binding to IKK-β thereby reducing ATP binding and NF-kβ activation (Yin, *et al.* 1998). Elevated FFA's have also been demonstrated to reduce insulin-stimulated phosphorylation of the IRS-1 and

PI(3)K activity (Salteil, *et al.* 2001, Nguyen, *et al.* 2005). A second requisite pathway for insulin signaling has been identified, initiated by the phosphorylation of the protooncogene Cbl. Cbl interacts with CAP, an adaptor protein containing three carboxy-terminal SH3 domains, and is recruited to the insulin receptor. Upon phosphorylation, the CAP-Cbl complex translocates to the lipid raft domains of the plasma membrane where it forms a tertiary complex with membrane flotillin. This complex through a series of reactions in the plasma membrane activates the G protein TC10, catalyzing the exchange of GTP for GDP. The activation of the G protein TC10 sends a second signal that functions independently, but parallel to the activation of PI(3)K, causing translocation of GLUT4 to the plasma membrane, followed by docking and fusion of the GLUT4 vesicle to the plasma membrane (Baumann, *et al.* 2000, Saltiel, *et al.* 2001).

Elevated free fatty acid levels have been associated with decreases in insulin mediated glucose disposal and inhibition of the insulin signaling cascade. Belfort et al. (2005), have recently shown a 60-70% decrease in insulin receptor phosphorylation and IRS-1 tyrosine phosphorylation, reduced association of PI-3 kinase with IRS-1 and impaired serine phosphorylation of Akt, all following FFA infusion of human subjects.

TNF- α release from adipocytes has consistently been shown to increase with obesity (Hotamisligil, *et al.* 1996, Lofgren, *et al.* 2000, Hirosumi, *et al.* 2002). Experiments to examine the modulatory effect of TNF- α on insulin dependent glucose transport utilizing human subcutaneous adipocytes were conducted by

Lofgren in 2000. In these studies, adipocytes from obese women and women with normal BMI's were treated with increasing concentrations of TNF- α. Insulin responsiveness, was measured as lipogenesis. Cells from obese women at the maximal stimulatory capacity of insulin for glucose transport, showed decreases in insulin responsiveness of one-third compared to cells from women of normal body mass indexes. There was no effect on insulin responsiveness at basal insulin concentrations. Multiple regression analysis indicated that 50% of the variation in insulin responsiveness was related to the effect of TNF- α secretion and fat cell volume.

In a later study with JNK knockout mice (Hirosumi, *et al.* 2002), it was found that obesity was associated with elevated JNK1 activity. $JNK1^{-/-}$ mice in this trial expressed increased protection from obesity-induced insulin resistance. Both TNF-α and increased FFA's are potent activators of JNK1 (Uysal, *et al.* 1997, Boden, *et al.* 1997). JNK1 phosphorylates IRS-1 at serine residue 307, which has an inhibitory effect on tyrosine phosphorylation and subsequent GLUT4 translocation. In murine models, a 60% reduction in insulin-stimulated autophosphorylation of the IR has been observed when endogenous IRS-1 from TNF-α treated cells, where serine phosphorylation predominantly occurred, was added to the cell culture (Hotamisligil, *et al.* 1996).

Experiments with human stromal cells from adipose tissue treated with 5 nmol/l TNF-α for 24 and 72 hours showed significant reductions in cellular GLUT4 concentrations of 49 and 82% respectively (Hauner, *et al.* 1995). In contrast, murine

models utilizing lean and obese, normal and TNF- α knockout mice, revealed no difference in the expression of GLUT4 protein, with western blots in adipose nor muscle tissue, indicating that TNF-α may not be a significant regulator of GLUT4 protein expression. However in this study, autophosphorylation of the IR was reduced by 70% in adipose, 35% in muscle, and 25% in liver tissue in obese TNF-$\alpha^{+/+}$ versus lean animals. Obese TNF-$\alpha^{-/-}$ mice were found to have similar levels of autophosphorylation of the IR to lean mice versus significantly reduced autophosphorylation in obese TNF-$\alpha^{+/+}$ mice (Uysal, *et al.* 1997). In studies with gold thioglucose injected (hyperphagic) mice, obese TNF-$\alpha^{-/-}$ versus obese TNF-$\alpha^{+/+}$ mice had significantly reduced glucose-stimulated plasma insulin levels by 50% and impaired glucose tolerance indicating that TNF-α plays a role in hypoinsulinemia and hyperglycemia (Ventre, *et al.* 1997).

In studies with 3T3 adipose cell cultures with both human and murine models, exposure of adipose cells for 72-96 hours with TNF-α caused significant reductions in IRS-1 and GLUT4 mRNA of greater than 80% and a 50% reduction in the amount of IR (Stephens, *et al.* 2004). Contrary to other experiments, however, no differences in the remaining GLUT4 translocation nor in the phosphorylation IR or IRS-1 were observed. The conclusions from this trial were that TNF-α has an effect on mRNA transcription of proteins involved in insulin signaling and transport, rather than functionality of proteins involved in insulin resistance.

In another trial, FFA treated 3t3-L1 adipocytes increased TNF-α secretion by about 80% versus untreated cultures, implicating TNF-α as the effector of FFA

induced inhibition of insulin activity (Nguyen, *et al.* 2005). Further strength to this hypothesis is the observation that with pre-treatment of 3T3-L-1 cultures with neutralizing antibodies against TNF-α or its receptors, restoration of insulin responsiveness by about 50% following FFA treatment occurs. In this same trial, blocking of TNF-α using neutralizing antibody pretreatment had no effect on FFA induced inhibition of phosphorylation of the IR or IRS-1. However, the use of anti-TNF-α antibody did prevent the degradation of IRβ and IRS-1 protein levels following FFA treatment of adipose cell cultures. The conclusion from this experiment was that TNF-α induced insulin resistance is at least partially accomplished by TNF-α degradation of components of the insulin signaling cascade (Nguyen, *et al.* 2005), which is in agreement with Stephens, *et al.* (2004). Other conclusions from this set of experiments were that FFA's induce JNK activation, resulting insulin resistance independently of TNF-α by inhibiting insulin-stimulated phosphorylation of IRβ and S^{473} , and T^{308} phosphorylation of Akt, another protein involved in the translocation of GLUT4 to the cell surface. TNF-α secretion is increased by JNK and can act either as an autocrine or paracrine effector, directly causing and/or amplifying insulin resistance.

Adiponectin

Adiponectin, also known as Acrp30 (for adipocyte complement-related protein) with a molecular weight of ~30 kDa, was first identified by Scherer, *et al.* in 1995. It is secreted into plasma circulation by fully differentiated adipocytes.

Adiponectin is not a complement protein, however through its globular domain it has homology with C1q, and also TNF-α. Two distinctly different receptors, AdipoR1 in the case of skeletal muscle and AdipoR2 in the case of hepatocytes, bind with adiponectin. Monomers referred as globular adiponectin (gAd) specifically bind to skeletal muscle receptors and full length hexamers or higher order complexes bind to hepatocyte receptors (Miner, *et al.* 2003).

AMPK (AMP-activated protein kinase) is the primary sensory mechanism of cellular energy status and is responsible for the regulation of changes in the ADP:ATP ratio. AMPK activates catabolic pathways resulting in the generation of ATP, concurrently downregulating ATP consuming processes, including gluconeogenesis and lipogenesis (Tomas, *et al.* 2002, Yamauchi, *et al.* 2002, Hardie, *et al.* 2004). AMPK activation is known to increase insulin-independent glucose uptake. Adiponectin stimulates phosphorylation of acetyl coenzyme A carboxylase (ACC) leading to inhibition of ACC-β activity, causing a reduction in malonyl-CoA expression, and a reduction in carnitine palmitoyltransferase 1 activity, all resulting in increased fatty-acid oxidation in skeletal muscle. Additionally, AMPK activation reduces the expression of glucose-6-phosphatase and phosphoenolpyruvate carboxykinase (PEPK) in hepatocytes causing downregulation in gluconeogenesis Hardie, *et al.* 1998, Winder, *et al.* 1999, Moller, *et al.* 2001, Yamauchi, *et al.* 2002).

Yamauchi, et al. 2002 treated C2C12 myocyte cultures with 10 and 25 μg/ml of globular adiponectin (gAd) and full length adiponectin resulting in a 2 fold increase in phosphorylation and thus activation of AMPK observed within 5 minutes

of treatment. These dosages are considered to be well within the normal physiological range for serum gAd. AMP was similarly increased by two fold within 5 minutes of treatment. Significant increases in ACC phosphorylation, glucose uptake, fatty-acid oxidation, and lactate production were observed in skeletal muscle. In hepatocytes full length, but not globular adiponectin caused an increase in the phosphorylation of AMPK and ACC, resulting in decreased expression of G-6-Pase and PEPCK, the regulators of gluconeogenesis. The conclusions were that increased serum adiponectin caused increased fatty-acid oxidation, insulin sensitivity, and glucose uptake in skeletal muscle and reduced hepatic glucose output, resulting in overall lowered plasma glucose and triglyceride levels.

Tomas, *et al.* (2002), in a similar design incubated rat gastrocnemius and soleus muscle cultures with 2.5 μg/ml gAd and observed a two fold increase in AMPK concentrations, a two fold increase in ACC, and a 68% reduction in malonyl CoA at 30 minutes of post-incubation. In this trial, glucose uptake in the absence of insulin increased by 50% and fatty-acid oxidation increased by 60%.

Elevated free fatty-acids have been reported to reduce the secretion of adiponectin by adipose cells (Nguyen, *et al.* 2005). In this trial, treated 3T3-L1 mouse adipocytes with 1 mM free fatty acids for 1 hour and resulted in a 15% reduction in adiponectin secretion in the presence of insulin and a 35% reduction in the expression of adiponectin secretion in the absence of insulin stimulation.

Improvements of 40% in the clearance rate of plasma free fatty-acids levels following lipid infusion in mice treated with adiponectin versus control animals were

reported by Fruebis in 2000. In a second study utilizing high fat diets, mice treated with gAd showed an 18% reduction in plasma free fatty-acids, a 27% reduction in plasma glucose, and a 14% reduction in plasma triglycerides in contrast to controls at 2 hours post-prandial. These differences were more pronounced at 4 hours post-prandial. Full length adiponectin treatment yielded positive, but smaller differences in the parameters measured in this trial.

TNF-α treatment of human adipocyte cell cultures (2.5 μg/ml TNF-α for 48 hours) has been reported to decrease adiponection secretion by 66% (Kappes, *et al.* 2000). Lord et al. 2005 also reported decreased adiponectin and receptor AdipoR2 mRNA expression in swine visceral stromal-vascular cell cultures treated with TNF-α. Bruun, *et al.* (2003) observed an ~50% decrease in adiponectin mRNA expression in human adipose tissue fragments treated with 10μg/L of TNF-α.

Others have reported strong correlations of reduced adiponectin expression in human subjects with increasing body mass index (BMI), insulin resistance, and HDL-cholesterol (Shand, *et al.* 2003, Stejskal, *et al.* 2003, Weiss, *et al.* 2003, Bruun, *et al.* 2003).

Cortisol

Glucocorticoid receptors (GR) regulate gene transcription for a number of metabolic processes and immune function. GR's located in the cytosol of target regulatory cells, when activated by glucocorticoid binding, translocate to the nucleus of the cell and bind to the regulatory regions of the DNA of target genes, where they

exert their effects on cell function. Serum cortisol concentrations are elevated as a direct effect of stress, including overcrowding, heat stress, and practices associated with intensive livestock management. Adrenal corticosteroids are released in response to stress from the adrenal cortex, initiated by secretion of corticotropin-releasing hormone (CRH) by neurosecretory neurons in the hypothalamus. CRH release causes the pituitary corticotropes to secrete adrenocorticotrophic hormone (ACTH), which acts on the adrenal cortex to synthesize and release glucocorticoids, progesterone, and androgens (Moberg, *et al.* 1991). In heat stress experiments, serum cortisol concentrations were reported to increase by 35% as temperatures increased from 30º C to 45º C (Elvinger, *et al.* 1991). Cortisol is also the primary hormone involved in fetal expulsion and in the initiation of lactogenesis. Serum cortisol concentrations begin to increase approximately 3 d prior to parturition and significantly spike on the day of parturition, followed by a rapid decrease to nadir levels within 24 hours following parturition (Priesler, *et al.* 2000). Glucocorticoids have an inhibitory effect on the expression of inflammatory cytokines inclucing TNF-α, IL-1, and IL-6, primarily by altering transcription of cytokine genes (Oconnor, *et al.* 2000).

The activation and deactivation of glucocorticoids is controlled by two 11β-hydroxysteroid dehydrogenases, with the type 1 form regulating activation and the type 2 form regulating deactivation of cortisol. Approximately 90% of circulating cortisol is bound to cortisol-binding protein and is inactive. 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) is widely expressed at the cellular level and is the

enzyme responsible for the conversion of endogenous 11β-hydroxycorticosterone to its inactive form, corticosterone in rodents or from cortisol to cortisone in humans and ungulate species. 11β-HSD2 is widely expressed in renal tissue and prevents cortisol from binding to the mineralocorticoid receptor (Trainer, *et al.* 2002).

11β-HSD type 1 is widely expressed in liver and adipose tissue and functions to recycle inactive cortisone back into the active form, cortisol. The relative cortisol contribution of hepatic and sphlachnic adipose tissue were measured by Andrew et.al, (2005) using hepatic vein catheterization and systemic infusion in human subjects to quantify relative 11β-HSD-1 activity. This trial found that approximately 50% of circulating cortisol is from hepatic and splanchnic origins, with adipose tissue regenerating approximately twice the cortisol than hepatic sources.

11β-HSD1 mRNA and protein expression have been shown to be upregulated in acquired obesity. In human twin studies with significant intrapair differences in BMI, elevated expression of serum 11β-HSD1 mRNA and protein have been observed. 11β-HSD1 was positively correlated with increased serum FFA concentrations during hyperinsulinemic conditions, suggesting a relationship between 11β-HSD1 protein overexpression and FFA induced insulin resistance. The importance of this study is that acquired obesity, independent of genetic factors, is positively associated with overexpression of 11β-HSD1 and the features of insulin resistance (Kannisto, *et al.* 2004). Khani and Tayek (2001) reported a two-fold increase in glucose production using a pituitary-pancreatic clamp procedure in response to cortisol infusion, primarily from increased hepatic gluconeogenesis.

This trial also observed significant increases in NEFA production in response to cortisol infusion, as well as increases in plasma concentrations of leucine, isoleucine, and phenyl-alanine, indicating a proteolytic effect of cortisol.

Chronic exposure to excessive doses of endogenous cortisol has been responsible for a number of deleterious effects on metabolic processes. Glucocorticoids (GC) have been shown to decrease intestinal absorption of Ca^{+2} in both humans and animals, by increasing the rate of degradation of 1,25 dihydroxyvitaminD_3 (calcitrol) at its mucosal binding site and decreasing the synthesis of vitamin D-dependent bovine intestinal calcium-binding, thus significantly inhibiting duodenal Ca^{+2} active transcellular transport (Manelli, *et al.*, 2000). GCs have also been shown to inhibit renal resorption of Ca^{+2} particularly with short term pulse doses, as occurs at parturition. Both of theses may have an affect on increasing parathyroid hormone (PTH) levels. PTH has been shown in some trials to increase with chronic exposure to high levels of GC, possibly as a direct stimulatory effect of GC, however more likely as a feedback response to reduced intestinal absorption and increased urinary excretion of calcium (Minelli, *et al.* 2000).

GC has also been shown to increase urinary excretion of calcium, presumably from inhibition of renal tubular reabsorption (Corsman, *et al.* 1994). In studies utilizing glycyrrhetinic acid (GA), a potent inhibitor of 11βHSD2 activity which reduces the inactivation of cortisol and conversion to receptor inactive cortisone, Ferrari 2002 found that urinary excretion of Ca^{2+} by 9.2% and serum ionized calcium

levels decreased by 6.3% and fractional Ca^{2+}/Na excretion ratios increased by ~38%, measured at day 7 of GA treatment in human subjects. Calcium reabsorption is normally dependent on the concentration of renal sodium transport proteins required to stimulate transport back across the distal convoluted tubule. Sodium transport protein expression is downregulated by aldosterone. Due to the decreased fractional Ca^{2+}/Na excretion ratios, Ferrari concluded that the inhibition of 11βHSD2 activity resulted in over stimulation of the mineralocorticoid receptor by cortisol, corresponding to decreased renal tubular reabsorption of calcium, and thus increased calcium excretion.

Glucocorticoid receptor expression in nuetrophils, lymphocytes and monocytes has been shown to be down-regulated in periparturient dairy cows in the third trimester of gestation in response to increasing levels of cortisol (Priesler, *et al.* 2000). Significant downregulation in the expression of the neutrophil adhesion molecule, L-selectin (CD62L), in response to elevated blood concentrations of cortisol has also been reported, resulting in decreased leukocyte trafficking and phagocytosis (Weber, *et al.* 2001).

Rat studies using a dietary ammonium chloride induced metabolic acidosis were shown to catabolize skeletal muscle. Increased protein degradation was the result of proteolysis of skeletal muscle tissue, mediated by an increase in adrenal glucocorticoid secretion. Glutamine release from muscle tissue breakdown was not increased leading the researchers to conclude that increases in serum glutamine levels were the result of hepatic glutamine synthesis. Glucocorticoids inhibit muscle

protein synthesis and increase protein degradation, which is subsequently converted to urea required for renal ammoniagenesis, which is subsequently utilized to regulate acid-base homeostasis in removal of excess H^+ ion during metabolic acidosis (May, *et al.* 1985).

In similar trials with human subjects using an NH_4Cl induced metabolic acidosis model, Sicuro et.al (1998) observed hypersecretion of cortisol with increases of 158% compared to non-acidotic controls. The administration of growth hormone resulted in suppression of cortisol levels to 26% below non-acidotic controls. This trial demonstrated the ability of growth hormone to effectively suppress glucocorticoid and mineralocorticoid activation, while increasing sodium bicarbonate reabsorption and renal H^+ excretion. Urine pH decreased immediately following NH_4Cl administration, however began to increase steadily as urine ammonia levels increased in response to cortisol release. Ammonia (NH_3) originating from cortisol induced skeletal muscle proteolysis is utilized as the primary proton transporter (as NH_4^+) for urinary excretion of excess H^+ in the regulation of acid-base balance.

HYPOTHESIS AND OBJECTIVES

Many of the perceptions regarding anionic diets arise from the fact that early attempts to utilize fertilizer grade anionic salts resulted in significant reductions in dry matter intakes. Secondly, a perception also exists that the decreased DMI from prepartum anionic diets leads to an increased incidence of metabolic and periparturient disorders. Therefore, my hypothesis was based around these

observations. The primary hypothesis was that anionic diets depress DMI thereby increasing the mobilization of adipose tissue. My second hypothesis was that anionic diets are associated with an increased incidence of postpartum disorders.

Another area of concern in the dairy industry is that increased body mass (beyond BCS 3.5) is associated with reduced DMI and lowered milk production in the subsequent lactation, as well as an increased risk for ketosis. Therefore, my third hypothesis was that increased BCS is associated with depressed DMI and an increase in the incidence of ketosis as measured by the mobilization of adipose tissue, utilizing plasma NEFA and BHB as markers of lipolysis. The fourth hypothesis is that animals with higher BCS return to positive energy balance slower that their less conditioned cohorts.

The objectives of this trial were to examine the effect prepartum dietary treatment and/or level of adiposity (BCS) and their potential to induce insulin resistance using the plasma parameters glucose, TNF-α, cortisol, adiponectin, insulin, calcium, BHB, and NEFA as explanatory variables. Another objective was to examine the effect of prepartum anionic diets and/or BCS on DMI in the postpartum period, as well as their effect on the incidence of postpartum disorders and subsequent milk production.

MATERIALS AND METHODS

Animals

Twenty-six Holstein and 18 Jersey multiparous cows from the Oregon State University Dairy Center were assigned in a randomized block design by calving date and breed to either a control group fed a traditional non-anionic diet or to a treatment group fed a diet containing a commercial anionic supplement (IMC-Agrico, Bannockburn, IL; DCAD = -711.65 Meq/100 g). The control and treatment diets were formulated to a dietary anion-cation difference (DCAD) of 21.3 and -14.3 Meq/100 g on a DM basis respectively, utilizing the equation ((Na%/0.023) + (K%/0.039) – (S%/0.016) – (Cl%/0.0355)). The control and experimental rations were fed in a total mixed ration (TMR) for a period of 21 d prior to parturition, followed by a common ration that was fed to both groups beginning at calving until 21 d post-parturition. The OSU Animal Care and Use Committee approved the research protocol prior to the commencement of the trial.

Average parity was 3.0 for the anionic diet and 2.95 for the control diet. Average parity by breed for control and treatment Holsteins was 2.55 and 2.64 respectively and was 3.44 for both control and treatment Jersey groups. Average parity for the combined groups by breed was 2.59 for the Holsteins and 2.44 for Jerseys.

Total mixed rations were delivered to animals once daily in individual feeders utilizing Calan® gates (American Calan Co. Northwood, NH) and animals

were acclimated to the individual feeders for a period of 7 d prior to the start of data collection. Orts were weighed and recorded daily, prior to the AM feeding.

Diets were formulated using "The Consulting Nutritionist" Ration Formulation Program (Dalex Computer Systems, Inc., Waconia, MN) to be isocaloric, isonitrogenous, and isoneutral detergent fiber (Table 2). Formulated and chemical composition of forages and concentrates, shown in Tables 3 and 4, consisted of corn silage, alfalfa hay, oat hay, steam flaked corn, soybean meal, and either a control mineral-vitamin premix or an anionic salt containing mineral-vitamin premix for the treatment group (Tables 5, 6, & 7). TMR analysis was conducted at 7 day intervals during the trial and a composite sample of the experimental and control diets were submitted to the Dairy One forage testing laboratory (Dairy One, Inc., Ithaca, NY) for proximate and chemical analysis (Table 4). Body condition scores (BCS) based on the average score of three observers using a five point scale and individual animal BWs were taken weekly (Edmonson 1989, Wildman 1982).

Prepartum energy balance was calculated for the last wk of pregnancy using BW and the 2001 NRC equation for maintenance with a 30% adjustment applied to account for the energy requirement of the gravid uterus in the last wk of pregnancy consistent with Moore and Goff 2000. Postpartum energy balance was calculated using the unadjusted NRC 2001 equation for maintenance.

$$\text{Energy Balance (Mcal/day)} = (NE_{L\ intake} - NE_{L\ maintenance} - NE_{L\ lactation})$$

$$NE_{L\ intake}\,(\text{Mcal}) = \text{DMI (kg)} \times NE_L$$

$$\text{Prepartum } NE_{L\ maintenance}\,(\text{Mcal}) = \text{BW (kg)}^{.75} \times 0.104\ \text{Mcal/kg}^{.75}$$

Postpartum $NE_{L\ maintenance}$ (Mcal) = BW $(kg)^{.75}$ x 0.08 Mcal/$kg^{.75}$

$NE_{L\ lactation}$ (Mcal) = milk (kg) x [(.029 x fat %) + (.0563 x prot %) + (.0395 x lact %)]

In contrast to Moore and Goff 2000, no adjustments were applied to account for efficiencies of energy supplied from the mobilization of body tissue for milk production during negative energy balance because our interest was in identifying the relative susceptibility of individual animals to mobilize skeletal muscle and adipose tissue as a response element to negative energy balance.

Ingredient and Chemical Composition Methods

Forages, concentrates, and total mixed rations were sampled weekly and dry matter was determined by drying at 60ºC for 48 hours in a forced-air oven and diets were adjusted based on moisture variance of silages. Monthly composites of feedstuffs were analyzed by the Dairy One Forage Laboratory, Ithaca, NY for crude protein (AOAC 984.13), degradable protein (Cornell Streptomyces griseus (SGP) enzymatic digestion), soluble protein (Cornell Sodium Borate-Sodium Phosphate Buffer Procedure, crude fat (Ether extraction – Foss Tecator Soxtex System HT6, Application Note AN 301), acid detergent fiber (AOAC 973.18), neutral detergent fiber (Van Soest 1991, J Dairy Sci 74:3583), and ash (AOAC Method 942.05). NFC (non-structural carbohydrates) was determined by difference using the equation; NFC= 100% – ((crude protein% + (neutral detergent fiber% –NDICP%)+ ether extract% + ash%)). The minerals assayed included calcium, phosphorus, magnesium,

potassium, sodium, zinc, copper, manganese, and iron and were analyzed using a Thermo Jarrell Ash IRIS Advantage Inductively Coupled Plasma (ICP) Radial Spectrometer (Thermo Jarrell Ash Procedure -"The Spectroscopist" Dec. 1994, Vol. 3. No. 1. pp 6-9). Sulfur was analyzed using a Leco Model SC-432 analyzer. Chloride was assayed using a Potentiometric titration with AgNO3 using Brinkman Metrohm 716 Titrino Titration Unit with silver electrode. Energy values (NE_L) was determined by prediction equations and relationships with other nutrients by Dairy One. Formulated energy values were determined using the 2001 NRC at 2X maintenance intake.

Table 2. Formulated Ingredient and Nutrient Composition of Diets (%)

Ingredient	Anionic Diet	Control Diet	Lactation Diet
Corn Silage	28.8	28.7	16.5
Grass Silage			15.1
Alfalfa Hay	15.2	15.2	19.1
Oat Hay	18.2	20.7	
Steam Flaked Corn	26.9	26.9	30.6
Soybean Meal	4.1	3.1	4.0
Cottonseed			9.7
Lactation Premix			5.0
Control Premix		5.4	
Anionic Premix	6.8		
Total	100.0	100.0	100.0

Nutrient Analysis (DM Basis)			
Dry Matter, %	56.8	56.9	54.6
Crude Protein	13.6	13.9	15.3
Rumen Undegradable Protein[1], %	5.53	6.08	5.58
Crude Fat, %	3.32	3.14	4.53
Ash, %	7.32	6.50	9.80
NDF, %	32.3	34.0	32.4
ADF, %	20.3	21.1	21.3
Net Energy – Lactation, Mcal/kg	1.68	1.65	.78
NFC, %	45.2	44.3	40.7
Calcium, %	0.93	0.74	0.63
Phosphorus, %	0.26	0.27	0.35
Magnesium, %	0.42	0.38	0.21
Potassium, %	1.20	1.21	1.29
Sodium, %	0.09	0.10	0.15
Chlorine, %	0.80	0.29	0.34
Sulfur, %	0.42	0.18	0.24
DCAD[2] Balance, Meq/100g	-14.24	15.9	15.0
Zinc, ppm	64.6	63.7	58.3
Iron, ppm	305	302	280
Copper, ppm	18.5	14.9	15.6
Manganese, ppm	76.7	62.9	76.2

[1]Calculated value from 2001 NRC.

[2]Dietary Anion–Cation Difference(DCAD), Meq/100 g
= (%Na/.023 + %K/.039) – (%S/.106 + %Cl/.0355)

Table 3. Average Chemical Composition of Forages (DM basis

	Alfalfa Hay	Corn Silage	Grass Silage	Oat Hay
Crude Protein,%	23.4	7.78	8.50	9.16
Crude Fat	1.96	2.83	3.34	3.42
ADF	27.2	28.6	42.1	35.0
NDF	34.8	45.9	62.9	55.3
NFC	34.1	38.7	21.6	25.9
NEL,Mcal/Kg.	1.43	1.52	1.11	1.27
Ca,%	1.34	.26	.38	.36
P	.27	.18	.24	.22
Mg	.41	.22	.14	.18
K	3.66	.75	2.09	1.73
S	0.30	0.09	0.13	0.24
Na	0.12	0.01	0.05	0.31
Cl	0.99	0.34	0.55	0.35
Zn,ppm	9	27	23	17
Cu	11.8	5.33	7.40	7.00
Mn	47.0	51.7	131.0	57.6
Fe	590	269	870	122
DCAD[1],Meq/100g	52.5	4.59	32.6	32.9

[1]Dietary Anion–Cation Difference(DCAD), Meq/100 g
= (%Na/.023 + %K/.039) – (%S/.106 + %Cl/.0355)

Table 4. Average Chemical Composition of Concentrates(DM basis)

	Corn/Barley	Whole Cottonseed	Soybean Meal	Lactating TMR
Crude Protein,%	8.84	22.85	51.72	15.78
Crude Fat	2.90	20.15	2.08	5.07
ADF	3.90	33.23	6.20	23.73
NDF	9.76	47.18	9.98	34.23
NFC	75.76	7.28	29.90	--
NEL,Mcal/Kg.	1.95	1.98	1.86	1.65
Ca,%	.05	.18	.36	.75
P	.26	.68	.73	.34
Mg	.11	.41	.32	.30
K	.32	1.06	2.26	1.57
S	.11	.26	.42	.21
Na	.01	.01	.00	.18
Cl	.09	.07	.04	.63
Zn,ppm	23.00	34.75	57.00	61.00
Cu	2.80	5.75	16.80	18.33
Mn	8.60	17.50	44.80	95.17
Fe	53.00	61.25	119.80	599.67
DCAD[1],Meq/100 g	-.79	10.02	30.91	17.54

[1]Dietary Anion–Cation Difference(DCAD), Meq/100 g
= (%Na/.023 + %K/.039) – (%S/.106 + %Cl/.0355)

Table 5. Ingredient Composition of Premixes (% DM basis)

	Control	Treatment
Animate®[1]		72.6
Distillers Grains	70.92	
Urea	7.89	
Limestone	7.69	16.35
Magnesium Oxide	5.04	0.15
Soybean Meal	3.75	
Grape Pomace	3.51	10.04
Vitamin E-50%	0.45	6.96
Selenium .2% Premix	0.35	0.26
Zinc Sulfate	0.17	0.12
Manganese Sulfate	0.13	0.09
Copper Sulfate	0.06	0.02
Vitamin A Premix	0.0220	0.0165
Vitamin D-3 Premix	0.0060	0.0045
E.D.D.I.	0.0020	0.0015
Cobalt Carbonate	0.0005	0.0005

[1]IMC-Agrico,Bannockburn, IL; DCAD = -711.65 meq/100g

Table 6. Formulated composition of prepartum premixes (% DM basis)

	Control	Anionic
CP, %	47.5	30.9
NPN	24.2	18.7
Crude Fat	0.82	4.33
ADF	13.7	11.1
NDF	36.2	22.2
NE_L[1], Mcal/kg	0.70	0.56
K,%	0.88	0.50
S	0.30	3.89
Na	0.24	0.16
Cl	0.13	9.83
Ca	3.36	8.01
P	0.65	0.46
Mg	3.24	3.11
Zn, ppm	715	571
Fe	199	491
Cu	173	138
Mn	440	359
Co	2.48	2.73
I	17.2	13.5
Se	7.74	6.16
A, KIU/kg	159	121
D	33	27
E	2.5	2.0
DCAD[2], Meq/100g	10.6	-500

[1]Calculated value from 2001 NRC.

[2] DCAD (Meq/100 g) = (%Na/.023 + %K/.039) – (%S/.106 + %Cl/.0355)

Table 7. Average Chemical Composition of Premixes (DM basis)

	Control Premix	Anionic Premix	Lactating Premix
Crude Protein,%	43.5	26.0	19.3
Ca	5.38	7.09	3.93
P	0.59	0.37	0.76
Mg	3.49	3.40	0.84
K	0.72	0.63	0.83
S	0.47	3.95	0.42
Na	0.80	0.26	2.19
Cl	0.42	8.08	3.33
Zn,ppm	810	663	617
Cu	158	181	191
Mn	488	611	724
Fe	957	847	917
DCAD[1] Meq/100 g	12.5	-447	-3.47

[1]Dietary Anion–Cation Difference(DCAD), Meq/100 g
= (%Na/.023 + %K/.039) – (%S/.106 + %Cl/.0355)

Sampling Procedures

Plasma samples were collected 4 hours postprandial via jugular venipuncture into evacuated tubes (Becton Dickson, Franklin Lanes, NJ) beginning on day -21 pre-parturition followed by collection on days -14, -11, -9, -7, -5, and then daily until parturition, and then on day of parturition, and post-parturition on days 1, 7, 14, and 21. Samples were divided, centrifuged, serum collected and frozen at -80° C for later analysis. Potassium EDTA was added to vacuum tubes used for β-hydroxybutyrate and NEFA assays. Sodium heparin was added as an anticoagulant to samples used for determination of calcium, magnesium, adiponectin, TNF-a, plasma insulin, and cortisol (Procedure No. 830; Stanbio Laboratories, Boerne, TX).

Sodium heparin with sodium floride was added as an anticoagulant to tubes used for the glucose assay (Procedure No. 1070; Stanbio Laboratories, Boerne, TX).

Midstream sampling of urine via vulva stimulation concurrent with blood sampling dates and urine pH was recorded with a pH meter (Corning model 20 pH meter; Corning Life Sciences, Acton, MA).

Assay Procedures

Prepartum diet was evaluated by treatment, by day, by breed, and body condition score for the effects on pre and postpartum expression of cortisol around the time of parturition, and the adipokines, TNF-α and adiponectin, and their potential effects and interactions on plasma insulin expression and glucose homeostasis. The effect of prepartum diet on the relative energy status of animals was evaluated using NEFA and BHB as markers of adipose tissue mobilization. Plasma levels of calcium, phosphorus and magnesium were assayed to evaluate the effects of anionic diets on the mobilization skeletal tissue at the onset of parturition as an indicator of susceptibility of animals to hypocalcemia.

Plasma glucose. (Sigma Procedure #510; St. Louis, MO), insulin, (Mercodia, Uppsala, Sweden; kit no. 31-3113), Plasma NEFA. (NEFA-C kit; #994-75409 Wako Chemicals, USA, Inc., Richmond, VA), BHB. (Autokit 3-HB, #417-73501, Wako Chemicals, USA, Inc., Richmond, VA ;), Plasma Cortisol. (DSL-10-2000, DSL Laboratories, Webster, TX; kit no.) were enzymatically assayed using a Bio-Tek Instruments, Inc. (Winooski, VT) EL-309 platereader.

Plasma calcium and magnesium were assayed using a Perkin-Elmer 5100 atomic absorption spectrophotometer. Chemical methods and diagnostic testing were performed during sample analysis in accordance with machine protocols.

A commercially available kit was used to determine tumor necrosis factor (TNF-α Screening Set, Pierce, Rockford, IL). Plates were prepared by incubated overnight with reconstituted lyophilized TNF-α coating antibody diluted 1:100 in carbonate/bicarbonate buffer. Blocking buffer was added to each well for one hour and the solution aspirated from the wells prior to the addition of samples. Samples were diluted to 1:100 with reagent diluent and standards were serially diluted. One hundred µl of diluted sample or standard was added to each well of the plate and incubated for 1 hour at room temperature. Plates were washed three times with wash buffer, prior to the addition of 100 µl of biotin labeled lyophilized TNF-α detection antibody reconstituted 1:100 in reagent diluent followed by an incubation for 1 hour at room temperature. Following washing three times with wash buffer, 100 µl of Streptavidin-HRP diluted 1:400 in reagent diluent was added to each well and the plates were incubated for 30 minutes. Plates were then washed three times with wash buffer and 100 µl of 3,3',5,5'-tetramethyl benzidine (TMB) was added to each well with an incubation of 20 minutes in the dark. The reaction was stopped by adding 100 µl of stop solution (0.18 M Sulfuric Acid) to each well. The absorbance was read with a KC Junior Plate Reader (Bio-Tek Instruments, Inc., Winooski, VT) at A_{450} minus A_{550}.

A western blotting procedure for bovine adiponectin had not previously been described in the literature; consequently several anti-bodies were tested before adequate binding occurred. For this procedure, aliquots of serum samples were sorted by block and run on 10 individual gels. An interassay standard was used to account for differences between gels. Protein separation utilized SDS-PAGE (10% percent sodium dodecyl sulphate-polyacrylamide gel electrophoresis), following the method described by Laemmli (1970). Serum samples were thoroughly mixed using a tissue homogenizer to ensure that proteins were not bound in a fibrinogen clump and to facilitate uniform pipetting. 20 µl of serum was diluted with 10 ml of a loading buffer (consisting of 62.5 mM Tris (pH 6.8), 2% SDS, 10% glycerol, 5% β-mercapto-ethanol, and 0.001% bromophenol blue) and 10 µl of water. Samples were denatured in a 100° C water bath for 2 minutes prior to loading onto gels. Denatured serum samples were electrophoresed using a Mini-protean II Electrophoresis Cell (Biorad, Hercules, CA) at 150 volts until the tracking dye ran into the bottom third of the gel. Gels were rinsed with distilled water and proteins transferred to methanol soaked PVDF membranes (Biorad, Hercules, CA) at 80 volts for 90 minutes. Gels were subsequently immersed in a blocking buffer consisting of 5% dried skim milk and 95% TTBS (100ml 10X TBS, 900 ml water, and 500 µl Tween-20) for 5 minutes, followed by five repeated washings for 5 minutes with TTBS. Membranes were then incubated with primary antibody [mouse anti-adiponectin, human monoclonal antibody (Chemicon® International, Temecula, CA)] (1:5000) in 5% milk/TTBS solution for 1 hour. Membranes were washed five times

with fresh TTBS and incubated with an anti-mouse secondary antibody conjugated with horseradish peroxidase (Biorad, Hercules, CA). Membranes were again washed five times in TTBS, treated with Immun-Star chemiluminesense reagents per manufacturers instructions (Biorad, Hercules, CA) utilizing the peroxidase-catalyzed oxidation of luminol resulting in the projection of light onto film. Membranes were exposed onto Biomax films (Eastman Kodak, Rochester, NY). A VersaDoc-1000 imaging System (Biorad, Hercules, CA) was used to quantify protein band density on the exposures.

Statistical Analysis

Data were analyzed as repeated measures using the MIXED procedure of SAS (SAS User's Guide 2001) for analysis of variance with cow within block by prepartum diet by breed by d (or wk) relative to parturition as random effects, and prepartum diet (control or anionic) and breed (Holstein or Jersey) as fixed effects in a randomized complete block design. In addition, the model included all interactions. For equally spaced repeated measures (DMI, milk yield, BW, and BCS), data were analyzed using an autoregressive (AR1) covariance structure. For unequally spaced repeated measures (d relative to parturition), Akaike's information criteria was used to select the best fit from three covariance structures, spatial power law, Gaussian, or spherical (SAS User's Guide 2001). The model used was $Y_{klmnop} = \mu + B_k + R_l + T_m + RT_{lm} + C_{(klm)n} + D_o + RD_{lo} + TD_{mo} + S_p + RS_{lp} + TS_{mp} + RTD_{lmo} + RTS_{lmp} + TDS_{mop} + RTDS_{lmop} + e_{klmnop}$, where μ = overall mean, B_k = kth block

(1,2,3…11), R_l = lth breed (Holstein or Jersey), T_m = mth treatment (control or anionic), $C_{(klm)n}$ = nth cow within the kth block, the lth breed, and the mth treatment. D_o = oth day or wk (a repeated measure), S_p = the pth BCS (3.0, 3.25, …,4.0), and e = the residual error. Results in the tables are reported as least square means. Differences in means for urine pH, plasma TNF-α, plasma insulin, and plasma calcium were significant for breed by treatment interactions. Significance was declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$. Stepwise regression analysis for plasma parameters was plotted by least square means against day and concentration as variables, on a logarithmic scale. All results are expressed as mean ± SEM obtained from ANOVA using the root mean square error to estimate the pooled standard error.

RESULTS

Body Weight, Body Condition Score, Urine pH, and Herd Health Disorders

Prepartum body condition score (Table 8) did not differ by breed ($P>0.11$), however Jersey cows averaged about 0.35 higher BCS than Holstein cows in the three wk period postpartum ($P<0.01$). Prepartum BW by breed was different between breeds, with average Holstein BW at 774 kg and the average Jerseys BW at 554 kg ($P<0.01$). Postpartum BW was different between breeds with average Holsteins weighing 675 kg and average Jerseys weighing 504 kg ($P<0.01$). Change in BW was different by breed with Holsteins losing an average of 98 kg and Jerseys losing 50 kg during the 21 d postpartum trial period ($P<0.01$).

Urine pH differed by dietary treatment (Table 8), with cows receiving the control diet testing at 8.2 and cows receiving the anionic prepartum diet testing at 6.2 ($P<0.01$).

Herd health parameters were not significantly different by treatment. There were 8 cases of milk fever for control cows and 5 cases for cows on the anionic diet. The incidence of retained placenta was slightly higher for the anionic diet at 5 cases for versus 3 for the control cows. The incidence of mastitis measured out to 60 d was numerically higher for control cows at 7 cases versus 5 for the cows on the prepartum anionic diet.

Table 8. Effect of Prepartum Diet by breed for body condition score, urine pH, body weight, and change in BW.

	Holsteins		Jerseys		*P*<			
Parameter	Control	Anionic	Control	Anionic	Pooled SE	Diet	Breed	Diet x Breed
Lactation #	2.55	2.64	3.44	3.44	0.19	0.89	0.02	0.90
Prepartum BCS	3.63	3.42	3.75	3.67	0.35	0.18	0.11	0.55
Postpartum BCS	3.14	3.13	3.50	3.41	0.05	0.53	0.01	0.68
Urine pH	8.24	6.09	8.17	6.30	0.17	0.01	0.55	0.22
Prepartum BW, kgs.	785	763	573	535	22.0	0.23	0.01	0.74
Postpartum BW, kgs.	684	667	519	489	17.9	0.25	0.01	0.75
Change in BW, kgs.	100	95.6	53.5	46.3	7.67	0.67	0.01	0.91

Dry Matter Intake

The effect of treatment, body condition score, day relative to parturition, and breed were evaluated against DMI. For all parameters, responses for DMI were similar to DMI as a percentage of BW, consequently I have chosen to report DMI in kg.

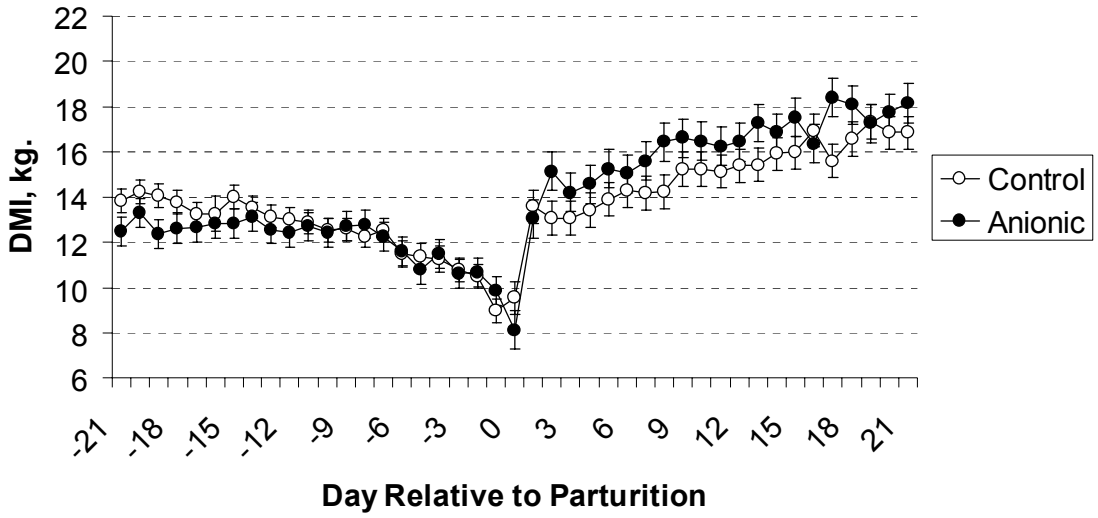

Figure 2. DMI by day relative to parturition for cows consuming a control or anionic diet during the prepartum period. Postpartum DMI was greater for the anionic diet ($P<0.05$).

Dry matter intake (kg or % of BW) was not different between dietary treatments for the prepartum period ($P>0.43$). Prepartum DMI differed by breed with Holstein cows consuming approximately 1.5 times more than Jerseys ($P<0.01$). DMI varied by day declining gradually from 17.8 kg at 21 d prior to parturition, to 9.21 kg. on the d before parturition ($P<0.01$). No effect of body condition score on DMI was observed in the 21 d prior to parturition ($P>0.39$). Interactions were not significant for prepartum DMI ($P>0.05$).

The effect of prepartum dietary treatment on postpartum DMI was significantly different by d ($P<0.05$). Postpartum DMI was approximately 4% lower for cows consuming the anionic diet during the prepartum period on d 1 following parturition and increased to 11% higher for cows fed the anionic diet in the prepartum period within 2 d postpartum. A margin of approximately an 8% increase in dry matter consumption of the lactating ration was maintained through d 21 postpartum for cows consuming the anionic diet during the prepartum period.

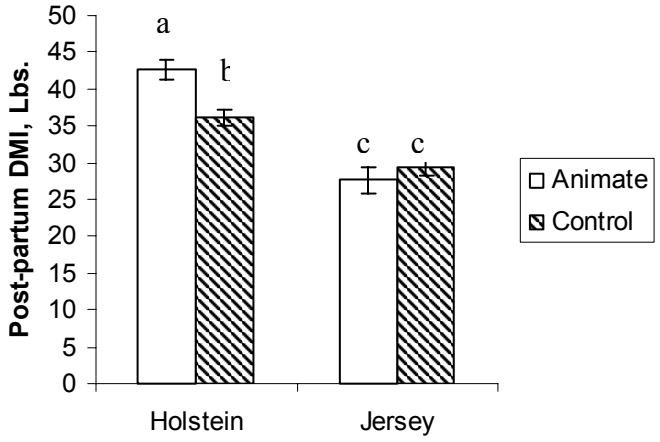

Figure 3. Postpartum DMI differed by treatment and breed ($P<0.01$). Means with different superscripts differ ($P<0.05$).

A breed by treatment interaction was observed for postpartum DMI of the lactating ration (Figure 4). Holstein cows fed the anionic diet in the prepartum period consumed 19.4 kg/d of dry matter per day in the postpartum period compared to Holstein

cows on the control diet, consuming 15% less dry matter per day at 16.4 kg ($P<0.05$). Dry matter intake was not different between treatments for Jerseys ($P>0.05$).

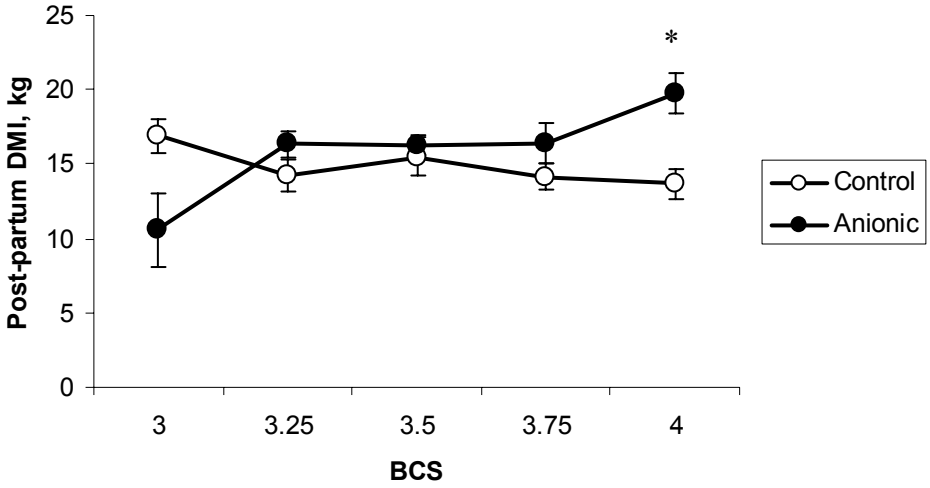

Figure 4. Postpartum DMI by body condition category. Prepartum diet by BCS category was significant ($P<0.01$). * Postpartum DMI differed at BCS 4.0 ($P<0.01$).

Cows fed the anionic diet in the prepartum period had lower DMI with body condition scores of less than 3.25 and higher postpartum DMIs once body condition score exceeded 3.25, when compared to cows fed the control diet.

Energy Balance

Energy balance was significantly different by wk relative to parturition ($P<0.01$), with the most severe negative energy balance occurring the wk following parturition, however no difference in treatment by week was observed. Cows with body condition scores of 3.25 or less had a better energy balance by 4 MCal/d than cows with higher BCS ($P<0.01$). There was an effect of breed on energy balance, with Jerseys more positive on net energy balance than Holsteins by approximately 3 MCal/d ($P<0.01$). There was a positive effect of treatment on energy balance ($P<0.05$), with cows fed the anionic diet averaging a positive energy balance advantage of 1.5 Mcal/d from the wk prior to parturition through 3 wks postpartum (Figure 6).

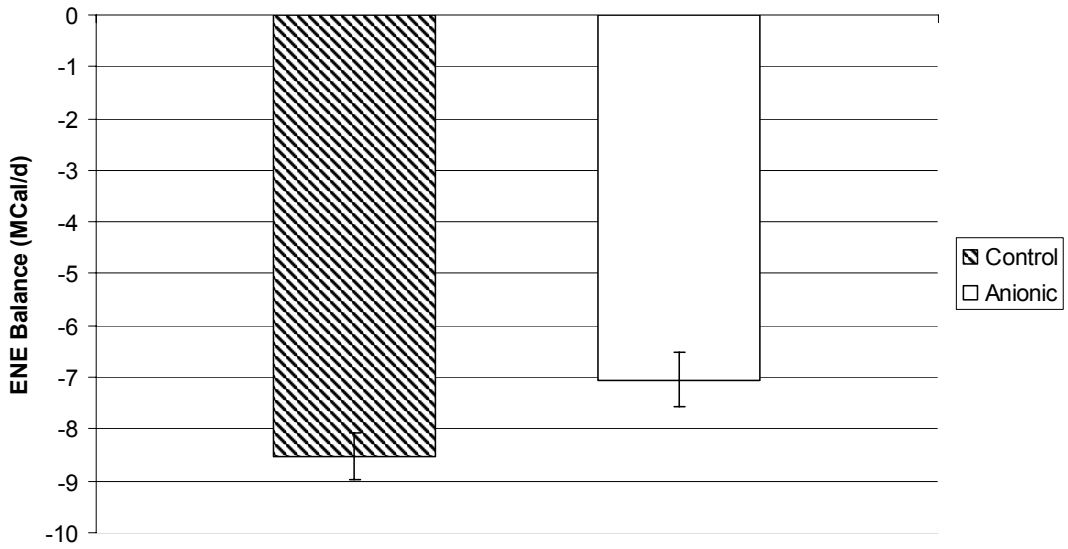

Figure 5. Energy balance by treatment differed ($P<0.05$).

Interactions for BCS by treatment ($P<0.05$) (Figure 7) and BCS by treatment by wk ($P<0.01$) were observed with cows receiving prepartum anionic diets experiencing lower energy balance depression than cows fed the control diet prepartum.

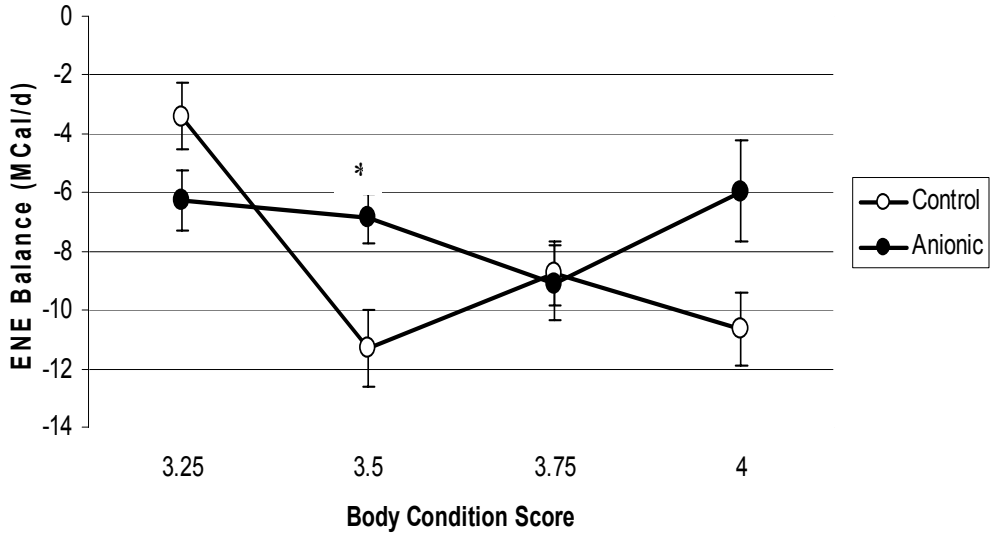

Figure 6. Energy balance differed by BCS and treatment ($P<0.05$) and by BCS by treatment by week ($P<0.05$). ENE balance differed at BCS 3.5 ($P<0.05$).

Milk Volume and Composition

A treatment by breed interaction was observed for milk volume in the 21 d following parturition ($P<0.001$). Holsteins fed the anionic diet produced 3.22 kg. more milk than Holsteins fed the control diet. There was no significant difference in the volume of milk produced by Jersey cows as an effect of dietary treatment (Figure 8). Energy corrected milk (ECM) yield was similar to unadjusted milk yield, with Holsteins on the anionic diet in the prepartum period averaging 46.2 kg. of ECM and 39.5 kg. of

ECM on the control diet. Jerseys consuming the anionic diet in the prepartum period averaged 27.3 kg. of ECM and averaged 29.9 lbs. of ECM on the control diet. The relationship of the treatment by breed interaction for DMI is similar for milk yield and energy corrected milk yield.

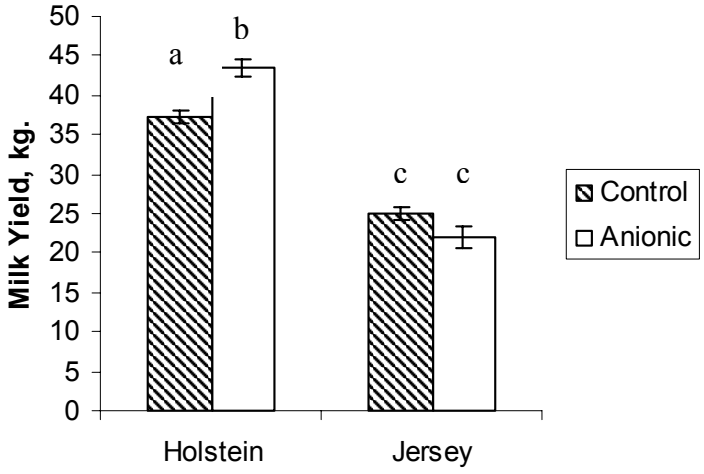

Figure 7. Milk yield by breed for Holsteins and Jersey cows fed an anionic diet (-□-) or control diet (-\-) ($P<0.01$). Means with different superscripts differ ($P<0.05$).

Milk yield was positively affected by body condition score ($P<0.01$), with optimal milk yield occurring at a prepartum body condition scores above 3.5 (Figure 9). Energy corrected milk yield by body condition score was similar to unadjusted milk yield by body condition score, with optimum energy corrected milk yield attained at a body condition score of 3.5. No additional benefit to exceeding a body condition score in excess of 3.5 in the prepartum period was observed.

Mean peak in milk production occurred at day 80 for the Holsteins and at day 73 postpartum for the Jerseys. No significant difference was observed in peak milk

(P<0.943) nor fat corrected milk based on prepartum dietary treatment (P<0.612). Peak milk was not affected by prepartum body condition score (P<0.57). First test day somatic cell count was not different as a result of prepartum dietary treatment (P<0.41).

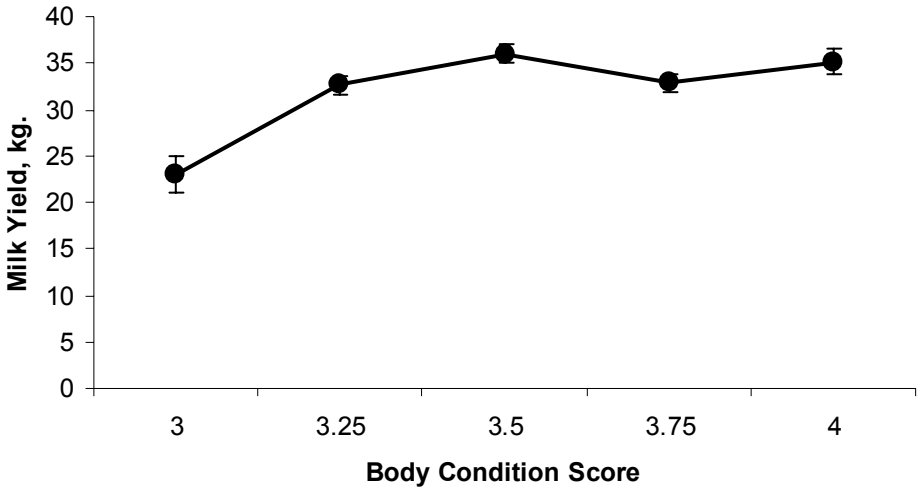

Figure 8. Milk yield by body condition score (P<0.01).

A breed by treatment interaction was observed for milk lactose percent (P<0.01). Milk lactose for Holstein cows fed the prepartum anionic diet was higher at 5.15% than for cows fed the prepartum control diet at 4.72%. Milk lactose averaged 4.79% for Jersey cows fed the prepartum anionic diet and 5.05% for cows fed the prepartum control diet (Figure 10). The volume of milk lactose produced was significantly different for Holsteins consuming the prepartum anionic diet, at 2.22 kg. per day, versus 1.75 kg. per day for Holsteins consuming the prepartum control diet, measured through the first 21 d

of lactation ($P<0.01$). Milk lactose was not affected by prepartum body condition score on a percentage of composition basis.

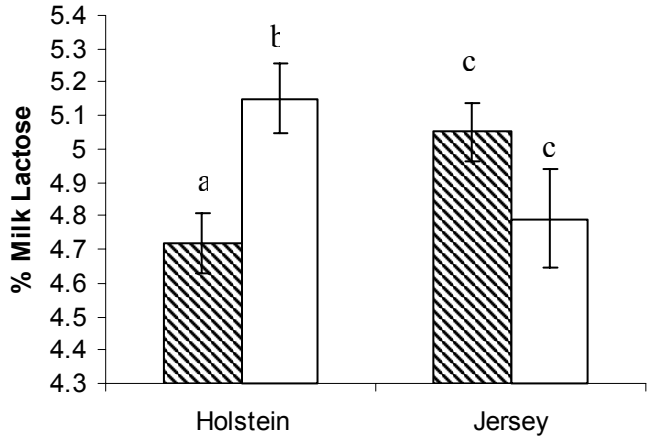

Figure 9. Percent milk lactose for Holstein and Jersey cows fed an anionic diet (-□-) or control diet (-\-) ($P<0.01$). Means with different superscripts differ ($P<0.05$).

Milk protein was not affected by prepartum dietary treatment nor prepartum body condition score, however there was a significant breed effect with Jerseys averaging 3.84% milk protein and Holsteins producing 3.03% milk protein ($P<0.01$).

Milk fat content was affected by breed on a percentage basis ($P<0.05$) and the volume of milk fat produced trended higher based on body condition score ($P<0.08$) (Figure 11). There was no effect of prepartum dietary treatment on milk fat percentage ($P<0.842$) nor absolute volume of milk fat produced ($P<0.69$).

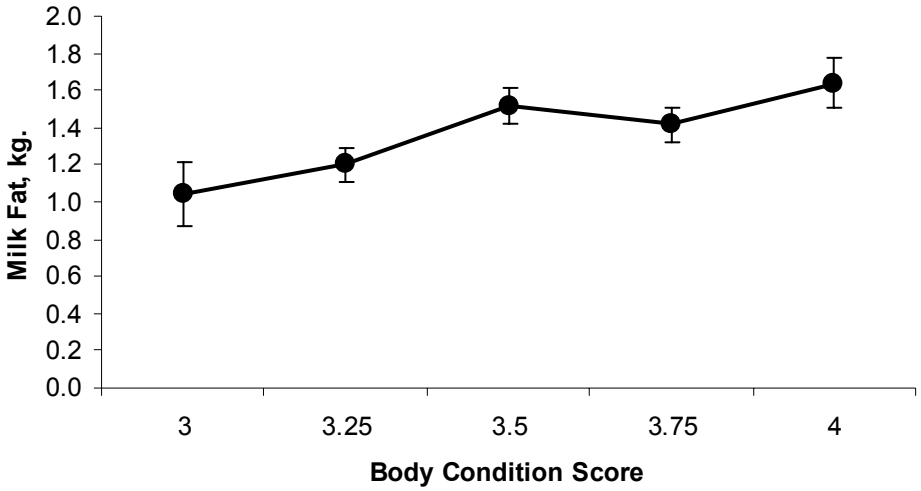

Figure 10. Milk fat volume by prepartum body condition score ($P<0.08$).

Table 9. DMI, milk yield, milk composition, and energy status of Holstein and Jersey cows receiving either a control or anionic prepartum diet.

	Holsteins		Jerseys			*P*<		
Parameter	Control	Anionic	Control	Anionic	Pooled SE	Diet	Breed	Diet x Breed
Prepartum DMI, kg/d	14.9	14.4	10.22	9.90	0.3	0.40	0.01	0.87
Prepartum DMI, %BW	1.68	1.63	1.16	1.12	0.04	0.41	0.01	0.88
Postpartum DMI, kg/d	16.4	19.4	13.4	12.5	0.5	0.18	0.01	0.01
Postpartum DMI, %BW	2.16	2.55	1.76	1.65	0.07	0.12	0.01	0.01
Milk Yield, kg/d	37.2	43.5	24.9	22.1	0.8	0.12	0.01	0.01
Fat %	4.17	3.74	4.61	4.93	0.23	0.84	0.02	0.21
Fat, kg/d	1.53	1.70	1.15	1.07	0.08	0.69	0.01	0.21
Protein %	3.01	3.05	3.64	4.04	0.13	0.19	0.01	0.28
Protein, kg/d	1.11	1.34	0.90	0.93	0.06	0.10	0.01	0.19
Lactose %	4.72	5.15	5.05	4.79	0.09	0.45	0.92	0.01
Lactose, kg/d	1.75	2.22	1.26	1.06	0.09	0.26	0.01	0.01
Peak FCM, kg/d[1]	44.3	48.7	35.8	34.7	3.0	0.61	0.01	0.36
ECM, kg/d[2]	39.5	46.2	29.9	27.8	1.7	0.28	0.01	0.05
Energy balance – Mcal/day[3]								
-1 wk prepartum	4.80	6.69	5.23	6.76	0.63	0.43	0.22	0.64
1 wk postpartum	-2.47	-1.50	-2.04	0.33	0.92	0.09	0.01	0.14
2 wk postpartum	-2.55	-1.94	0.64	3.11	0.82	0.31	0.01	0.14
3 wk postpartum	1.19	2.17	2.58	3.93	0.61	0.75	0.05	0.81

[1]Fat corrected milk (FCM) = (0.4 x kg of milk) + (15 x kg of fat)

[2]Energy corrected milk (ECM) = (kg milk × 0.3246) + (kg fat × 12.86) + (kg protein × 7.04)

[3]Energy Balance (Mcal/day) = $NE_{L\ intake}$ - ($NE_{L\ maintenance}$ + $NE_{L\ lactation}$)

Glucose

Plasma glucose concentrations were significantly different by day relative to parturition for all cows. Average prepartum glucose concentrations were 57 mg/dL. Average postpartum concentrations were 19.5% lower at 46 mg/dL. Plasma glucose concentrations trended higher by day relative to parturition (Figure 12), for Jerseys vs. Holsteins in the prepartum period, however the trend reversed in the postpartum period with Jersey's trending significantly lower than Holsteins in the postpartum period ($P<0.05$).

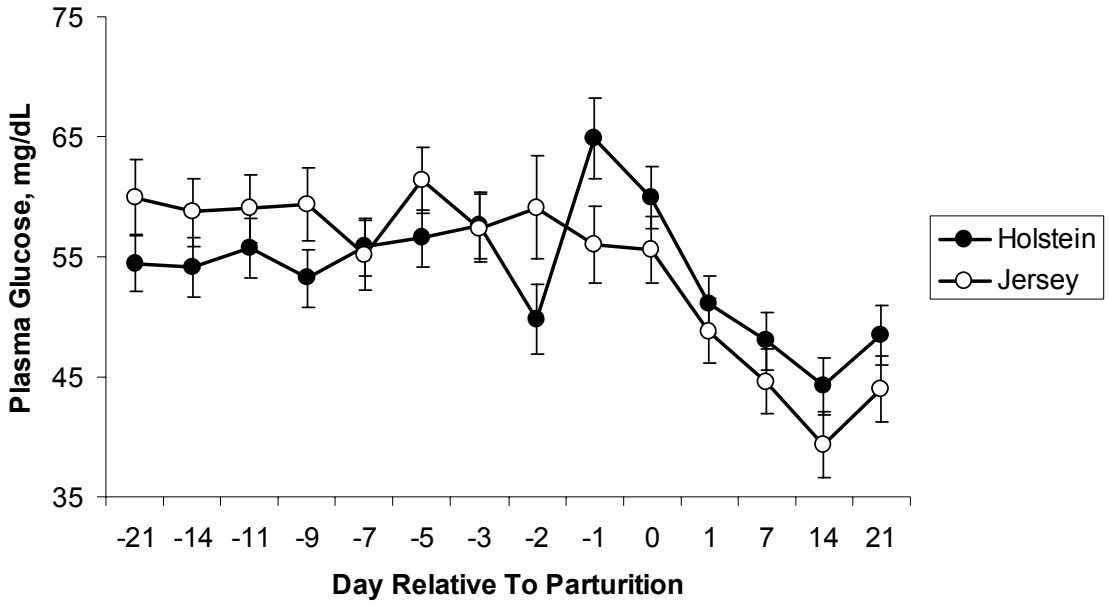

Figure 11. Plasma glucose by d relative to parturition for Holstein and Jersey cows fed an anionic (-o-) or control diet (-●-) ($P<0.05$).

There was a tendency for cows fed the anionic diets in the prepartum period with body condition scores of less than 3.25 to have lower plasma glucose concentrations ($P<0.07$).

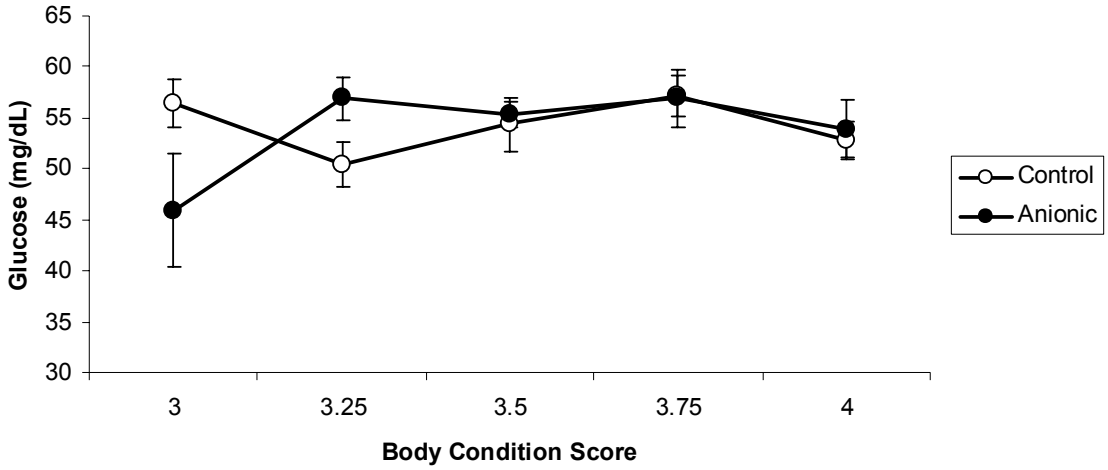

Figure 12. Plasma glucose by body condition score for cows fed an anionic prepartum diet (-o-) or a control diet (-●-) was suggestive of an interaction for cows below a body condition score of 3.25 ($P<0.065$).

Insulin

A breed by treatment interaction was observed for plasma insulin, with lower plasma insulin concentrations observed for Holsteins fed the anionic prepartum diet at 0.15 μg/L, compared with 0.34 μg/L for Holsteins fed the control prepartum diet (Figure 14). Jerseys fed anionic diet had higher plasma insulin concentrations than Jerseys fed the control diet, at plasma insulin concentrations of 0.68 μg/L and 0.51 μg/L respectively ($P<0.01$).

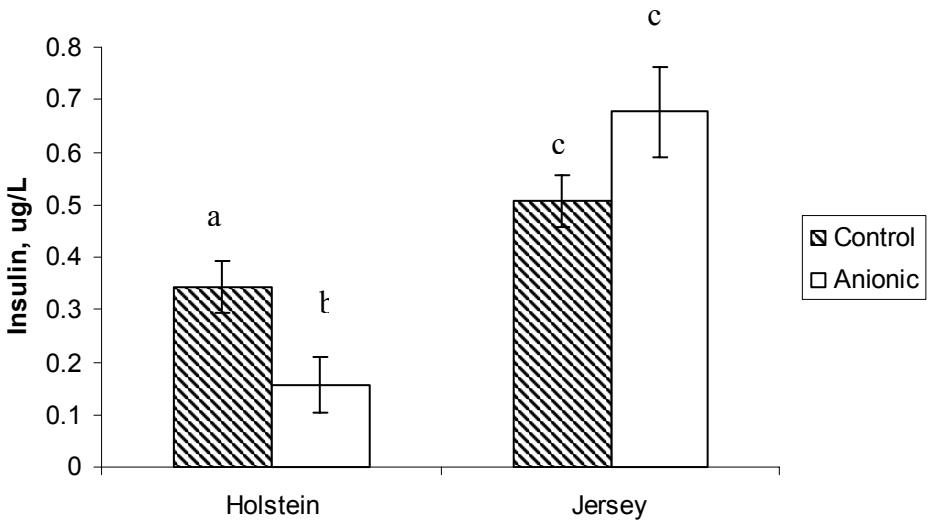

Figure 13. Plasma insulin concentration by breed for cows fed anionic prepartum diet (-□-) or a control diet (-\-) ($P<0.01$). Means with different superscripts differ ($P<0.05$).

Mean insulin concentrations were significantly higher for Jerseys than Holsteins by day relative to parturition ($P<0.05$), with plasma concentrations approximately 58% higher for Jerseys (Figure 15).

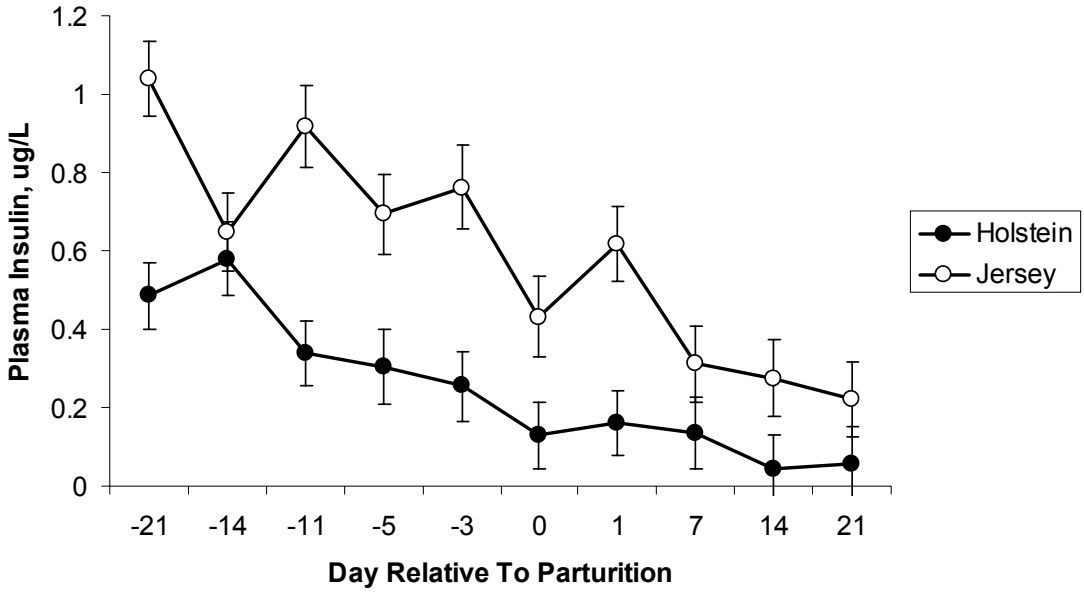

Figure 14. Plasma insulin concentration by d relative to parturition for Holstein (-●-) and Jersey cows (-o-) ($P<0.05$).

A significant interaction of treatment by body condition score for plasma insulin concentration was observed. A reduction in plasma insulin concentration occurred as body condition score increased (Figure 16). Cows fed the control prepartum diet had 13 times higher plasma insulin concentrations than cows fed the anionic diet prepartum at body condition score 4.0 ($P<0.05$).

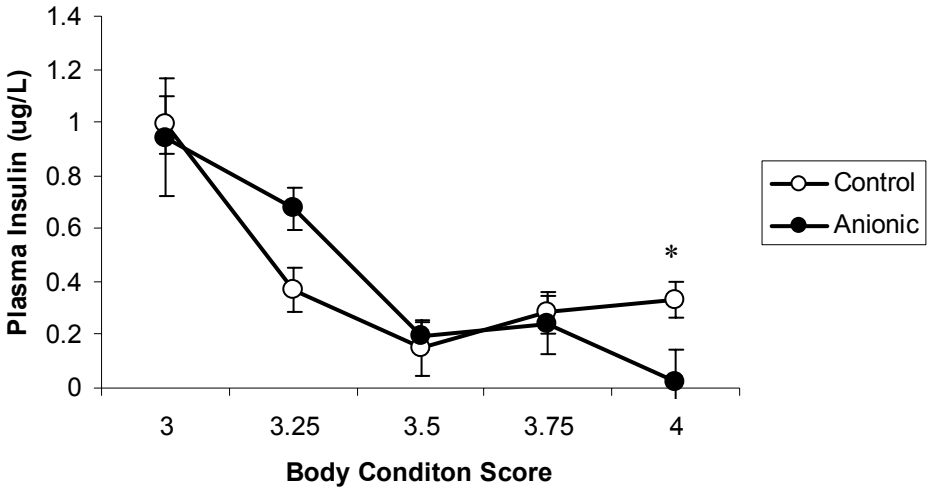

Figure 15. Plasma insulin concentration by body condition score for cows fed an anionic prepartum diet (-●-) or a control diet (-○-) (P<0.05). Insulin differed at BCS 4.0 (P<0.05).

NEFA

NEFA concentration was different by day relative to parturition (P< 0.01). NEFA gradually increased regardless of treatment, by a factor of five fold from day -21 through parturition, and decreased from calving through day 21 post-calving (Figure 17). Prepartum NEFA concentrations were 162 μmol/L 21 d prior to parturition, gradually increasing to a peak of 708 μmol/L at calving. This was followed by a gradual decrease to 452 μmol/L by day 21 post-calving. Differences between breeds and dietary treatments were not significant.

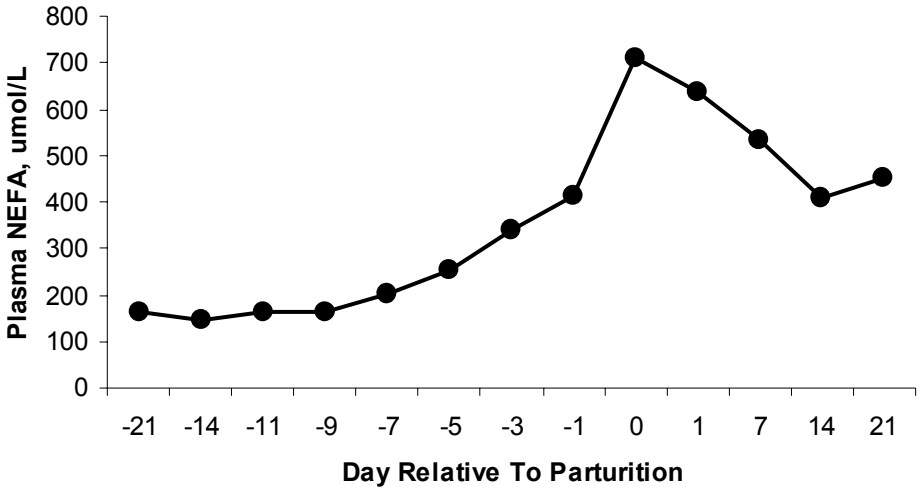

Figure 16. Plasma NEFA concentration by d relative to parturition ($P<0.01$).

β-hydroxybutyrate

β-hydroxybutyrate (BHB) varied significantly by day relative to parturition ($P<0.01$), with a gradually increasing trend from day -1 relative to parturition at 6.88 µmol/L, increasing to a peak of 11.49 µmol/L at day 14 postpartum, and then decreasing. The increase was 177% of nadir levels (Figure 19).

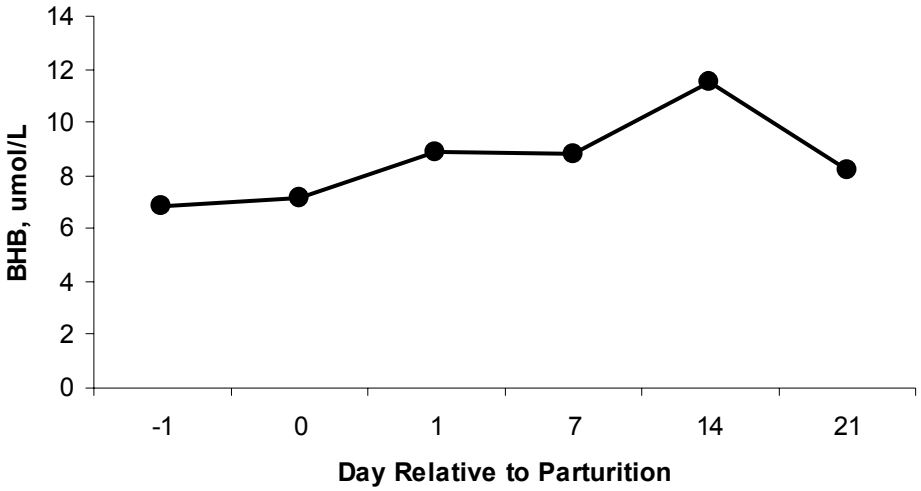

Figure 17. β-hydroxybutyrate by d relative to parturition ($P<0.01$).

BHB concentrations were observed to average 66% lower for Holsteins versus Jerseys. Holsteins had mean BHB concentrations of 6.86 µmol/L compared to 10.28 µmol/L for Jerseys for the period from day -1 prior tp parturition to 21 d postparturition (Figure 19).

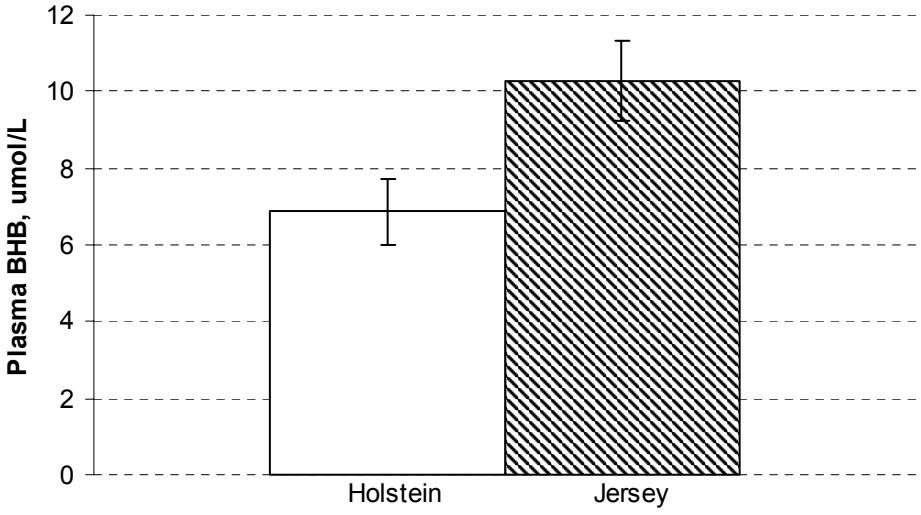

Figure 18. Plasma BHB concentrations by breed for Holstein (-○-)and Jersey (-●-) cows ($P<0.05$).

BHB concentrations were significantly affected by body condition score by treatment ($P<0.02$), with the higher concentrations being observed at a body condition score of 3.0 on the anionic diet at 20.58 µmol/L. BHB concentrations steadily declined on the anionic diet in relation to increasing body condition score to a low of 3.02 µmol/L. Animals fed the control diet prepartum had BHB concentrations of 5.90 µmol/L at a body condition score of 3.0, increasing to a peak of 11.92 µmol/L at a body condition score of 4.0. The crossover point for this interaction was observed to occur at a body condition score of approximately 3.6 (Figure 20).

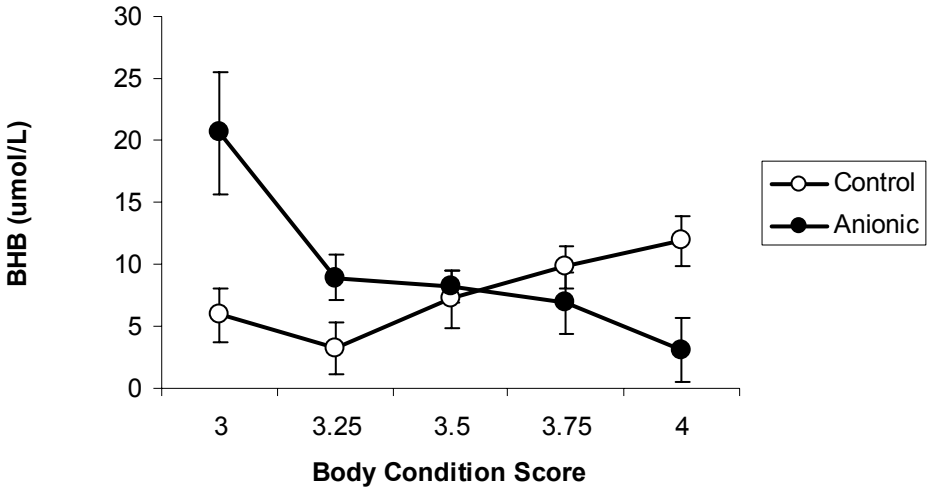

Figure 19. BHB concentrations by body condition score for cows fed an anionic prepartum diet (-●-) or a control diet (-○-) ($P<0.05$). BHB differed at BCS 3.0, 3.25, and 4.0 ($P<0.05$).

TNF-α

There was a significant breed by treatment interaction observed for plasma TNF-α concentrations ($P<0.05$). Holstein cows fed the anionic prepartum diet had higher plasma TNF-α concentrations at 9921 pg/ml, compared to 5691 pg/ml for Holstein cows fed the control prepartum diet. For Jersey cows fed the control prepartum diet, the reverse was true with Jerseys consuming the control prepartum diet expressing higher concentrations of TNF-α at 11148 pg/ml compared to 6715 pg/ml for Jerseys consuming the anionic prepartum diet (Figure 21).

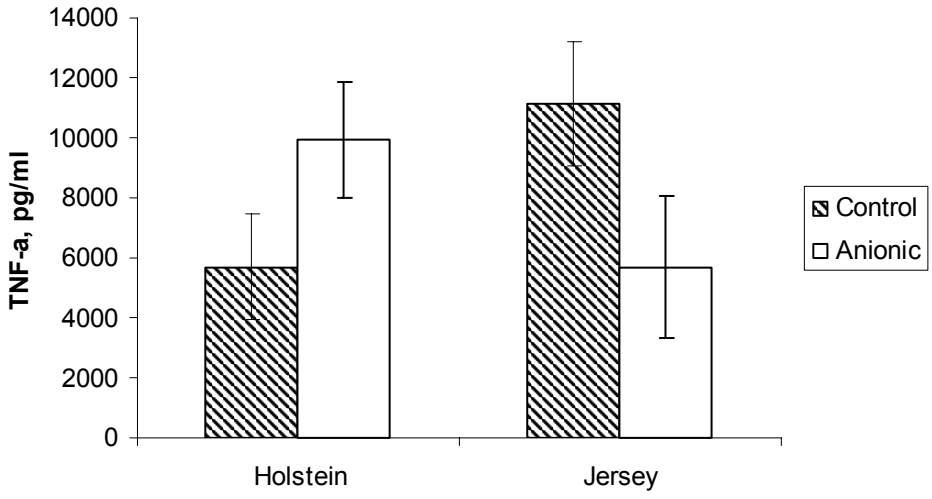

Figure 20. Mean plasma TNF-α concentration by treatment for Holstein (-□-) and Jersey cows (-\-) differed for the period -7 d prepartum through day 14 postpartum ($P<0.05$). A breed by treatment by day effect was not observed ($P<0.76$).

An interaction was observed for body condition score by treatment ($P<0.01$). Animals fed the anionic diet during the prepartum period increased in plasma TNF-α concentration as body condition score increased from 3.0 to 4.0. Mean TNF-α were

zero at a body condition score of 3.0 increasing to 23298 pg/ml at a body condition score of 4.0. An equal but opposite effect on mean plasma TNF-α concentrations was observed for cows fed the control diet during the prepartum period. Plasma TNF-α concentrations were 22,220 pg/ml for cows with a body condition score of 3.0 decreasing to 3844 pg/ml at a body condition score of 4.0 (Figure 22).

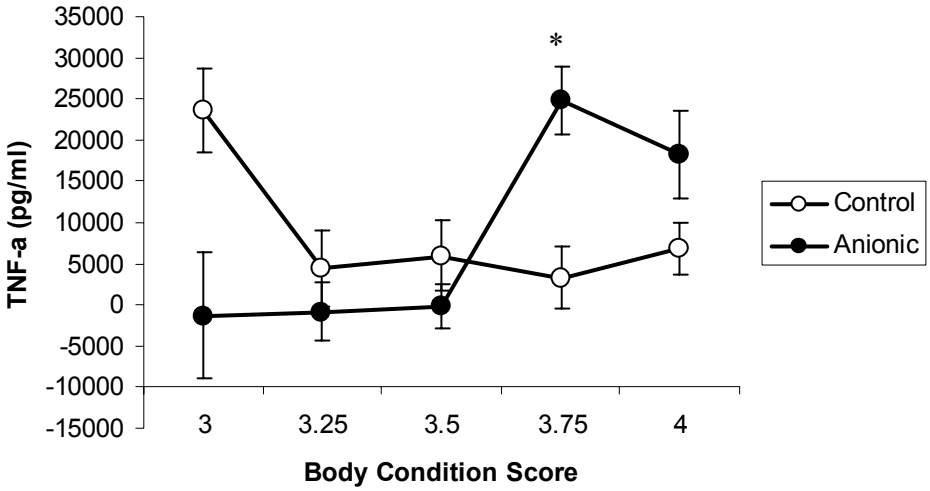

Figure 21. Mean plasma TNF-α concentration by body condition score for cows fed an anionic (-●-) or a control diet (-○-) ($P<0.01$). * TNF-α differed at BCS 3.0 and 3.5 ($P<.05$)

Adiponectin

The effect of prepartum dietary treatment (P>0.48), breed (P>0.59), day (P>0.16), and body condition score (P>.89) was not significantly different for serum concentrations of adiponectin (Figure 23). Relative plasma levels of adiponectin were numerically lower for the 4.0 body condition score category by 19.5% and there

was a numerically decreasing tendency as body condition score increased from 3.0 to 4.0.

Figure 22. Western blot of the relative expression of adiponectin. No difference was observed for dietary treatment (P>0.48), breed (P>0.59) or BCS (P>0.16).

Cortisol

Plasma cortisol concentrations were significantly different by day relative to parturition (P<0.001). Plasma cortisol levels averaged 1.50 µg/dL from day -7 through day -1 prepartum, followed by an abrupt spike in concentration of the day of parturition at 2.71 µg/dL, representing a 181% increase. Mean plasma cortisol concentrations abruptly dropped to 1.55 µg/dL on day 1 following parturition and continued to decline through day 14 postpartum (Figure 24).

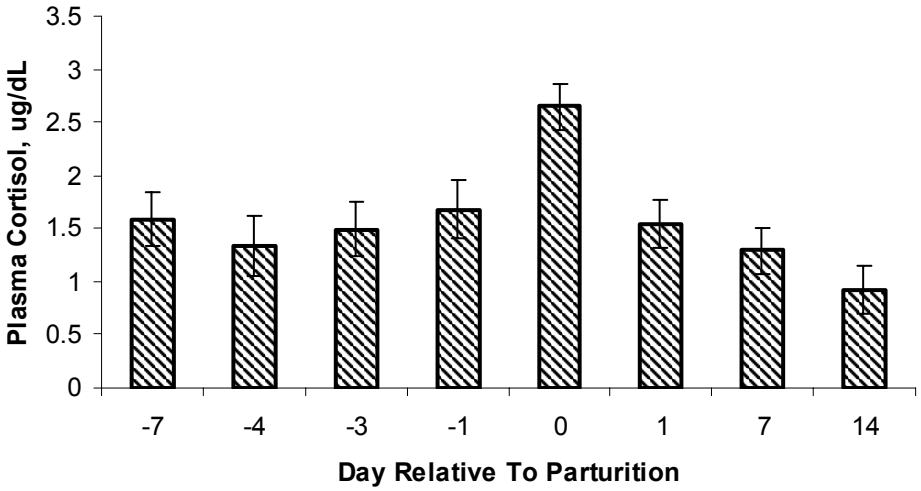

Figure 23. Mean plasma cortisol by d relative to parturition (P<0.001).

Plasma cortisol levels were not affected by treatment ($P<0.936$) nor were they affected by body condition score $P<0.209$). Treatment by BCS was not significant after removal of outliers ($P<0.267$).

Calcium

Plasma calcium was significantly different by day relative to parturition ($P<0.01$). Mean plasma calcium levels ranged from 7.75 mg/dL in the wk prior to parturition and steadily decreased to 6.07 mg/dL on day 1 postparturition. The trend reversed on day 1 postparturition, with an increase in plasma calcium concentration to 8.21 mg/dL by day 7 postparturition (Figure 25).

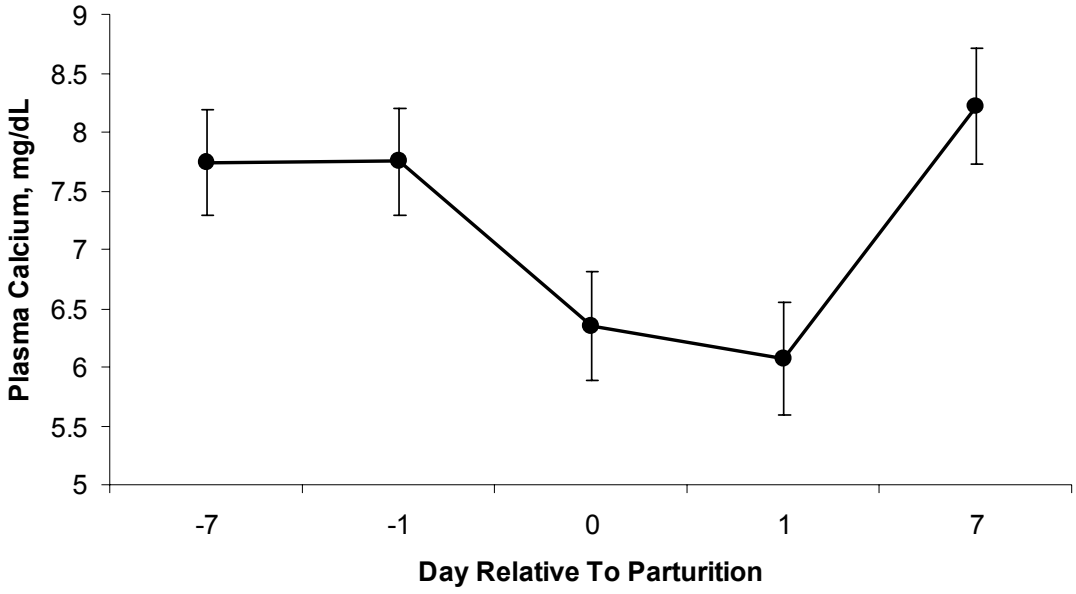

Figure 24. Mean plasma calcium concentration by d relative to parturition ($P<0.01$).

Plasma calcium concentrations were affected by prepartum dietary treatment ($P<0.05$). Mean plasma calcium concentrations for cows fed the anionic diet were 7.69 mg/dL and 6.76 mg/dL for cows fed the control diet (Figure 26).

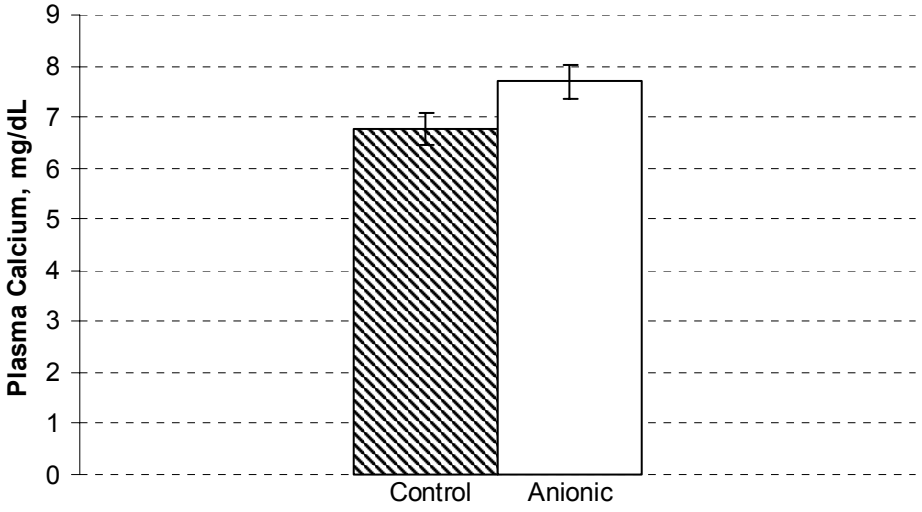

Figure 25. Plasma calcium concentrations by dietary treatment ($P<0.05$).

Plasma calcium concentrations were also significantly different by breed ($P<0.05$), with Holsteins averaging 7.76 mg/dL and Jerseys averaging 6.69 mg/dL for the period from 7 d prepartum through day 7 postpartum (Figure 27).

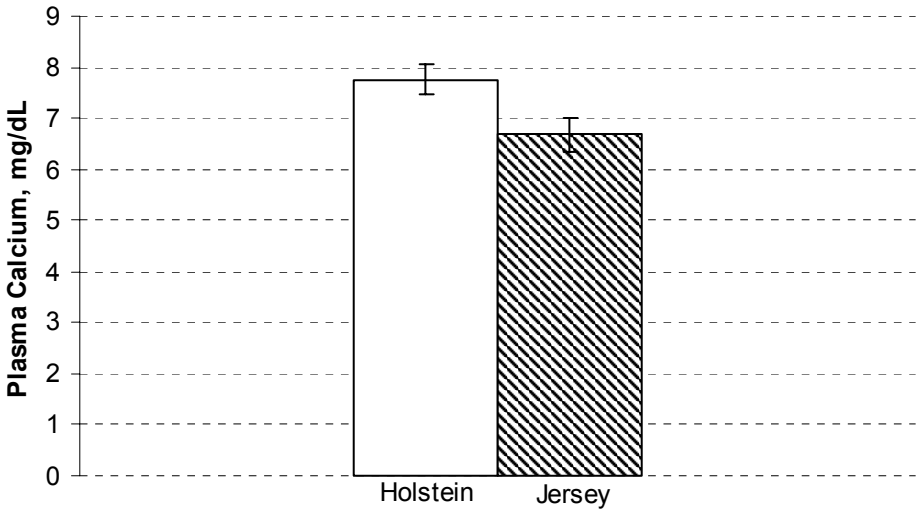

Figure 26. Mean plasma calcium concentration differed by breed from d 7 prepartum through d 7 postpartum ($P<0.05$).

Magnesium

Plasma magnesium concentrations were different by day relative to parturition ($P<0.06$). Magnesium concentrations increased from a mean concentration of 1.85 mg/dL the 7 d immediately prior to parturition to 2.20 mg/dL on the day of parturition representing a 16% increase. Plasma magnesium concentrations decreased to 1.81 mg/dL by the day following parturition and subsequently to 1.64 by day 7 postpartum (Figure 28).

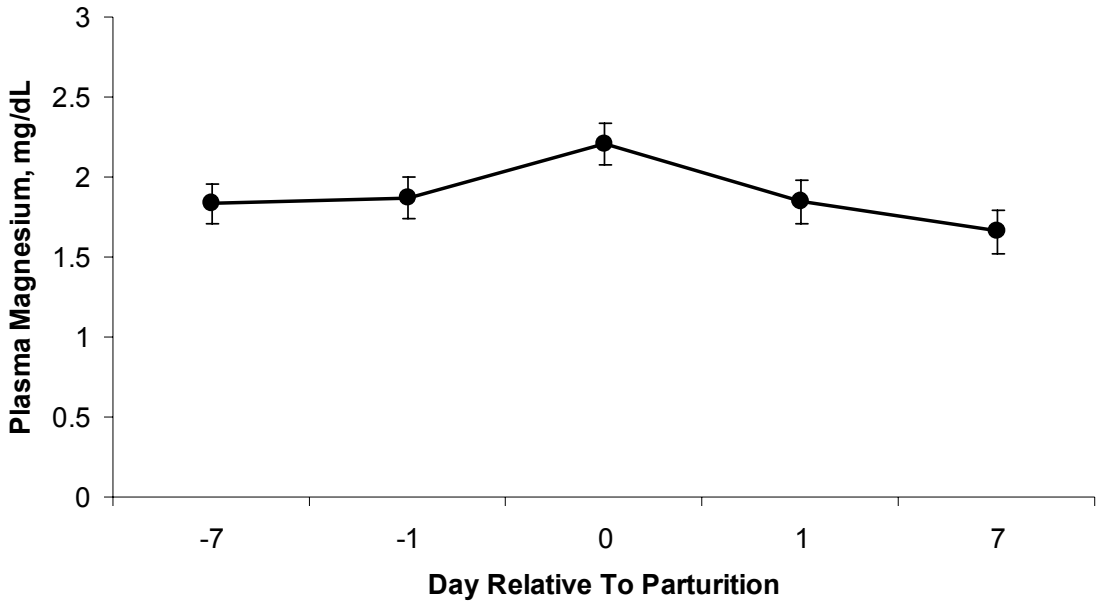

Figure 27. Plasma magnesium concentrations tended to be higher on d of parturition regardless of prepartum dietary treatment ($P<0.06$).

A trend was observed for plasma magnesium concentration by dietary treatment ($P<0.06$) with animals fed the anionic diet having higher plasma magnesium concentrations than animals fed the control diet at 1.99 mg/dL and 1.77 mg/dL respectively, an increase of approximately 12.5% for animals fed the anionic diet (Figure 29).

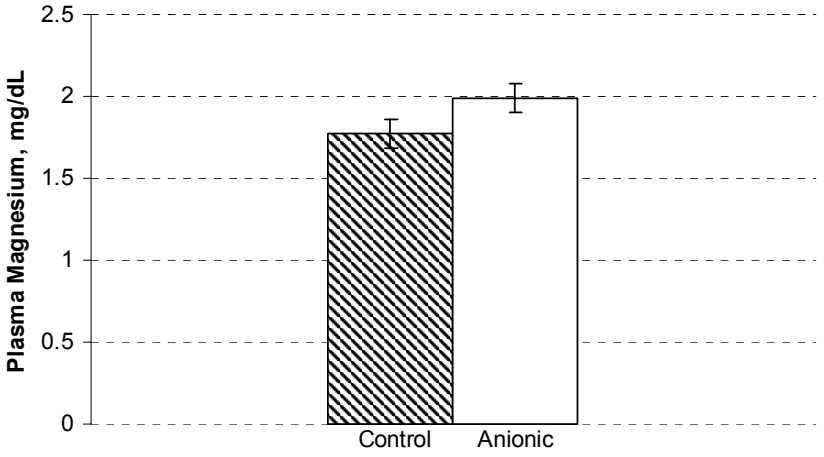

Figure 28. Mean plasma magnesium concentrations for cows fed an anionic prepartum diet (-□-) compared to a control diet (-\-) from d -7 prepartum through d 7 postpartum ($P<0.06$).

Periparturient Disorders

The incidence of postpartum disorders was examined, both as individual events and for the likelihood of having any one of the disorders. The events examined were ketosis, retained placenta, metritis, milk fever, and mastitis before 30 DIM. Single events were not different between treatment groups (Table 10). When examined together, cows on the anionic prepartum diet were less likely to have one of the five postpartum disorders with an odds ratio of 2.29 to 1 using chi square analysis ($P<0.05$).

Table 10. The incidence of postpartum disorders by prepartum dietary treatment.

Postpartum Disorder	Control	Anionic	Odds Ratio	*P*<
Total Milk Fever	13	10	2.08:1	NS
Clinical	9	6	--	NS
Subclinical	4	4	--	NS
Retained Placenta/Metritis	4	1	4.86:1	NS
Total Ketosis	7	3	3.18:1	NS
Clinical	4	2	--	NS
Subclinical	3	1	--	NS
Total Disorders	24	14	2.29:1	0.05

Table 11. Prepartum and postpartum plasma parameters of Holstein and Jersey cows receiving either a control or anionic diet during the prepartum period.

	Holstein		Jersey			*P*		
Parameter	Control	Anionic	Control	Anionic	Pooled SE	Diet	Breed	Diet x Breed
Prepartum								
Glucose, mg/dl	53.7	59.2	57.5	57.3	1.22	0.05	0.45	0.05
NEFA, µmol/L	227	206	188	195	16.5	0.81	0.37	0.62
BHB, mg/dl	6.58	5.79	7.34	7.27	0.18	0.26	0.05	0.40
Ca, µg/dl	8.89	8.62	6.56	7.71	0.43	0.70	0.06	0.16
Mg, µg/dl	1.85	2.05	1.47	2.05	0.10	0.07	0.38	0.36
TNF-α, pg/m	4765	12932	8064	10409	1780	0.05	0.88	0.24
Cortisol, µg/dl	1.53	1.63	1.38	0.86	0.11	0.62	0.30	0.39
Insulin, µg/l	0.45	0.32	0.71	0.60	0.07	0.31	0.05	0.96
Adiponectin, (ref)	70.4	71.8	71.9	87.3	8.94	0.44	0.42	0.51
Postpartum								
Glucose, mg/dl	53.1	50.2	50.0	48.6	0.93	0.19	0.15	0.67
NEFA, µmol/L	535	527	583	463	32.2	0.41	0.92	0.47
BHB, mg/dl	7.79	5.45	9.56	10.2	0.74	0.47	0.05	0.28
Ca, µg/dl	6.45	8.49	6.00	6.97	0.32	0.75	0.47	0.05
Mg, µg/dl	1.82	1.95	1.76	2.0	0.10	0.19	0.97	0.69
TNF-α, pg/m	3411	1223	9595	8573	1660	0.11	0.63	0.05
Cortisol, µg/dl	1.36	1.17	1.25	2.04	0.14	0.34	0.28	0.13
Insulin, µg/l	0.06	0.04	0.10	0.10	0.04	0.11	0.22	0.05
Adiponectin, (ref)	86.6	90.6	96.0	87.3	8.39	0.81	0.75	0.51

DISCUSSION

In this study, I tested the hypothesis that prepartum anionic diets positively affect post-partum DMI and that level of adiposity, measured by body condition score and body weight, may also affect DMI and pre- and postpartum energy balance in Holstein and Jersey cows. I evaluated several markers of the mobilization of adipose tissue, plasma BHB, NEFA, and glucose, as well as the serial expression of cortisol, adiponectin, TNF-α, and insulin, and their potential effect on energy balance. I also investigated the effect of anionic prepartum diets on post-parturient metabolic disorders, body weight loss, and subsequent milk production.

The results indicated a significant increase in milk yield for Holstein cows ($P<0.01$), as well as energy corrected milk ($P<0.01$) from the consumption of an anionic prepartum diet. The main concern was that this increase could be at the expense of body reserves, however there was no significant effect of treatment on energy balance, and in fact cows on the anionic diet returned to positive energy balance faster than their cohorts on the control diet. DMI did not differ in the prepartum period as a result of animals consuming an anionic prepartum diet. This is in agreement with reports from Goff & Horst (1998) where increased DMI was observed with anionic prepartum diets using HCl as dietary acidifier, however there have been a number of conflicting early trials using anionic prepartum diets, presumably where fertilizer grade sources of anions were used for acidification, and DMI actually decreased compared to non-anionic controls in the prepartum period (Moore, *et al.* 2000, Vagnoni, *et al.* 1998, Joyce, *et al.* 1997, Horst, *et al.* 1994,

Oetzel, *et al.* 1993). I observed a carryover effect into the postpartum period for Holsteins receiving the anionic diet in the prepartum period resulting in an additional 3.71 kg per day of DMI for Holsteins ($P<.01$), thus providing more than enough net energy (an additional 6.14 Mcal) to account for the 6.4 kg increase in milk yield. The source of anions for dietary acidification used in our trial was a highly processed form complexed with distillers grains (Animate®), to improve palatability. Indeed, it would appear that the increase in average milk yield to day 21 post-parturition is the result of increased DMI rather than at the expense of body reserves. Surprisingly, this did not translate to an increase in peak fat corrected milk.

Plasma calcium was significantly higher in cows receiving the anionic prepartum diet by approximately 14% ($P<0.05$). Cows averaged 6.6 mg/dL of plasma calcium on the anionic prepartum diet versus 5.5 mg/dL on the control diet at 1 day following parturition. Cows are considered clinically hypocalcemic, when serum calcium concentrations fall below 5.5 mg/dL and subclinically hypocalcemic at serum concentrations below 7.5 mg/dL (Goff, *et al.* 1997), consequently the anionic diet did not completely eliminate the risk for milk fever, however in relation to the control diet, improved postpartum calcium status would imply reduced metabolic risk for hypocalcemia. I would have expected to have seen a significant reduction in clinical hypocalcemia (<5.5 mg/dL) as a result of feeding the prepartum anionic diet, however animal numbers would need to be 36 per treatment group to attain significance. It is possible the -14 DCAD of the anionic prepartum diet was not sufficiently negative to prevent all cases of milk fever, however urine pH's were

within the accepted normal recommendation for anionic diets (Moore, *et al.* 2000, Goff, *et al.* 2003, Joyce, *et al.* 1997). I was not able to reduce potassium levels below 1.1% as recommended by Horst (1997) and others, due to a preponderance of high potassium forages in the Pacific Northwest, which could have induced an increased risk factor for hypocalcemia by compromising PTH function.

Plasma NEFA concentrations were unaffected by dietary treatment, nor were they affected by body condition score. Goff (2006) reported an increase in plasma NEFA and BHB concentrations in response to severity prepartum DMI depression in the week preceeding parturition, leading to decreased neutrophil (PMN) myeloperoxidase activity and an increased incidence of metritis and retained fetal membranes. BHB concentrations were similarly not directly affected by dietary treatment, however cows had higher serum BHB concentrations on the anionic prepartum diet until body condition score reached 3.5 and at that point and above serum BHB concentrations increased dramatically for the control diet. Cows with BHB concentrations of 12 mg/dL are generally considered to be subclinically ketotic and cows with serum BHB concentrations in excess of 15 mg/dL are considered to be clinically ketotic. Cows with plasma BHB concentrations >12 mg/dL are 8 times more likely to have a displaced abomasums (LDA) (LeBlanc, *et al.* 2005). The BHB results indicated 3 cases of ketosis for cows consuming the anionic prepartum diet at 14 DIM, with 2 of the 3 being clinical cases. For the cows consuming the control prepartum diet, there were 7 cases of ketosis at 14 DIM, with 4 of the 7 being considered clinical. The results indicate approximately a 50% reduction in the

incidence of ketosis for cows consuming an anionic prepartum diet. Kremer et al. (1993) reported an association of increased severity of intramammary infections related to elevated serum BHB concentrations from decreased energy balance postpartum. The results were generally in agreement with both Goff (2006) and Kremer (1993) yielded 11 cases of milk fever in cows with an average BCS of 3.7, 5 cases of mastitis within the first 21 days of lactation with an average BCS of 3.8, and 5 cases of retained fetal membranes in cows with an average BCS of 3.6. Cows experiencing milk fever were on the average 11% below the mean as a percent of BW for DMI in the prepartum period. My conclusion from this observation is that overconditioned animals may benefit from a reduced incidence of ketosis on a prepartum anionic diet in addition. Reduced prepartum DMI has been shown to be a risk factor for subclinical ketosis and displaced abomasum (LeBlanc, *et al.* 2004).

Plasma cortisol concentrations were not different by dietary treatment, nor by body condition score. I observed the same pattern of an increase in plasma cortisol concentration approximately 3 d prior to parturition with a significant spike on the day of parturition, followed by a rapid decrease to nadir levels within 24 hours following parturition reported by Priesler, *et al.* (2000) and Weber, *et al.* (2001).

The most striking observations were with plasma TNF-α and insulin levels. Both TNF-α and insulin were significantly affected based on body condition score and I observed a body condition score by treatment interaction for each hormone. Insulin had a tendency to decrease with increasing body condition, in agreement with research observations in other species, particularly rats and humans (Nguyen, *et al.*

2005, Uysal, *et al.* 1997). By treatment, plasma insulin differences followed a generally declining trend, however insulin levels were significantly higher for body condition scores above 3.5 for the control diet versus the anionic diet. TNF-α levels were the lowest at body condition scores below 3.5 on the anionic diet and markedly increased as body condition scores moved towards obesity. For control diets the opposite effect was observed. TNF-α has been shown in human and rodent trials to increase with increasing adiposity (Hotamisligil, *et al.* 1996, Hirosumi, *et al.* 2002) and TNF-α has been shown to have a modulatory effect on insulin dependent glucose transport in subcutaneous human adipocytes cultures (Lofgren, *et al.* 2000). Furthermore, elevated FFA and TNF-α concentrations are associated with elevated JNK1 activity, leading to reduced insulin responsiveness (Uysal, *et al.* 1997, Boden, *et al.* 1997). Hotamisligil, *et al.* (1996) reported a close association with increased adiposity and TNF-α expression, blocking insulin receptor activity and precluding glucose from entering the cell. TNF-α has been shown to increase lipolysis in human and rodent models (Zhang, *et al.* 2002, Hauner, *et al.* 1995, Hotamisligil, *et al.* 1995). While this trial did not measure plasma triglyceride levels, my observations were that BHB levels generally were below 14.6 mg/dL, which is the breakpoint for subclinical ketosis, would indicate that excessive lipolysis did not occur in this population. The only animals in this trial with elevated BHB above the ketosis breakpoint had BCS's below 3.0 and were fed the anionic prepartum diet.

Serum adiponectin was not affected by dietary treatment. This is contrary to work done in obese adolescent human subjects by Cianflone, et al., (2005), where

there was a significant effect observed when Adiponectin was regressed against percentage of ideal BW ($P<0.03$; $r^2=.211$), a similar indexing procedure to body condition scoring. The explanation for this observation could be that animals in this trial were not considered at the extreme end of the range of obesity. Acute obesity in dairy cattle occurs at body condition scores in excess of 4.0. Plasma levels for adiponectin in our trial were numerically lower for the 4.0 body condition score category by 19.5% and there was a numerically decreasing trend as body condition score increased from 3.0 to 4.0, also in agreement with Cianflone, *et al.* (2005).

Elevated free fatty-acids have been reported to reduce the secretion of adiponectin by adipose cells (Nguyen, *et al.* 2005), however in this trial, elevation of NEFA in relation to increasing BCS did not occur, consequently differences in serum levels due to serum FFA levels could not be determined.

Adiponectin has been shown to modulate the effects of TNF-α on insulin sensitivity in both human and rhodent species, however no correlation between TNF-α and adiponectin expression was found in the population of cows that were tested. A relationship between serum adiponectin and plasma glucose levels was not observed. Perhaps if I had tested subjects above a BCS of 4.0, effects may have been more pronounced.

CONCLUSIONS

In this trial, the hypothesis was that prepartum anionic diets would have detrimental effects on energy balance and DMI, however my findings would indicate that impaired insulin responsiveness, indicating an increase in lipolysis did not occur with the prepartum anionic diets, as would have been evident as an elevation in plasma NEFA levels. While I observed an increase in postpartum plasma TNF-α levels with animals fed the prepartum anionic diet, the observed levels did not appear to cause cachexia in more heavily conditioned animals, however this was not true with underconditioned animals fed the prepartum anionic diet. BHB levels were reduced as BCS increased above 3.5 on the anionic prepartum diet, but not the control diet, indicating that anionic supplements may have a modulatory effect on adipose tissue mobilization, and may have application for animals predisposed to excessive weight gain, as occurs with reproductive failures and extended prior lactations. The observation that there was an increase in postpartum DMI and energy corrected milk for the first 21 days of lactation as a result of feeding the prepartum anionic diet, coupled with the observation that peak fat corrected milk production was not different between treatment groups, lead to the conclusion that the effects of prepartum anionic diets do not carry-over into lactation beyond the transition period.

Economic losses to the dairy industry related to postpartum disorders exceed $600 million (USD) annually (Guard, *et al*. 1996, Grohn, *et al*. 1995). The likelihood for cows to have postpartum disorders was more than double for cows consuming the control prepartum diet versus the anionic diet. These data suggest that feeding anionic

supplements in the close-up dry period may have benefits beyond the prevention of hypocalcemia, particularly for the prevention of ketosis with overconditioned animals. Anionic diets typically cost $21(USD) more than conventional non-anionic prepartum diets for a 21 days prepartum feeding period. Extrapolating the data from Guard et al., (1996) and Grohn et al., (1995), the average cost of postpartum disorders per cow per lactation is over $67(USD). A 50% reduction in the incidence of postpartum disorders would provide a substantial return on investment on the additional cost of an prepartum anionic diet. The additional revenue from milk produced, based on Holsteins, would provide a net return over feed costs of approximately $7.50(USD) for the 21 day period following parturition, resulting in a total return on investment for an anionic prepartum diet approaching 2:1.

This study was indicative of the potential for prepartum anionic diets to modulate adipose tissue mobilization on overconditioned animals. Further research should evaluate the effects of prepartum anionic diets at levels indicative of obesity up to a BCS of 4.5. Statistical power analysis should be conducted to select the appropriate sample size for each class of BCS. The insulin assay used in this study lacked sensitivity and an alternative to the Mercodia elisa kit should be investigated. Plasma triglyceride levels could provide additional data for the evaluation of lipolysis. This trial utilized two breeds to in order to investigate differences in responses of Jersey and Holstein cows to prepartum anionic diets, however a single breed study would provide more statistical relevance. TNF-α is associated with catabolism of skeletal muscle. The potential for TNF-α to initiate a release of skeletal

muscle glutamine and alanine, both gluconeogenic precursors, and their effect on energy status should also be investigated.

BIBLIOGRAPHY

Andrew, R., J. Westerbacka, J. Wahren, H. Yki-Jarvinen, and B.R. Walker. 2005. The contribution of visceral adipose tissue to splanchnic cortisol production in healthy humans. Diabetes 54: 1364-1370.

Baird, G.D. 1982. Primary ketosis in the high producing dairy cow: clinical and subclinical disorders, treatment, prevention, and outlook. J. Dairy Sci. 65:1.

Bareille, N. and P. Faverdin. 1996. Lipid metabolism and intake behavior of dairy cows: effects of intravenous lipid and a-adrenergic supplementation. J Dairy Sci 79: 1209-1220.

Baumann, C.A., Ribon, V., Kanzaki, M., Thurmond, D., Mora, S., Shigematsu, S., Bickel, P., Pessin, J.E., and A. Saltiel. 2001. CAP defines a second signaling pathway required for insulin-stimulated glucose transport. Nature 407: 202-207.

Belfort, R., L. Mandarino, S. Kashyap, K. Wirfel, T. Pratipanawatr, R. Berria, R.A. DeFronzo, and K. Cusi. 2005. Dose-response effect of elevated plasma free fatty acid on insulin signaling. Diabetes 54:1640-1648.

Bell, A.W. 1995. Regulation of organic nutrient metabolism during transition from late pregnancy to early lactation. J. Anim. Sci. 73:2804-2819.

Blaak, E.E.. 2003. Fatty acid metabolism in obesity and type 2 diabetes mellitus. Proceedings of the Nutrition Society 62:753-760.

Block, E., H. Takagi. 1986. Effect of manipulating anion-cation balance on calcium metabolism in ruminants. J. Dairy Sci. 69 (suppl. 1), 115.

Bobe, G., J.W. Young, and D.C. Bietz. 2004. Invited review: pathology, etiology, prevention, and treatment of fatty liver syndrome in dairy cows. J Dairy Sci. 87: 3105 – 3124.

Boden, G. 1997. Role of fatty acids in the pathogenesis of insulin resistance and NIDDM. Diabetes 46: 3-10.

Bruun, J.M., A.S. Lihn, C. Verdih, S.B. Pedersen, S. Toubro, A. Astrup, and B. Richelsen. 2003. Regulation of adiponectin by adipose tissue-derived cytokines: in vivo and in vitro investigations in humans. Am J Physiol Endocrinol Metab 285:E527-E533.

Bremmer, D.R., S.L. Trower, S.J. Bertics, S.A. Besong, U. Bernabucci, and R.R. Grummer. 2000. Etiology of fatty liver in dairy cattle: effects of nutritional and hormonal status on hepatic microsomal triglyceride transfer protein. J Dairy Sci 83:2239 – 2251.

Burton, J.L., and R.J. Erskine. 2003. Immunity and mastitis. Some new ideas for an old disease. Vet. Clin. Food Anim. 19:1-45.

Byers, F.M. and G.T. Schelling. Lipids in Ruminant Nutrition. The Ruminant Animal; Digestive Physiology and Nutrition. 1988. Waveland Press, Prospect Heights, IL.

Campfield, L.A., F. J. Smith, Y. Guisez, R. Devos, P. Burn. 1995. Recombinant mouse ob protein: evidence for a peripheral signal linking adiposity and central neural networks**.** Science 269:5546-549.

Cianflone, K., H. Lu, J. Smith, W. Yu, H. Wang, and M. Rosenbloom. 2005. Adiponectin, acylation stimulating protein, and complement C3 are altered in obesity in very young children. Clin Endocrinol. 62 (5): 567-572.

Curtis, C.R., H.N. Erb, C.J. Sniffen, R.D. Smith, and D.S. Kronfeld. 1985. Path analysis of dry period nutrition, postpartum metabolic and reproductive disorders, and mastitis in holstein cows. J. Dairy Sci. 68:2347.

Czech, M.P. 2000. Lipid rafts and insulin action. Nature 407:147-8.

Del Aguila, L.F., K.P. Claffey, and J.P. Kirwan. 1999. TNF-α impairs insulin signaling and insulin stimulation of glucose uptake in C_2C_{12} muscle cells. Am J Physiol 276:E849-E855.

Doris, R., R.G. Vernon, M.D. Houslay, E. Kilgour. 1994. Growth hormone decreases the response to anti-lipolytic agonists and decreases the levels of Gi2 in rat adipocytes. Biochem J. 297: 41–45.

Drackley, J.K., J.J. Veenhuizen, M. J.Richard, J.W. Young. 1991. Metabolic changes in blood and liver of dairy cows during either feed restriction or administration of 1,3-butanediol. J. Dairy Sci. 74:4254.

Dufva, G.S., E.E. Bartley, A.D. Dayton, and D.D. Riddell. 1983. Effect of niacin supplementation on milk production and ketosis of dairy cattle. J. Dairy Sci. 66:2329.

Edmonson, A. J., I. J. Lean, L. D. Weaver, T. Farver, and G. Webster. 1989. A body condition scoring chart for Holstein Dairy Cows. J. Dairy Sci. 72:68–78.

Fahey, G.C. and L.L. Berger. 1988. Carbohydrate nutrition in ruminants. The Ruminant Animal; Digestive Physiology and Nutrition. 1988. Waveland Press, Prospect Heights, IL.

Francois Elvinger F., R.P. Natzke, and P. J. Hansen. 1992. Interactions of heat stress and bovine somatotropin affecting physiology and immunology of lactating cows. J. D. Sci. 75:449-462.

Fain, J., A.K. Madan, M.L. Hiler, P. Cheema, and S.W. Bahouth. 2004. Comparison of the release of adipokines by adipose tissue, adipose tissue matrix, and adipocytes from visceral and subcutaneous abdominal adipose tissues of obese humans. Endocrinology 145:2273-2282.

Feinstein, R., H. Kanety, M.Z. Papa, B. Lunenfeld, and A. Karasik.
1993. Tumor necrosis factor-α suppresses insulin-induced tyrosine phosphorylation of insulin receptor and its substrates. J. Biol. Chem. 268:26055–26058.

Ferrari, P., M.G. Bianchetti, A. Sansonnens, and F.J. Frey. 2002. Modulation of renal calcium handling by 11β-hydroxysteroid dehydrogenase type 2. J Am Soc Nephrol 13: 2540-2546.

Fruebis, J., T. Tsao, S. Javorschi, D. Ebetts-Reed, M.R. Erickson, F.T. Yen, B.E. Bihain, and H.F. Lodish. 2001. Proteolytic cleavage product of 30-kDa adipocyte complement-related protein increases fatty acid oxidation in muscle and causes weight loss. Proc Natl Acad Sci 98: 2005-2010.

Frühbeck, G. 2002. Physiology and pathophysiology of energy storage. Heart and Metabolism 17:35-39.

Godden, S.M., S.C. Stewart, J.F. Fetrow, P. Rapnicki, R. Cady, W. Weiland, H. Spencer, and S.W. Eicker. 2003. The relationship between herd rBST-supplementation and other factors and the risk for removal of cows in Minnesota Holstein dairy herds. Pp. 55-64. Proc. Of the Four-State Nut. Conf., LaCrosse, WI.

Goff, J.P., R. Ruiz, and R.L. Horst. 2004. Relative acidifying activity of anionic salts commonly used to prevent milk fever. J Dairy Sci. 87: 1245-1255.

Goff, J.P., and R.L. Horst. 1998. Use of hydrochloric acid as a source of anions for prevention of milk fever. J Dairy Sci. 81: 2874-2880.

GröhnY.T., S.W. Eicker, V. Ducrocq, and J.A. Hertyl. 1997. Effect of diseases on the culling of Holstein dairy cows in New York state. J Dairy Science 81:966-978.

Grunfeld, C., C. Zhao, J. Fuller, A. Pollock, A. Moser, J. Friedman, and K. Feingold. 1996. Endotoxin and cytokines induce expression of leptin, the ob gene product in hamsters. J Clin Invest 97:2152-2157.

Guard, C. 1996. Fresh cow problems are costly: culling hurts the most. Page 100 in Proc. Ann. Conf. Vet., Cornell Univ., Ithaca, NY.

Halaas, J.L., C. Boozer, J. Blair-West, N. Fidahusein, D.A. Denton; J.M. Friedman. 1997. Physiological response to long-term peripheral and central leptin infusion in lean and obese mice**.** Proc Nat Acad Sci 94: 8878-8883.

Hammon, D.S., and J.P. Goff. 2006. Immune function and energy status in Holstein cows with uterine infections. Page 30-36 in Proc of the Mid-South Ruminant Nutr Conf.

Hayirli, A., R. R. Grummer, E. V. Nordheim, and P. M. Crump. 2002. Animal and dietary factors affecting feed intake during the prefresh transition Period in Holsteins. J. Dairy Sci. 85:3430–3443.

Hardie, D.G. 2004. The AMP-activated protein kinase pathway – new palyers upstream and downstream. J of Cell Science 117: 5479-5487.

Hauner, H., T. Petruschke, M. Russ, K. Rohrig, and J. Eckel. 1995. Effects of tumour necrosis factor alpha (TNF-α) on glucose transport and lipid metabolism of newly-differentiated human fat cells in cell culture. Diabetes 38:764-771.

Hillers, J. K., P. L. Senger, R. L. Darlington, and W. N. Fleming. 1984. Effects of production, season, age of cow, dry days, and days in milk on conception to first service in large commercial dairy herds. J. Dairy Sci 67:861.

Hirosumi, J., Tuncman, G., Chang, L., Gorgun, C., Uysal, K.T., Maeda, K., Karin, M., and G.S. Hotamisligil. 2002. A central role for JNK in obesity and insulin resistance. Nature 420: 333-336.

Hoggard, N., P. Haggerty, L. Thomas, and R. Lea. 2001. Leptin expression in placental and fetal tissues: does leptin have a functional role? Biochem Soc Trans 29: 57-63.

Horst, R.L., J.P. Goff, T.A. Reinhardt, and D.R. Buxton. 1997. Strategies for preventing milk fever in dairy cattle. J Dairy Sci. 80: 1269-1280.

Horst, R.L., J.P. Goff, and T.A. Reinhardt. 1994. Symposium: calcium metabolism and utilization. J Dairy Sci. 77: 1936-1951.

Hotamisligil, G.S., P. Peraldi, A. Budavari, R. Ellis, M.F. White, and B.M. Spiegelman. 1996. IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF-α and obesity-induced insulin resistance. Science 271: 665–668.

Janeway, C., P. Travers, M. Waldport, and M. Shlomchik. 2001. Immunobiology, Fifth Edition. Garland Publishing, New York, NY.

Johnson, J.A., Z. Han, K. Otani, H. Ye, Y. Zhang, H. Wu, Y. Horikawa, S. Misler, G.I. Bell, and K. Polonsky. 2004. RyR2 and calpain-10 delineate a novel apoptosis pathway in pancreatic islets. J of Biol Chem 279:24794-24802.

Joyce, P.W., W.K. Sanchez, and J.P. Goff. 1997. Effect of anionic salts in prepartum diets based on alfalfa. J Dairy Sci. 80: 2866-2875.

Kannisto, K., K.H. Pietilainen, E. Ehrenborg, A. Rissanen, J. Kaprio, A. Hamsten, and H. Yki-jarvinen. 2004. Overexpression of 11β-hydroxysteroid dehydrogenase-1 in adipose tissue is associated with acquired obesity and features of insulin resistance: studies in young adult monozygotic twins. J. of Clin. Endocrin. & Metab. 89: 4414-4421.

Kappes, A. and G. Löeffler. 2000. Influences of ionomycin, dibutyrl-cycloAMP and tumour necrosis factor-alpha in intracellular amount and secretion of apM1 in differentiating primary human preadipocytes. Horm Metab Res 32:548-554.

Khani, S. and J.A. Tayek. 2001. Cortisol increases gluconeogenesis in humans: its role in the metabolic syndrome. Clinical Science 101: 739-747.

Kharroubi, I., L. Ladriere, A. Cardozo, Z. Dogusan, M. Cnop, and D. Eizirik. 2004. Free fatty acids and cytokines induce pancreatic β-cell apoptosis by different mechanisms: role of nuclear factor kB and endoplasmic reticulum stress. Endocrinology 145: 5087-5096.

Kim, J.K. Kim, Y., Fillmore, J.J., Chen, Y., Moore, I., Lee, J., Yuan, M., Li, Y.W., Li, Karin, M., Perret, P., Shoelson, S.E., and Shulman, G.I. 2001. Prevention of fat-induced insulin resistance by salicylate. J. Clin. Invest. 108: 437-446.

Kimura, K., J.P. Goff, M.E. Kehrli and T.A. Reinhardt. 2002. Decreased neutrophil function as a cause of retained placenta in dairy cattle. J. Dairy Sci. 85:544-550.

Kirwan, J.P., P. Catalano et al. 2002. TNF-α is a predictor of insulin resistance in human pregnancy. Diabetes. July; 51 (7): 2007-13.

Klucinski, W., E. Miernik-Degorska, W. Degorski, S. Targowski, and A. Winnicka. 1988. Effect of ketone bodies on mitogenic response of bovine milk lymphocytes. J. Vet. Med. A35:626.

Kremer, W.D., E.N. Noordhuizen-Stassen, F.J. Grommers, Y.H. Schukken, R. Heeringa, and A. Brand. 1993. Severity of experimental escherichia coli mastitis in ketonemic and nonketonemic dairy cows. J. Dairy Sci. 7:3428.

Laemmli U.K. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature, 1970, 227:680.

Lanna, D.P.D., and D.E. Bauman. 1999. Effedt of somatotropin, insulin, and glucocorticoid on lipolysis in chronic cultures of adipose tissue from lactating cows. J Dairy Sci 82:60-68.

LeBlanc, S.J., K.E. Leslie, and T.F. Duffield. 2005. Metabolic predictors of displaced abomasums in dairy cattle. J Dairy Sci. 88: 159-170.

Lennon, E.J. and W.F. Piering. 1970. A comparison of the effects of glucose ingestion and NH_4Cl acidosis on urinary calcium and magnesium excretion in man. J. Clin. Invest. 49: 1458-1465.

Lofgren, P., V. Van Harleman, S. Reynisdottir, E. Naslund, M. Ryden, S. Rossner, P. Arner. 2000. Secretion of tumor necrosis factor-alpha shows a strong relationship to insulin-stimulated glucose transport in human adipose tissue. Diabetes 49:688-692.

Lord, E., S. Ledoux, B.D. Murphy, D. Beaudry, and M.F. Palin. 2005. Expression of adiponectin and its receptors in swine. J. Anim. Science 83:565-578.

Manelli, F. and A. Giustina. 2000. Glucocorticoid-induced osteoporosis. TEM 11:79-85.

Markusfeld O. 1985. Relationship between overfeeding, metritis, and ketosis in high yielding dairy cows. Vet. Record pp:489-491.

Mathews, C.K., K.E. Van Holde, K.G.Ahern. 1999. Biochemistry. Addison-Wesley Longmann, San Francisco, CA.

May, R.C., R.A. Kelly, and W.E. Mitch. 1985. Metabolic acidosis stimulates protein degradation in rat muscle by a glucocorticoid-dependent mechanism. J Clin Invest 77:614-621.

Miner, J., J. Byatt, C. Baile, G. Krivi. 1992. Adipsin expression and growth in rats as influenced by insulin and somatotropin. Phys. and Behavior vol. 54:207-212.

Miner, J.L. 2004. The adipocyte as an endocrine cell. J. Anim. Sci. 82:935-941.

Moberg, G.P. 1991. How behavioral stress disrupts the endocrine control of reproduction in domestic animals. J. Dairy Sci. 74: 304-311.

Moller, D.E. 2001. New drug targets for type 2 diabetes and the metabolic syndrome. Nature 414: 821-827.

Moore, D., V. Ishler. 1997. Managing dairy cows during the transition period: focus on ketosis. Vet. Med. 1061.

Moore, S.J., M.J. Vanderhaar, B.K. Sharma, T.E. Pilbeam, D.K. Beede, H.F. Bucholtz, J.S. Liesman, R.L. Horst, and J.P. Goff. 2000. Effects of altering dietary cation-anion difference on calcium and energy metabolism in peripartum cows. J. Dairy Sci. 83:2095-2104.

Nguyen, M.T., H. Satoh, S. Favelyukis, J. Babendure, T. Imamura, J. Sbodio, J. Zalevsky, B. Dahiyat, Nai-Wen Chi, and J.M. Olefsky. 2005. JNK and TNF-a mediate free fatty acid-induced insulin resistance in 3T3-L1 adipocytes. J Biochemistry. Manuscript M504611200 August 5, 2005.

National Research Council. 2001. Nutrient Requirements of Dairy Cattle. 7th Revised Edition. Natl. Acad. Sci., Washington DC.

Oetzel, G. 1991. Meta-analysis of nutritional risk factors for milk fever in dairy cattle. J Dairy Sci 74:3900-3912.

Oikawa, S., N. Katoh, F. Kawawa, and Y. Ono. 1997. Decreased serum apolipoprotein B-100 and Aa-1 concentrations in cows with ketosis and left displacement of the abomasums. AJVR, Vol 58, No. 2.

Ouchi, N., et al. 1999. Novel modulator for endothelial adhesion molecules: adipocytes-derived plasma protein adiponectin. Circulation 100 (25):2473-6.

Overton, T. R. 2000. Transition cow programs – the good, the bad, and how things get ugly. Page 5 in Proc. 2001 Tri-State Northwest Dairy Shortcourse, Washington State University, Oregon State University, Idaho State University, Skamania, WA.

Overton, T.R. 2004. Managing the metabolism of transition cows. Proc of the 2nd Mid-Atlantic Nut. Conf., Timonium, MD.

Overton, T. R., and M.R. Waldron. 2004. Nutritional management of transition dairy cows: strategies to optimize metabolic health. J Dairy Sci. 87: (E Suppl.): E105-E119.

Pelleymounter, M; M.J. Cullen; M. B. Baker; R. Hecht; D. Winters; T. Boone; F. Collins. 1995. Effects of the obese gene product on body weight regulation in ob/ob mice. Science 269:540-543.

Piepenbrink, M.S., and T.R. Overton. 2000. Liver metabolism and production of periparturient dairy cattle fed rumen-protected choline. J. Dairy Sci. 83 (Suppl. 1):257. (Abstr.)

Priesler, M.T., P.S. Weber, R.J. Templeman, R.J. Erskine, H. Hunt, and J.L. Burton. 2000. Glucocorticoid receptor down-regulation in neutrophils of periparturient cows. AVJR 61: 14-19.

Priesler, M.T., P.S. Weber, R.J. Templeman, R.J. Erskine, H. Hunt, and J.L. Burton. 2000. Glucocorticoid receptor expression profiles in mononuclear leukocytes of periparturient dairy cows. J Dairy Sci 83: 38-47.

Qian, H., D.B. Hausman, M.M. Compton, R.J. Martin, M.A. Della-Fera, D.L. Hartzell, and CA. Baile. 2001. TNF-α induces and insulin inhibits caspase 3-dependent adipocyte apoptosis**.** Biochemical and Biophysical Research Communications 284**:**1176-1183.

Reid, I.M., C.J. Roberts, and D.G. Baird. 1980. The effects of underfeeding during pregnancy and lactation on structure and chemistry of bovine liver and muscle. J. Agric. Sci. 94:239.

Rogers, C.A., A.C. Fitzgerald, M.A. Carr, B.R. Covey, J.D. Thomas, and M.L. Looper. 2004. On-farm management decisions to improve beef quality of market dairy cows. J. Dairy Sci. 87:1558-1564.

Rukkwamsuk, T., T. Kruip, G.A. Meijer, T.Wensing. 1999. Hepatic fatty acid composition in periparturient dairy cows with fatty liver induced by intakeof a high energy diet in the dry period. J Dairy Sci 82:280-287.

Rukkwamsuk, T., Wensing, T., and M.J. Geeling. 1998. Effect of overfeeding during the dry period on regulation of adipose tissue metabolism in dairy cows during the periparturient period. J Dairy Sci 81:2904-2911.

Sakai, T., Hayakawa T., M. Hamakawa, K. Ogura, and S. Kubo. 1993. Therapeutic effects of simultaneous use of glucose and insulin in ketotic dairy cows. J. Dairy Sci. 76:109.

Saltiel, A.R., and R. Kahn. 2001. Insulin signaling and the regulation of glucose and lipid metabolism. Nature 414: 799-806.

Salway, J.G. 1999. Metabolism at a glance. University of Surrey, Guildford, UK. pp: 78-81.

Sanchez, W.K., Block, E. 2000. Update on DCAD: milking and transition cows. Proc of 2000 California Animal Nutrition Conference. pp: 106-125.

SAS User's Guide: Statistics, Version 8 Edition. 2001. SAS Inst., Cary, NC.

Scherer, P.E., S. Williams, M. Fogliano, G. Baldin, and H.F. Lodish. 1995. A novel serum protein similar to C1q, produced exclusively in adipocytes. J. Biol. Chem. 270: 26746-26749.

Schvarcz E., Palmer M., Aman J., Berne, C. 1995. Hypoglycemia increases the gastric emptying rate in healthy humans. Diabetes Care 18:674-676.

Senger, P. L., **2003.** Fertility factors—which ones are really important? Proc West Dairy Mgmt Conf 6:89-105.

Shand, B.I., R.S. Scott, P.A. Elder, and P.M. George. 2003. Plasma adiponectin in overweight, nondiabetic individuals with or without insulin resistance. Diabetes, Obesity and Metabolism 5: 349-353.

Shepard, P.R. and B.B. Kahn. 1999. Glucose transporters and insulin action. N Engl J Med 341:248–257.

Shpigel, N., R. Chen, Y. Andivar, E. Bogin. 1996. Use of corticosteroids alone or combined with glucose to treat ketosis in dairy cows. JAVMA Vol 208, No. 10.

Sicuro, A., K. Mahlbacher, H.N. Hulter, and R. Krapf. 1998. Effect of growth hormone on renal and systemic acid-base homeostasis in humans. AJP Renal Physiol 274: 650-657.

Smith, T.R., A.R. Hippen, D.C. Beitz, and J.W. Young. 1997. Metabolic characteristics of induced ketosis in normal and obese dairy cows. J Dairy Sci 80:1569-1581.

Stich, V. and M. Berlan. 2004. Physiological regulation of NEFA availability: lipolysis pathway. Proceedings of the Nutrition Society 63:369-374.

Stedman's Medical Dictionary, 27th Edition. 2000. Lippincott, Williams and Wilkins, Baltimore, MD.

Stefan, N., and A. Fritsche. 2000. Acute stimulation of leptin levels in humans during hyperglycemic hyperinsulinemia. Influence of free fatty acids. Diabetes: May 2000.

Stejskal, D., and J. Bartek. 2003. Adiponectin in patients with various stages of coronary heart disease – comparison of its concentration in coronary arteries and peripheral venous circulation. Biomed. Papers 147:161-166.

Stephens, J., J. Lee, P. Pilch. 1996. Tumor Necrosis Factor-α-induced insulin resistance in 3T3-L1 adipocytes is accompanied by a loss of insulin receptor substrate-1 and GLUT4 expression without a loss of insulin receptor-mediated signal transduction. Journal of Biochemistry. January 272(2):971-976.

Stunkard, A.J. 1957. The effect of the intravenous administration of various nutrients on the gastric hunger contractions of a man with severe brain damage. Am. J. Clin. Nutr. 5:203

Tanaka, Y. and H.F. DeLuca. 1973. The control of 25-hydroxyvitamin D metabolism by inorganic phosphorus. Arch Biochem Biophys. 154 : 566-574.

Templeman, R., J. Burton, et al. 2002. Genetic variation in bovine neutrophil sensitivity to glucocorticoid challenge. Actaa Agric. Scand., Section A, Animal Sci. April 52:189-202.

Tomas. E., T. Tsao, A. Saha, H.E. Murrey, C.C. Zhang, S.I. Itana, H.F. Lodish, and N.B. Ruderman. 2002. Enhanced muscle fat oxidation and glucose transport by Acrp30 globular domain: acetyl-CoA carboxylase inhibition and AMP-activated protein kinase activation. Proc Nat Acad Sci 99: 16903-16313.

Trainer, P. 2002. Corticosteroids and pregnancy. Semin Reprod Med. Nov; 20(4):375-80.

USDA-APHIS. 2002. Reference of dairy cattle health and health management practices in the United States, Part III. National Animal Health Monitoring Service, Ft. Collins, CO.

Uysal, K.T., Weisbrock, S.M., Marino, M.W., and G.S. Hotamisligil. 1997. Protection from obesity-induced insulin resistance in mice lacking TNF-α function. Nature 389: 610-614.

Vagnoni, D.B., and G.R. Oetzel. 1998. Effects of dietary cation-anion difference on the acid-base status of dry cows. J Dairy Sci. 81: 1643-1652.

Ventre, J., Doebber, T., Wu, M., Mac Naul, K., Stevens, K., Pasparakis, M., Kollias, G., and D. Moller. 1997. Targeted disruption of the tumor necrosis factor-α gene. Diabetes 46: 1526-1531.

Vernon, R.G., E. Finley. 1985. Regulation of lipolysis during pregnancy and lactation in sheep. Biochem. J. 230: 651.

Vernon, R.G., E. Finley, P. Watt. 1991. Adenosine and the control of adregenig regulation of adipose tissue lipolysis during lactation. J. Dairy Science 74:695-705.

Warne, J.P., 2003. Tumour necrosis factor α: a key regulator of adipose tissue mass. Journal of Endocrinology 177:351-355.

Weber, P.S., S. Madsen, G. Smith, J. Ireland, J. Burton. 2001. Pre-translational regulation of neutrophil L-selectin in glucocorticoid-challenged cattle. Vet. Immunology and Immunopathology 83:213-240.

Weiss, R., S. Dufour, A. Groszmann, K. Petersen, J. Dziura, S.E. Taksali, G. Shulman, and S. Caprio. 2003. Low adiponectin levels in adolescent obesity: a marker of increased intramyocellular lipid accumulation. J of Clin Endocrin and Met 88: 2014-2018.

White, R.T., Damm, D., Hancock, N. Rosen, B.S., et. al. 1992. Human adipsin is identical to complement factor D and is expressed at high levels in adipose tissue. J. Biol. Chem. 267: 9210-9213.

Wildman, E. E., G. M. Jones, P. E. Wagner, R. L. Boman, H. F. Troutt, Jr., and T. N. Lesch. 1982. A dairy cow body condition scoring system and its relationship to selected production characteristics. J. Dairy Sci. 65:495–501.

Wright, E.M., Turk, E. Abbel, B. Mundlos, S. and J. Dyer. 1991. Molecular genetics of intestinal glucose transport. J Clin Invest 88:1435-40.

Yamamoto, Y. et al. 2002. Correlation of the adipocytes-derived protein adiponectin with insulin resistance index and serum high-density lipoprotein-cholesterol, independent of body mass index, in the Japanese population. Clinical Science 103, 137-142.

Yin, M., Yamamoto, Y., and R. Gaynor. 1998. The anti-inflammatory agents aspirin and salicylate inhibit the activity of IkB kinase-β. Nature 396: 77-80.

Yamauchi, T., J. Kamon, Y. Minokashi, Y. Ito, S. Uchida, S. Yamashita, M. Noda, S. Kita, K. Ueki, K. Eto, Y. Akanuma, P. Froguel, F. Foufelle, P. Ferre, D. Carling, S. Kimura, R. Nagai, B.B. Kahn, and T. Kadowaki. 2002. Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. Nature Med. 8: 1288-1295.

Yokota, T., K. Oritani, I. Takahashi, J. Ishikawa, A. Matsuyama, N. Ouchi, T. Funahashi, A. Tenner, Y. Tomiyama, and Y. Matsuzawa. 2000. Adiponectin, a new member of the family of soluble defense collagens, negatively regulates the growth of myelomonocytic progenitors and the functions of macrophages. Blood 96: 1723 – 1732.

Yokata, T., C.S. Meka, K.L. Medina, H. Igarashi, P.C. Comp, M. Takahashi, M. Nashida, K. Ortani, J. Miyagawa, T. Funahashi, Y. Tomiyama, Y. Matsuzawa, and P.W. Kincade. 2002. Paracrine regulation of fat cell formation in bone marrow cultures via adiponectin and prostaglandins. J Clin Invest 109:1303-1310.

Zhang, H., M. Halbleib, F. Ahmad, V.C. Manganiello, A.S. Greenberg. 2002. Tumor necrosis factor-α stimulates lipolysis in differentiated human adipocytes through activation of extracellular signal-related kinase and elevation of intracellular cAMP. Diabetes 51: 2929-2935.