

Evaluation of weaning stress in beef calves

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ABSTRACT

Conventional techniques within the beef cattle industry involve weaning the calf from the dam when the calf is about 205 days of age. Weaning induces a stress-response that is implicated in reducing the health and productivity of newly weaned calves. Our goal was to evaluate the impact of weaning on the stress immune responses of beef calves. To that end, we 1) evaluated novel methods to quantify physiological markers of stress, 2) compared immune function and growth of calves grazing legume versus grass forages, and 3) compared the effects of abrupt versus two-stage weaning on calves. In study 1, calf, yearling, and adult beef cattle were used to assess the accuracy and precision of handheld glucometers in quantifying bovine blood glucose concentration. Precision Xtra® and ReliOn® glucometers were used chute side to quantify blood glucose concentrations in cattle and were compared to an accepted plasma glucose analysis on the same samples for validation. The Precision Xtra® glucometer was more accurate and precise than the ReliOn® glucometer. In study 2, weaned heifers were used to compare the immunomodulatory effects of grazing alfalfa versus fescue over a 30 day grazing period. No differences were detected in the interferon gamma (IFN γ) production and weight gain between the heifers on alfalfa and fescue. In study 3, effects of two-stage (fenceline) and abrupt weaning were compared. Calf weights, immune cell function, antibody production, blood glucose concentrations, fecal cortisol concentrations, and gene expression (FAS, IL-4, IL-10, and IFN γ) were measured pre- and post-weaning. On the day after weaning, the abruptly weaned calves had higher blood glucose concentrations than fenceline weaned calves. Fecal cortisol

concentration and gene expression of FAS and IL-4 increased in both groups after weaning, but no differences were detected between the weaning treatments. Gene expression of IL-10 and IFN γ did not change over time. No date, treatment or treatment*date effect was detected for total weight gain or IFN γ production within the non-stimulated and the mitogen-stimulated whole blood samples.

DEDICATION

I dedicate this work to: my father, Craig Landa, whose lessons in veterinary medicine have molded my life's interests and passions; my mother, Rebecca Landa, who has always supported and guided me during challenging times; and my Aunt, Melissa Cassidy, whose grace and generosity cannot be described with words. I am blessed to have a supporting and loving family that brightens my life each day. This work and my success would be impossible without them.

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Chapter 1

Introduction

Novel methods to improve cattle health and management practices within the beef cattle industry are desirable in decreasing production costs while simultaneously advancing meat quality and cattle productivity. Specifically, aims to minimize the stress response elicited by weaning on calves and the provision of quality nutrients in forage-based diets are common challenges that impact the beef industry. Research relating to techniques to reduce calf stress at weaning, improve calf health, and the effects of differing forages on cattle productivity has provided nutritional and disease-prevention insight, but many unknowns still remain. The present thesis compiles findings of already published work in these areas and furthers the understanding of methods to improve efficiency in diagnostic care of cattle, assessing the effects of legume versus grass forage on heifer immune function and growth, and evaluating the effect of gradual weaning on reducing the stress response in newly weaned calves.

In study 1, beef calf, yearling, and adult cattle were used to analyze the accuracy and precision of two handheld glucometer systems in quantifying bovine whole blood glucose concentrations. Precision Xtra® and ReliOn® Confirm glucometers were used to analyze whole blood glucose concentration in all ages of cattle. Glucose concentrations measured by these two glucometers were compared to the golden standard of plasma glucose analysis on the same blood samples. The glucometer whole blood and plasma glucose comparisons were used to assess the ability of these systems to provide rapid chute-side analysis of bovine whole blood glucose concentrations.

Comparisons of the effects of legume versus grass forages on heifer immune function and weight gain were evaluated in study 2. Angus heifers were grazed on non-endophyte infected fescue (*Festuca arundinacea* Shreb.) or alfalfa (*Medicago sativa* L.) for a 30 day period. During this grazing period, heifer weights were monitored and blood samples were taken to compare interferon gamma production by leukocytes. Interferon gamma production was analyzed in non-stimulated and pokeweed mitogen-stimulated blood samples before heifers were placed on their allotted pasture and then again after 15 and 30 days of grazing on their respective pasture.

The third study in the current thesis describes the effects of a gradual (fenceline) weaning technique on newly weaned calf immune function, stress, and weight gain in comparison to a conventional abrupt weaning technique. Fenceline-weaned calves were separated from their dams by a single fence for 6 days prior to weaning. Abruptly weaned calves had no intermediary stage to gain independence from their dams. Calves in both weaning treatments were vaccinated on the day after weaning to compare antibody production against a primary and secondary antigenic exposure. Fecal cortisol, weight gain, interferon gamma production in non-stimulated and pokeweed mitogen-stimulated blood, and immune gene expression were monitored in response to the weaning treatments.

Chapter 2

Literature Review

Introduction

Livestock producers strive to produce quality meat in substantial quantity to meet consumer demand. In order to accomplish the highest production and quality of meat, the industry constantly modifies management protocols to improve profitability and meet production goals. The beef cattle sector of the livestock industry is no stranger to the constant search for improvements in management practices. Weaning management practices for beef calves are among those that are undergoing constant alterations in aims to produce higher quality meat and maintain a profitable industry. Improved weaning procedures can promote the growth of healthier and heavier calves that produce more revenue at auction with enhanced quality of meat end-products.

Factors of interest in this review include weaning strategies and grazing options for forage-raised beef. The discussion of these factors will be presented in two parts. Part one will discuss weaning in terms of how it can impact productivity early in the life of beef cattle. Part two will introduce some different forage types available for grazing in southwest Virginia. Forage nutritive content and availability are important for sustaining prolonged performance from weaning onward.

PART I – Weaning in Beef Calves

Weaning and its Purpose

In beef cattle production, weaning is defined by the complete physical separation of the dam and her calf. The weaning process involves the termination of milk feeding, and often a change in solid feeds and living environment for the weaned calf (Price, Harris et al. 2003). In natural circumstances, weaning of the cow-calf pair is a much more gradual process than the one that is implemented in beef cattle production systems. During the natural weaning process, the calf exhibits decreased suckling while the mother produces less milk and reduces mothering behavior as the calf ages (von Keyserlingk and Weary 2007). The reduction in mothering behavior by the dam, and thereby her increased independence, may be explained by the parent-offspring conflict theory (Trivers 1974). In this theory, when the calf is young, the provision of maternal care to the calf largely benefits the calf in terms of its chances for survival while costing the dam little in terms of her ability to produce additional offspring. However, as the offspring matures, the dam benefits more from withholding care and conserving her energy for producing future offspring (Trivers 1974). These behavioral and relationship changes lead to eventual weaning and separation of the cow-calf pair by the time the calf is 14 months of age (Enríquez, Ungerfeld et al. 2010).

In conventional beef production, the weaning process is expedited through the use of abrupt separation of the cow-calf pair when the calf is approximately 210 days of age (Hudson, Banta et al. 2010). The abrupt weaning protocol has been implemented to increase the overall production potential of the dam, and thereby increasing the overall production potential of the beef herd. In the beef industry, producers strive to achieve one calf per year per cow. To accomplish this, cows must be re-bred within three months after calving (Freetly, Nienaber et al.

2006). Once re-bred, the cow must dedicate energy to lactation in order to sustain the calf by its side in addition to providing nutrients and energy for the developing fetus (Freetly, Nienaber et al. 2006). Fetal nutrient requirements are the greatest during late gestation (Battaglia and Meschia 1978). Therefore, producers aim to complete weaning prior to late term gestation.

Hudson and colleagues (2010) compared the effects of conventional (210 days of age) versus late (300 days of age) weaning on dams' body condition score (BCS) and body weight (BW). The cows that supported the late-weaned calves had lower BCS, BW, and subsequent reproductive performance than dams of conventionally-weaned calves (Hudson, Banta et al. 2010). This trend can be partially explained by the 100.5 Mcal increase in energy demand to support a late-weaned calf into the 8th, 9th, and 10th month of lactation. However, if calves are weaned at 210 days of age, that energy demand is avoided, thereby preserving resources for maintenance of a healthy BCS and BW in the dam (Hudson, Banta et al. 2010). The cumulative effect of late weaning and increased energy demand of the late-term fetus can impose detrimental effects on dams' health and production capacity.

A cow's BCS at calving is an important factor affecting the length of postpartum interval and pregnancy rates (Wiltbank, Rowden et al. 1964; Selk, Wettemann et al. 1988). Low BCS at calving will result in a longer interval to first estrus and decreased fertility of that estrus cycle (Wiltbank, Rowden et al. 1964). This situation is avoided through the practice of weaning the calf at approximately 210 days of age, thereby increasing overall production of the beef industry.

Weaning-Related Stress

Abrupt weaning is implemented as a general husbandry practice in cow-calf beef production systems. Abrupt separation of the cow-calf pair imposes physical, psychological, and

nutritional stressors on the calves involved (Lynch, Earley et al. 2010). Stress can be defined as any external or internal change that disrupts the internal physiological environment (Sheridan, Dobbs et al. 1994). Stressors may be physical or psychological, and are often associated with exposure to novel experiences and environmental conditions (Grandin 1997).

The abrupt weaning procedure consists of many novel conditions that impose stress on calves. Contributing to the multitude of stressors related to weaning is the common practice of immediately transporting calves by truck and trailer to a new facility after separation from their dams. The associated transportation process is one of the most widely recognized stressors in beef cattle (Arthington, Eichert et al. 2003). Some of the potential stressors that are associated with transportation of newly-weaned calves include noise, vibration, novel social regrouping, crowding, climatic factors (e.g., temperature), restraint, and feed and water deprivation (Swanson and Morrow-Tesch 2001). The newly-weaned calves are also commonly exposed to novel antigens as well as an increased pathogen and environmental stress load at the new location (Arthington, Eichert et al. 2003).

Physiological Impact of Stress

The calf responds to the multi-faceted, stress-related stimuli of weaning and transportation processes through physiological changes that result in a shift away from the homeostatic condition. Mitchell et al. (1988) determined that the animal's physiologic response to a stressor consists of two phases. One phase involves a stage of "perception of events." In this stage, poor handling practices, novelty, and anxiety are implicated in producing negative responses that can cause the activation of the hypothalamic-pituitary-adrenal (HPA) axis (Mitchell, Hattingh et al. 1988; Grandin 1997; Jacobson and Cook 1998). The other phase

involves a sympathetic adrenal medulla response that can occur when neurogenic stimulation, such as transport, is imposed on the cattle (Mitchell, Hattingh et al. 1988). Combinations of perceived environmental stress and neurogenic-associated stress can produce a mixed response within these two phases.

Physiological changes such as increased body temperature and increased heart and respiration rates are responses that are commonly observed in conjunction with the stress response and HPA activation (Sheridan, Dobbs et al. 1994). The HPA axis affects perception in the brain, and involves the release of hypothalamic corticotrophin-releasing factor and vasopressin, which stimulates the anterior pituitary to secrete adrenocorticotrophic hormone (ACTH). Circulating ACTH causes the adrenal cortex to produce glucocorticoids which are an essential component of the stress-adaptive mechanism of the body and are potent immunosuppressive agents (Aich, Shakiba et al. 2007). Glucocorticoids are potent inhibitors of inflammation by altering immune cell trafficking, effector cell activity, and inhibiting pro-inflammatory gene expression (Buckbinder and Robinson 2002; De Bosscher, Vanden Berghe et al. 2003).

Norepinephrine and epinephrine are two hormones that are also produced upon HPA activation. These hormones interact with alpha and beta-adrenergic receptors to mediate the adaptive cardio-vascular and metabolic effects under the conditions of stress (Sheridan, Dobbs et al. 1994). Beta-adrenergic receptors are expressed on T and B lymphocytes, macrophages, neutrophils, and natural killer (NK) cells (Sheridan, Dobbs et al. 1994). The beta-adrenergic receptors are stimulated in response to increased concentrations of norepinephrine, thereby elevating the production of cyclic AMP and increasing the activation state of these mononuclear cells (Sheridan, Dobbs et al. 1994).

Norepinephrine increases the total number of white blood cells (WBC) in circulation; specifically increased numbers of neutrophils, eosinophils, and mononuclear cells (Kent and Ewbank 1986; Murata, Takahashi et al. 1987). Lynch, et al. (2010) reported that calves that were weaned and moved to a new environment had a transient neutrophilia, impaired neutrophil phagocytic function, decreased peripheral lymphocyte count, and altered immunophenotypes (Lynch, Earley et al. 2010). Murata et al. (1987) also reported a decrease in T-lymphocyte populations in calves that were subjected to four hours of transport.

Under stressful conditions such as transportation, neutrophils and macrophages are thought to have an enhanced rate of respiratory burst, thereby producing a greater concentration of reactive oxygen species which leads to a condition of oxidative stress within the body (Wernicki, Urban-Chmiel et al. 2006). Markers of oxidative stress are increased in calves subjected to stress as compared to control calves (Chirase, Greene et al. 2004). Oxidative stress has negative impacts on the proper functioning of the body as it reduces the antioxidant defense capacity (Chirase, Greene et al. 2004). Despite the evidence that oxidative stress results from physical stressors, it is unlikely that the degree of oxidative stress resulting from weaning is severe enough to be deleterious to tissues (Burke, Scaglia et al. 2009). Therefore, oxidative stress cannot be considered the direct link to the manifestation of disease that is commonly observed with recently weaned beef calves, but it must still be considered in the physiological reaction to the stressful stimuli associated with conventional weaning procedures.

The physiological response to stressful stimuli is substantial and has great potential to negatively affect animals' health and performance. Under stressful conditions, the body undergoes a shift away from the homeostatic condition while it spends energy allocating resources to producing stress-related hormones, releasing protein and nutrient reserves, and

altering the function of the immune system. The body's multi-faceted response to stress often results in an increased susceptibility to infection from pathogens and development of a disease.

Impact of Stress on Calf Health

The combination of the weaning-related stress, naïve immune systems, and lack of prior exposure to novel environments and pathogens increases newly-weaned calves' susceptibility to disease (Swanson and Morrow-Tesch 2001). The interaction between stress and disease susceptibility is complex. Both the duration and nature of the stressor play a role in determining whether there is enhanced or decreased severity of disease (Hodgson, Aich et al. 2005). The additive impacts of stress paradigms (e.g., environmental, social, transport, etc. stress experienced independently or together) have been shown to adversely affect the survival rate of diseased animals (Sheridan, Dobbs et al. 1994). Hodgson, et al. (2005) provided evidence that stress paradigms increased disease severity. In Hodgson et al.'s study, mortality due to respiratory disease was twice as high in calves experiencing a combination of social reorganization and transport than in calves subjected to transportation alone (Hodgson, Aich et al. 2005). In beef cattle, the combination of weaning and transport stress results in weight loss and increased susceptibility and severity of disease (Arthington, Eichert et al. 2003; Hodgson, Aich et al. 2005)

Bovine respiratory disease (BRD) is the most common disease in the beef cattle industry (Staples and Haugse 1974; Swanson and Morrow-Tesch 2001; Hodgson, Aich et al. 2005). In addition to cost of therapy during the clinical disease, calves with BRD also have decreased feed efficiency final body weight, average daily gain, carcass weight, and USDA quality grades (Gardner, Dolezal et al. 1999; Aich, Shakiba et al. 2007).

Respiratory infections usually occur when a primary viral infection overcomes compromised host defenses. Once the primary viral infection is established, the probability and severity of a secondary bacterial infection is increased (Hodgson, Aich et al. 2005). A primary viral infection followed by a secondary bacterial infection is termed the viral-bacterial synergy. This type of viral-bacterial synergy model creates the most virulent and fatal infections of BRD (Hodgson, Aich et al. 2005).

Stressful stimuli have the potential to hinder the overall health status of an animal. The overall stress load can further affect the severity and incidence of disease. Heavier loads of stress have been indicated to enhance susceptibility to disease and have been implicated in the viral-bacterial synergy model of disease. Young animals with naïve immune systems and a lack of exposure to novel environments and pathogens are even more vulnerable to the effects of stress. The negative effects of stress on the health status of the animal must be recognized so that management systems can be implemented in order to improve the overall health and productivity of newly weaned calves.

Change in Behavior as a Response to the Stress of Weaning

Behavioral responses to weaning are predictable and remain detectable for 3-5 days after separation (Haley, Bailey et al. 2005). These deviations from normal behavior provide evidence that the traditional method of weaning by abrupt separation has a negative effect on the well-being of beef cattle (Haley, Bailey et al. 2005). Changes in behavior in response to abrupt weaning include decreased eating and lying down and increased walking and vocalizations (Veissier, Le Neindre et al. 1989; Price, Harris et al. 2003; Boland, Scaglia et al. 2008). The increased vocalization and walking behavior of calves after abrupt weaning can be explained by

the natural process that would foster reunion with their dams if they were physically separated. The weaning process, social separation, and termination of milk feeding or a combination of all these factors are the sources that induce behavioral changes of calves (Price, Harris et al. 2003).

Price, et al. (2003) observed vocalization frequencies to be the greatest during the first three days after weaning, with the most dramatic change in behavior observed on day two (20 to 30 hours post-weaning). The highest vocalization rates on day two suggested that motivation of the calves to contact their dams had peaked at that time (Price, Harris et al. 2003). Additionally, calves weaned by conventional abrupt techniques, were observed to spend less time grazing than calves weaned by intermediate-weaning techniques for the first four days following weaning (Boland, Scaglia et al. 2008).

The behavioral responses to weaning are associated with health risks to calves. Increased time spent walking can lead to fatigue, muscle damage or other injuries. Decreased feed and water intake can result in weight loss and dehydration. Together, all of the behavior changes in response to abrupt weaning can further increase an animal's susceptibility to injury and disease.

Alternative Weaning Methods

While the definition of weaning remains the same, weaning techniques to separate the calf from its dam can differ. In addition to abrupt weaning, two alternative weaning techniques have been suggested to reduce the stress response of calves at weaning; fence-line weaning, and a two-stage nose clip technique.

Fence-line weaning and nose clips are two prospective weaning techniques that have been implemented into research projects aiming to identify protocols to increase productivity of the beef cattle industry (Price, Harris et al. 2003; Haley, Bailey et al. 2005; Boland, Scaglia et al.

2008; Burke, Scaglia et al. 2009). These techniques are accomplished in two stages. The first stage is the prevention of suckling while the second stage involves the complete separation of the cow-calf pair (Haley, Bailey et al. 2005). Fence-line weaning involves the physical separation of the cow-calf pair through the use of a single fence line for one to two weeks before the calf is completely separated from all contact with its dam. While separated by the fence, the calf may no longer suckle, but it still has the ability to vocalize, visualize, and smell its dam. The nose-clip technique differs from fence-line weaning because calves remain with their dams until weaning. However, a device is attached to the calves' noses to prevent suckling of their dams. These two-stage weaning techniques promote the cessation of suckling and adjust the calves to separation from their dams, and thus may be less stressful than abrupt weaning.

Behavioral Change in Response to Alternative Weaning Methods

The alternative weaning techniques described above, fence-line and nose clip, give rise to less dramatic behavioral changes in calves than abrupt weaning (Haley, Bailey et al. 2005). Fence-line weaned calves did not display decreased eating during separation from their dams (Price, Harris et al. 2003). When observing behavior of fence-lined weaned calves, Price et al. (2003), reported that the proportion of time in which calves were within 3 m of the fence decreased from the first day to the fifth day after separation. Over these five days, the distance that fence-line weaned calves traveled from the fence while grazing increased daily until the calves failed to consistently return to the fence (Price, Harris et al. 2003). Fence-line weaning of calves may encourage a more natural and gradual establishment of their independence from their dams.

Nose-clip and fence-line weaned calves spend more time grazing in the post weaning period as compared to calves that are abruptly weaned (Boland, Scaglia et al. 2008). Compared to nose-clip and fence-line weaned calves, abruptly weaned calves exhibit greater pacing along fence lines after remote separation, as measured by pedometers (Boland, Scaglia et al. 2008). The increased pacing in abruptly weaned calves suggests a greater level of distress (Boland, Scaglia et al. 2008). During the week after weaning, calves weaned in two stages had greater average daily gain (ADG) than calves weaned by abrupt separation (Haley, Bailey et al. 2005). The greater ADG in two-stage weaned calves can be partially attributed to their reduced pacing and subsequent increased grazing time than the abruptly weaned calves. Calves weaned in two stages tend to vocalize less, walk less, and spend more time eating and resting/lying after separation than calves that are weaned by the traditional method of abrupt separation.

Fence-line weaning may promise greater production potential than the nose clip technique. When comparing the ADG of fence-line and nose-clip-weaned calves, the nose-clip calves had lower ADG (Boland, Scaglia et al. 2008). This trend may be partially explained by the decrease in grazing behavior of calves after the insertion of the nose-clip. The novelty of the nose-clip may result in the calf showing a decreased tendency to graze, ultimately leading to decreased ADG. Therefore, fence-line weaning may be the most effective weaning method in maintaining calf growth after weaning (Boland, Scaglia et al. 2008).

Techniques to Measure Stress-Response to Weaning

The body's physiologic response to stress is complex thus quantifying the magnitude of stress response is not straightforward. The selection of the specific physiologic byproducts, or parameters, for quantification requires consideration of factors that will generate valid results.

Important considerations include: time sensitivity of the samples, handling requirements to minimize sample degradation, confounding variables (e.g., handling stress), and accuracy of assays to analyze the parameter of interest. Several parameters have been utilized by the clinical and research community to quantify the stress response. Two parameters that can be used to quantify stress response of cattle include fecal cortisol metabolites and blood glucose concentration (Kahrer, Möstl et al. 2005; Abeni, Calamari et al. 2007).

In response to stress, cortisol (a glucocorticoid) is produced and released into circulation. Glucocorticoids are steroid hormones that are produced to facilitate the “fight or flight” response to a stressor. Cortisol concentrations can be measured in blood samples. However, changes in cortisol can be caused by a variety of factors, not limited to the stress of weaning. One such factor is the process of collecting the blood sample itself. The blood collection process is stress inducing because calves must be driven into the chute system, restrained in head gates, and subjected to venipuncture. These procedures result in a rapid release of cortisol into the bloodstream, and could potentially confound interpretation of cortisol values.

Measurement of fecal cortisol metabolites can be utilized to eliminate the confounding changes of cortisol in circulation during blood sample procurement. Fecal cortisol metabolites mirror the cortisol concentrations present in the blood stream 12 hours prior to fecal collection (Kahrer, Möstl et al. 2005). Cows subjected to transport-related or injury-related stress had higher fecal cortisol metabolite concentrations than control cows. (Mostl, Maggs et al. 2002; Kahrer, Möstl et al. 2005).

Another glucocorticoid-related parameter that is used to quantify the stress response is blood glucose concentration. Glucocorticoid production increases gluconeogenesis, thereby increasing circulating glucose to provide an additional source of energy for the CNS and other

cells (Aich, Shakiba et al. 2007). Because of the physiological impacts of glucocorticoid release, researchers have measured blood glucose concentrations as an indicator of an animal's response to stress (Swanson and Morrow-Tesch 2001).

When monitoring the physiological response to high stocking densities and transport in cattle, elevated plasma cortisol and glucose concentrations have been detected (Tarrant, Kenny et al. 1992). Gruber et. al (2010) reported that cattle showing behavioral signs of stress after transport had greater plasma glucose and lactate concentrations at slaughter than non-stressed cattle. Plasma concentrations of glucose and insulin have also been found to be higher in restraint and isolation-stressed calves than in non-stressed calves after 80 and 100 minutes of stressor application (Apple, Kegley et al. 2005). Blood glucose is also elevated in cattle undergoing multiple re-grouping and re-penning arrangements and in response to surgery (Gupta, Earley et al. 2005; Mudroñ, Rehage et al. 2005).

Blood glucose analysis involves specific collection and handling protocols that must be followed in order to minimize the loss of glucose in the sample over time. If not handled properly, glucose can be rapidly metabolized by other cells in the blood collection tube. Some commonly used techniques to maintain the integrity of the sample include: using blood collection tubes coated with sodium fluoride to minimize cell metabolism rates, keeping samples on ice, and separating the plasma from the red blood cells through immediate centrifugation. The advent of "chute side" analysis of blood glucose with hand held glucometers may provide an accurate method to determine blood glucose concentrations while limiting the disadvantages of sample collection and subsequent analysis in the laboratory.

PART II – Forages for Weaned Calves

Introduction

Proper nutrient provisions are critical for stressed animals. Dietary energy must be sufficient to support a properly functioning immune system, especially during times of stress. Differing compositions of roughage and concentrate in the diet are suggested to impact the morbidity, mortality and ADG rates of stressed calves. Rivera et al. (2005) described that morbidity from BRD decreased as dietary roughage concentration increased [morbidity (%) = $49.59 - 0.0675 \times \text{roughage} (\%)$; $P=0.003$], whereas ADG [ADG (kg) = $1.17 - 0.0089 \times \text{roughage} (\%)$; $P<0.001$], and DMI [DMI (kg/d) = $5.34 - 0.0135 \times \text{roughage} (\%)$; $P<0.001$] were affected negatively by increasing dietary roughage concentration. If cattle producers aim to wean their calves onto pasture instead of feedlot, forage type that is offered has the potential to impact calf productivity after weaning. Two common forage types that are implemented into grazing systems of Southwest Virginia include tall fescue and alfalfa. The nutritive value, persistence, and subsequent impact on animal performance of these two forages will be discussed in detail below.

Tall Fescue

Tall fescue (*Lolium arundinaceum* (Schreb.) Darbysh) is the most abundant cool season forage grass in the USA and is widely used in the United States for grazing cattle (Bouton, Latch et al. 2002; Browning 2004). Under appropriate conditions, tall fescue may be produced in large quantities at minimum cost to the producer. Cattle producers commonly aim to reserve a portion of their tall fescue fields as a stockpile for winter feeding. In comparison to other winter forage

systems, stockpiled tall fescue extends grazing availability into the winter and minimizes hay feeding (Allen, Fontenot et al. 1992).

The chemical composition of tall fescue is of especially high quality during the fall and early spring (Beck, Gunter et al. 2006). However, common varieties of tall fescue contain ergot alkaloids produced by fungal endophytes (*Neotyphodium coenophialum*, *Neotyphodium lolii*) that are present in the intercellular spaces of the plant (Gunter and Beck 2004). Tall fescue plants containing these fungal endophytes are termed endophyte positive (E+) tall fescue. The endophyte infection of tall fescue provides the plant with agronomic attributes that make it attractive as a forage, including an ability to withstand drought, produce forage in winter, and withstand intense defoliation (Gunter and Beck 2004). These characteristics provide an economical feed source for fall and spring calving herds by reducing supplementary feed expenses in the winter (Janovick, Russell et al. 2004; Curtis and Kallenbach 2007). Despite providing an acceptable feed source for winter grazing, E+ tall fescue can pose negative effects on cattle performance in the summer by reducing growth rates and elevating body temperatures (Beck, Gunter et al. 2008). The toxicity of E+ tall fescue presents livestock producers with a dilemma. Producers must decide whether to grow current E+ cultivars for stand persistence, risking reduced animal performance due to the inherent toxins or to graze their cattle on a less persistent forage that is less of a health risk to their cattle (Bouton, Latch et al. 2002). To cater to the needs of livestock producers, new tall fescue cultivar types are being offered. Producers now have the options of grazing their livestock on E+, endophyte-free (E-), or novel endophyte (NE) tall fescue cultivars.

Endophyte infection of tall fescue has a negative effect on livestock by inducing fescue toxicosis (Bacon 1988; Porter 1995). Fescue toxicosis in livestock is especially of concern in the

summer months (Bacon 1988). When exposed to heat, cattle grazing E+ fescue exhibit an increase in rectal temperature, respiration rate, and a decrease in food intake, accompanied by a shift of grazing time to night periods (Bond, Powell et al. 1984; Rhodes, Paterson et al. 1991; Osborn, Schmidt et al. 1992). The results of fescue toxicosis are reduced growth rates and hyperthermia under conditions conducive to heat stress in cattle (Osborn, Schmidt et al. 1992; Thompson, Fribourg et al. 1993; Paterson, Forcherio et al. 1995; Al-Haidary, Spiers et al. 2001). The response to the consumption of E+ fescue is rapid. In the warm months, grazing on E+ fescue for just four hours can start causing vasoconstriction of the caudal artery and lead to hyperthermia in cattle (Aiken, Kirch et al. 2007). The caudal artery constriction in cattle consuming an E+ diet is also observed with a concurrent 20% reduction in heart rate compared with cattle fed an E- diet (Aiken, Kirch et al. 2007). Reduction in blood flow further restricts ability to dissipate core body heat to peripheral tissues, particularly if the animal is exposed to high ambient temperature and humidity (Aiken, Kirch et al. 2007). Fescue toxicosis further affects the value of cattle at market. Average discounts at market for cattle showing signs of fescue toxicosis are \$7.49 for each 45.4 kg of BW (Gunter and Beck 2004).

Endophyte-free tall fescue cultivars are available for livestock producers who are in search of a cool season grass to offer their cattle herds. The E- fescue cultivar does not cause fescue toxicosis in cattle and is a safe alternative to feed during the summer. Excellent animal performance, with reported gains of 1.8 kg/day, are associated with cattle grazing E- fescue (Thompson, Fribourg et al. 1993). However, E- cultivars do not tolerate drought and are susceptible to overgrazing (Gunter and Beck 2004). Complete stand loss has been observed in E- fields subjected to four years of grazing with cattle (Gunter and Beck 2004). Despite offering excellent performance, the inability of E- fescue to persist in drought conditions and withstand

grazing pressure makes it an economically unacceptable and thus a nonviable alternative to E+ fescue (Gunter and Beck 2004).

Novel endophyte (NE) tall fescue is another management option for producers. The NE tall fescue consists of novel, nontoxic endophytes that produce very little or no ergot alkaloids (Bouton, Latch et al. 2002; Parish, McCann et al. 2003). Hill and Belesky concluded that the selection of endophytes that produced low levels of ergopeptine alkaloids, especially ergovaline, in tall fescue plants resulted in little or no loss in plant fitness (Hill, Belesky et al. 1991). Not only does NE tall fescue provide the plant persistence advantages of E+ tall fescue, it also promotes the animal performance advantages of E- tall fescue (Bouton, Latch et al. 2002; Gunter and Beck 2004). Unlike true E+ tall fescue, NE fescue does not result in an increase in body temperature or fescue toxicosis in ruminants (Bouton, Latch et al. 2002). Additional benefits include reduction in respiratory rates and rectal temperatures, and smoother hair coats in cattle consuming NE fescue than cattle consuming E+ fescue (Gunter and Beck 2004). However, replacement of E+ fescue pastures with NE fescue can be a time consuming and costly task that may prevent farmers from implementing NE fescue into their grazing management systems.

Out of all of the three different endophyte infection types of tall fescue, the NE infected fescue results in a longer growing season and increased animal performance in comparison to the other endophyte-infection types of tall fescue cultivars (Beck, Gunter et al. 2008). However, implementation of new forages in pasture can be very time demanding and costly to the producers. Producers may have to work through up to 10 years of cattle sales before they make back the monetary investments required to plant a new forage type in their pastures (Zhuang, Marchant et al. 2005). Because of these costly factors, it is not always a feasible option to

implement NE fescue into already developed forage systems. If establishment of NE fescue is a feasible option for producers, they will eventually be rewarded with a greater average net return than they would experience with E+ fescue (Bouton, Latch et al. 2002). In summary, NE tall fescues offer potential benefits related to decreased risk of stand establishment of annual forage crops, longer growing season, and acceptable animal performance in comparison to the other endophyte-infection types of tall fescue cultivars (Beck, Gunter et al. 2008).

Alfalfa

Alfalfa (*Medicago sativa* L.) is a highly nutritious forage legume adapted throughout most of the USA (Brummer and Bouton 1991). Incorporation of alfalfa into forage systems is advantageous because of its high yield potential and its leguminous ability to fix nitrogen. Alfalfa contains more nonstructural carbohydrates and higher concentrations of crude protein than grasses (Elizalde, Merchen et al. 1999; Fisher, Mayland et al. 2002). Cattle have been shown to prefer forages that have greater total nonstructural carbohydrate contents (Fisher, Mayland et al. 2002). These nutritive characteristics, therefore, encourage increased grazing and increased production as a result of the addition of alfalfa to grazing systems.

When offered free access to tall fescue or alfalfa, cows preferred and selected alfalfa, the forage species that yielded greater daily intake potential (Utsumi, Cangiano et al. 2009). Despite the positive attributes of the implementation of alfalfa in grazing systems, every alfalfa cultivar is associated with the occurrence of bloat in ruminants (Majak, Hall et al. 1995; Sen, Makkar et al. 1998).

Bloat in cattle is positively associated with protein fractions in the alfalfa (Majak, Hall et al. 1995). Protein fractions decrease with advancing stages of alfalfa plant maturity, thereby

leading to a decreased probability of bloat as alfalfa plants age (Majak, Hall et al. 1995). Providing condensed tannin supplements and continuous availability to alfalfa reduce the occurrence of bloat and support enhanced cattle productivity through grazing alfalfa rather than grazing grass alone (Majak, Hall et al. 1995; Vasconcelos and Galyean 2008). Mineral bloat blocks and pasture management systems that promote continuous and rapid ruminal clearance (more bypass, less gas) are also likely to reduce the incidence of bloat (Majak, Hall et al. 1995).

Alfalfa is of interest in grazing systems for beef cattle because increased feed intake and animal performance has been observed in ruminants grazing legume in addition to grass diets. This improvement in performance is thought to be partially attributed to the positive associative effects of legumes, which have a faster digestion and passage rate compared with grasses (Moseley and Jones 1979; Andrighetto, Bailoni et al. 1993). Grazing of alfalfa in grass mixtures increased calf and total cow/calf weight gains in comparison with grazing of smooth bromegrass (Hermann, Russell et al. 2002). Inclusion of alfalfa in systems for stocker cattle in Virginia improved gains by 7 kg during the stocker phase and had carryover effects that improved total gains during the finishing phase (Allen, Fontenot et al. 2000). Grazing systems including alfalfa lead to an increase in steer gains during the winter phase compared with gains of steers that grazed stockpiled tall fescue alone (Allen, Fontenot et al. 2000).

Implications

Management systems are of vital importance in minimizing stress and maximizing production potential in cattle. Weaning technique alone can have impacts upon animal performance over the long run (Schoonmaker, Fluharty et al. 2001). Reduced stress at weaning will subsequently decrease the incidence of morbidity and mortality rates of newly weaned

calves. Behavior and biological measures can be used to analyze and monitor the stress response of beef calves to weaning protocols. Two-stage weaning techniques have proven to decrease stress-like behavior during the weaning process. Low stress weaning-management protocols in addition to provision of quality forage should improve the well-being and productivity of cattle.

Chapter 3

Point of care glucometer systems for quantifying bovine blood glucose concentrations in the field for both clinical, research, and stress monitoring applications

Abstract

Blood glucose concentrations are commonly utilized to evaluate both metabolic and disease status of animals. A common challenge to accurate evaluation of blood glucose concentration is that red blood cells continue to utilize glucose after collection of blood samples. This necessitates either the rapid separation of plasma/serum or the use of specialized tubes to inhibit glucose metabolism. Therefore, a trial was conducted to compare the accuracy of two handheld glucometers for field measurement of glucose in cattle. Initially, blood or plasma glucose concentrations were evaluated on calf (n=18), yearling (n=18), and yearling (n=9) pasture-based beef cattle. Blood collected via jugular venipuncture was immediately measured on hand-held glucometers (Precision Xtra®, Abbott Laboratories; ReliOn® Confirm, ReliOn) while plasma samples measured (Beckman Coulter AU 480 Chemistry Analyzer) at the Virginia-Maryland Regional College of Veterinary Medicine (VMRCVM) clinical pathology lab served as the golden standard. Plasma glucose concentrations ranged from 74.3-79.3 mg/dl with a mean of 76.8 mg/dl. The Precision Xtra® had a higher coefficient of determination ($r^2 = 0.709$, $P < 0.001$) relative to the gold standard as compared to the ReliOn® Confirm ($r^2 = 0.416$, $P < 0.001$). On matched pair analysis, the average coefficient of variation for the repeated measures of the Precision Xtra® (2.9%) was higher than that of the ReliOn® Confirm (9.5%).

Introduction

The measurement of blood glucose concentration in cattle serves a variety of purposes in both clinical and research settings. In a clinical setting, cattle disorders such as ketosis and neonatal hypoglycemia occur in cattle with a negative energy balance (Duffield, Kelton et al. 1997; Panciera, Boileau et al. 2007). While in a negative energy balance, cattle experience reduced blood glucose concentrations. The measurement of blood glucose in cattle expressing clinical signs of these disorders is common in the diagnostic tests performed by the attending veterinarian. Diagnostics involving such glucose-related disorders can be enhanced through the use of point of care testing through handheld glucometer systems. Point of care testing can increase the efficiency of the diagnosis through reduced sample handling, transport, and laboratory analysis time when determining blood glucose concentrations. This associated increase in efficiency will save both money and time resources during the diagnosis of ketotic or hypoglycemic cattle. Additionally, the subsequent time to treatment of these cattle is reduced because of the reduced time of diagnosis required with point of care testing.

Furthermore, blood glucose concentration is analyzed in bovine research trials involving nutrition and stress (Mudroñ, Rehage et al. 2005; Delany, Macmillan et al. 2010). Blood glucose concentration is related to energy status, making it an analyte of interest when testing new diets. An increased concentration of glucose in circulation suggests that more energy resources are available. Blood glucose concentrations are also increased in the stress response. This increase in blood glucose concentration provides a source of energy to the cells to sustain the fight or flight response that results from stressful stimuli. For example, after cattle are exposed to transport, they have an increase in blood glucose concentration (Tarrant, Kenny et al. 1992).

Surgical stress also results in increased blood glucose concentrations in both hypoglycemic and normoglycemic cattle (Mudroñ, Rehage et al. 2005).

Whole blood, serum, and plasma glucose concentrations can be used to analyze glucose concentrations in cattle. Concentrations of glucose in whole blood are different than concentrations in plasma or serum because red cells in the blood metabolize glucose. Therefore, whole blood glucose concentrations are generally lower than the concentrations of glucose in plasma or serum (where the blood cells have been separated out from the extracellular fluid). The recommended equation to account for the differences in whole blood and plasma/serum concentration is: $serum/plasma[glucose] = \frac{whole\ blood\ [glucose]}{(1.0 - (0.0024 \times Hct\ in\ \%))}$ where “Hct”=hematocrit (Stockham and Scott 2002).

An experiment was conducted to determine if hand-held glucometer systems could be used for quick, reliable, and real-time analysis of bovine whole blood glucose concentrations. The objective of this study was to evaluate the accuracy, precision, and ease-of-use of two hand-held glucometer systems in comparison to an accepted standard for analysis of plasma glucose concentration.

Experimental Procedures

Two handheld glucometers, the ReliOn® Confirm (ReliOn) and the Precision Xtra® (Abbott), were chosen at random to measure blood glucose. Two phases were implemented in the overall design of this study. Animal health was monitored throughout the trial and all animal handling procedures were approved by the Virginia Tech Animal Care and Use Committee.

Phase I – Validation of Glucometer Accuracy

The objective of this phase was to validate the accuracy of the ReliOn® Confirm and the Precision Xtra®. This phase was conducted using two groups of cattle during the summer of 2010. The first group of cattle consisted of nine pasture-based beef heifers located at Virginia Polytechnic Institute and State University's Hoot Owl Farm (80°26' West longitude; 37°12' North latitude). The second group of cattle consisted of eighteen Angus-cross cows and eighteen Angus-cross calves located at Shenandoah Valley Agriculture Research and Extension Center (Steeles Tavern, VA, 79°20' West longitude; 37°92' North latitude). Cattle of different ages were used to mimic the natural population that would be sampled in real-life settings. Analyzing cattle of all ages also served to test the ability of the glucometers to detect a range of blood glucose concentrations because of the natural variation among young and older animals (i.e. calves would be expected to have a higher blood glucose concentration than older cattle).

All blood samples were collected via jugular venipuncture between 7:00-9:00 AM. Blood samples were collected in lithium heparin coated tubes for immediate whole-blood testing on the glucometers. Plasma samples were collected by one of two techniques. When at Hoot Owl, blood samples were collected in sodium fluoride-coated tubes (n=9) and placed immediately on ice until delivered to the Veterinary Teaching Hospital (VTH) for plasma glucose analysis within 45 minutes of collection. When at the Steeles Tavern location, blood samples were collected in lithium heparin-coated tubes with silicone gel separators (n=36). Samples were immediately centrifuged at 250 x g for 20 minutes to separate the red blood cells from the plasma with the silicone gel separator in the tube. After centrifugation, samples were placed on ice until delivered to the VTH for plasma glucose analysis within 150 minutes of collection.

Phase 2 – Validation of Glucometer Precision

The precision of the ReliOn® Confirm and the Precision Xtra® were determined by taking duplicate readings of the same blood sample. This phase was conducted using nine pasture-based beef heifers located at Virginia Polytechnic Institute and State University's Hoot Owl Farm (80°26' West longitude; 37°12' North latitude). The blood samples were obtained via jugular venipuncture and collected in lithium-heparin coated tubes. Once collected, the blood was immediately applied to glucose test strips and analyzed on the glucometers two times in a row on the same sample.

Statistical Analysis

Phase 1:

Normal probability plots were generated to verify that glucose concentrations followed a normal distribution. Least squares mean bias estimates for Precision Xtra® and Relion® compared to VTH plasma values were estimated and tested for statistical significance using mixed-model ANOVA. The linear model included glucometer and age as fixed effects with animal ID within age as a random effect. Residual plots were inspected to assess model adequacy (ie, errors followed a normal distribution with constant variance). Deming regression was applied to test for both systematic and proportional bias simultaneously. Statistical significance was set to $P < 0.05$. All analyses were performed using SAS version 9.2 (Cary, NC, USA).

Phase 2:

Coefficients of variation were generated for each of the glucometers and checked for normality using normal probability plots. Subsequently, a mean CV with a corresponding 95%

confidence interval was computed. Additionally, Precision Xtra® and Relion® CVs were compared using a paired t-test. Statistical significance was set to $P < 0.05$. All analyses were performed using SAS version 9.2 (Cary, NC, USA).

Results

Phase I - Validation

Blood glucose concentrations as measured by the Precision Xtra® had a higher coefficient of determination ($R^2 = 0.709$) as compared to the ReliOn® (R^2 of 0.416). Mean plasma glucose concentration was 6.4 mg/dl higher than the mean Precision Xtra® blood glucose concentration and 26.0 mg/dl higher than the mean ReliOn® blood glucose concentration. Analysis with each glucose quantification method revealed higher concentrations of glucose in circulation of the calves in comparison to the yearling and adult cattle, respectively (Table 3.1).

When plotted for deming regression in comparison to the standard, the deming line represents the relationship of the glucometer detection of whole blood glucose concentrations to the gold standard detection of plasma glucose concentrations. The deming line is compared to the line of equality which represents the line that would be created if the whole blood and plasma glucose concentrations were read identically between the two analysis techniques. The closer relationships between the deming line and the line of equality represent the more accurate glucometer system. The deming line relating to Precision Xtra®'s analysis of whole blood glucose concentrations was much closer to the line of equality than the deming line relating to the ReliOn®'s analysis of whole blood glucose concentrations (figures 3.1 and 3.2, respectively). Also reflected on the ReliOn® deming line is the trend that the higher blood glucose

concentrations have a larger difference from the line of equality than the lower blood glucose concentrations. Based on this data and the comparison against the standard, the Precision Xtra® is more accurate in its analysis of the blood glucose concentration of cattle.

Table (3-1) Glucose concentrations of calves, yearlings and cows

Group	Blood or Plasma Glucose concentration (mg/dl)			Standard error
	VTH ¹	Precision Xtra® ²	Relion® ²	
Calves	91.3	80.7	56.9	1.9
Yearlings	74.1	70.4	51.3	2.6
Cows	64.9	59.9	44.1	1.9

Values are LS means with pooled standard error

¹ Plasma glucose concentration

² Whole blood glucose concentration

All glucose concentrations between the different glucometer methods and animal ages differed (P<0.0001)

Figure (3-1) Comparison of blood glucose concentrations from Precision Xtra® to plasma glucose concentrations

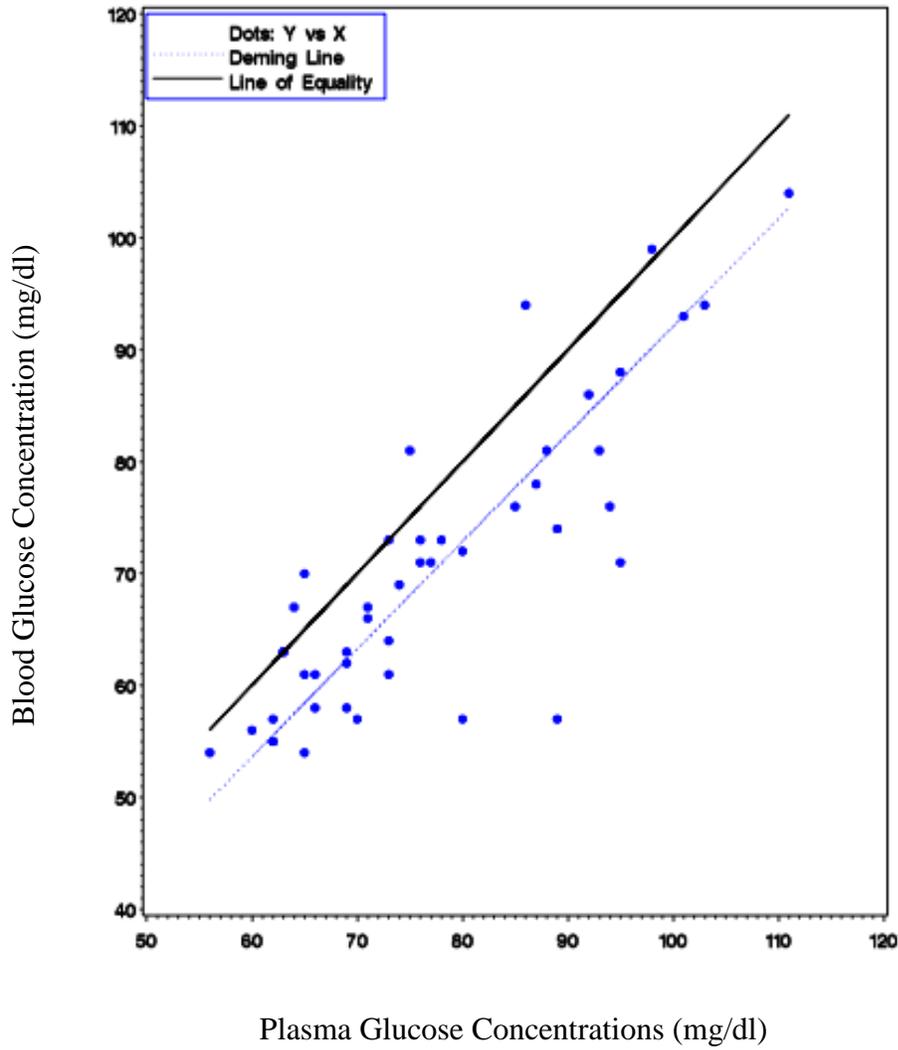
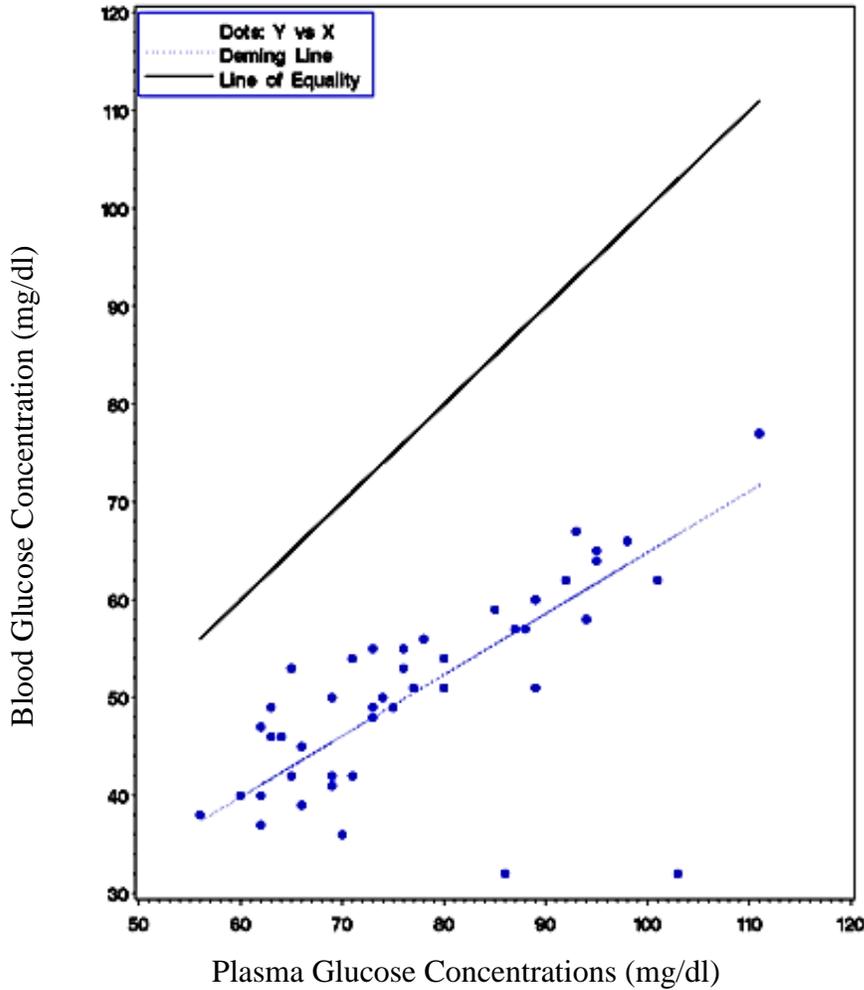
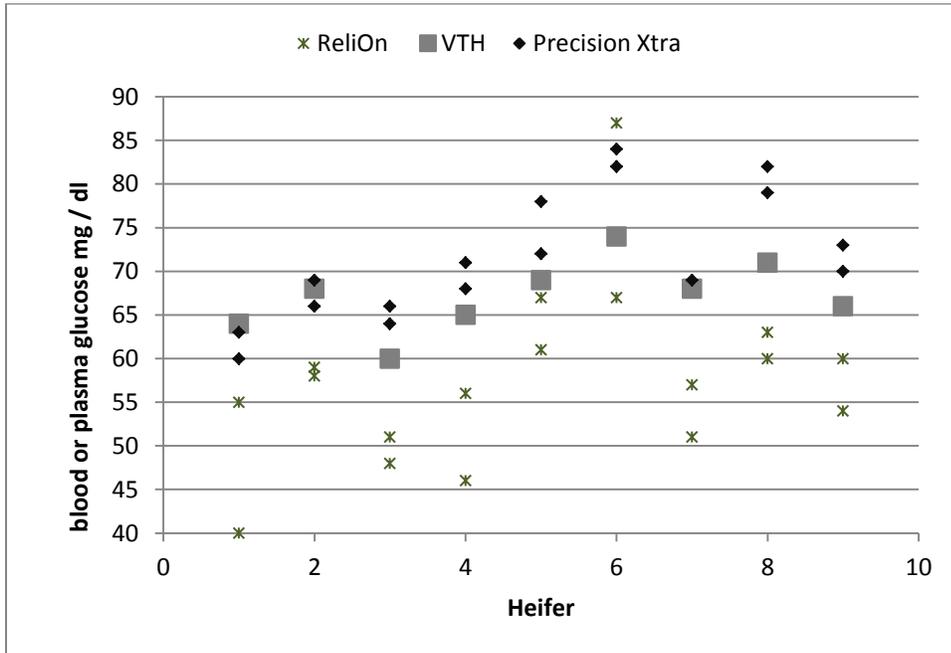


Figure (3-2) Comparison of blood glucose concentrations from ReliOn® to plasma glucose concentrations



When testing the repeatability of the glucometers, the average coefficient of variation (CV) between the consecutive readings for the Precision Xtra® was 2.9% with a 95% confidence interval (CI) of 1.8-3.9%. The average CV between the consecutive readings in the ReliOn® was 9.5% with a 95% CI of 3.9-14.9% (Figure 3.3). Based on this data, the Precision Xtra® glucometer has a higher degree of repeatability in comparison to the ReliOn®.

Figure (3-3) Duplicate whole blood glucose concentrations from the Precision Xtra® and ReliOn® in relation to the plasma concentrations



Discussion

In cattle production, quick and reliable methods are needed to quantify glucose concentration in the systemic circulation for accurate diagnosis of diseases. Voyoda and Erdogan (2010) compared the Optimum Xceed® handheld glucometer to conventional laboratory techniques when quantifying bovine blood glucose concentrations for detection of subclinical ketosis in dairy cattle. The Optimum Xceed® read higher glucose concentrations than the laboratory method, but the two methods were related with a $P < 0.001$ (Voyvoda and Erdogan 2010). In the present study, blood glucose concentrations as measured by both glucometers were lower than plasma concentrations measured by standard analysis. Plasma glucose concentrations are expected to be higher than whole blood glucose concentrations (Stockham and Scott 2002). However, some glucometers are programed to use an equation to convert the whole blood

glucose concentration detected to a calculated plasma glucose concentration displayed on the screen. It is important to note that the glucometers used in the present study depicted a whole blood glucose concentration without any conversions to a plasma glucose concentration.

Handheld glucometers can be utilized for reliable, quick, chute-side monitoring of whole blood glucose concentrations in cattle based on the results presented by Voyada and the current study. This capability will improve the efficiency in veterinary diagnostics and monitoring of disease in addition to use in stress and energy balance in research studies.

As with many technological units, there are limits within the optimum range of operation and physical endurance of the glucometers. Temperature limitations are one negative aspect to the functionality of the handheld glucometer systems. For example, the Precision Xtra® has an optimum operating temperature of 10-50⁰ C while the ReliOn® has an optimum operating temperature of 10-40⁰ C. These operating temperatures pose limitations on successful blood glucose analysis when the glucometers are being used in cold weather, such as in the winter. Another potential limitation for prolonged use of the glucometers is the battery life. If the battery life were to terminate during a sampling time, monitoring may not be able to be continued if backup systems were not in place. This could be avoided, however, by having spare batteries on hand.

Conclusion

The Precision Xtra® handheld glucometer system can be used to effectively monitor bovine blood glucose concentrations in chute-side applications. Even with immediate analysis on the glucometer, whole blood glucose concentrations were consistently lower than the plasma

concentrations determined by the accepted golden standard of glucose analysis. The use of point of care testing with handheld glucometers may improve diagnostic capabilities and efficiency while reducing costs in regards of veterinary care of cattle with glucose-related disorders.

Chapter 4

Effect of grass or legume-based forages on immune function of beef heifers

Abstract

In areas with moderate climates, such as Southwest Virginia, both grasses and legumes are utilized in grazing systems to support maintenance and growth of beef cattle. A trial was conducted to evaluate immune function and weight gain of yearling heifers grazed on either legumes or grass for a 28 day period in early fall. During a 28 day pilot study, Angus heifers were grazed on either tall fescue (n=14) or alfalfa (n=14) pasture. Blood samples were collected and body weights were recorded on d -3, 15 and 28 days after the heifers were placed on their respective grazing treatments. Interferon gamma (IFN γ) production by lymphocytes was utilized to evaluate immune function. Lymphocytes were non-stimulated or stimulated with pokeweed mitogen or concanavalin A. Weight gain was also compared between heifers in both grazing treatments. No differences were detected in the control or stimulated IFN γ production within the different grazing treatments. Similarly, no differences were detected within the weight gain between the heifers in the different grazing treatments.

Introduction

Beef calves undergo changes in environmental, dietary, and social conditions at weaning (Swanson and Morrow-Tesch 2001). These changes produce a stress response in the calves, thereby increasing their susceptibility to disease (Mitchell, Hattingh et al. 1988). Proper dietary nutrient provision is critical for stressed animals. Forage-based diets are increasingly utilized in

beef cattle production systems for newly weaned calves. Dietary energy from forage must be sufficient to support proper growth and a properly functioning immune system, especially during times of stress. A forage-based system is particularly well suited for areas with moderate climates, such as Virginia. In such a moderate climate, common nutritive forage species include both grasses and legumes. The focus of this chapter is to compare the impact of a grass, tall fescue (*Lolium arundinaceum* (Schreb.) Darbysh) and a legume, alfalfa (*Medicago sativa* L.), on the immune function and growth performance of Angus heifers.

Tall fescue is the most predominant cool season, perennial grass grown in the USA for forage. Tall fescue covers over 14 million hectares (ha) in the United States (Thompson, Stuedemann et al. 2001). Most naturalized fescue, termed endophyte-infected fescue (E+), contains ergot alkaloids produced by fungal endophytes (*Neotyphodium coenophialum*) present in the intercellular spaces of the plant (Gunter and Beck 2004). While these alkaloids promote plant hardiness in harsh environmental conditions, they present undesirable consequences for the grazing cattle. Two such consequences resulting from grazing on E+ fescue include reduced weight gain and heat stress (Osborn, Schmidt et al. 1992; Thompson, Fribourg et al. 1993; Paterson, Forcherio et al. 1995; Al-Haidary, Spiers et al. 2001). Heat stress (panting, excessive salivation, and standing in mud near the water source) may be observed in ambient temperatures as low as 30°C, at which point the risk for mortality increases (Beck, Gunter et al. 2008).

Alternatives to E+ fescue are available. The alternate options include endophyte-free (E-) tall fescue or tall fescue infected with a novel endophyte (NE). The E- cultivars circumvent the negative impact on animal performance of E+ fescue. However, the E- fescue does not withstand grazing or drought/hot conditions. Thus, NE fescue cultivars have become more commonly implemented into grazing systems. Nontoxic endophytes in the NE cultivars provide

the plant with the persistence advantages of E+ tall fescue while simultaneously providing the animal performance advantages of E- fescue (Bouton, Latch et al. 2002; Parish, McCann et al. 2003; Gunter and Beck 2004; Beck, Gunter et al. 2008).

Although NE fescue seems to be the most beneficial fescue cultivar, stand establishment is costly in both time and monetary resources. Most beef cattle producers have E+ fescue in their pastures, which must be eliminated prior to implementation of NE fescue. The process of eliminating existing E+ fescue and replacement with NE fescue may require two or more years, during which time the land is unavailable for grazing during periods of new plant growth. This investment translates into short term revenue loss and producers may not recoup their investment for nearly a decade (Zhuang, Marchant et al. 2005). Thus, for producers with small profit margins, implementation of NE fescue is not always feasible.

Another major forage option is alfalfa, which offers many benefits to producers and their beef cattle. Alfalfa is a highly nutritious legume that has been adapted throughout most of the USA. The inclusion of alfalfa into forage systems is advantageous because of its high yield potential and its leguminous ability to fix nitrogen. Alfalfa is additionally advantageous in ruminant production systems because it contains nutritive components, such as soluble proteins and nonstructural carbohydrates that result in increased feed intake and animal performance in comparison to grazing grass alone (Fisher, Mayland et al. 2002). Despite the positive attributes of the implementation of alfalfa in grazing systems, every alfalfa cultivar is associated with the occurrence of bloat in ruminants (Majak, Hall et al. 1995; Sen, Makkar et al. 1998).

In addition to primary nutrients (protein, energy, vitamin, and minerals), plants can also contain secondary compounds that have positive or negative effects on grazing animals. Bloat is one example of a negative effect grazing animals experience from secondary plant compounds.

Bloat in cattle is positively associated with protein fractions in the alfalfa (Majak, Hall et al. 1995). Protein fractions decrease with advancing stages of alfalfa plant maturity, thereby leading to a decreased probability of bloat as alfalfa plants age (Majak, Hall et al. 1995). Providing condensed tannin supplements and continuous availability to alfalfa reduce the occurrence of bloat and support enhanced cattle productivity through grazing alfalfa rather than grazing grass alone (Majak, Hall et al. 1995; Vasconcelos and Galyean 2008). The continuous provision of mineral bloat blocks in alfalfa fields will also reduce the incidence of bloat in cattle.

Bloat is not the only physiological change induced by the secondary compounds in plants. Legumes, such as alfalfa, possess mitogenic components that activate the mammalian immune system (Skene and Sutton 2006). Of interest, pokeweed mitogen (PWM) and concanavalin a (ConA) are two mitogenic compounds that are isolated from American Pokeweed (*Phytolacca americana*) and Jack-Bean (*Canavalia ensiformis*), respectively. These mitogenic compounds are commonly utilized to stimulate lymphocytes in vitro. Likewise, the author is aware of opinions that cattle on alfalfa grow faster, yet morbidity from common maladies like shipping fever is also higher in cattle on alfalfa.

Differing compositions of roughage and concentrate in the diet are suggested to impact the morbidity and mortality rates of stressed calves. Diets containing higher concentrations of roughage are associated with less morbid calves with lower ADG rates (Rivera, Galyean et al. 2005). Furthermore, Macarulla et al. (1992) observed reduced immunocompetence with hypertrophic spleens and thymuses in newly weaned mice fed diets containing a legume in comparison to mice fed a control diet without legume. Hypertrophic spleens and thymuses in these mice could indicate an increased activation of the immune system. The larger concentrate

to roughage ratio and the leguminous qualities of alfalfa may explain the testimonies that cattle on alfalfa pasture grow quicker, but experience an increased incidence of morbidity.

Therefore, a thirty day pilot study was conducted with the objective of comparing the effects of grazing legume (alfalfa) versus grass (E- tall fescue) in weaned Angus heifers. The hypothesis being tested was that growth rates and immune cell activity would be higher in the heifers grazed on alfalfa compared to those grazed on E- tall fescue. Immune function differences were determined through quantifying the lymphocyte production of IFN γ from the calves in the two grazing groups.

Experimental Procedures

Experimental Design

Twenty-eight commercial Angus heifers (initial weight and age: 228.2 ± 31.6 kg; 223 ± 10.6 d) reared at Virginia Polytechnic Institute and State University's Kentland Farm (81°5' West longitude; 37°25' North latitude) were blocked by age and allotted to graze either established alfalfa (ALF) or fescue (FES) pasture. Forage samples were collected from each pasture included in this study and processed for crude protein, neutral detergent fiber (NDF), and acid detergent fiber (ADF). All heifers were placed on their respective grazing treatment for 28 days, during which blood samples were procured and weight gain was monitored. The ALF heifers were on fescue with free access to bloat blocks (Sweetlix, Mankato, MN) for one week prior to being placed on alfalfa pasture to acclimate the heifers to the bloat block. Bloat block were continually offered throughout the duration of the time that heifers in the ALF group were grazing the alfalfa. Heifers in the ALF group were rotated to a new 0.65 hectare alfalfa-based pasture every 4 days. Heifers in the FES group were rotated to new 0.65 hectare fescue-based

pasture every 7 days. On day -3, heifers were weighed and blood was collected for baseline data. Heifers were bled via jugular venipuncture on the right side of the neck into two 10 mL lithium heparin-coated blood collection tubes (Becton Dickinson, Franklin Lakes, NJ). Filled blood tubes were inverted 8 times and kept at ambient temperature until they were used for the interferon gamma stimulation assay described below. Heifers were placed on their respective treatment forage on day 0. On days 15 and 28, heifers were weighed and blood was collected from all heifers using the same collection and sample handling protocol that was used on day -3. The only difference to the bleeding protocol is the side of the neck from which blood samples were collected was changed at each sampling time (i.e. blood was collected from the left side of the neck on day 15 and the right side of the neck on day 28). Heifer health was monitored twice daily throughout the trial and all animal handling procedures were approved by the Virginia Tech Animal Care and Use Committee.

Forage Sample Collection and Processing

Samples were obtained from alfalfa (n=5) and fescue (n=5) pastures on day 14 using hand-held plant clippers. Starting at one corner of each pasture, and working towards the diagonal corner, handful-sized samples were clipped at random intervals at 1-2 inches from the ground. Samples were placed in cloth forage quality bags and returned to the lab. At the lab, forage samples from each pasture were placed in pre-weighed paper bags. Bagged samples were then placed in a 60⁰C drying oven (Fisher Scientific Isotemp Oven, Model 738F) for 48 hours. Dried bagged samples were then weighed to record the dry matter weight. Dried forage samples were then ground down to 1mm diameter using a Thomas Scientific, Thomas Wiley Mill, Model 4 grinder. Ground samples were stored in separate air-tight containers per pasture.

For NDF and ADF analysis, half of a gram of ground samples were placed into pre-weighed Ankom F57 filter bags. Bags were then sealed within 1 cm from the open edge using a heat sealer. Sample was then spread uniformly inside the filter bag by lightly shaking and flicking the bag. Two filter bags were filled per pasture for duplicate analysis of NDF and ADF.

Determination of NDF

Bagged samples were digested using a neutral detergent solution to isolate NDF in the dried forage samples. Specific reagents and procedures implemented in this analysis are described in appendix 1. Amount of NDF was determined as a percentage of the dry weight of the 1mm-ground samples.

ADF Determination

Bagged samples that had previously been analyzed for NDF were further digested using an acid detergent solution to isolate ADF in the dried forage samples. Specific reagents and procedures implemented in this analysis are described in appendix 2. Amount of ADF was determined as a percentage of the dry weight of the 1mm-ground samples.

Crude Protein Analysis

Crude protein analysis was completed by Cumberland Valley Analytical Services, Incorporated (Mauganscille, MD). Crude protein was determined as a percentage of the dry matter in the 1mm-ground forage samples.

Whole Blood Stimulation Assay for Determination of Interferon gamma (IFN γ)

Upon arrival to the lab, blood tubes and all necessary equipment were sprayed with 70% ethanol and placed into a sterile culture hood with laminar air flow. Using aseptic technique, 1 mL of whole blood from each heifer was added to each of 9 wells of a 24-well flat-bottomed culture plate. To three of the nine wells, 100 μ l of culture media (RPMI 1640 with 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin) was added. These three wells served as the control samples for this assay. To another three of the nine wells, 100 μ l of pokeweed mitogen (PWM) diluted in culture media was added for a final concentration of 5 μ g/ml of PWM. To the remaining three wells per heifer, 100 μ l of concanavalin A (ConA) diluted on culture media was added for a final concentration of 5 μ g/ml of ConA. The plates with whole blood plus complete media, PWM, and ConA were incubated for 24 hours at 37°C with 5% CO₂. After the incubation, contents of the wells were transferred into 1.5 mL microcentrifuge tubes and centrifuged for 20 minutes at 3000 x g. Supernatant of the centrifuged samples was stored at -20°C until further analysis,

Supernatant IFN γ ELISA

A Bovine IFN γ Screening Kit (Thermo Scientific, Rockford, IL, lot number: LE144142) was utilized to measure IFN γ in supernatant. Standards and control, PWM-, and ConA-stimulated samples were analyzed in duplicate following the recommended assay procedures detailed in appendix 3. Samples stimulated with PWM had to be diluted at an initial concentration of 1:20 in reagent diluent to fall within the range of detection within the standard curve. Dilutions for PWM-stimulated samples ranged from undiluted to 1:100 so they could be detected on the dynamic range of the standard curve. Duplicate samples with a correlation of

variance (CV) greater than 5% were re-run for appropriate quantification. Sample concentrations were determined using the equation of a four parameter logarithmic standard curve. Standard range in the assay was from 31.25-2000 pg/mL.

Statistical Analysis

A logarithmic transformation was applied to the IFN γ concentrations (control, PWM, and CONA) because data were skewed. Accordingly, data were summarized as geometric least squares means with a corresponding 95% confidence interval. Baseline measurements were compared between the grazing treatments using a mixed model ANOVA. Effects of grazing treatment on 10/15/10 (day 15) and on 10/28/10 (day 28) were assessed using mixed-model analysis of covariance (ANCOVA). The linear model included baseline measurements (covariate) from 9/27/10 (day -3), and sampling date and grazing treatment as fixed effects. Similarly, initial (baseline) body weights were compared between grazing treatments using mixed model ANOVA. Weight gain was compared between grazing treatments using mixed model ANCOVA. The linear model included baseline weight (covariate) and grazing treatments as fixed effects. For each analysis, residual plots were inspected to verify model adequacy (ie, the errors followed a normal distribution with constant variance). Statistical significance was set to $P < 0.05$. All analyses were performed using SAS version 9.2 (Cary, NC, USA).

Results

Forage Nutrient Analysis

Crude protein, NDF, and ADF are all presented as a percentage of the dry matter of the forage sample analyzed (table 4.1). The average crude protein percentage in the alfalfa fields was 9.2% higher than that of the tall fescue fields. Conversely, the average NDF in the alfalfa fields was 16.2% less than that of the tall fescue fields. The average ADF of the alfalfa fields was 2.1 % less than that of the tall fescue fields.

Table (4-1) Nutrient analysis of E- fescue and alfalfa fields

Forage	Crude Protein	NDF	ADF
E- Fescue ¹	14.9±1.0	61.1±1.7	33.7±2.6
Alfalfa ¹	24.2±1.3	44.9±3.7	31.6±3.0

¹ Mean values with standard error of the E- fescue (n=5) and alfalfa (n=5) pastures sampled

Whole Blood Stimulation

No difference was detected in the IFN γ production of the non-stimulated samples from the ALF and FES heifers three days before they were placed on their respective treatments (P=0.36). Likewise, no difference was detected in the IFN γ production of the non-stimulated samples between the ALF and FES heifers on days 15 (P=0.46) and 28 (P=0.61). Baseline samples stimulated with PWM were not different between the ALF and FES groups before treatment was administered (P=0.08). Furthermore, no difference was detected between the

IFN γ production of the PWM-stimulated samples between the ALF and FES heifers on days 15 (P=0.55) and 28 (P=0.75). For the samples stimulated with ConA, no difference was detected between the ALF and FES heifers before they were placed on their grazing treatment (P=0.09). Likewise, no difference was detected in the IFN γ production of the ConA-stimulated lymphocytes between the ALF and FES heifers on days 15 (P=0.41) and 28 (P=0.36). The observations listed above are compiled in table 4.2. Furthermore, no date effect was detected in IFN γ production of ALF heifers throughout the 28 day grazing period in the nonstimulated (P=0.56), PWM (P=0.28), or ConA (P=0.39) blood treatments. Again, no date effect was detected in IFN γ production of heifers grazing fescue throughout the 28 day grazing period in the nonstimulated (P=0.90), PWM (P=0.17), or ConA (P=0.26) blood treatments.

Table (4-2) IFN γ production of heifers on alfalfa and fescue pasture with non-stimulated or stimulated (PWM and ConA) blood treatments

Blood Treatment	Day	IFN γ Concentration ¹		SEM ²	P-Value
		Alfalfa Pasture	Fescue Pasture		
	-3*	3.56	3.31	0.23	0.36
Non-stimulated	15	3.24	3.42	0.16	0.46
	28	3.31	3.43	0.16	0.61
	-3*	9.49	8.77	0.31	0.08
PWM	15	8.17	8.01	0.19	0.55
	28	8.45	8.36	0.19	0.75
	-3*	4.76	4.26	0.27	0.09
ConA	15	4.29	4.54	0.21	0.41
	28	4.52	4.24	0.21	0.36

* baseline samples served as a covariate for the analysis of this data

¹IFN γ concentrations in pg/mL after log₁₀ transformation

²Standard error of the mean

Weight Gain

There was no difference detected in the initial weights of the heifers in the FES and ALF groups (P=0.16). After the twenty-eight day grazing treatments, there was no difference detected

in the weight gains between the heifers in the ALF and FES grazing groups (P=0.91). Initial weights and weight gains of the heifers in the different grazing treatments are depicted in table 4.3.

Table (4-3) Body weights and gain of heifers on alfalfa and E- fescue

Variable	Treatment		SEM ¹	P-value
	Alfalfa	Fescue		
Initial Weight (Kg)*	212.8	225.7	24.4	0.16
Weight Gain (Kg)	8.6	8.4	3.4	0.91

Weight gain for a 30 day grazing period

*Initial weight served as a covariate for the analysis of this data

¹Pooled standard error of the mean.

Discussion

Mitogenic components found in many legume species, such as PWM and ConA, are activators of the mammalian immune system (Skene and Sutton 2006). Alfalfa is a main example of a legume that is commonly utilized in ruminant diets (Francis, Kerem et al. 2002). There are conflicting accounts on the impact of legume secondary components in ruminal dry matter digestion and weight gain rates (Mader and Brumm 1987). Further analysis is needed to determine if the lack of differences detected in this study are a true representation of the immunological and productivity trends of cattle grazing grass versus legume forages. Results reported from this analysis could be due to a lack of mitogens within alfalfa. If mitogens are part

of the composition of alfalfa, lack of absorbance from the intestine could provide further reasoning as to the lack of differences detected in this study. Another possibility to the lack of differences detected in this analysis could be a result of decomposition of alfalfa mitogens by rumen microbes.

Conclusion

All heifers were maintained on fescue pastures before placement on their respective forages for this study. No differences were detected between any of the grazing treatments on heifer immune function or weight gain. Although laboratory methods using mitogens isolated from legumes have proven to stimulate immune cells, trials are still needed to determine if there are differences in immune cell function and productivity of cattle grazing grass versus legume forages. Lack of differences detected in IFN γ production from legume-grazing heifers in this study could be a result of a lack of mitogen absorbance from the intestine. Another possibility is that microbes in the rumen may destroy the mitogenic properties of the alfalfa, thereby reducing its effect on immune cells if absorbed through the intestine. If absorbed through the intestine while remaining intact, the immunostimulatory effects of alfalfa mitogens may be transient and occur within the first few days. In future studies, sampling during the first week of legume grazing may provide additional insight to the effects of alfalfa-associated mitogens on immune cell function. Finally, the immunostimulatory effect of vegetative versus mature alfalfa requires further investigation.

Chapter 5

Influence of two-stage weaning on improving immune system function and overall herd health in beef calves

Abstract

A common challenge within the beef production industry is reducing disease and maximizing productivity of newly weaned calves. Weaning is a stressful event for beef calves and is associated with decreased average daily gains (ADG) and increased morbidity and mortality. A trial was conducted to evaluate the stress response through analysis of blood glucose, glucocorticoid production, immune gene expression, weight gain, antibody production, and interferon gamma (IFN γ) production of calves weaned by either abrupt or fenceline techniques. Forty crossbred Angus calves (20 heifers and 20 steers; 214.49 \pm 23.21 kg; 197.53 \pm 13.83 days old) were blocked by sex and age and allotted into abrupt (AB) or fenceline (FL) weaning treatments. Fenceline calves were separated from their dams by a single fence on day -6. All calves were weaned from their dam on day 0 and vaccinated with a tetanus toxoid vaccine and a bovine viral diarrhea (BVDv) vaccine on day 1. Blood was collected from all calves on day -6, 1, 15, and 30. Blood and fecal samples were analyzed on days -6 and 1 for blood glucose and fecal cortisol concentrations, respectively. Gene expression of FAS, IL-4, IL-10, and IFN γ was analyzed using qPCR and IFN γ production in non-stimulated and pokeweed mitogen (PWM)-stimulated blood samples were analyzed from samples on days -6 and 1. Serum samples from day 1, 15, and 30 were analyzed for IgG₁ and IgG₂ antibodies against the tetanus toxoid vaccine. Serum samples from days 1 and 30 were analyzed with a serum neutralization assay for antibody production against type 1 (NACL) and type 2 (125C) BVDv. Abruptly weaned (AB) calves had

lower weight gain the day after weaning than fenceline (FL) calves ($P=0.0001$). Fecal cortisol concentrations increased on the day after weaning in both groups ($P<0.0001$), but no treatment effect was observed. A date effect was detected for an increase in expression of FAS and IL-4, but no treatment effect was detected. Gene expression of IL-10 and IFN γ did not change over time. No date, treatment or treatment*date effect was detected for total weight gain or IFN γ production within the non-stimulated and the PWM-stimulated samples. Likewise, no treatment or treatment*date effects were detected in antibody production against the tetanus toxoid or BVD, although a date effect was detected for an increase in anti-tetanus IgG $_1$ and IgG $_2$ between days 1-15 and days 1-30. In summary, differences in immune function were not detected between calves that were fenceline or abruptly weaned.

Introduction

Conventional weaning practice in the beef industry involves abrupt separation of the cow-calf pair. Weaning is strategically timed to maximize the reproductive performance of dams. Dams are usually re-bred within three months of calving (Freetly, Nienaber et al. 2006). Once re-bred, the nutritional requirements of the dams must be allocated to the suckling offspring and developing fetus. Since fetal nutrient requirements are greatest during the later stages of gestation (Battaglia and Meschia 1978), beef cattle producers aim to separate calves from their dams prior to this time, thereby, allowing the dam's nutritional resources to be dedicated to the energy-demanding fetus.

The most common weaning practice is to abruptly separate calves from their dams. This practice is referred to as "abrupt weaning," and occurs when the calf is about 210 days of age (Hudson, Banta et al. 2010). Abrupt separation of the cow-calf pair imposes physical,

psychological, and nutritional stressors on calves (Lynch, Earley et al. 2010). Specific stressors associated with the weaning of beef calves include: commingling with beef calves from differing farms, exposure to novel antigens, crowding, nutritional changes, injury, pathogens, and environmental pressure (Arthington, Eichert et al. 2003). Calves respond to the multi-faceted stressors associated with weaning with physiological changes that result in a shift away from the body's homeostatic condition.

The stressors associated with abrupt weaning have been implicated in physiological responses that activate the hypothalamic-pituitary-adrenal (HPA) axis (Grandin 1997; Jacobson and Cook 1998). HPA activation commonly involves increased body temperature, heart, and respiration rates (Sheridan, Dobbs et al. 1994). During the stress response, the HPA axis and the sympathetic nervous system elicit the release of glucocorticoids and norepinephrine (Carroll and Forsberg 2007). These hormones negatively affect calf weight gain and immune function (Hickey, Drennan et al. 2003). Glucocorticoids are potent inhibitors of inflammation by altering immune cell trafficking, effector cell activity, and inhibiting pro-inflammatory gene expression (Buckbinder and Robinson 2002; De Bosscher, Vanden Berghe et al. 2003).

Alterations in immune gene expressions in response to weaning stress have been implicated as contributory to morbidity of newly weaned calves. The neutrophil apoptotic inducer, FAS, has been shown to decrease in bovine blood neutrophils treated with 10^{-5} M dexamethasone (a synthetic stress-related hormone) in vitro (Chang, Madsen et al. 2004). Conversely, oxidative stress increases FAS expression and neutrophil apoptosis in mice (Denning, Takaishi et al. 2002). In-vivo once-daily dexamethasone treatments of cattle resulted in the decrease of L-selectin, a lymphocyte homing receptor, expression in mononuclear cells (Burton and Kehrli 1996). When analyzed in supernatant, lymphocyte production of IFN γ also

decreases in response to weaning-related stress in calves (Hickey, Drennan et al. 2003). Such alterations in gene and cytokine expressions result in decreased immune capability for combating pathogens and disease.

The alterations in gene and cytokine expression from immune cells in combination with the weaning-related stress, naïve immune systems, and lack of prior exposure to novel pathogens increases the newly-weaned calves' susceptibility to disease (Swanson and Morrow-Tesch 2001). Bovine respiratory disease (BRD) is the most common cause of morbidity and mortality in recently weaned calves (Staples and Haugse 1974; Swanson and Morrow-Tesch 2001; Hodgson, Aich et al. 2005). Disease will negatively affect cattle performance through decreasing final body weight, average daily gain, carcass weight, and USDA quality grades of meat (Gardner, Dolezal et al. 1999; Aich, Shakiba et al. 2007). The severity of the stress paradigm can increase severity of disease (Hodgson, Aich et al. 2005). Severe stress paradigms that include environmental, social, transport, and other stressors (experienced independently or together) reduce survival rates of infected animals (Sheridan, Dobbs et al. 1994). The stressors, alterations in immune function, and decreased resistance to diseases resulting from weaning are topics that must be addressed when considering options to improve the productivity of the beef cattle industry. Intermediary weaning techniques provide one option for improving the health and productivity of newly weaned beef calves.

Fenceline weaning is an example of an intermediary weaning technique that provides a period of adjustment to the separation of the calves from their dams. Calves are separated from their dams via a fence for 3-7 days where they are unable to suckle, but remain able to smell and have vocal contact with their dams. Following this period, calves are completely separated from

their dams. Fenceline weaning provides an opportunity for calves to gain independence from their dams before complete separation.

Our objectives were to compare cortisol release, antibody production in response to primary and secondary antigen exposure, IFN γ production, and immunological gene expression in calves that were subjected to abrupt versus fenceline weaning procedures. Cortisol concentrations were measured in the calves' feces on the day after weaning. Fecal cortisol concentrations represent the cortisol concentrations present in circulation 12 hours pre-collection (Mostl, Maggs et al. 2002). This experiment was conducted to test the hypothesis that: abrupt weaning results in increased cortisol release compared to fenceline weaning. This increase in cortisol release is associated with alterations in antibody production, reduced IFN γ production, and altered gene expression.

Experimental Procedures

Animals and design

Forty Angus crossbred heifers (n=20) and steers (n=20) with an initial weight and age of 214.5 ± 23.2 kg and 197.5 ± 13.8 days, from the Shenandoah Valley Agriculture and Extension Center (Steele's Tavern, VA) were blocked by sex and age and were randomly allotted within blocks into fenceline (FL) or abrupt (AB) weaning treatments. The FL calves were physically separated from their dams via a fence for 6 days prior to complete weaning from their dams. The AB calves were weaned at the same time as the FL calves, but were not given a transition period to promote independence from their dams before weaning. All calves were maintained on fescue paddocks, post-weaning for the duration of the study (30 days). On day 1, all calves were administered 1 mL Tetanus Toxoid Vaccine (Fort Dodge Laboratories Inc., Fort Dodge, IA) and

2 mL Triangle 4 + Type II BVD Vaccine (Bovine Rhinotracheitis-virus Diarrhea-parainfluenza-3-respiratory Syncytial Virus Vaccine) (Fort Dodge, Overland Park, KS). The Tetanus Toxoid Vaccine was used to induce an immune response to a novel antigen while the Triangle 4 + Type II BVD vaccine was used to induce a secondary immune response to a prior immunization with a modified-live-BVD vaccine.

Timeline and Sample Collection

Calves were weighed on d-6, 1, 15 and 30. Fecal samples were collected on d -6 and d 1 for determination of fecal cortisol. Blood samples were collected on d – 6 and d 1 for determination of blood glucose. Serum samples were collected on d 1, 15 and 30 for determination of anti-tetanus and anti-BVDv antibodies. Subsets of calves (n=11, 6 heifers, 5 steers) from each treatment group were bled on d -6 and d 0 for complete blood cell counts, quantification of IFN γ production in non-stimulated and PWM-stimulated whole blood and measurements of gene expression. Calf health was monitored daily throughout the trial and all animal and handling procedures were approved by the Virginia Tech Animal Care and Use Committee.

Forage Sample Collection and Processing

Forage samples (n=6) with a strip length of 10.4 - 12.3 ft and a strip width of 2.5 ft were collected using a Swift 3 pt. hitch forage plot harvester (Swift Machine and Welding, 1881 Chaplin St. West, Swift Current, Saskatchewan, Canada) from each pasture included in this study. These samples were processed for crude protein, neutral detergent fiber (NDF), and acid detergent fiber (ADF). Forage samples from each pasture were placed in pre-weighed paper

bags. Bagged samples were then placed in a 60⁰C drying oven (Fisher Scientific Isotemp Oven, Model 738F) for 48 hours. Dried bagged samples were then weighed to record the dry matter weight. Dried forage samples were then ground down to 1mm diameter using a Thomas Scientific, Thomas Wiley Mill, Model 4 grinder. Ground samples were stored in separate air-tight containers per pasture.

For NDF and ADF analysis, half of a gram of ground samples were placed into pre-weighed Ankom F57 filter bags. Bags were then sealed within 1 cm from the open edge using a heat sealer. The sample was then spread uniformly inside the filter bag by lightly shaking and flicking the bag. Two filter bags were filled per pasture for duplicate analysis of NDF and ADF.

Determination of NDF

Bagged samples were digested using a neutral detergent solution to isolate NDF in the dried forage samples. Specific reagents and procedures implemented in this analysis are described in appendix 1. Amount of NDF was determined as a percentage of the dry weight of the 1mm-ground samples.

ADF Determination

Bagged samples that had previously been analyzed for percent ADF were further digested using an acid detergent solution to isolate ADF in the dried forage samples. Specific reagents and procedures implemented in this analysis are described in appendix 2. Amount of ADF was determined as a percentage of the dry weight of the 1mm-ground samples.

Crude Protein Analysis

Crude protein analysis was completed by Cumberland Valley Analytical Services, Incorporated (Mauganscille, MD). Crude protein was determined as a percentage of the dry matter in the 1mm-ground forage samples.

Whole Blood Stimulation Assay for determination of Interferon gamma (IFN γ)

Blood samples were collected via jugular venipuncture into one 10 mL lithium-coated blood tube and 1.5 mL EDTA-coated blood collection tube (Becton Dickinson, Franklin Lakes, NJ). Blood tubes were inverted 8-10 times immediately after blood collection. Samples in lithium heparin coated tubes were kept at ambient temperature for 150 minutes until they were processed in the laboratory.

Upon arrival to the lab, blood tubes and all necessary equipment were sprayed with 70% ethanol and placed into a sterile culture hood with laminar air flow. Using aseptic technique, 1 mL of whole blood from each heifer was added to 6 wells of a 24-well flat-bottomed culture plate. To three of the six wells, 100 μ l of culture media (RPMI 1640 with 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin) was added. These three wells served as the control samples for this assay. To the remaining three wells, 100 μ l of pokeweed mitogen (PWM) diluted in culture media was added for a final concentration of 5 μ g/ml of PWM. The plates with whole blood plus complete media or PWM were incubated for 24 hours at 37°C with 5% CO₂. After the incubation, contents of the wells were transferred into 1.5 mL microcentrifuge tubes and centrifuged for 20 minutes at 3000 x g. Supernatant of the centrifuged samples was drawn off and pooled into separate 1.5 mL microcentrifuge tubes for the stimulation or control treatment. Pooled supernatant was stored at -20°C until further analysis using the IFN γ Bovine Screening Kit (Thermo Fisher Scientific Inc., Waltham, MA).

Supernatant IFN γ ELISA

A Bovine IFN γ Screening Kit (Thermo Scientific, Rockford, IL, lot number: LE144142) was utilized to measure IFN γ in supernatant. Standards plus control and PWM-stimulated samples were analyzed in duplicate following the recommended assay procedures detailed in appendix 3. Samples stimulated with PWM had to be diluted at an initial concentration of 1:20 in reagent diluent to fall within the range of detection within the standard curve. Dilutions for PWM-stimulated samples ranged from undiluted to 1:100 in reagent diluent so they could be detected on the dynamic range of the standard curve. Duplicate samples with a correlation of variance (CV) greater than 5% were re-run for appropriate quantification. Sample concentrations were determined using the equation of a four parameter logarithmic standard curve. Standard range in the assay was from 31.25-2000 pg/mL.

Fecal Collection

Feces were collected from each calf while they were restrained in the head gate of the chute. Fecal samples were placed in 50 mL conical tubes and placed on ice. Upon arrival to the laboratory, fecal samples were stored at -20°C until they were subjected to the cortisol extraction procedure.

Extraction of fecal cortisol

The extraction was based on the procedure from Mostl and Maggs (Mostl, Maggs et al. 2002). Frozen feces were placed at 4°C overnight to thaw. Once thawed, 0.5 g of each wet fecal sample was weighed out into 15 mL conical tubes and 5 mL of 80% methanol was subsequently

added to the tube. Samples with methanol were then vortexed for 1-2 minutes then centrifuged for 15 minutes at 2500 x g. Vortexed fecal plus methanol samples were stored at -20°C until analysis with a competitive radioimmunoassay technique.

Cortisol Radioimmunoassay

This assay was based on the Coat-A-Count Cortisol procedures from Siemens Diagnostic Product Corporation (Los Angeles, CA). First, four 12x75 mm polypropylene tubes not coated with antibody were labeled “T” in duplicate for total counts and nonspecific binding (NSB). Twelve cortisol antibody-coated tubes were labeled A through F in duplicate (with concentrations of 0, 5, 10, 25, 50, and 100 ng/mL) for a standard comparison to determine the concentration of cortisol in the fecal samples. Two cortisol antibody-coated tubes were labeled for each sample for cortisol analysis in duplicate. Into the NSB and A tubes, 25 µl of the 0 µg/dL was added. To the remaining standard tubes, 25 µl of the appropriate standard was added. Fifty microliters extracted fluid fecal samples were added to each appropriate tube. To all tubes, 1 mL of the radiolabeled ¹²⁵I cortisol was also added. All tubes were then incubated for 47 minutes in a 37°C water bath. After incubation, solutions were completely decanted from all tubes except for the “T” tubes. A foam decanting rack was used to hold the tubes for 2-3 minutes in the inversed position so that they could completely decant. While still in the foam decanting rack, the tubes were struck sharply on absorbent paper to shake off the residual droplets. Each tube was then counted for 1 minute in a gamma counter. Cortisol concentrations in the samples were determined by comparing the average counts of the duplicate readings to the curve created by the counts of the various standards.

Quantification of Whole Blood Glucose Concentration

Blood samples were collected via jugular venipuncture into lithium-heparin coated tubes. Immediately after collection, blood was applied to glucose test strips and analyzed for the blood glucose concentration using the Precision Xtra® glucometer that was validated in chapter III.

Tetanus Toxoid-Specific ELISA – Sample Processing

Blood samples were collected via jugular venipuncture into two 10 mL blood collection tubes without any additives (Becton Dickenson, Franklin Lakes, NJ). Samples were kept at ambient temperature for 150 minutes and then were centrifuged for 20 minutes at 3000 x g. Serum was collected from the blood tubes and stored at -20°C until analysis.

The positive control that was used in this assay consisted of pooled serum from a heifer that had been hyperimmunized by 5 consecutive biweekly vaccinations with the tetanus toxoid vaccine. For quantification of the IgG₂ isotype, hyperimmunized serum and calf serum samples were heat treated in a 55°C water bath for 30 minutes to heat reduce complement binding. Both IgG₁ and IgG₂ isotypes of anti-tetanus toxoid titers were measured by use of an ELISA method.

Tetanus Toxoid-Specific ELISA

A tetanus toxoid-specific ELISA method was used to determine IgG₁ and IgG₂ isotype production in response to immunization with the tetanus toxoid vaccine. All standards and samples were analyzed in duplicate using the procedure described in appendix 7. Calf serum samples were diluted at 1:20 in assay buffer and compared against a standard range from 1:20 to 1:1280 of hyperimmunized serum in assay buffer for IgG₁ analysis. Heat treated calf serum was diluted at 1:50 in assay buffer and compared against a standard range from 1:1 to 1:1024 of heat

treated hyperimmunized serum in assay buffer for IgG₂ analysis. Duplicate samples with a CV greater than 10% were re-run for appropriate quantification. Titers were calculated as percent positivity of the sample concentrations was calculated using the equation of the logarithmic curve generated by the standard curve on each plate.

BVDv Serum Neutralization Sample Collection and Handling

Blood samples were collected via jugular venipuncture into two 10 mL blood collection tubes (Becton Dickenson, Franklin Lakes, NJ) without any additives. Samples were kept at ambient temperature for 150 minutes until they were centrifuged for 20 minutes at 3000 x g. Serum was collected from the blood tubes and stored at -20°C until analysis.

BVDv Serum Neutralization Assay

Titers against NADL, a cytopathic type 1 BVDv strain, and 125C, a cytopathic type 2 BVDv strain, were determined by a serum neutralization assay. The assay was completed by the Department of Pathology at The Auburn University College of Veterinary Medicine (M. Daniel Givens, Alumni Professor and Coordinator of Animal Health Research). The quantity of virus added to each dilution of serum tested was within the quality control limits of 100 to 500 cell cultures infective doses to the 50% endpoint per well on back titration.

Gene Expression: RNA Extraction

Blood was collected into PAXgene Blood RNA tubes (Becton Dickinson, Franklin Lakes, NJ) by venipuncture and tubes were kept at ambient temperature until return to the laboratory. Tube were stored at 4°C for 5 days blood collection and then equilibrated to room

temperature overnight. RNA was extracted using the PAXgene Blood RNA Kit (Qiagen Inc., Valencia, CA) following the manufacturer's instructions (appendix 8). The isolated RNA samples were stored at -80°C until quantification and transcription into cDNA.

RNA Quantification

RNA samples were evaluated for total RNA concentration by following the manufacturer-recommended techniques and supplies of the fluorescence-based Quanti-iT RiboGreen Kit (Invitrogen Corporation, Carlsbad, CA). The stock RNA standard of $100\ \mu\text{g}/\text{mL}$ was diluted 1:50 ($20\ \mu\text{l}$ RNA standard in $980\ \mu\text{l}$ nuclease-free 1X TE buffer) to achieve a final concentration of $2\ \mu\text{g}/\text{mL}$ RNA standard. Standards were added to the plate in duplicate starting with the $2\ \mu\text{g}/\text{mL}$ concentration and serially diluted 1:2 seven times to end with a RNA concentration of $0.03125\ \mu\text{g}/\text{mL}$. The RNA samples of interest were diluted 1:100 in 1X TE Buffer followed by the duplicate addition of $100\ \mu\text{l}$ of diluted sample per well. Two wells were left without sample or TE buffer to serve as blanks. Once all standards and samples were on the plate, $100\ \mu\text{l}$ of diluted ribogreen (1:200 in 1X TE buffer) was added to each well. Because of the light sensitivity of ribogreen, the plates were covered in tin foil before being loaded into the plate reader. Plates were loaded into a plate reader (SpectraMax M5 Multi-Mode Microplate Reader, Molecular Devices, Downingtown, PA) and fluorescence was read at 480 nm emission and 520 nm excitation. The OD values of the blank wells were subtracted from all other wells. RNA concentrations of the samples were determined using a linear standard curve generated from the standards used on the plate.

Complimentary DNA (cDNA) Production

Total RNA from the samples isolated using the PAXgene RNA Isolation kit were reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). A weight of 200 ng from each RNA sample was isolated and combined with RNase-free water to achieve a final volume of 10 μ l. The isolated 200 ng of RNA per sample was added to a master mix containing 2 μ l of 10x RT Buffer, 0.8 μ l of 25x dNTP Mix, 2 μ l of 10x RT Random Primers, 1 μ l of MultiScribe Reverse Transcriptase and 4.2 μ l of Nuclease-Free Water. Samples were then mixed, briefly centrifuged, and placed in a thermal cycler. Thermal cycler conditions for cDNA transcription included: 10 minutes at 25°C, 120 minutes at 37°C, and 8 seconds at 85°C. Samples were stored at -20°C until analysis by qPCR.

qPCR

Gene expression bovine-specific 18S, β -actin, FAS, IL-4, IL-10, IFN γ , and L-selectin (Applied Biosystems, Foster City, CA) were analyzed using the TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA) for real-time PCR. Each cDNA sample was run in duplicate for each gene. Gene analysis for 18S and β -actin served as house-keeping genes for this assay. Reactions occurred in MicroAmp Optical 96-well Reaction Plates (Applied Biosystems, Foster City, CA). Each well had 1 μ l of 20x TaqMan Gene Expression Assay for the respective gene of interest, 10 μ l of 2x TaqMan Universal PCR Master Mix, 1 μ l of the 200 ng cDNA template diluted 1:10 in RNase/DNase-free water, and 8 μ l of RNase/DNase-free water. A master mix control was used for each gene with RNase/DNase-free water substituting cDNA to ensure that there was no contamination within the mixtures. Once samples were added

to the wells, plates were sealed with MicroAmp Optical Adhesive Film (Applied Biosystems, Foster City, CA). PCR amplifications were performed using the 7300/7500 Applied Biosystems System (Applied Biosystems, Foster City, CA). Thermal cycling conditions included a primary incubation at 95°C for 10 minutes and then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Gene expression was measured using relative quantification by comparing the threshold cycle (Ct) of the sample to the average Ct of the endogenous controls (18S and β -actin) resulting in a Δ Ct.

Statistical Analyses

All data were graphed before statistical analysis to evaluate distribution and assess normality. For each analysis, residual plots were inspected to verify model adequacy (ie, the errors followed a normal distribution with constant variance). Statistical significance was set to $P < 0.05$. All analyses (except those for the neutrophil to lymphocyte ratios) were performed using SAS version 9.2 (Cary, NC, USA).

Weight Gain

Normal probability plots showed that initial weight, weight again after weaning, and total weight gain followed an approximately normal distribution. Accordingly data were summarized as means \pm standard deviation. Effects of weaning treatment on weight gain after weaning and separately on total weight gain were assessed using mixed-model ANCOVA. The linear model included initial weight (covariate) and treatment as fixed effects. Blocks based on a combination of sex and age constituted the random effect.

Whole Blood Stimulation IFN γ Concentrations

A logarithmic transformation (base e) was applied to the interferon measurements (control and PWM) because data were skewed. Accordingly, data were summarized as geometric least squares means with a corresponding 95% Confidence interval [This is one way of doing it. If you summarized the data another way, just delete this sentence]. Effects of weaning treatment on interferon measurements on day -6 (9/23/10) and on day 1 (9/30/10) were assessed using mixed-model ANOVA. The linear model included day, treatment, and day*treatment as fixed effects. Sex and calf within treatment constituted the random effects.

Fecal Cortisol

Normal probability plots showed that fecal cortisol measurements followed an approximately normal distribution. Accordingly data were summarized as means \pm standard deviation. Effects of weaning treatment on fecal cortisol measurements on day -6 (9/23/10) and on day 1 (9/30/10) were assessed using mixed-model ANOVA. The linear model included day, treatment, and day*treatment as fixed effects. Blocks based on a combination of sex and age, and calf within treatment constituted the random effects.

Whole Blood Glucose

Baseline glucose concentrations on day -6 (9/23/10) were compared between the weaning groups using a 2-sample t-test and used as the covariate in the analysis of glucose concentrations on day 1. Glucose concentrations on day 1 (9/30/10) were compared between the groups using mixed model analysis of covariance. The linear model for blood glucose analysis included baseline concentration and treatment as fixed effects and sex and calf id within treatment as random effects.

Tetanus IgG₁ and IgG₂

A logarithmic transformation (base e) was applied to the titers because data were skewed. As a result, data were summarized as geometric least squares means with a corresponding 95% confidence interval. Effects of weaning treatment on the log titers (separately for IgG₁ and IgG₂) on day 1 (9/30/10), day 15 (10/15/10), and day 30 (11/01/10) were assessed using mixed-model ANOVA. The linear model included day, treatment, and day*treatment as fixed effects. Blocks based on a combination of sex and age, and calf within treatment constituted the random effects.

BVD_v

A logarithmic transformation (base e) was applied to the titers because data were skewed. As a result, data were summarized as geometric least squares means with a corresponding 95% confidence interval. Effects of weaning treatment on the log titers (separately for BVDV1 and BVDV2) on day 1 (9/30/10) and on day 30 (11/01/10) were assessed using mixed-model ANOVA. The linear model included day, treatment, and day*treatment as fixed effects. Blocks based on a combination of sex and age, and calf within treatment constituted the random effects.

qPCR

Gene expression data from qPCR analyses were normalized to 2 housekeeping genes (18S and β -actin (β -a)). For each gene of interest, 2 delta CTs were generated as follows: CT for gene of interest – CT for each house keeping gene. Subsequently, an average delta CT was obtained from the 2 delta CTs. Normal probability plots showed that the average delta CTs for each gene followed an approximately normal distribution. Effects of weaning treatment on the averaged delta CT on day -6 (9/23/10) and on day 1 (9/30/10) were assessed using mixed-model

anova. The linear model included day, treatment, and day*treatment as fixed effects. Sex and calf within treatment constituted the random effects.

Results

Forage Nutrient Analysis

All six pastures in this study consisted of E+ fescue with an average 3,738 estimated dry kg of forage/hectare. The average crude protein, NDF and ADF concentrations were 14.9 ± 0.8 , 59.7 ± 1.3 and 30.1 ± 0.6 percent, respectively on a dry matter basis.

Weight Gain

There was no difference detected in the initial weights of the calves in the AB and FL treatment groups ($P=0.64$). On the day after weaning (day 1), the FL calves gained 7.8 Kg more than the AB calves ($P=0.0001$). On day 30, there was no difference detected in the overall weight gains between the calves in the AB and FL treatment groups ($P=0.81$). Initial weights and weight gains of the calves in the different weaning treatments are depicted in table 5-1.

Table (5-1) Body weights and gain of calves weaned (day 0) by abrupt and fenceline techniques

Variable	Treatment		SEM ²	P-Value
	Abrupt	Fenceline		
Initial Weight (Kg)	212.9	216.1	5.3	0.64
Gain (day -6 – day 1) (Kg)*	0.4 ^a	8.2 ^b	1.2	0.0001
Gain (day -6 – day 30) (Kg)*	10.9	10.5	1.4	0.81

*Initial weight served as a covariate for the analysis of weight gain

²Pooled standard error of the mean

^{a,b}Uncommon superscripts within a row signify differences between treatments with P=0.0001

Whole Blood Stimulation

Neutrophil to lymphocyte ratios were determined by analysis of complete blood cell counts collected on days -6 and 1. No difference was detected in the mean neutrophil to lymphocyte ratios between the AB (0.36±0.04) and FL (0.33±0.04) calves on day -6 (P=0.37). Again, no difference was detected in the mean neutrophil to lymphocyte ratios between the AB (0.40±0.06) and FL (0.32±0.06) calves on the day following weaning (P=0.37).

No difference was detected between the AB and FL groups in IFN γ production of the nonstimulated blood samples on day -6 (P=0.76) or day 1 (P=0.83) (table 5-2). Similarly, no difference was detected in the IFN γ production of the PWM-stimulated blood samples between the AB and FL groups on day -6 (P=0.87) or day 1 (P=0.75). The amount of PWM-stimulated IFN γ production increased in both groups of calves between d-6 and d 0 (P<0.001).

Table (5-2) Least squares means of IFN γ production and lymphocyte percent in whole blood from abrupt and fenceline-weaned calves pre- (day -6) and post-weaning (day 1)

	IFN γ (pg/ml) production							
	Day -6		Day 1		SE	P-Value ¹		
	Abrupt	Fenceline	Abrupt	Fenceline		T	D	TxD
Nonstimulated*	4.07	3.95	4.58	4.49	0.30	0.72	0.07	0.95
PWM*	6.58 ^a	6.64 ^a	7.68 ^b	7.79	0.23	0.78	<0.0001	0.88

* Value after log₁₀ transformation

¹P-value is probability of significance for treatment (T), day (D), and treatment x day (TxD)

^{a,b}Within a variable, means with an uncommon superscript differ by day (P<0.0001)

Fecal Cortisol

Fecal cortisol concentration was influenced by date (P<0.0001), but not by treatment (P=0.93). No difference was detected in fecal cortisol concentrations of the AB and FL calves before (P=0.84) or after (P=0.72) weaning (table 5-3).

Table (5-3) Fecal cortisol concentration of the abrupt and fenceline-weaned calves pre- (day -6) and post-weaning (day 1)

Variable	Day -6		Day 1		SEM	P-value ¹		
	Abrupt	Fenceline	Abrupt	Fenceline		T	D	TxD
Fecal Cortisol (µg/dL)	10.5 ^a	10.3 ^a	11.8 ^b	12.1 ^b	0.6	0.9	<0.0001	0.5

¹P-value is probability of significance for treatment (T), day (D), and treatment x day (TxD)

^{a,b}Within a variable, means with an uncommon superscript differ by day

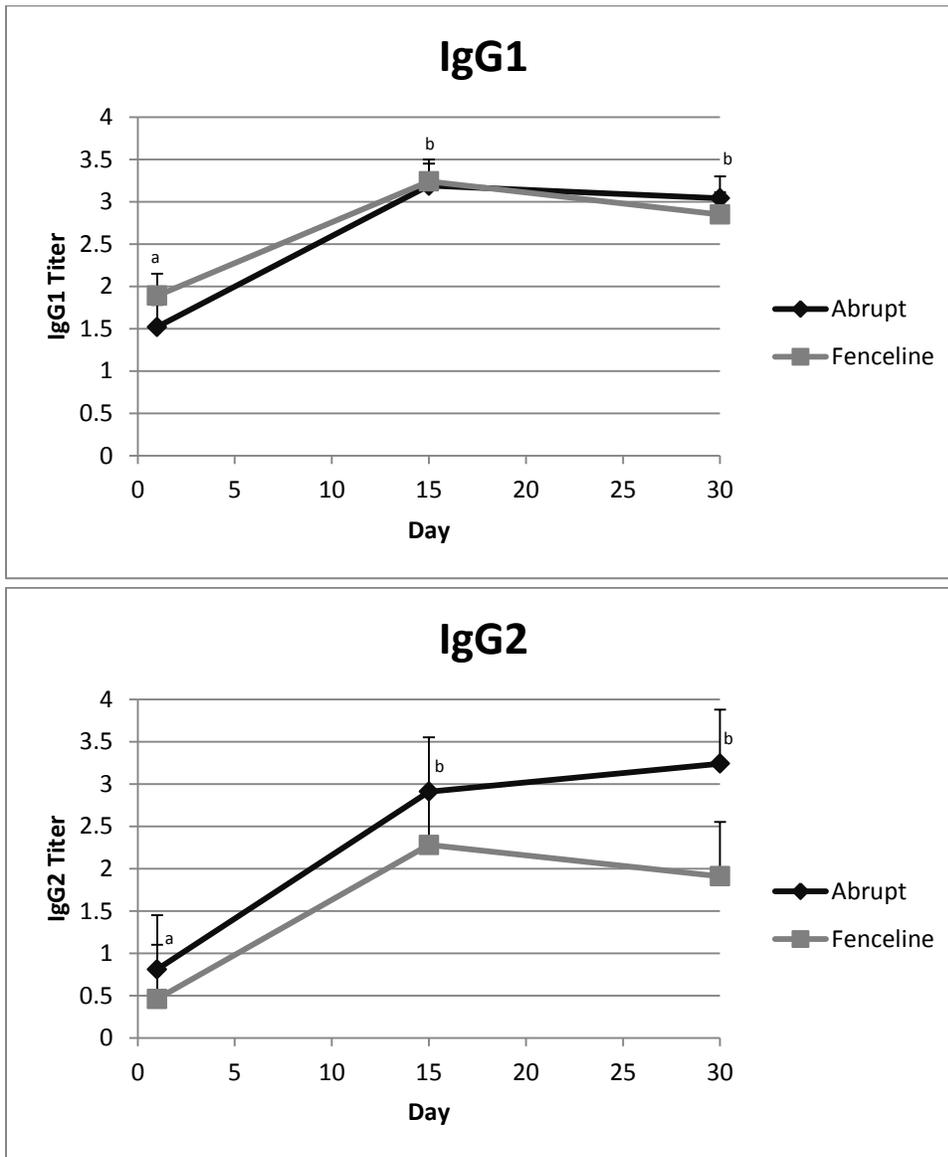
Whole Blood Glucose Concentrations

On the day after weaning, the average blood glucose concentration for calves subjected to the low-stress (fence-line) weaning was lower (66.3±12.7 mg/dl) than that of the calves subjected to the high-stress (abrupt) weaning (75.5±13.0 mg/dl) (P=0.03).

Tetanus Toxoid-Specific Titers

A difference in IgG₁ and IgG₂ antibody response to the tetanus toxoid vaccine administered at day 1 was not detected between the AB and FL calves (figure 5-1). A date effect was detected for an increase in both the IgG₁ and IgG₂ isotypes of anti-tetanus toxoid antibodies between days 0 to 30 (P<0.001).

Figure (5-1) IgG1 and IgG2 titers* against tetanus toxoid vaccination for abrupt and fenceline weaned calves from days 1 to 30



Calves were vaccinated with tetanus toxoid (Fort Dodge) on the day after weaning (day 1)
 *Values expressed as least squares means of the \log_{10} transformation of the percent of tetanus toxoid-specific antibodies from serum samples in comparison to hyperimmunized calf serum
^{a,b}Uncommon superscripts indicate difference in means between days ($P < 0.001$)

BVDv Serum Neutralization Titers

No difference was detected between the BVDv type 1 or type 2 antibody response of the AB and FL weaning treatments (table 5-4). A date effect was detected for an increase in antibody response to BVDv type 1 in the AB group from day 0 to day 30 (P<0.05).

Table (5-4) Type 1 (NADL) and type 2 (125C) BVDv SN titers¹ for abrupt and fenceline weaned calves from days 1 to 30

Virus Isotype	log ₁₀ (BVDv Titer)				SEM ²	P-Value ³		
	Day -6		Day 30			T	D	T*D
	Abrupt	Fenceline	Abrupt	Fenceline				
Type 1	6.7 ^a	6.7	7.2 ^b	6.9	0.4	0.6	0.02	0.1
Type 2	4.1	3.8	4.5	4.3	0.2	0.3	0.02	0.7

Calves were vaccinated against BVDv types 1 and 2 one month before and the day after weaning (day 1)

²Standard error of the mean

³P-value is probability of significance for treatment (T), day (D), and treatment x day (TxD)

^{a,b}Within a row, uncommon superscripts signifies that means differ by day (P<0.05)

qPCR

Results from qPCR analysis of target gene relative quantification (Δ Ct) to the mean Ct values of the endogenous controls (18S and β -actin) are depicted in table 5-5 as the least squares means for each target gene Δ Ct from both treatments at days -6 and 1. An overall treatment effect was detected with an increase among FAS expression (P=0.04), but no treatment effect was detected for FAS individually at day -6 (P=0.12) or day 1 (P=0.09). A date effect was

detected for an increase in FAS from day -6 to day 1 for the AB (P=0.01) and FL (P=0.008) groups. There was also a date effect detected for an increase in IL-4 expression from day -6 to day 1 in the FL group (P=0.02), but not for the AB group (P=0.10). No treatment*date interaction was detected for either FAS or IL-4. No treatment effect, date effect, or treatment*date effect was detected for IL-10 or IFN γ gene expression.

Table (5-5) Δ Ct of target genes against the mean Ct from the endogenous control genes (18S and β -actin) for abrupt and fenceline-weaned calves pre- (day -6) and post-weaning (day 1). Genes of interest include: FAS (neutrophil apoptotic marker), IL-4 (Th2 cytokine), IL-10 (Th2 cytokine), and IFN γ (Th1 cytokine)

Gene	Target Gene Δ Ct				SE	P-Value ¹		
	Day -6		Day 1			T	D	TxD
	Abrupt	Fenceline	Abrupt	Fenceline				
FAS	4.85 ^a	5.16 ^a	5.38 ^b	5.71 ^b	0.14	0.04	0.0006	0.93
IL-4	13.50 ^a	13.51 ^a	13.92 ^b	14.11 ^b	0.27	0.78	0.008	0.61
IL-10	9.62	9.94	9.99	10.07	0.44	0.63	0.55	0.78
IFN γ	16.82	16.18	16.55	16.88	0.28	0.59	0.42	0.08

¹P-value is probability of significance for treatment (T), day (D), and treatment x day (TxD)
^{a,b}Uncommon superscripts within a row indicate differences in means with P<0.05

Discussion

In conventional beef cattle production, weaning induces a multi-faceted stress response in the newly weaned calf. The calf responds to the weaning stress-related stimuli through

physiological changes that shift away from the body's homeostatic condition. Glucocorticoids, such as cortisol, increase in concentration in response to stress and prove essential components of the stress-adaptive mechanism of the body (Aich, Shakiba et al. 2007). Glucocorticoid production increases gluconeogenesis, thereby increasing circulating glucose concentrations to provide an additional source of energy for the CNS and other cells (Aich, Shakiba et al. 2007). Because of the physiological impacts of glucocorticoid release, researchers have measured blood glucose concentrations as an indicator of an animal's response to stress (Swanson and Morrow-Tesch 2001). Abruptly weaned calves in the present study had higher blood glucose concentrations on day 1 than the calves weaned by the intermediary fenceline technique. The observed increase in blood glucose concentration on the day following weaning is accordance with the literature that stressed cattle develop elevated plasma cortisol and glucose concentrations (Tarrant, Kenny et al. 1992).

Measurement of fecal cortisol metabolites mirror the cortisol concentrations present in the blood stream 12 hours prior to fecal collection (Kahrer, Möstl et al. 2005). Fecal cortisol metabolites have been proven to increase in cattle subjected to transport and injury-related stress in comparison to control cattle (Mostl, Maggs et al. 2002; Kahrer, Möstl et al. 2005). However, the competitive-binding EIA fecal cortisol metabolite quantification method validated by Möstl et al. (2002) did not produce a standard curve for analysis when performed in this lab. To compensate for this, the concentration of fecal cortisol in the feces collected the day after weaning was analyzed through an RIA technique. Non-metabolized fecal cortisol concentrations were hypothesized to still remain a representative measure of cortisol concentrations in circulation 12 hours before fecal collection. In the present study, fecal cortisol concentrations increased in all calves after weaning, but were not different between the calves that were

subjected to hypothesized high stress (abrupt) and low stress (fenceline) weaning. The lack of increase in fecal cortisol in response to the high stress weaning technique detected in the present study is not in accordance to the literature where an increase in fecal cortisol metabolite concentration was observed in stressed calves (Mostl, Maggs et al. 2002; Kahrer, Möstl et al. 2005).

Fecal cortisol concentration analysis through an RIA technique is not validated in cattle and proved to only detect a low concentration of cortisol in the fecal samples. Cortisol that is not metabolized while in circulation may also be expelled from the body at a different rate than the cortisol metabolites. Therefore, it is possible that collection of feces 12 hours post-weaning may not be the most accurate representation of non-metabolized cortisol in circulation at the time of weaning. Furthermore, the detection range of this RIA technique ranges from 0 ng/mL to 100 ng/mL. Cortisol concentrations from 10 ng/mL to 50 ng/mL lie in the dynamic range of the standard curve, thereby providing the most accurate detection of the sample concentration. The cortisol concentrations that were detected in the fecal samples analyzed in this study ranged from 5.9 ng/mL to 21.7 ng/mL. These concentrations lie below or in the lower range of the dynamic portion of the curve. This may have resulted in a decrease in the accuracy of quantification of the fecal cortisol concentrations between the two weaning groups. However, the increases detected in fecal cortisol on day 1 from both weaning treatments indicate that the calves did experience a stress-induced physiological response to weaning that occurred on day 0.

During the stress response, glucocorticoids alter immune cell trafficking and gene expression (Buckbinder and Robinson 2002; De Bosscher, Vanden Berghe et al. 2003). Glucocorticoids specifically act through their receptors on antigen-presenting cells (APCs) to suppress IL-12, the production of the main inducer of Th1 responses. Suppressed production of

IL-12 results in decreased IFN γ in circulation. With decreased IFN γ , IL-4 is increasingly released by T cells, thereby further increasing Th2 activity through the production of IL-10 and IL-13 in response to stress (Elenkov 2004). The FAS gene represents neutrophil apoptosis, which is hypothesized to increase in response to stress. The two housekeeping genes (18S and β -actin) remained unchanged in expression pre- and post-weaning and between the weaning treatments, allowing for reliable analysis of the target genes of interest. A date effect of increased FAS and IL-4 expression was observed from day -6 to day 1. A treatment effect with an increase in FAS was detected, but the expression of IL-4 did not differ between weaning treatments. No differences were detected in IL-10 or IFN γ gene expression. The lack of differences detected in gene expression on the day after weaning between weaning treatments could be representative of the lack of stress resulting from the weaning methods used in this study. Stress responses of the AB and FL calves may have been of similar magnitudes, thereby resulting in similar gene expression in response to weaning. Previous analysis within this laboratory on gene expression resulting from abrupt and fenceline weaning remained unsuccessful in detecting differences between weaning treatments three days after weaning (Gilberte 2010). The increase of FAS and IL-4 observed after weaning in this study provides evidence that immunomodulatory effects are occurring in response to the stress of weaning. Future trials including a group of calves subjected to a synthetic glucocorticoid (i.e. dexamethasone) injection could serve as a positive control to represent alterations in gene expression during the stress response.

Among the multi-faceted immunomodulatory effects of glucocorticoids is the inhibition of lymphocyte proliferation and cytotoxicity, and the secretion of TNF α , IL-2, and IFN γ (Elenkov 2004). The neutrophil to lymphocyte ratio in circulation has been found to increase

(i.e. neutrophils increase and lymphocytes decrease in circulation) in calves in response to weaning stress (Lynch, Earley et al. 2010). However, the neutrophil to lymphocyte ratio in the calves in this study remained unchanged in response to weaning. This is an indicator that the different weaning treatments did not induce a strong or noticeably different stress response to the calves this study.

Stimulation with the legume-derived mitogen PWM is commonly used in vitro to stimulate immune cell activity (Bosward, Dhand et al. 2010). The stimulation with PWM resulted in an increased amount of IFN γ than was detected in non-stimulated supernatant. However, weaning did not seem to induce a strong enough stress response in the calves to result in reduced IFN γ concentration in non- or PWM-stimulated supernatant. The lack of differences among AB and FL IFN γ production after weaning may indicate that the weaning methods themselves did not induce significantly different stress responses in the different treatment groups.

When Th2-mediated humoral immunity is increased as a result of glucocorticoid release, there is a shift in the IgG₁:IgG₂ ratio of isotype antibody production. When Th2 immunity activity is enhanced, IL-4 induces secretion of IgG₁ (Isakson, Pure et al. 1982). IFN γ blocks IgG₁ production, but is inhibited in response to stress (Finkelman, Katona et al. 1988). Therefore, the IgG₁:IgG₂ ratio should increase (greater IgG₁ concentration:lower IgG₂ concentration) with an increase in Th2 activity in response to the stress-induced glucocorticoid release. When exposed to the novel tetanus toxoid antigen via vaccination on day 1, calves in the high stress AB weaning group were expected to have an increase in anti-tetanus IgG₁ production in comparison to FL calves. When analyzed using an IgG₁-specific anti-tetanus ELISA, calves in both the AB and FL treatments increased in IgG₁ titers in response to

vaccination. However, no differences were detected in IgG₁ production between the AB and FL calves from day 0-30. Furthermore, IgG₁ production against the novel tetanus toxoid antigen peaked at day 15 for both AB and FL calves with no changes between day 15 and 30. The anti-tetanus IgG₂ titers also increased from the day of vaccination (day 1) to day 15 with no changes up until day 30 for either weaning treatment.

Titers to a secondary immunization against BVDv types 1 and 2 (administered to all calves on day 1) were determined by a serum neutralization assay. If the AB calves were more stressed at weaning, their antibody production against BVDv was hypothesized to be less than that of the FL calves. However, no difference in antibody production from day 1-30 was detected for BVDv types 1 or 2 between the weaning treatments. The weaning protocols implemented in this study likely did not induce profound differences in the physiological stress response of AB and FL calves, thereby allowing for equal immune responses within treatments to antigen exposure.

The physiological stress response to weaning is accompanied by behavioral changes in calves. Changes in behavior in response to abrupt weaning include decreased eating and lying down and increased walking and vocalizations (Veissier, Le Neindre et al. 1989; Price, Harris et al. 2003; Boland, Scaglia et al. 2008). One factor resulting from these behavioral changes is reduced weight gain of the stressed calf. Haley et al. (2005) observed that calves weaned in two stages (i.e. fenceline weaning) had greater average daily gain (ADG) than calves weaned by abrupt separation. In the present study, AB calves gained 7.8 Kg less than FL calves from day -6 to day 1. The reduced weight gain of the AB calves on day 1 is likely a result of dehydration and reduced grazing in comparison to the FL calves during the 24 hours between weaning and weighing. However, on day 30, calf weight gain was the same for the AB and FL calves,

meaning that the reduced weight gain seen on day 1 in the AB calves was compensated for quickly.

Nutritive forage is important for promoting maximum weight gain in newly weaned calves. In the present study, the calves were weaned onto E+ fescue. In a study completed by Boland et al. (2010) calves weaned onto E+ fescue gained an average of 0.68 Kg/d for a 42 day grazing period. The calves in the present study gained less (0.36 Kg/d) over a 30 day grazing period on E+ fescue.

Conclusion

No differences were detected between the abrupt or fenceline weaning methods in terms of weight gain or antibody production against primary and secondary antigen exposure. Calves in each weaning treatment remained healthy throughout the 30 day post-weaning period during which they were monitored. A stress response likely occurred to the weaning in both groups as is represented by an increase in fecal cortisol concentrations and alterations in FAS and IL-4 gene expression. The weight gain of AB calves differed from FL calves on the day after weaning. However, the indicators of an exaggerated response to the stress of weaning were not detected in AB calves in comparison to the FL calves.

The calves in this study were comprised from the same herd of cows and were never subjected to transportation to a new facility after weaning. The FL calves were separated from the AB calves during the six days of fenceline separation, but all calves were reunited at weaning. These situations did not subject the calves to commingling with calves from different farms or herds. These conditions reduce the weaning stress-related stimuli experienced by the calves in this study in comparison to the weaning stress experienced by calves that are weaned

by conventional methods in the beef industry. Future trials with the implementation of a positive control treatment group with a synthetic glucocorticoid injection or the inclusion of a transportation stage may provide more information on if fenceline weaning techniques reduce calf stress, morbidity, and mortality after weaning in comparison to the conventional abrupt weaning technique.

Chapter 6

Summary and Conclusion

Beef cattle producers aim to provide rapid diagnostic care and treatment to sick animals, as well as environmental and nutritional conditions that minimize stress, thereby increasing productivity and health while of the herd. Cow-calf producers especially are conscious of the health and rate of growth of their young animals. Larger calves bring in more revenue at sales than do small calves. Therefore, reducing incidences of morbidity and providing optimum dietary nutrients during stressful stages, such as weaning, are constant aims of the beef cattle producers.

Literature is available with recommendations for forage types, physiological responses to stress, alternative weaning techniques, and techniques to reduce the incidences of common maladies, such as bovine respiratory disease, for newly weaned calves. The current thesis supplies a compilation of such current literature, while expanding the field of knowledge through investigations of novel diagnostic techniques, effect of forage on immune function, and impact of weaning technique on health and productivity of newly weaned calves.

The implementation of handheld glucometer systems into use for chute-side analysis of bovine blood glucose concentration is a recent innovative technique in improving veterinary diagnostics for neonatal disorders and ketosis in cows. Because blood glucose concentrations increase during the physiological response to stress, glucometers can also be used to monitor and compare stress responses in research applications. In the current thesis, the Precision Xtra® glucometer was validated for chute-side analysis of bovine blood glucose concentrations. The use of a handheld glucometer reduces the need for tedious sample handling protocols and decreases time of analysis, thereby also reducing time lapse to clinical treatment, during the determination of blood glucose concentrations.

Heifers grazed on either alfalfa or fescue forage did not have different production of interferon gamma in non-stimulated or pokeweed mitogen stimulated blood. The weight gain of heifers on each forage type was the same throughout the 30 day trial. Previous observations have detected an increased incidence of morbidity with quicker weight gain in cattle grazing alfalfa in comparison to those grazing grass. These observations in addition to the accepted use of legume-derived mitogens during *in vitro* stimulation of immune cells lead to the hypothesis that calves on alfalfa would have increased interferon gamma production and gain more weight than those on fescue. Indicators of this pattern were not recognized in the present study. The lack of differences detected between the heifers on alfalfa and fescue could be resultant from the time of year and/or the limited time (30 days on pasture) during which this study was completed.

Finally, work described herein provides no evidence that gradual (i.e. fenceline) weaning assuages the stress response observed with conventional abrupt weaning techniques. Although blood glucose concentrations differed between weaning treatments on the day after weaning, other indicators of altered physiological responses were not detected between abrupt and fenceline-weaned calves. Weight gain was less in abrupt calves than fenceline weaned calves on the day after weaning, indicating a difference in behavior resulting from the different weaning techniques. Weight gain and antibody production against antigen exposure was the same between calves in both groups, providing evidence that both groups of calves would have equal weights at auction and abilities to combat disease. Future investigations with a positive control group injected with a synthetic glucocorticoid or the implementation of a transportation stage may provide enhanced insight to calf immune function and stress resulting from conventional weaning procedures.

In conclusion, new systems, such as glucometers, have promise to improve the efficiency of clinical and research applications within the beef cattle industry. However, specific changes in immune cell activity that result from weaning stress as well as successful mechanisms to reduce calf morbidity through proper forage diets and alternative weaning techniques are areas that are still lacking in empirical data.

APPENDICES

Appendix A: Determination of Neutral Detergent Fiber (NDF)

Reagents

1. Neutral-detergent solution (NDS)
 - 18.0 L Deionized Water
 - 1200.0g Neutral Detergent Dry Concentrate (Ankom # FND20C)
 - 200.0 ml Triethylene glycol
2. Sodium sulfite (Na_2SO_3)
3. Heat-stable alpha amylase (activity = 340,000 Modified Wohlgemuth Units/ml)
4. Acetone (CH_3COCH_3)

Procedure

1. Add 2000 ml of ambient NDS into a digestion vessel (Ankom 200 Fiber Analyzer Model A200, Ankim Technology, Macedon, NY)
2. Place plastic bag suspender with bagged samples into the solution in the digestion vessel
3. Turn *agitate* and *heat* on and close the lid and seal the digestion vessel
4. After 60 min have elapsed turn *agitate* and *heat* off and open the valve slowly and exhaust hot solution before opening the lid
5. After the solution has exhausted, close the valve and add 2000 ml of 90° – 100°C H_2O and 4 ml alpha-amylase
6. Lower the lid to the digestion vessel but do not tighten.
7. Turn *agitate* on and leave the *heat* off for 5 minutes
8. Exhaust liquid in digestion vessel and then close valve
9. Repeat Steps 5 – 8
10. Repeat steps 5 – 8 without adding alpha-amylase
11. Remove filter bags from bag suspender and gently press out excess water
12. Place filter bags in beaker and soak in acetone for 3 minutes
13. Remove filter bags from acetone bath and lightly press out excess acetone
14. Spread bags out and allow acetone to evaporate completely in fume hood
15. Complete drying in an oven at 105°C for at least 4 hours
16. Remove bags from oven and place directly into sealable plastic bag containing a desiccant and flatten to remove air
17. Cool bags to ambient temperature and weigh filter bags with samples
18. Record weight

Appendix B: Determination of Acid Detergent Fiber (ADF)

Reagents

5. Acid detergent solution (ADS)
200g Hexadecyltrimethylammonium bromide ($\text{CH}_3(\text{CH}_2)_{15}\text{N}(\text{CH}_3)_3\text{Br}$)
10 L 1 N sulfuric acid (H_2SO_4)
6. Acetone (CH_3COCH_3)

Procedure

1. Add 2000 ml ADS into digestion vessel
2. Place plastic bag suspender with bagged samples into the solution in the digestion vessel
3. Turn *agitate* and *heat* on and close the lid and seal the digestion vessel
4. After 60 min have elapsed turn *agitate* and *heat* off and open the valve slowly and exhaust hot solution before opening the lid
5. After the solution has exhausted, close the valve and add 2000 ml of 90° – 100°C H_2O
6. Turn *agitate* on and leave the *heat* off for 5 minutes
7. Exhaust liquid in digestion vessel and then close valve
8. Repeat Steps 4 – 7 twice
9. Place filter bags in beaker and soak in acetone for 3 minutes
10. Remove filter bags from acetone bath and lightly press out excess acetone
11. Spread bags out and allow acetone to evaporate completely in fume hood
12. Complete drying in an oven at 105°C for at least 4 hours
13. Remove bags from oven and place directly into sealable plastic bag containing a desiccant and flatten to remove air
14. Cool bags to ambient temperature and weigh filter bags with samples
15. Record ADF bag weight
16. Subtract ADF bag weight from the bag weight after 0.5 g 1mm ground sample had been added in the preparation step
17. Divide value determined in step 16 by 0.5 then multiply that value by 100 to determine percent ADF of sample placed in filter bag
18. Divide value determined in step 17 by the average micro dry matter weight of the forage sample to determine the percent of ADF in the dry matter of the forage sample

Appendix C: Bovine IFN γ ELISA Protocol

Reagents and Materials

1. D-PBS: Dulbecco's Phosphate Buffered Saline Packs (Thermo Scientific Product # 28374)
2. Carbonate-Bicarbonate Buffer (Thermo Scientific Product # 28382)
3. Blocking Buffer: 4% BSA and 5% Sucrose in D-PBS; 0.2 μ m filtered
4. Reagent Diluent: 4% BSA in D-PBS; 0.2 μ m filtered
5. Wash Buffer: 0.05% Tween-20 in D-PBS (made fresh daily)
6. ReactiBind 96-well EIA Strip Plates (Corner Notch Product # 15031)
7. Plate Sealers for 96-well EIA plates (Thermo Scientific Product # 15036)
8. ImmunoWare Reagent Reservoirs (Thermo Scientific Product # 15075)

Procedure

1. Dilute coating antibody 1:100 in carbonate/bicarbonate buffer
2. Seal plates and incubate overnight at room temperature
3. Aspirate coating antibody solution and add 300 μ l blocking buffer to each well
4. Seal plates and incubate for 1 hour at room temperature
5. Aspirate blocking buffer and add 100 μ l/well standards (0, 31.25, 62.5, 125, 250, 500, 1000, 2000 pg/ml) and control-, PWM-, and ConA-stimulated samples were added in duplicate
6. Seal plates and incubate for 1 hour at room temperature
7. Aspirate standards and samples and wash three times with 300 μ l/well wash solution
8. Dilute detection antibody 1:100 in reagent diluent
9. Add 100 μ l diluted detection antibody to each well
10. Seal plates and incubate for 1 hour at room temperature
11. Aspirate detection antibody and wash three times with 300 μ l/well wash solution
12. Dilute SA-HRP 1:400 in reagent diluent
13. Add 100 μ l/well diluted SA-HRD reagent
14. Seal plates and incubate for 30 minutes at room temperature
15. Aspirate and wash three times with 300 μ l/well wash buffer
16. Add 100 μ l substrate solution to each well
17. Seal plate and incubate in the dark for 20 minutes at room temperature
18. Stop the reaction by adding 100 μ l stop solution to each well
19. The plate absorbance was read at A₄₅₀ minus A₅₅₀ (SpectraMax M5 Multi-Mode Microplate Reader, Molecular Devices, Downingtown, PA).
20. Sample concentrations were determined using the equation of a four parameter logarithmic standard curve.

Appendix D

Mean environmental conditions recorded at Kentland Farm (Blacksburg, VA) over the entire data collection period in September and October, 2010

Climatic Condition	Daytime ¹		Nighttime ²	
	September	October	September	October
Temperature, °C	21.7	15.9	14.3	9.0
Relative Humidity, %	58.3	59.4	82.7	82.8
Soil Temperature, °C	17.7	13.2	18.1	13.6
Sunshine, kilowatts/m ²	0.558	0.331	0.120	N/A
Wind Speed, km/hr	7.3	7.8	1.8	3.5
THI ³	68.0	60.0	57.8	49.1
Precipitation (0.01 in)	0.540	0.105	0.635	0.492

¹Mean daytime conditions from 08:00 – 18:59

²Mean nighttime conditions from 19:00 – 07:59

³Temperature Humidity Index = $(0.8 \times \text{Temperature}) + [(\% \text{ Relative Humidity}/100) \times (\text{Temperature} - 14.40)] + 46.4$

Appendix E

Composition of mineral supplied¹ to heifers grazing E- tall fescue at Kentland Farm, Blacksburg, VA

Item	As-fed basis
Calcium, minimum, %	8.50
Calcium, maximum, %	10.20
Phosphorus, minimum, %	7.90
Salt, minimum, %	16.50
Salt, maximum, %	19.80
Magnesium, minimum, %	12.00
Sulfur, minimum, %	0.05
Potassium, minimum, %	1.00
Iodine, minimum, ppm	60
Copper, minimum, ppm	3000
Cobalt, minimum, ppm	50
Selenium, minimum, ppm	60
Zinc, minimum, ppm	1300
Manganese, minimum, ppm	1200
Vitamin A, minimum, IU/lb	100000
Vitamin D, minimum, IU/lb	25000
Vitamin E, minimum, IU/lb	250

¹Supplied by Southern State Cooperative, Inc., Richmond, VA 23260

Appendix F

Composition of bloat block^{1,2,3} supplied to heifers grazing alfalfa at Kentland Farm, Blacksburg, VA

Guaranteed Analysis	As-fed basis
Crude protein, minimum, %	4.00
Crude fat, minimum, %	0.05
Crude fiber, maximum, %	12.50
Salt, minimum, %	19.50
Salt, maximum, %	23.00
Potassium, minimum, %	1.80
Iodine, minimum, ppm	43
Selenium, minimum, ppm	13

¹Active drug ingredient, Poloxalene (6.6%)

²Contains ethoxyquin (0.03%) and BHT (0.095%) as preservatives

³Sweetlix, P.O. Box 8500, Mankato, MN 56002

Appendix G: Tetanus Toxoid-Specific ELISA

Reagents and Materials

1. Assay Buffer
 - 29.2g Trizma Base (Sigma T-1503), 20 mmol/l
 - 17.9 g NaCl (Sigma S-9625), 0.3 mol/l
 - 1g Bovine Serum Albumin (Sigma A-4503)
 - 1ml Tween 80 (Sigma P-8074)
 - Dissolve in 1L ddH₂O, pH to 7.5 with (about 17 ml 1M HCl)
 - Filter thru Sep-Pak C18
2. Coating Buffer
 - 1.59g Na₂CO₃
 - 2.39g NaHCO₃
 - Dissolve in 1L ddH₂O and pH to 9.6 with 1M HCl
 - Filter thru Sep-Pak C18
3. Second Coating Buffer
 - 3.146 g Trizma Base
 - 23.3 g NaCl
 - 13 g BSA
 - 1.3 g Sodium azide
 - Dissolve in 1.3L ddH₂O pH to 7.5 with 1M HCl
4. Wash Solution
 - 0.5ml Tween 20
 - Dissolve in 2.5L ddH₂O
5. TMB Substrate
6. 2 M H₂SO₄
7. Tetanus Toxoid Vaccine (Fort Dodge Animal Health Inc., Fort Dodge, IA)
8. Positive control serum from hyperimmunized heifer
9. Fetal Bovine Serum
10. Reservoirs for multi-channel pipetting
11. 15 ml conical tubes for dilutions of positive serum

Procedure

- 1) Coat half of the plate with 100µL/well Tetanus Toxoid vaccine diluted 1:10 in the coating buffer
- 2) Coat the other half of the plate with just the coating buffer
- 3) Cover plate and incubate overnight at room temperature
- 4) Decant coating solution
- 5) Add 300µL/well of blocking buffer
- 6) Cover the plate and incubate overnight
- 7) Wash plate 3 times with washing buffer

- 8) Dilute hyperimmunized serum in assay buffer to create standards and pipette onto the plate:
 - a. For IgG₁:
 - i. In duplicate, add 200 μ L of 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, and 1:1280 of positive serum samples diluted in assay buffer to both the vaccine-coated and non-vaccine coated sides of the plate
 - b. For IgG₂:
 - i. In duplicate, add 200 μ L of 1:1, 1:4, 1:16, 1:64, 1:256, and 1:1024 of heat-treated positive serum samples diluted in assay buffer to both the vaccine-coated and non-vaccine coated sides of the plate
- 9) Add calf serum samples onto the plate
 - a. For IgG₁
 - i. In duplicate, add 200 μ l of serum samples diluted 1:20 in assay buffer to both the vaccine-coated and non-vaccine-coated sides of the plate.
 - b. For IgG₂
 - i. In duplicate, add 200 μ l of heat-treated serum samples diluted 1:50 in assay buffer to both the vaccine-coated and non-vaccine-coated sides of the plate.
- 10) Keep the 4 bottom left wells free for negative control and blank wells
- 11) Add 200 μ l fetal bovine serum (FBS) to the negative control wells (in duplicate)
- 12) Keep blank wells empty at this point
- 13) Cover and incubate the plates overnight at 4°C with gentle shaking.
- 14) Wash plates 3 times with 300 μ l/well wash solution
- 15) Dilute the appropriate IgG₁- or IgG₂-HRP antibody 1:1000 with PBS + 0.05% Tween 20
- 16) Add 100 μ l/well of the diluted antibody to all wells (except blank)
- 17) Cover and incubate the plate for 2 hours at 4°C with gentle shaking
- 18) Wash plate 3 times 300 μ l/well with wash buffer
- 19) Add 200 μ l ddH₂O to blank wells
- 20) Add 100 μ l of TMB substrate to each well (except blank wells)
- 21) Immediately cover plate in tin foil and incubate for 15 minutes at room temperature
- 22) Stop the reaction by adding 100 μ l/well 2N H₂SO₄ to each well (except blank wells)
- 23) Read absorbance at 450nm
- 24) Subtract the blank well OD values from all other wells
- 25) Subtract any response on the non-vaccine-coated side of the plate from the corresponding readings on the vaccine-coated wells
- 26) Calculate percent positivity for each sample by using the equation of a logarithmic curve generated in Excel from dilutions of the positive control.

Appendix H: PAXgene Blood RNA Kit Protocol

Manual Purification of Total RNA from Whole Blood Collected into PAXgene Blood RNA Tubes

PreAnalytix – 50(cat.no. 762164)

Before Starting

- Equilibrate PAXgene blood RNA tubes with blood sample to room temperature and keep at room temperature for an additional 2 hours before starting the procedure
- Set the temperature of the shaker-incubator to 55° C
- Buffer BR2 may form a precipitate upon storage. If necessary, warm to 37° C to dissolve
- Buffer BR4 is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96-100%) as indicated on the bottle to obtain a working solution.
- If using the RNase-Free DNase Set of the first time, prepare DNase I stock solution.
 - o Dissolve the solid DNase I in 550 µl of the RNase-Free water provided with the set. Take care that no DNase is lost when opening the vial. Do not vortex reconstituted DNase I. DNase I is especially sensitive to physical denaturation. Mixing should only be carried out by gently inverting the tube.

Equipment Needed – Not Supplied in Kit

- PAXgene blood RNA tubes (cat. no. 762165)
- Ethanol (96-100%)
- Pipets (10 µl – 4 ml)
- Sterile, aerosol-barrier, RNase-free pipet tips
- Graduated cylinder
- Centrifuge capable of attaining 3000-5000 x g, and equipped with a swing-out rotor and buckets to hold PAXgene blood RNA tubes
- Vortex mixer
- Crushed ice
- Permanent pen for labeling
- Variable speed microcentrifuge capable of attaining 1000-8000 x g, and equipped with a rotor for 2 mL microcentrifuge tubes
- Shaker-incubator capable of incubating at 55° C and 65° C and shaking at ≥400 rpm, not exceeding 1400 rpm
- Label 1 lilac column with 1 tube, 1 microcentrifuge tube, 1 red column with 7 tubes and one more microcentrifuge tube for each animal

Procedure

1. Turn Shaker Incubator on to 55° C

2. Centrifuge the PAXgene Blood RNA Tube for 10 minutes at 3000 x g using a swing-out rotor.
3. Remove supernatant by decanting or pipetting.
4. Add 4 ml RNase-free water to the pellet and close the tube using a fresh secondary BD hemogard closure supplied with the kit.
5. Vortex until the pellet is visibly dissolved
6. Centrifuge the blood tube for 10 minutes at 3000 x g using a swing-out rotor.
7. Remove and discard the entire supernatant
8. Add 350 µl Buffer BR1, and vortex until pellet is visibly dissolved
9. Pipet the sample into a 1.5 ml microcentrifuge tube. Add 300 µl Buffer BR2 and 40 µl proteinase K.
10. Mix by vortexing for 5 seconds
11. Incubate for 10 minutes at 55° C using a shaker incubator at 400 rpm
12. After incubation, set the temperature of the shaker-incubator to 65° C
13. Pipet the lysate directly into a PAXgene Shredder spin column (lilac) placed in a 2 mL processing tube
 - a. First add 600 µl of lysate, centrifuge for 3 minutes at 16,000 x g
 - b. add the remaining lysate to the same shredder and spin column and centrifuge for 5 minutes at 16,000 x g
14. Carefully transfer the entire supernatant of the flow-through fraction to a fresh 1.5 ml microcentrifuge tube without disturbing the pellet in the processing tube.
15. Add 350 µl ethanol (96-100%)
16. Mix by vortexing and centrifuge briefly (1 second at 500-1000 x g) to remove drops from the inside of the tube lid
17. Pipet 600 µl sample into the PAXgene RNA spin column (red) placed in a 2 ml processing tube.
 - a. Keep remaining sample
18. Centrifuge for 1 minute for 1 minute at 16,000 x g.
19. Place spin column in a new 2 ml processing tube and discard the old processing tube containing containing flow-through.
20. Pipet the remaining sample into the PAXgene RNA spin column
21. Centrifuge for 1 minute at 16,000 x g.
22. Place the spin column in a new 2 mL processing tube and discard the old processing tube containing flow-through
23. Pipet 350 µl Buffer BR3 into the PAXgene RNA spin column
24. Centrifuge for 1 minute at 16,000 x g.
25. Place the spin column in a new 2 ml processing tube, and discarding the old processing tube containing the flow-through
26. Add 10 µl DNase I stock solution to 70 µl Buffer RDD, for each sample, in a 1.5 ml microcentrifuge tube.

- a. For 24 samples, 270 μ l DNase I stock solution to 1890 μ l Buffer RDD
27. Mix by gently flicking the tube, and centrifuge briefly to collect residual liquid from sides of the tube
28. Pipet 80 μ l of the DNase I incubation mix to each sample directly onto the PAXgene RNA spin column membrane
29. Let sit on benchtop for 15 minutes
30. Pipet 350 μ l Buffer BR3 into the PAXgene RNA spin column and centrifuge for 1 minute at 16,000 x g.
31. Place the spin column in a 2 ml processing tube and discard the old processing tube containing the flow-through
32. Pipet 500 μ l Buffer BR4 to the PAXgene RNA spin column and centrifuge for 1 minute at 16,000 x g.
33. Place the spin column in a new 2 ml processing tube and discard the old processing tube containing the flow-through
34. Add another 500 μ l Buffer BR4 to the PAXgene RNA spin column and centrifuge for 3 minutes at 16,000 x g.
35. Discard the tube containing the flow-through and place the PAXgene RNA spin column in a new 2 ml processing tube
36. Centrifuge for 1 minute at 16,000 x g.
37. Discard the tube containing the flow-through
38. Place the PAXgene RNA spin column in a 1.5 ml microcentrifuge tube
39. Pipet 40 μ l Buffer BR5 directly onto the PAXgene RNA spin column membrane
 - a. Wet the entire membrane to obtain maximum efficiency.
40. Centrifuge for 1 minute at 16,000 x g to elute the RNA
41. Repeat the elution steps (steps 39-40) as described, using 40 μ l Buffer BR5 and the same microcentrifuge tube
42. Incubate the eluate for 5 minutes at 65° C in the shaker-incubator without shaking
 - a. Do not exceed incubation time or temperature
43. Chill immediately on ice
44. If the RNA samples will not be used immediately, store at -70° C. Since the RNA remains denatured after repeated freezing and thawing, it is not necessary to repeat the incubation at 65° C

Appendix I

Mean environmental conditions recorded at Shenandoah Valley Agricultural Research and Extension Center (Steele's Tavern, VA) over the entire data collection period in August, September, and October, 2010

Climatic Condition	Daytime ¹			Nighttime ²		
	August	September	October	August	September	October
Temperature, °C	17.7	21.9	15.9	16.4	15.8	10.3
Relative Humidity, %	71.6	55.5	55.5	91.29	78.7	79.0
Soil Temperature, °C	21.2	18.9	16.3	21.4	19.9	16.9
Sunshine, kilowatts/m ²	0.381	0.398	0.307	0.005	0.005	0.005
Wind Speed, km/hr	6.1	8.1	8.7	2.9	4.3	4.5
THI ³	62.9	68.1	59.9	61.3	60.1	51.4
Precipitation (in)	6.3	6.3	5.2	4.4	4.4	2.7

¹Mean daytime conditions from 08:00 – 18:59

²Mean nighttime conditions from 19:00 – 07:59

³Temperature Humidity Index = $(0.8 \times \text{Temperature}) + [(\% \text{ Relative Humidity}/100) \times (\text{Temperature} - 14.40)] + 46.4$

Appendix J

Mean (\pm SD) hematocrit (%) for abrupt and fenceline calves on day -6 and 1

Treatment	Day -6	Day 1
Abrupt	42.1 \pm 1.6	42.1 \pm 2.9
Fenceline	40.9 \pm 2.5	40.6 \pm 2.5

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