Increases in Cortisol due to Weaning Stress and the Subsequent Alterations to Immune Function in Beef Calves

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Jessica M. Gilbertie

Abstract

Weaning is defined as the physical separation of the cow-calf pair and the end of milk feeding. Natural weaning occurs between 7 and 14 months and is a gradual process. However, domestic weaning occurs between 6 and 8 months and occurs rapidly. Calves that are abruptly separated from their dam respond with increased vocalization and walking, and decreased eating and resting. The psychological stress the calf undergoes during weaning causes elevated glucocorticoid and catecholamine hormone concentrations that may predispose to increased morbidity and/or mortality from infectious diseases such as Bovine Respiratory Disease Complex. As an attempt to counter these changes, alternative weaning methods have been implemented and normally occur in two stages. Two-stage weaning begins with the cessation of milk feeding for approximately one week with the calf maintaining some contact with their dam and then permanent separation occurs. One of these methods uses a single fence to separate the cow-calf pair; this process allows the calf to see, hear and smell their dam, but does not allow the calf to suckle from its dam.

Increases in cortisol, a glucocorticoid, have been linked to immunological alterations. Most notably, elevated cortisol concentrations decrease neutrophil function by down regulating the gene expression of CD62L and Fas. Cortisol also alters lymphocyte phenotype by decreasing γδ T cells and increasing αβ T cells in the circulation. Lastly, increases in cortisol can modify T cell cytokine production. The cytokines IL-12 and IFNγ are secreted from T helper 1 cells while T helper 2 cells secrete IL-4 and IL-10; these T cells subsets also inhibit one another. During

higher cortisol concentrations, these T cells are biased toward T helper 2 cytokine production. All these changes in immune function can lead to increased susceptibility to disease around the time of weaning. Therefore, two trials were conducted to test the hypotheses that abrupt weaning results in elevated concentrations of cortisol and subsequently alters immunological functions, and that fenceline weaning alleviates the increase in cortisol and alterations to immune function associated with weaning.

In the fall of 2008, 12 Angus and Angus-X heifers (186±21 kgs; 174±16 days of age) were blocked by age and weight and randomly allotted into two groups, fenceline and abrupt. Blood samples were taken on day -7, 0, 7, 14, 21, and 42; fecal samples were taken on day -7, 0, and 3. All calves were weighed on day -7, 0, 7, 14, and 42. On day -1 all calves were separated from their dam and transported for 2 hours to another facility. On day 0 all calves were vaccinated with *Brucella abortus* (strain RB51). Serum was analyzed for IFNγ and IL-4 as well as IgG_1 and IgG_2 specific antibodies to RB51. Fecal samples were analyzed for cortisol metabolites. Both IgG_1 and IgG_2 antibodies to RB51 increased from day 0 to day 14 ($P<0.05$), however no differences were detected between treatment groups. Fecal cortisol metabolites were higher on day 0 in abruptly weaned calves ($P< 0.001$) but did not differ between groups on day -7 or day 3. Fenceline calves had higher concentrations of IFNγ in the serum on day -7 and day 0 as compared to the abruptly weaned calves ($P<0.04$).

In the fall of 2009, forty-four Angus and Angus-X calves (19 heifers and 25 steers; 181±27 kgs; 148±17 days old) were blocked by age and gender and randomly allotted within block into two treatment groups, fenceline (FL) and abrupt (AB). Approximately half the fenceline calves were separated from their dams by a single fence at day -7 and the rest of the fenceline group at day -6; all calves were removed from their dam at day 0. Calves were

vaccinated with *Histophilus somni* on day 1. Blood samples were taken at day -6, 1, 3, 8, 15, and 22. Fecal samples were taken on day -7, -6, 1 and 3. All calves were weighed on day -7, 0, 8, and 22. Serum samples were analyzed for IgG_1 and IgG_2 specific-*H. somni* antibodies, white blood cells were analyzed for lymphocyte phenotypes, and gene expression using 18S as the housekeeper gene. Fecal samples were analyzed for cortisol metabolites. Abruptly weaned calves had higher concentrations of cortisol metabolites in the feces than fenceline calves at day 1 ($P<0.0001$). No difference in average daily gain or *H. somni* specific antibodies between treatment groups was detected. There was a treatment*date interaction in lymphocyte and neutrophil populations ($P<0.05$); neutrophils from fenceline calves dropped from day -6 to day 1, but increased from day 1 to day 3, while abrupt calves decreased from day -6 to day 3. Lymphocytes from fenceline calves increased from day -6 to day 1, but decreased from day 1 to day 3, while lymphocytes from abrupt calves increased from day -6 to day 3. No difference in treatment groups was detected for lymphocyte phenotypes or gene expression; however, a date effect was detected. The CD4 and CD8 cell populations increased over time ($P<0.0001$) and WC1 and TcR1 decreased over time ($P=0.0243$ and $P=0.0027$ respectively) for both treatment groups. A decrease was detected over time for expression of GAPDH and CD62L ($P<0.0001$). The gene expression for the cytokines IFNγ, IL-4 and IL-10 had no change over time. Results from the two studies suggest that fenceline weaning decreases the cortisol response associated with cow-calf separation, but does not have a significant effect on immunological parameters measured in this study.

Dedication

I dedicate this work to my Grandmother Olive Phillips who allowed me to develop a passion for the care and management of animals that lead me to pursue a career in veterinary medicine, to my parents, Betsey and Michael, who always encouraged me to follow my academic dreams and to my boyfriend, Matt Kovatch, who always has and always will support me in whatever educational endeavors I chase.

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Table of Contents

List of Appendices

List of Tables

List of Figures

List of Abbreviations

AB	Abrupt weaning
ACTH	Adrenocorticotropic hormone
α_4-integrin	Alpha 4 integrin
αβ	Alpha beta
ADG	Average daily gain
ALP	Alkaline phosphatase
APC	Allophycocyanin
APC	Antigen-presenting cell
BAFF	B cell-activating factor of the TNF family
Bcl-2	B cell lymphoma 2
β_2-AR	Beta 2 adrenoceptors
β_2-integrin	Beta 2 integrin
BRD	Bovine respiratory disease
BSA	Bovine serum albumin
BUN	Blood urea nitrogen
C3	Complement component 3
C5	Complement component 5
C9	Complement component 9
CC	Beta chemokine
CCK	Cholecystokinin
CCL	Beta chemokine ligand
CCR	Beta chemokine receptor
CD2	Cluster of differentiation 2
CD3	Cluster of differentiation 3
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
CD14	Cluster of differentiation 14
CD18	Cluster of differentiation 18
CD28	Cluster of differentiation 28
CD31	Cluster of differentiation 31
CD40	Cluster of differentiation 40
CD40L	Cluster of differentiation 40 ligand
CD62L	Cluster of differentiation 62 ligand or L-selectin
CD86	Cluster of differentiation 86
cDNA	Complementary deoxyribonucleic acid
CNS	Central nervous system
Con-A	Concanavalin A
COX-2	Cyclooxygenase-2

CR3	Complement receptor 3
CRH	Corticotrophin-releasing hormone
CRP	C-reactive protein
Ct	Threshold cycle
CXC	Alpha chemokine
CXCL	Alpha chemokine ligand
CXCR	Alpha chemokine receptor
DAP5	Death-associated protein 5
Daxx	Death associated protein 6
DC	Dendritic cell
ddHOH	Double-distilled water
DEX	Dexamethasone
DNA	Deoxyribonucleic acid
dNTP	Nucleotides
D-PBS	Dulbecco's phosphate buffered saline
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immunosorbent spot
EtOH	Ethanol
Fab	Antibody variable region
FADD	Fas-Associated protein with Death Domain
Fas	Apoptosis-stimulating fragment
FasL	Apoptosis-stimulating fragment ligand
FBS	Fetal bovine serum
Fc	Antibody constant region
FITC	Fluorescein isothiocyanate
FL	Fenceline weaning
FLASH	Caspase 8 associated protein 2
FSC	Forward scatter
γδ	Gamma delta
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GATA-3	GATA-bindingprotein3
GC	Glucocorticoid
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GR	Glucocorticoid receptor
H_2SO_4	Sulfuric acid
HOH or H_2O	Water
HPA axis	Hypothalamo-pituitary adrenal axis
HRP	Horse radish peroxidase
ICAM-1	Intracellular adhesion molecule 1

ICAM-2	Intracellular adhesion molecule 2
IFNγ	Interferon-gamma
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-1β	Interleukin 1 Beta
IL-2	Interleukin 2
IL-3	Interleukin 3
IL-4	Interleukin 4
IL-5	Interleukin 5
IL-6	Interleukin 6
IL-8	Interleukin 8
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-13	Interleukin 13
IL-17	Interleukin 17
IL-22	Interleukin 22
IRAK	Interleukin-1 receptor-associated kinase
JAK	Janus tyrosine kinases
$KHCO_3$	Potassium chloride
LFA-1	Lymphocyte function-associated antigen 1
LPS	Lipopolysaccharide
LTα	Lymphotoxins alpha
LTβ	Lymphotoxins beta
LY96	Lymphocyte antigen 96
MBL	Mannose binding lectin
MHC	Major histocompatibility complex
MSH	Melanocyte-stimulating hormone
MYD88	Myeloid differentiation primary response gene (88)
Na_2CO_3	Sodium carbonate
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
NEFA	Non-esterified fatty acids
NFkB	Nuclear factor kappa B
NH_4Cl	Ammonium chloride
NK cells	Natural killer cells
NOD	Nucleotide-binding oligomerization domain-containing protein
NSB	Non-specific binding

OD	Optical density
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline tween-20
PCR	Polymerase chain reaction
PECAM-1	Platelet/endothelial cell adhesion molecule 1
PMN	Polymorphonuclear
POD	Beta peroxidase
PRR	Pattern recognition receptor
PSST	Subunit of NADH:ubiquinone oxidoreductase
PVL	Panton-Valentine leukocidin
PVN	Paraventircular nucleus
qPCR	Real-time or quantitative polymerase chain reaction
RIP	Receptor interacting protein
RNA	Ribonucleic acid
ROS	Reactive oxygen species
R-PE	R-phycoerythrin
RPMI	Roswell Park Memorial Institute
RT	Reverse transcription
SAP	Serum amyloid protein
SD	Standard deviation
SE	Standard error
SNS	Sympathetic nervous system
SRID	Single radial immunodiffusion
SSC	Side scatter
ssRNA	Single-stranded RNA
STAT-1	Signal-transducing activators of transcription 1
STAT-4	Signal-transducing activators of transcription 4
STAT-6	Signal-transducing activators of transcription 6
TAK	TGFβ-activated kinase
TANK	TRAF family member-associated NFκB activator
T-bet	T-box expressed in T cells
Tc	T cytotoxic cells
TcR1	T cell receptor 1
TE	Tris ethylenediaminetetraacetic acid
TGFβ	Transforming growth factor beta
Th	T helper cells
Th1	T helper 1
Th2	T helper 2
Th3	T helper 3

Th17	T helper 17
Treg	T regulatory
THI	Temperature humidity index
TLR	Toll-like receptor
TMB	Tetramethylbenzidine
TNFα	Tumor necrosis factor alpha
TRAF6	TNF receptor-associated factor 6
VP	Vasopressin
WC1	Workshop cluster 1

Chapter 1
Introduction

In the beef cattle industry, weaning is the separation of the cow-calf pair when the calf is approximately 205 days of age (Fluharty, Loerch et al. 2000). Usually, weaned calves are abruptly separated from their dam and transported from the cow-calf farm to a marketing facility where they will experience comingling, crowding, restraint, novel exposure to pathogens, nutritional changes, injury, pathogens and environmental pressure (Arthington, Eichert et al. 2003). The stress of weaning and subsequent transport causes elevation of stress hormones like glucocorticoids and catecholamines (Hickey, Drennan et al. 2003). These hormones alter immune function and metabolism, increase susceptibility to disease, especially of the respiratory tract, and, ultimately, these changes may result in the calf's death (Boland, Scaglia et al. 2008). Alternative weaning methods have been developed to decrease the stress associated with weaning. These methods occur in two stages which terminate suckling but allow the calf to maintain some contact with the dam before permanent separation; two of these methods are nose clips and fenceline separation (Boland, Scaglia et al. 2008). Previous studies have investigated differences in behavior and weight gain associated with alternative weaning methods, but none have addressed the differences in the immune response (Price, Harris et al. 2003). This thesis compiles literature concerning stress-related alterations to immune function and investigates the immunological differences between traditional and alternative weaning methods. Experiments were conducted in the fall of 2008 and 2009 to test the hypotheses that 1) abrupt weaning increases cortisol concentrations and alters immune function by shifting the response to a Th2 bias as measured by increased IL-4 and IL-10, decreased IFNγ, increased antigen-specific IgG_1, decreased antigen-specific IgG_2, increased αβ T cells, decreased γδ T cells, and decreased CD62L; 2) fenceline weaning decreases the Th2 response observed in abrupt weaning.

In the fall of 2008, heifer calves were weaned by abrupt separation or fenceline contact and subsequently transportation to a new location. Fecal samples were utilized to evaluate cortisol metabolites as an indicator of stress. Cortisol can be measured in the blood; however restraint and venipuncture are necessary for blood collection and increases cortisol. Therefore, non-invasive methods have been developed to measure cortisol metabolites in the feces. The concentration of cortisol metabolites in the feces reflects the cortisol production 12 hours earlier, thus fecal collection took place the day after weaning (Mostl, Maggs et al. 2002). Calves were weighed for the duration of the study. Serum samples were analyzed for IgG antibodies specific to the *Brucella abortus* vaccine RB51 to determine the effect of stress on antibody production. Serum samples were also assessed for the cytokine IFNγ to examine the Th1/Th2 paradigm.

An experiment was conducted in the fall of 2009 that used the results from the fall 2008 study to further investigate the immunological differences of weaning methods. Steer and heifer calves were weaned by abrupt separation or fenceline contact. No transportation was implemented. Fecal samples were utilized to evaluate cortisol metabolites as an indicator of stress. Serum samples were assayed to measure antibody concentration to a *Histophilus somni* vaccine. White blood cells taken from whole blood samples were used to analyze the lymphocyte markers CD4, CD8, WC1, TcR1, and CD21. The markers CD4 and CD8 were used to represent T helper and T cytotoxic cells respectively; both these of these T cells bear the αβ T cell receptor. The markers WC1 and TcR1 were used to represent the T cell bearing the γδ T cell receptor. The marker CD21 was used to represent the B cell population. Since increases in cortisol have been associated with increases in αβ T cells, decreases in γδ T cells and decreases in B cells, these markers were chosen to monitor these respective cell populations (Burton and Kehrli 1996; Nonnecke, Burton et al. 1997; Menge and Dean-Nystrom 2008). RNA samples

from white blood cells were analyzed for gene expression of GAPDH, CD62L, IFNγ, IL-4 and IL-10. Since increases in cortisol are associated with alteration to neutrophil function (CD62L) and cytokine expression (IFNγ, IL-4 and IL-10) these genes were chosen to monitor those changes.

Chapter 2
Literature Review

Introduction

In the beef cattle industry, a calf is weaned off its dam around 205 days of age (Fluharty, Loerch et al. 2000). Weaning is a stressful event that causes reduced immune function and increased susceptibility to disease (Hickey, Drennan et al. 2003). Calves undergo various stressors including physiological stress, such as comingling, restraint and novel exposure to pathogens and physical stress, such as hunger, injury, disease and environmental pressure. The most recognized stressor is transportation (Arthington, Eichert et al. 2003). Conversely, fence line contact between dam and calf during weaning lessens behavioral stress and minimizes weight loss as compared with abrupt weaning (Price, Harris et al. 2003). The stress involved with weaning causes an increase in glucocorticoids, especially cortisol (Hickey, Drennan et al. 2003). Glucocorticoids (GCs) are considered immunosuppressive and impact neutrophil function, lymphocyte phenotypes and cytokine production (Elenkov 2004).

Glucocorticoids affect both the innate and adaptive immune response. Bovine neutrophils are sensitive to glucocorticoids because they exhibit high expression of glucocorticoid receptors (Burton, Madsen et al. 2005). Glucocorticoid activities are mediated by intracellular glucocorticoid receptors (GR) that are located in the cytosol of target cells. Upon binding of glucocorticoids, GRs become activated, dissociate from its protein complex and translocate into the nucleus. Once in the nucleus the GR binds to DNA or other transcription factors and thereby inhibits or enhances expression of GC responsive gene (Preisler, Weber et al. 2000; Burton, Madsen et al. 2005). This translocation is associated with decreases in expression of two genes that regulate neutrophil behavior, including migration (CD62L) and programmed cell death (Fas) (Burton, Madsen et al. 2005). Correlations between serum cortisol

concentrations and blood neutrophil counts have been reported, suggesting that cortisol causes GR translocation and decreased neutrophil migration (Preisler, Weber et al. 2000). Also, glucocorticoids delay neutrophil apoptosis through alteration of Fas gene expression by GRs (Chang, Madsen et al. 2004). Changes to the neutrophil occur to direct the cell's priorities to extracellular matrix remodeling in the event that tissues become damaged. However, the shift in neutrophil function has been shown to decrease antimicrobial defenses such as migration, margination, and respiratory burst (Burton, Madsen et al. 2005). Neutrophils are the first line of defense; therefore if these cells cannot get to the site of infection the calves are more susceptible to disease.

In the adaptive response, GCs affect T lymphocytes. Generally, T lymphocytes can split into two major subsets, helper and cytotoxic. T-helper (Th) lymphocytes have been classified as either Th1 cells, which primarily produce IL-2, IL-12, IFN-γ and TNF-α, or Th2 cells, which primarily produce IL-4, IL-10 and IL-13 (Brown, Rice-Ficht et al. 1998; Elenkov 2004). Other Th classifications are Th3, Treg, and Th17. Cytokines responses of Th1 and Th2 classifications inhibit one another. The cytokines IFN-γ and IL-12 inhibit Th2 cell activity, while IL-4 and IL-10 inhibit Th1 cell activity. The cytokines IL-4 and IL-10 inhibit macrophage activation, T cell proliferation, and production of pro-inflammatory cytokines (Elenkov 2004; Janeway, Murphy et al. 2008).

Glucocorticoids suppress Th1 cytokine production and thus shift the immune response to Th2 cytokine production. Glucocorticoids act on antigen-presenting cell (APC) receptors to inhibit IL-12 production, which is the main inducer of Th1 responses. Interleukin-12 enhances IFN-γ production and inhibits IL-4 production. Thus, by suppressing IL-12, GCs upregulate the Th2 response (Elenkov 2004).

Antibody responses in cattle associated with Th1 or Th2 clones can be linked to specific IgG subclasses. Bovine IL-4 has been shown to induce IgG_1 and IgE, while bovine IFNγ has been shown to induce IgG_2 over IgG_1 production (Estes and Brown 2002). Therefore, an increase in IgG_2 would support a shift to a Th1 bias.

The stress from weaning can lead to the release of glucocorticoids that can affect the immune system by shifting the Th1/Th2 response and decreasing the function of neutrophils (Elenkov 2004; Burton, Madsen et al. 2005). These changes in immune function lead to an increase in disease susceptibility and morbidity in the calf (Boland, Scaglia et al. 2008). Understanding the changes in the immune response post weaning is important in managing calves on the farm, therefore studies that look at the results of weaning stress on the immune response should be considered.

Weaning & Stress

Weaning of young mammals is defined as the physical separation of a mother from its offspring along with a cessation of suckling and a change to solid food (Price, Harris et al. 2003). In nature, the loss of this dependence on the dam occurs gradually. The mother gradually reduces milk production and the young slowly increases solid food intake (von Keyserlingk and Weary 2007). Domesticated animals, however, are weaned by abruptly separating the mother and young, ending milk feeding. In farmed animals, this permanent separation occurs at a younger age and is normally accompanied by changes in social and physical environments (Weary, Jasper et al. 2008). Natural weaning in cattle occurs between 7 and 14 months of age and the cow-calf pair remains in contact for several months after the end of suckling (Enríquez, Ungerfeld et al. 2009). However, domestic weaning takes place around 6-7 months of age and the cow-calf pair does not remain in contact. This early, abrupt weaning leads to altered behavior and decreased weight gain (Enríquez, Ungerfeld et al. 2009).

Abrupt weaning causes a break in mother-young bonds at an age when the young are still suckling frequently and the social bond is still strong. When this bond is broken too early, periods of vocalization, locomotion, and depression occur. Other behaviors include altered eating and sleeping habits, and changes in heart rate and body temperature. In nature, this behavior would reunite the young with its mother, but in artificial weaning the mother and offspring are not reunited (Newberry and Swanson 2008). Along with the behavioral changes, abrupt weaning also results in hormonal changes noted by increased concentrations of stress hormones such as cortisol and noradrenaline (Lefcourt and Elsasser 1995; Hickey, Drennan et al. 2003).

The hormonal changes that occur during the separation of the cow-calf pair are complex and these changes alter behavior and ultimately immune function (Pollock, Rowan et al. 1992; Lefcourt and Elsasser 1995; MacKenzie, Drennan et al. 1997; Arthington, Eichert et al. 2003; Hickey, Drennan et al. 2003). The maternal bond is formed and maintained by neurotransmitters like oxytocin, gonadal steroids, prolactin, opioids, and dopamine. However, when this bond is severed these hormones decrease and other hormones, such as cortisol and norepinephrine, increase (von Keyserlingk and Weary 2007). One of the most important hormones in the maternal bond is oxytocin which through dopaminergic and opioid receptors is responsible for the rewarding and addictive features of social contact and nursing (Newberry and Swanson 2008) and is associated with suckling in most mammalian species (Weary, Jasper et al. 2008). Oxytocin is required to form a strong mother-young bond at birth. Suckling calves have higher concentrations of oxytocin, gastrin, CCK, and insulin in comparison to calves that were not suckling (Lupoli, Johansson et al. 2001). While suckling increased oxytocin levels in offspring, oxytocin knockout mice showed a decreased level of distress during abrupt separation from their mother; this distress was indicated by less vocalization (Lim and Young 2006). Conversely, when injected with oxytocin, weaned rat pups decreased their vocalizations. However, oxytocin injection did not completely eliminate vocalizations (Weary, Jasper et al. 2008). Oxytocin may be a major contributor to the mother-offspring bond. When oxytocin is absent at birth, the young do not form a strong attachment to their mother. Also, when oxytocin was supplemented at weaning, distress was reduced indicating that a supplement of oxytocin may help replace what is lost from the severed bond. Further investigation into stress hormone concentrations and immunological parameters post weaning are needed to determine if oxytocin supplementation at weaning is beneficial.

A slow decrease in hormones, such as oxytocin occurs during natural weaning as the young have progressively less contact with their dam and the frequency of suckling decreases. This slow process results in a steady decrease of oxytocin, prolactin and opioid release induced by cow-calf interactions. Subsequently, gonadal steroids increase as oxytocin decreases and gene expression of the receptors for oxytocin is predicted to decrease (Newberry and Swanson 2008). However, if weaning or separation of the cow-calf is abrupt there is an immediate decrease in these hormones, leading to distress behaviors like vocalization and locomotion.

Due to the stressful results of abrupt weaning, alternative weaning methods are used to decrease the calves' distress; these normally occur in two stages. The first stage prevents nursing of the cow-calf pair and the second stage separates the cow-calf pair permanently (Haley, Bailey et al. 2005). These two-stage weaning methods include nose clips and fence line contact. Fence line weaning occurs when the calf is moved into an adjacent paddock removing physical contact and ending suckling, but allowing the calf to maintain social contact with the dam. Alternatively, nose clips allow calves to maintain physical contact with their mother while preventing suckling (Boland, Scaglia et al. 2008).

Fence line weaning has been shown to reduce the time calves spend walking and vocalizing, and increase the time calves spend eating and lying down in comparison to their abruptly weaned counterparts (Stookey 1997). Price et al (2003) observed that fence line weaning improved both the behavior and weight gain of the calves. The abruptly weaned calves spent less time grazing and resting and more time walking; they also had higher frequencies of vocalization. Fence line weaned calves also gained 95% more weight than the abruptly separated calves in the first 2 weeks post weaning and the former were still heavier 10 weeks post weaning (Price, Harris et al. 2003). By comparison, abruptly weaned lambs exhibited

greater agitation represented by continuous motion, restlessness and vocalization compared to lambs weaned in two-stages. Vocalizations were most notable during the first day post separation and decreased to zero by the third day (Schichowski, Moors et al. 2008).

Another two-stage weaning technique is the addition of anti-suckling devises called nose clips. During stage 1, the calf receives a nose clip and remains with its dam; stage 2 occurs approximately 7 days later when the nose clip is removed and the calf is permanently separated from its dam. Hailey et al (2005) reported that after permanent separation (stage 2), calves that had a nose clip pre-separation (stage 1), vocalized 96.6% less and spent 78.9% less time walking, 23% more time eating, and 24.1% more time resting than abruptly weaned calves (control). Calves with nose clips had decreased weight gain after the addition of the nose clip (stage 1), but had greater average daily gain after permanent separation (stage 2) compared to their abruptly weaned counterparts. However, average daily gain over the course of the experiment did not differ for this study. In other studies, compared to abruptly weaned or fence line weaned calves, calves weaned with nose clips had decreased or no weight gain (Boland, Scaglia et al. 2008; Burke, Scaglia et al. 2009; Enríquez, Ungerfeld et al. 2009). The decreased or no weight gain associated with nose clip weaning could be due to the obstruction to grazing by the nose clip itself. Overall, weaning calves with anti-suckling devices decreases the distress post permanent separation, but negatively affects the overall weight gain of the calf.

Amongst the benefits of alternative weaning methods such as fence line weaning and the utilization of nose clips, weaning younger calves is another alternative. Gain to feed ratios are reported higher in early weaned calves (~3 months old) in comparison to their abruptly weaned counterparts (~10 months old) (Arthington, Spears et al. 2005). Early weaned calves are heavier at the time that normal weaning would have occurred and are subsequently heavier at slaughter.

Calves weaned earlier also showed greater potential to produce intramuscular fat, increasing carcass quality (Rasby 2007). Early weaning in pigs also resulted in less active behavior indicated by decreased vocalizations (Sumner, D'Eath et al. 2008). These studies support the notion that early weaned calves are less distressed than calves weaned later and this weaning method can be used to alleviate weaning related stress. The decrease in behavioral stress during early weaning may be a result of a weaker bond between dam and offspring because the bond had less time to develop. Early weaned calves may also be less dependent on the oxytocin increase from the bond with their mother and milk feeding.

One of the most significant effects of weaning stress is an increased incidence of disease. Mortality from Bovine Respiratory Disease (BRD) was twice as high in abruptly weaned, transported calves than unweaned, transported calves (Hodgson, Aich et al. 2005). Increases in stress-related hormones, such as cortisol, may alter immune function leaving the calves more susceptible to infectious disease from pathogens like BRD.

Abrupt weaning causes distress in the young animal. Calves that are abruptly weaned show stressful behaviors such as increased vocalization and walking, decreased eating and resting, increased cortisol and norepinephrine levels and increased incidence of disease. Alternatives to abrupt separation decrease the stress response; those include fence line weaning, the addition of an anti-suckling device, and weaning calves at an earlier age. These alternative methods have decreased the behavioral response associated with distress. Understanding the neurobiology of social attachment and detachment and the effects thereof is important in reducing the stress response in the calf.

Neurohormones

As previously described, stress from weaning results in an increase in cortisol and noradrenaline in response to abrupt weaning and these hormones negatively affect weight gain and immune function. The two areas of the brain affected by the stress response are the hypothalamo-pituitary adrenal (HPA) axis and the sympathetic nervous system. Stimulation of the HPA axis results in release of glucocorticoids from the adrenal gland, while stimulation of the sympathetic nervous systems results in release of norepinephrine (Tilbrook and Clarke 2006).

Stressors, such as weaning, stimulate the paraventircular nucleus (PVN) of the hypothalamus to release corticotrophin-releasing hormone (CRH) and vasopressin (VP) into the hypophyseal blood supply (Tilbrook and Clarke 2006; Tausk, Elenkov et al. 2008). Corticotrophin-releasing hormone and vasopressin stimulate cells, called corticotrophs, of the anterior pituitary gland to secrete adrenocorticotrophic hormone (ACTH); lipotropin, beta-endorphin, met-enkephalin, and melanocyte-stimulating hormone (MSH) production is also stimulated by CRH. The hypothalamic hormones CRH and VP work together to stimulate the production of ACTH, however VP cannot stimulate ACTH independently (Charmandari, Tsigos et al. 2005; Carroll and Forsberg 2007). Subsequently, ACTH production then stimulates the adrenal cortex to produce glucocorticoids. Once glucocorticoids are released, they can negatively feedback to decrease the release of CRH, VP, and ACTH (Tilbrook and Clarke 2006). Glucocorticoids then influence the immune response through intracellular receptors distributed on the cells of the immune system.

Corticotrophin-releasing hormone also causes the sympathetic nervous system (SNS) to produce norepinephrine by stimulating noradrenergic neurons in the central nervous system (CNS) (Tausk, Elenkov et al. 2008). Norepinephrine and epinephrine increase heart rate,

constrict blood vessels, dilate bronchioles, and increase metabolism. More specifically, epinephrine increases heart contractions, blood flow to the muscles and brain, relaxes smooth muscle, and converts glycogen to glucose in the liver. Norepinephrine also increases blood pressure. Catecholamines also influence the HPA axis by increasing neurohormone release, ACTH release, and cortisol (Carroll and Forsberg 2007).

The sympathetic nervous system and HPA axis work together to regulate the stress response and maintain homeostasis within the body. Weaning among other stressors like transportation and castration have been shown to increase the hormones attributed with the stress response in cattle and other livestock species. Lefcourt et al., 2005 detected an increase in catecholamine concentrations, norepinephrine and epinephrine mainly in the first day post weaning. Similarly, Hickey et al., 2003 observed increased norepinephrine concentrations during weaning which coincided with previous findings. Additionally, Hickey et al., 2003 found an increase in cortisol concentrations associated with abrupt weaning.

Weaning increases stress related hormones, but so do other stressors. Yagi et al., 2004 observed a peak increase in plasma cortisol in the first two hours of transportation in lactating dairy cattle. Buckham Sporer et al., 2007 observed a 321% increase in cortisol 4.5 hours after the onset of transportation as compared to 24 hours prior to shipping in beef bulls. An increase in cortisol after shipping has also been observed in goats and camels as well (Kannan, Terrill et al. 2000; Saeb, Baghshani et al. 2010). Along with weaning and transportation, castration is also an established stress model. Fischer et al., 2006 noted an increase in cortisol post-castration in calves, with the castration method influencing the level of the stress response.

Stressors activate both the sympathetic nervous system and hypothalamic-pituitary adrenal axis evident by an increase in catecholamines and glucocorticoids. The increase in stress

hormones in response to weaning, transportation and castration significantly affects the animal's immune function. Increase in norepinephrine and cortisol are associated with alterations in acute phase proteins, neutrophil-lymphocyte ratios, white blood cell counts, and interferon gamma among others (Church and Hudson 1999; Hickey, Drennan et al. 2003; Arthington, Qiu et al. 2008; Kojima, Kattesh et al. 2008). These immunological alterations lead to increased susceptibility to disease and, sometimes, fatalities. The next section will summarize stress responses on the immune system.

The Immune System: An Overview

The immune system is made up of a vast number of cells and molecules with the purpose of protecting the host from pathogenic organisms, while not attacking the host. In general the immune system can be divided into two parts, innate and acquired immunity (Lippolis 2008). Innate immunity is the first line of defense against invading pathogens; it occurs quickly and nonspecifically. Acquired immunity is antigen-specific and occurs less quickly over time (Salak-Johnson and McGlone 2007; Lippolis 2008). The strength and duration of the immune response is important in overcoming disease; a too weak or too strong response can damage the host. Therefore, a balanced immune system is of the utmost importance in maintaining homeostasis (Lippolis 2008).

Innate Immunity is the first line of defense against invading pathogens and includes the physical barriers: skin, mucosal secretions, tears, urine, and stomach acid, as well as cellular barriers (Carroll and Forsberg 2007). The cells of innate immunity are monocytes, macrophages, dendritic cells (DCs), mast cells, neutrophils, eosinophils, basophils and natural killer cells (Janeway and Medzhitov 2002; Carroll and Forsberg 2007; Janeway, Murphy et al. 2008). Of these cells, the phagocytic cells are neutrophils, monocytes, macrophages and DCs; activation of the cells occurs at the site of infection where they internalize the pathogen and possibly destroy it (Janeway and Medzhitov 2002). Also, the innate immune system can "talk" with the acquired immune system through costimulatory molecules. The costimulatory molecules CD80/CD86 are expressed on the surface of antigen presenting cells (APCs) along with CD40 and MHC class II receptors. These molecules bind to CD28 on T cells and act as secondary signals for T cell activation (Janeway and Medzhitov 2002; Lippolis 2008).

Acquired (adaptive) immunity is antigen-specific and includes B and T lymphocytes which are associated with humoral and cell-mediated immunity respectively. Bone marrow derived lymphocytes are B lymphocytes, which, when stimulated, differentiate into plasma cells that synthesize and secrete antibodies (or immunoglobulins). Thymus derived lymphocytes are T lymphocytes, which interact with APCs to develop effector and memory responses (Saker 2006). Antibodies produced by B cells recognize whole antigens, conversely T cells only recognize fragments of antigens (Lippolis 2008). Also, B cells can recognize antigens through their own receptors while T cells need the antigen to be processed and presented via APCs (Banchereau and Steinman 1998).

The four main tasks of the immune system are to recognize the presence of infections, utilize the effector functions to contain the infections, regulate the response to keep the immune system under control, and form memory to protect the host from recurring disease. These tasks must work in concert to formulate a successful immune response and clear the infection (Janeway, Murphy et al. 2008).

The first task is to recognize foreign antigen; this is accomplished through pattern recognition receptors (PRRs). These receptors recognize pathogen-associated molecular patterns (PAMPs), which are repetitive molecular structures on the surface of pathogens. One of these receptors is a free protein called the mannose binding lectin (MBL), which can activate the lectin pathway of the complement system. The phagocytes have PRRs as well; among those are the macrophage mannose receptor and the scavenger receptor which initiate phagocytosis. These phagocytic receptors can also activate innate immunity, like migration of neutrophils to the site of infection, or acquired immunity, like expression of co-stimulatory molecules that aid in antigen presentation through the use of Toll-like receptors (Janeway, Murphy et al. 2008).

Toll-like receptors (TLRs) are a family of cell surface receptors that recognize PAMPs and activate innate and acquired immunity. There are ten TLRs (TLR1-TLR10) that recognize different pathogens and they can be expressed on the cell surface or in intracellular vesicles. Toll-like receptor-4 was the first to be identified and it recognizes lipopolysaccharide (LPS), a cell wall component of gram-negative bacteria. Upon recognition of LPS, TLR4 works with CD14 and LY96 to induce inflammatory cytokine and costimulatory molecule production. While TLR4 recognizes gram-negative bacteria, TLR2 recognizes both gram-positive bacteria through the presence of lipoteichoic acid and peptidoglycan and gram-negative bacteria through the presence of lipoproteins. Both TLR2 and TLR4 mainly recognize bacteria, but TLR3 is stimulated through virus-derived double-stranded RNA resulting in the production of interferon cytokines. Some of the other TLRs are TLR9 which recognizes unmethylated CpG DNA, TLR8 which recognizes G-rich oligonucleotides, TLR7 which recognizes ssRNA, and TLR5 which recognizes flagellin (Takeda, Kaisho et al. 2003; Janeway, Murphy et al. 2008).

Toll-like receptors are expressed on the surface of immune cells and in epithelial cells of the mucosal surface of the respiratory and intestinal tract. Expression on TLRs can be regulated by the type of infection and cytokines. When TLRs are activated they elicit both an innate and acquired immune response. In acquired immunity, TLRs activate immature DCs by causing the expression of costimulatory molecules CD80/CD86/CD40 and proinflammatory cytokine production. Upon binding with LPS, the TLR4 receptor signals the nucleus to activate the NFκB transcription factor (Takeda, Kaisho et al. 2003; Janeway, Murphy et al. 2008).

Toll-like receptors are located in the cellular membrane, but there are receptors also present in the cytosol called NOD1 and NOD2. These receptors can activate the same inflammatory pathways through NFκB as TLRs and are located in epithelial cells and phagocytic

cells. Since phagocytic cells also express TLRs, NODs signals act together with TLR signals, but in epithelial cells NODs are the primary responders. Lipopolysaccharide is recognized on the surface of the cell by TLR4, but in the cytoplasm NOD1 and NOD2 recognizes LPS (Takeda, Kaisho et al. 2003). When TLRs or NODs recognize LPS, they activate the adapter protein MyD88 which activates protein kinases within the cell (i.e. IRAK, TAK, and TRAF) that amplify the signal and ultimately cause the activation of NFκB. The protein complex NFκB is a transcription factor that, upon activation, enters the nucleus and influences DNA transcription for both innate and adaptive immunity. Activation of NFκB by either TLRs or NODs results in the production of cytokines and chemokines which lead to the activation of innate immunity and the expression of costimulatory molecules CD80, CD86, and CD40. All these changes are accomplished through alterations to transcription by NFκB (Janeway, Murphy et al. 2008).

After immune recognition occurs through the pattern recognition receptors, effector functions must be elicited to contain or eliminate the infection. This clearance is accomplished through innate immunity first and then acquired immunity. After the pathogen has entered the tissue and been recognized by the phagocytic cells residing there, an innate immune response is initiated.

To clear infections, the immune system must communicate with itself. The immune system achieves this by utilizing cellular receptors, cytokines, chemokines, and adhesion molecules. Adhesion molecules are used for cell migration, phagocytosis and cellular cytotoxicity. For the most part, adhesion molecules are expressed on the surface of leukocytes and endothelial cells allowing for communication between the two. Cytokines are small molecular weight messengers secreted by cells to act as “hormones” of the immune system. They can be autocrine, paracrine, or endocrine and are produced by almost all cells. Their

function is cell dependent, but they mostly affect cell activation, division, apoptosis, and movement. Cytokines produced by leukocytes are called interleukins (IL). Cytokines that cause proliferation and differentiation of stem cells are called colony-stimulating factors. Cytokines interfering with viral replication are called interferons. And lastly, cytokines involved in chemoattraction are called chemokines (Parkin and Cohen 2001).

Macrophages and dendritic cells located in the tissues produce cytokines and chemokines in response to bacterial recognition by a cellular receptor. The secreted products IL-1β, IL-6, TNFα, and the chemokine CXCL8 (formerly IL-8) are used to mobilize neutrophils, mobilize proteins and energy, increase body temperature, initiate DC migration to regional lymph nodes, and trigger acute phase protein productivity in the liver. An increase in body temperature, or a fever, is caused by an increase in IL-1β, IL-6 and TNFα which induces prostaglandin E2 synthesis mediated by the enzyme cyclooxygenase-2 (COX-2). Prostaglandin E2 subsequently acts on the hypothalamus to increase heat production. Lipopolysaccharide and other bacterial components cause fever independently of IL-1β, IL-6 and TNFα by directly acting on COX-2. This increased body temperature is beneficial because pathogen growth is decreased and increased T cell proliferation, T helper cell activation, cytotoxic T cell activity, and antibody production. However, the most important effect of IL-1β, IL-6 and TNFα is the production of acute-phase proteins (Colditz, Schneider et al. 2007; Janeway, Murphy et al. 2008).

Acute-phase proteins are synthesized in the liver in response to neutrophil and macrophage cytokine release and include haptoglobin, serum amyloid protein (SAP), C-reactive protein (CRP), fibrinogen, and mannose binding lectin (MBL). These proteins are secreted into the plasma from the liver. Acute-phase proteins recognize a broad range of PAMPs and only require the presence of cytokines for production. Haptoglobin prevents the loss of iron by

forming complexes with hemoglobin; these complexes restrict the amount of free iron available for bacterial growth. The function of SAP is still unknown. A pathogen recognition molecule, CRP, binds to phosphocholine in bacterial and fungal cell wall components. Another pathogen recognition molecule is MBL. When CRP and MBL bind to pathogens they opsonize them for phagocytosis and activate complement (Petersen, Nielsen et al. 2004; Janeway, Murphy et al. 2008).

Acute-phase proteins have been noted in activating the complement system, which consists of three pathways: classical, lectin and alternative. Although the three pathways are triggered by different methods, they all activate C3, the central component of the system. Antibodies stimulate the classical pathway. The lectin pathway is initiated by the binding of PAMPs to lectin proteins; this pathway consists of three members: MBL, ficolin H and ficolin L. The alternative pathway is always "turned on." Once the complement system has been activated, C3b attaches to the antigen as a flag or marker for phagocytosis. Activation of C3 by all pathways leads to the assembly of C5-C9 which forms a transmembrane pore or a membrane-attack complex in the antigen. This complex bores a hole in the surface of the pathogen that causes cell death by osmotic lysis. Host cells possess a complement receptor which prevents C3 attack; however microbes lack this receptor making them susceptible. Other complement functions are opsonization by C3b and release of C3a and C5a which act as anaphylatoxins. Anaphylatoxins initiates vascular permeability, smooth muscle contraction and mast cell degranulation. Anaphylatoxin, also acts as a chemokine for neutrophils and monocytes (Parkin and Cohen 2001; Carroll 2004).

Neutrophils are the first line of defense against invading pathogens. These cells play a key role in innate immunity and the inflammatory response and they accumulate within the first

few hours after pathogen invasion. Neutrophils are continuously produced in the bone marrow and circulate in the blood until they are recruited by macrophages and DCs to tissues. Chemokines, adhesion molecules and endothelial cells, are used to recruit neutrophils from the bloodstream to the site of infection. Neutrophils which are not recruited, which represent the majority, undergo apoptosis and are ingested by macrophages (Parkin and Cohen 2001; Kobayashi, Voyich et al. 2003; Appelberg 2007; Janeway, Murphy et al. 2008).

When chemokines, particularly TNFα, are released by macrophages or DCs they upregulate P-selectin first and then predominately E-selectin on the surface of endothelial cells. The adhesion molecule L-selectin on neutrophils, and other leukocytes, binds to the E-selectin of the endothelium and leads to the formation of tight bonds. These bonds then dissociate and the neutrophil is released to the next selectin-bearing endothelial cell causing a process called rolling. Rolling slows down the neutrophils in the bloodstream allowing them to localize on the endothelium next to the inflamed site (Parkin and Cohen 2001; Paape, Bannerman et al. 2003; Kelly, Hwang et al. 2007). Chemokines also upregulate integrins, such as β_2-integrin and α_4-integrin on neutrophils, that aid the cells in their attachment to the endothelium. Intracellular adhesion molecule 1 and 2 (ICAM-1 and ICAM-2) on the endothelium, which are also upregulated by chemokines, bind the integrins (CR3 and LFA-1) present on neutrophils to aid in adhesion (Kelly, Hwang et al. 2007; Janeway, Murphy et al. 2008). Neutrophils roll along the endothelium, adhere to the site of inflammation and extravasate from the circulation through the junctions between the endothelial cells. Markers on leukocytes and endothelial cells like CD31 or PECAM-1 allow the leukocytes to squeeze through the intracellular junction penetrating the basement membrane of the endothelial cell with enzymatic aid. Once in the tissue, neutrophils are aided by chemokines CXCL8 and CCL2 toward the infection. Neutrophils extravasate into

the tissues within the first 6 hours of the inflammatory response, but other leukocytes, such as monocytes and lymphocytes, can undergo similar migration later (Parkin and Cohen 2001; Janeway, Murphy et al. 2008).

Once neutrophils have arrived at the site of infection, they phagocytose the pathogen. Neutrophils also have pattern recognition receptors, like TLRs, and although neutrophils can directly recognize pathogens, opsonization of pathogens by antibodies or complement immensely enhances phagocytosis. Phagocytosis by neutrophils or other phagocytes occurs by engulfing the organism with projections of the membrane called pseudopodia and forming a membrane-bound vesicle called a phagosome. The phagosome fuses with the granules in the neutrophil forming a phagolysosome. A respiratory burst then occurs inside the phagolysosome killing the organism by using reactive oxygen species (ROS), including superoxide, hydrogen peroxide, and hypochlorous acid. Production of ROS is oxygen-dependent, however there is an oxygen-independent reaction that involves cationic proteins and enzymes such as myeloperoxidase and lysozyme contained within the neutrophil's granules (Parkin and Cohen 2001; Kobayashi, Voyich et al. 2003; Paape, Bannerman et al. 2003). This mechanism of pathogen clearance is effective; however some bacteria possess genes that counteract destruction. *Staphylococcus aureus* has the Panton-Valentine leukocidin (PVL) gene that causes neutrophil cell lysis. Lysing the cell releases the ROS into the host causing extensive tissue damage. However, infections with *Streptococcus pneumonia* or *Mannheimia haemolytica*, which do not have the PVL gene, result in neutrophil apoptosis that does not release the ROS (Kobayashi, Voyich et al. 2003; Janeway, Murphy et al. 2008). *Mannheimia haemolytica* produces a leukotoxin that at different concentrations affects leukocytes. At low concentrations, the leukotoxin induces respiratory burst and degranulation of the leukocytes. At moderate

concentrations, the leukotoxin stimulates the cells to undergo apoptosis and at higher concentrations, the cells die. The *M. haemolytica* leukotoxin acts on PMNs more strongly than PBMCs (Narayanan, Nagaraja et al. 2002).

The innate immune response, involving neutrophils and macrophages, is the first response to a naïve infection. After phagocytosis, APCs need to present the antigen to T cells for the development of an acquired immune response. If the infection is not naïve, then memory T and B cells can react to the infection without the aid of APCs.

During infection, neutrophils and macrophages work together. When a neutrophil ingests an antigen, it kills the pathogen and undergoes apoptosis; a macrophage will ingest the apoptotic cell. The macrophage thereby gains the neutrophils granules for increased antimicrobial activity against intracellular pathogens and the host is not damaged by the release of the ROS into the extracellular environment of the host (Appelberg 2007).

Neutrophils and monocytes are phagocytic cells located in the circulation. A monocyte gives rise to either a macrophage or a dendritic cell (monocytes-derived cells), which are both professional phagocytes (Savina and Amigorena 2007; Randolph, Jakubzick et al. 2008). Monocytes circulate in the blood and frequently migrate into the tissue where they differentiate to become macrophages (Janeway, Murphy et al. 2008). Upon antigen recognition, macrophages secrete chemokines that influence or recruit other immune cells to the site of infection. Macrophages eliminate the pathogen through antimicrobial killing or ingesting and then presenting the antigen to T cells. After antigen presentation, the microbicidal and phagocytic activities of macrophages and dendritic cells are upregulated or downregulated by the cytokines and antibodies produced by activated T and B cells (Gordon 1998). Like neutrophils, macrophages ingest the antigen into a phagosome which matures into a phagolysosome.

However, macrophages do not possess the potent antimicrobials that are present within the neutrophil's granules. Macrophages use glycosidases and proteases as lysosomal degradative enzymes and a acidification of the phagosome aids in antimicrobial killing (Savina and Amigorena 2007).

Neutrophils and macrophages both digest microbes and aid in cell mediated immunity by presenting digested antigens to T lymphocytes. However, neutrophils are more adept at antimicrobial breakdown and macrophages are more adept at antigen presentation. Macrophages are also more capable phagocytes because, unlike neutrophils, they can ingest multiple pathogens. Dendritic cells, another subset of professional phagocytes, are the most adept at antigen presentation. These cells have minimal antimicrobial activity; they have a neutral pH, limited proteolytic activity, and low levels of oxidation. Dendritic cells have enough antimicrobial activity to limit microbial proliferation and break down the organism into proteins suitable for antigen presentation, but this breakdown is slower. In macrophages and neutrophils, the breakdown of the pathogen only takes a few minutes; but in DCs it takes a few hours. Antimicrobial breakdown is a slower process in DCs but it gives the cell a better chance to present suitable peptide to T cells (Savina and Amigorena 2007).

For acquired immunity to progress, T cells must be presented with a processed antigen in order to take action against the infection. For this to occur, an APC must be an intermediary between the antigen and the T cell. Immature DCs located in most tissues capture antigens via phagocytosis and process them. When activated by their TLRs, DCs shift their functionality from phagocytosis, to antigen presentation by upregulating costimulatory molecules CD80/CD86/CD40 and migrating to the lymphoid organs, such as the spleen and the lymph nodes where they present antigens to naïve T cells (Banchereau and Steinman 1998). Migration

occurs, after TLR stimulation, by downregulating CCR6 receptor, an inflammatory chemokine, and upregulating CCR7 receptor, a lymphoid chemokine. This shift causes the dendritic cell to be attracted to the lymphoid tissue rather than the site of inflammation (Lippolis 2008).

Before activation by an antigen, naïve T cells have no functional activity; T cells need to be presented with a processed antigen by APCs to develop an immune response. Dendritic cells that have migrated to the peripheral lymphoid organs display antigen fragments on their surface bound to molecules of the major histocompatibility complex (MHC). There are two types of MHCs, MHC class I and MHC class II. The MHC class I molecule is expressed on all immune cells, while the MHC class II molecule is only expressed on antigen presenting cells (i.e. DCs, macrophages and B cells). For a successful activation to occur, the MHC molecule on the APC must bind to the T cell receptor (TCR). The CD80/CD86 molecule must bind to the CD28 on the T cell, and the CD40 molecule on the APC must bind to the CD40L on the T cell. If the costimulatory molecules CD80/CD86 and CD40 do bind to their respective receptors then T cell activation will not occur. Recognition of viral proteins in the cytosol is accomplished by MHC class I molecules that present the viral proteins to cytotoxic T cells (Tc) expressing the cell surface marker CD8. Helper T cells (Th), which express cell surface marker CD4, recognize the MHC class II molecule. Once activated by APCs, cytotoxic T cells kill host cells infected with a viral pathogen, while helper T cell subsets produce cytokines that direct the immune system toward a Tc-mediated response (T helper 1 cells), B cell-mediated response (T helper 2 cells), and neutrophil-mediated response (T helper 17 cells). When naïve Th cells are activated by DCs they can mature into different types of Th cells with unique cytokine profiles (Banchereau and Steinman 1998; Janeway, Murphy et al. 2008; Kaiko, Horvat et al. 2008; Lippolis 2008).

T cell maturation is determined by the cytokine profile existing during DC stimulation and directs the immune response in different directions. If the mature DC is in the presence of IL-12, then the naïve T cell differentiates into a mature Th1 cell producing interferon gamma (IFNγ) and tumor necrosis factor beta (TNFβ); they enhance protection against intracellular bacteria, viruses, and cancerous cells. In the presence of IL-4, DCs induce Th2 cell differentiation which directs the immune system against extracellular targets like parasites through secretions the cytokines IL-4, IL-5, IL-10 and IL-13. Parasitic infections are also eliminated by increasing eosinophils in blood, increasing immunoglobulin (Ig) E production by B cells, and increasing mast cell proliferation and degranulation. Interleukin-6 induces Th17 cells producing IL-17, IL-6, and IL-22. Extracellular bacteria are cleared by using the cytokine IL-17, secreted from Th17 cells, to stimulate neutrophil recruitment, epithelial barrier function, and expression of antimicrobial peptides (Banchereau and Steinman 1998; Kaiko, Horvat et al. 2008; Lippolis 2008).

Cell mediated immunity involves T lymphocytes, but the other side of acquired immunity is humoral immunity which involves B lymphocytes. The primary role of B cells is to mature into antibody-secreting plasma cells; however they can also act as APCs. These two roles are called T cell dependent and T cell independent. Antibodies enhance the innate immune response by neutralizing toxins, preventing adherence of organisms to mucosal surfaces, activating complement, opsonizing bacteria for phagocytosis, and flagging infected cells for attack by killer cells. Antibodies are normally secreted by mature B cells, however in naïve B cells they are membrane-bound and act as receptors. As such, the antibodies internalize and process antigens for presentation by MHC class II to T cells. The T cell recognizes the antigen and produces cytokines or B cell growth factors causing B cell maturation to antibody-secreting plasma cells.

When CD40 on the B cell binds to CD40L on the T cell an immunoglobulin isotype switch occurs. A B cell upon initial antigen stimulation will produce IgM, but after T cell interaction B cells undergo a class switch producing IgG and other isotypes. The B cells that bind antigen proliferate more than the others. After the isotype switch, some of the activated B cells become long-living memory cells and react quickly upon stimulation with the same antigen. This is called a T cell dependent response, but B cells can also act independently from T cells. Only antigens having repeating epitopes that bind multiple different B cell receptors can cause an independent reaction. This multiple binding causes the B cell to be directly activated and begins producing IgM. However, this IgM is not antigen specific and the B cell produced it will not become a long-living memory cell (Parkin and Cohen 2001).

The end products of B cell activation are antibodies or immunoglobulins; which are composed of a two types of proteins, a heavy chain and a light chain connected by disulfide bonds. The disulfide bonds link each heavy chain to a light chain, and link the heavy chains to one another. Antibodies are also made up of two regions, the constant region (Fc) and the variable region (Fab) that resembles a "Y" shape. The variable region, or the arms of the "Y", are the antigen binding regions which vary between antibody molecules to become antigen specific. This mechanism allows antibodies to bind many different antigens. The constant region, or the stem of the "Y", comes in five main forms. Each form activates a different effector function. These five different forms are IgM, IgD, IgG, IgA, and IgE (also called isotypes) and are characterized by their heavy chain (μ, δ, γ, α, and ε respectively). The IgG isotype is the most abundant antibody in plasma and has four subclasses (IgG_1, IgG_2, IgG_3, and IgG_4) in humans. The other isotype functions are as follows: IgA is secreted by mucosal lymphoid tissues, IgE is involved in host defense against parasites and allergic responses, IgM is

the first antibody to appear on the B cell surface and the first to be secreted, and IgD is on the surface of mature naïve B cells, but its function is unknown (Janeway, Murphy et al. 2008).

Humoral and cell mediated immunity work together to mount a successful immune response. However, livestock management practices can be utilized to manipulate the immune system to offer better protection to the animal; this is achieved through vaccination. Vaccination is a process through which adaptive immunity to a specific pathogen is induced by injecting a vaccine (a killed or nonpathogenic form of a pathogen) into an animal. The ideal vaccine mounts long-lasting protection against the live pathogen without causing illness or death in the host. Vaccines differ in their status; killed or attenuated, the addition of adjuvants, and the frequency and route of administration. Killed vaccines are stable and easy to produce, but require large amounts of antigen to stimulate immunity, often require adjuvants to enhance immunity, and require multiple injections due to the short lived immune response. Live attenuated vaccines offer long-lasting immunity that mimic natural infection with minimal side effects. However, live vaccines are difficult to store because they are temperature sensitive. Both live and killed vaccines have advantages and disadvantages, and the decision to use one or the other is based on the management program being utilized (Osburn and Stott 1989; Janeway, Murphy et al. 2008).

Vaccination is important in managing animal production systems; however the condition of the animal to be vaccinated should be considered. Neonates and young animals are especially problematic when it comes to vaccination. At birth, calves have a fully developed, but immature immune system. Calves, up to four months of age, have a larger number of phagocytes than adult cattle; however, the function of these cells is diminished. Complement is also lower in calves until they reach 6 months. Maturation of the immune system in cattle is a slow process

with full development being reached between 5 and 8 months of age (Barrington and Parish 2001; Chase, Hurley et al. 2008; Cortese 2009). Lymphocyte populations change in the calf until 6 months of age; B cells and αβ T cells increase over time, while γδ T cells decrease with age. The result of this naïve immune system is that calves are more susceptible to disease then adults. Therefore, calves must rely on passive immunity from their dam for protection from infections. Colostrum is the mammary secretion containing antibodies and immune cells that is only available in the first 24 hours after birth. The results of colostrum ingestion disappear in the calf between 3-5 weeks of age (Barrington and Parish 2001). Vaccination of the cow prior to calving increases colostral antibodies and allows the producer to offer specific protection to their calf crop. Another vaccination assumption is that vaccinating a calf when maternal antibodies are still present in their system decreases the efficacy of the vaccine. However, this assumption depends upon the type of vaccine; a vaccine that induces cell-mediated immunity will be more efficacious than one inducing humoral immunity (Barrington and Parish 2001; Chase, Hurley et al. 2008; Cortese 2009).

Innate and acquired immunity work in concert to minimize disease. Clearance of infection requires recognition of the antigen, utilization and regulation of the effector functions, and formation of a memory response. When pathogens migrate into the tissues they are recognized by receptors on epithelial and phagocytic cells. Those receptors cause the cell to release cytokines and chemokines to bring other immune cells like neutrophils to the site of infection. Neutrophils, macrophages, and dendritic cells phagocytize the invading pathogens and degrade them. Dendritic cells then present antigens to the T cells for development of a memory response. Cytokines, secreted by T cells, direct the immune system in a specific direction and cause B cells to produce antibodies that are antigen specific. Antibodies, acute phase proteins,

and complement all increase phagocytosis and aid in pathogen clearance. All these responses to infection/exposure with a foreign antigen are important in the healthy animal. However, they can be manipulated with immunomodulators such as vaccines and stress hormones such as glucocorticoids, which will be discussed next.

Stress Hormones and the Th1/Th2 Balance

Adaptive immunity involves T-helper (Th) lymphocytes that are either Th1 or Th2 cells. Th1 cells primarily produce IL-2, IL-12, IFN-γ and TNF-α. Th2 cells primarily produce IL-4, IL-10 and IL-13. Fragments of antigens presented by APCs are recognized by Th cells with the MHC class II molecule. After antigen recognition, Th cells stimulate B cell antibody class switching. Differentiation to a Th1 or Th2 cell depends upon the cytokine profile produced by the APCs (Elenkov 2004).

When dendritic cells or macrophages present antigens to naïve T cells in the presence of IFNγ and/or IL-12 the T cell differentiates into a Th1 cell. The cytokines, IFNγ and/or IL-12, stimulate the Janus tyrosine kinases (JAK) signal-transducing activators of transcription (STAT) intracellular signaling pathway. Specifically, the STAT1 transcription factor is produced which initiates T-bet production. The T-bet transcription factor switches on the IFNγ gene in the naïve T cell and induces expression of the IL-12 receptor. Antigen presenting cells produce IL-12 that acts on the IL-12 receptor and activates the STAT4 signaling pathway to further promote Th1 differentiation. After all these transcription factors are produced, the naïve T cell becomes a committed Th1 cell that will produce large amounts of IFNγ upon antigen recognition. This IFNγ secretion will also cause more Th1 cells to be produced (Brown, Rice-Ficht et al. 1998; Estes and Brown 2002; Elenkov 2004; Janeway, Murphy et al. 2008; Rautajoki, Kylaniemi et al. 2008).

Macrophage activation by Th1 cells is important in intracellular pathogen clearance; consequently the Th1 cytokine IFNγ and CD40L on the Th1 cell work together to activate macrophages. In most cases, macrophages can kill the pathogen themselves; however, some pathogens can chronically infect macrophages. The binding of a Th1 CD40 ligand to the CD40

receptor on the macrophage in the presence of IFNγ increases the cell's antimicrobial ability. More specifically, this synergy allows the macrophage to fuse the lysosomes with the phagosome more effectively. Production of IFNγ by Th1 cells activate macrophages by increasing phagocytosis, MHC class I and II expression and IL-12, nitric oxide, and superoxide production. The upregulation of these functions helps the macrophage clear the intracellular pathogen. If the macrophage cannot clear the infection Th1 cells use the Fas ligand and LTα to kill these cells. The pathogen is then released and taken up by fresh macrophages. Secretion of IL-2 by Th1 cells induces T cell proliferation and the release of Th1 cytokines causing further Th1 differentiation. Th1 cells also induce new monocyte production by releasing IL-3 and GM-CSF that act on hematopoietic stem cells in the bone marrow. Another function of Th1 cells is to transport monocytes from the circulation to the site of infection by producing TNFα, LTβ and CCL2. Other Th1 effector functions are activation of CD8, NK cells, nitric oxide, and other inflammatory mediators. Increased macrophage activation is the most important effect of Th1 differentiation (Janeway, Murphy et al. 2008).

Similar to Th1 production, Th2 production is initiated by the cells of the innate immune system. Dendritic cells and macrophages secreting IL-4 during antigen presentation initiate the upregulation of STAT6 production in the naïve T cell; STAT6 then induces the synthesis of GATA-3, which activates genes for several Th2 cytokines. Utilizing this effect, GATA-3 maintains Th2 differentiation. The Th2 effector functions are less understood than Th1, but they play a role in clearance of extracellular pathogens like parasites and allergic responses (Estes and Brown 2002; Janeway, Murphy et al. 2008; Rautajoki, Kylaniemi et al. 2008).

Responses by Th1 and Th2 cells upregulate their own production and inhibit the differentiation of each other. The Th1 cytokines, IFN-γ and IL-12, inhibit Th2 cell activity and

activate more Th1 cell production, while the Th2 cytokines, IL-4 and IL-10, inhibit Th1 cell activity and upregulate Th2 production. Because IL-4 and IL-10 inhibit macrophage activation, T cell proliferation, and production of pro-inflammatory cytokines, they Th2 cells are considered anti-inflammatory (Elenkov 2004; Janeway, Murphy et al. 2008).

One of the other effector functions of Th1 and Th2 cells is immunoglobulin class switching. Antibody isotype class switching occurs by changing the heavy chain of the antibody. The interaction between the CD40 ligand on the T cell and the CD40 molecule on the B cell in the presence of Th1 and Th2 cytokines results in isotype switching. Production of IFNγ by Th1 cells induces production of IgG_2 while Th2 cells, that produce IL-4, induce production of IgG_1 and IgE. This effector function of both Th1 and Th2 cells allows antigen-specific antibodies to be produced that direct the immune system toward the more favorable mechanism for clearance of that specific pathogen (Estes 1996; Brown, Rice-Ficht et al. 1998; Estes and Brown 2002; Crawley, Raymond et al. 2003).

Stress hormones alter the differentiation of naïve T cells. Glucocorticoids have been found to suppress Th1 cytokine production by shifting the immune response to Th2 cytokine production. Glucocorticoids act on cytoplasmic/nuclear receptors on antigen-presenting cell (APC) receptors to inhibit IL-12 production, which is the main inducer of Th1 responses. Interleukin-12 enhances IFN-γ production and inhibits IL-4 production. Thus, by suppressing IL-12, GCs up regulate the Th2 response (Elenkov 2004). Glucocorticoids also bind to their receptor on T cells to downregulate the expression of the IL-12 receptor. GCs decrease the production of IL-12 by APCs and decrease the IL-12 receptor in T cells optimizing the downregulation of Th1 cells. Also, by decreasing IL-12, GCs subsequently decrease IFNγ. GCs also decrease IL-18 production which works together with IL-12 to produce a Th1 response.

Glucocorticoids also act directly on Th2 cells by upregulating their IL-4, IL-10 and IL-13 production. Since IL-4 and IFNγ upregulate their own production and downregulate one another these alterations by GCs are amplified. Therefore, GCs cause a Th2 bias by decreasing Th1 cytokine production in APCs and T cells and increasing Th2 cytokine production in T cells (Elenkov 2004; Rautajoki, Kylaniemi et al. 2008)

Catecholamines also have a significant effect on T helper cells by shifting the immune system to a Th2 driven response. Norepinephrine and epinephrine inhibit IL-12 production and enhance IL-10 production. Catecholamines stimulate β_2-adrenoceptors (AR) expressed on Th1 cells (but not Th2 cells). The β_2-AR agonists inhibit IFNγ production by Th1 cells, but do not affect IL-4 production by Th2 cells. Through this mechanism, catecholamines diminish Th1 induced immunity. For example, NK cell activity is diminished through the β_2-AR expressed on the NK cell and through the inhibition of IL-12 and IFNγ, which are essential to NK cell activity (Elenkov and Chrousos 1999; Elenkov and Chrousos 2002; Elenkov 2008).

Stressors such as weaning, transportation, and castration have been shown to increase glucocorticoid and catecholamine hormone concentrations with subsequent effects on IFNγ, a Th1 related cytokine. Hickey et al (2005) observed an increase in cortisol and norepinephrine in response to abrupt weaning with a subsequent decrease in IFNγ production in the serum. Gupta et al (2007) concluded that transportation increased plasma cortisol concentration and decreased IFNγ production. Fischer et al (1997) observed an increase in cortisol and a decrease in IFNγ production post castration. All three stressors indicate an effect of stress hormones on Th1 response, possibly switching the immune response to Th2. However, none of these studies evaluated the expression of Th2 cytokines like IL-4, therefore, it is unknown if the down-regulation in IFNγ is due to an increase in IL-4.

Stress Hormones and Neutrophil Function

Neutrophils play an important function in innate immunity because they are the first line of defense against invading pathogens. However, hormones released upon stress disrupt their function. Neutrophils remain in the blood stream but will migrate across the epithelial layer when signaled by chemokines that are released from inflamed tissues. Once at the site of infection, neutrophils phagocytose foreign particles and undergo respiratory burst, once these functions are accomplished the neutrophil undergoes apoptosis and never reenters the blood stream. All these functions are altered in the presence of stress hormones like glucocorticoids. Neutrophils are affected by glucocorticoids through the expression of glucocorticoid receptors (GRs), with blood neutrophils expressing higher amounts of GRs than neutrophils in the bone marrow. Under normal conditions, the GR is located in the cytoplasm with a high affinity hormone binding site. In the presence of glucocorticoids inside the cell, the GR binds the hormone and becomes activated and translocates to the nucleus. Inside the nucleus, the GR alters hormone sensitive genes particularly those involved with migration (CD62L) and programmed cell death (Fas). Decreased migration and apoptosis increases the number of neutrophils circulating in the blood; therefore increases in glucocorticoids leads to a neutrophilia. Neutrophil bactericidal activity is depressed which is linked to increased susceptibility to disease. Glucocorticoid receptor activation also down regulates itself, which makes these neutrophilic changes short lived (Burton, Madsen et al. 2005).

Stressful events, such as transportation and calving, have been examined in relation to decreased neutrophil function. Dairy cows in the periparturient period, or the two weeks surrounding calving, have elevated cortisol concentrations and susceptibility to disease increases. At parturition, when cortisol is increased, genes encoding for neutrophil apoptosis are down

regulated; the proteins encoded by these genes are Fas-associated protein with death domain (FADD), death associated protein 6 (Daxx), Caspase 8 associated protein 2 (FLASH), receptor interacting protein (RIP), apoptosis-stimulating fragment (Fas), apoptosis-stimulating fragment ligand (FasL) and death-associated protein 5 (DAP5). Cortisol upregulates the expression of the anti-apoptotic genes B cell lymphoma 2 (Bcl-2), TNF receptor-associated factor 6 (TRAF6), TRAF family member-associated NFκB activator (TANK) and B cell-activating factor of the TNF family (BAFF) and the chemokine IL-8. Coordination between down regulating pro-apoptotic genes and up regulating anti-apoptotic genes leads to pronounced neutrophil survival and neutrophilia, resulting in increased circulating neutrophils in correlation with a cortisol surge (Chang, Madsen et al. 2004; Burton, Madsen et al. 2005).

Decreased apoptosis is only one of the functions affected by glucocorticoids and parturition; expression of migratory genes is affected as well. Genes relating to vascular adhesion and migration, CD62L and CD18, are downregulated in correlation with elevated cortisol concentrations. Another gene, PSST (subunit of NADH:ubiquinone oxidoreductase), involved in regulation of reactive oxygen species, is decreased during stress. When ROS are produced inside the cell they damage the organelles and cause the release of cytochrome *c* that activates the apoptosis-inducing caspase pathway. Increases in cortisol surrounding parturition diminish the ability of neutrophils to migrate to infected tissues and decreases the bactericidal activity and phagocytosis of these neutrophils. These changes could play a key role in the increased susceptibility to disease surrounding stress (Burton, Kehrli et al. 1995; Weber, Madsen et al. 2001; Weber, Toelboell et al. 2004; Burton, Madsen et al. 2005).

Parturition-related stress has a profound impact on neutrophils gene expression, but transportation-related stress has similar effects. As previously discussed, road transportation in

cattle, sheep, and camels increases both glucocorticoids and catecholamines and ultimately alters neutrophil function. Buckham Sporer et al., 2007 observed a down-regulation in the Fas and CD62L gene in response to increase in cortisol levels 4.5 hours into transportation. This study, however, also observed a depression in CD62L is followed by a rebound where CD62L expression profoundly increased 9.75 hours into transportation. Yagi et al., 2004 also indicated a decrease in surface expression of the adhesion molecules CD62L and CD18 in neutrophils. However, Sporer et al., 2008 observed no change in the expression of CD62L and Fas after transportation.

The results from the previous studies conclude that the type of stressor and the timing of sample collection results in significant differences. Transportation and parturition stress result in increased levels of cortisol and an increased susceptibility to disease. The influences of cortisol may alter immunological parameters, such as neutrophil function, that lead to increased morbidity from infectious diseases.

Synthetic Glucocorticoids

Dexamethasone (DEX) is a synthetic glucocorticoid that is 25 times more potent than naturally occurring cortisol (Lo, Lee et al. 2005). Researchers favor treatments with DEX because the dose and timing of the injections can be manipulated to fit an experimental protocol better. Many researchers have used DEX as a model for glucocorticoid effects on immunological parameters in cattle, including neutrophil gene expression, lymphocyte phenotypes, and acute phase proteins (Roth and Flaming 1990).

As discussed previously, neutrophil dysfunction occurs during stress induced by parturition and transportation. But, researchers have also used DEX to induce a similar response of neutrophils in vivo. Burton et al., 1996 observed a down regulation in CD62L and CD18, important neutrophil surface markers for adhesion, in response to DEX injection. Prior to the treatment with DEX, 98% of cells expressed CD62L and only 17% displayed CD62L 40 hours after DEX treatment. During parturition, decreased expression of CD62L is correlated with increased concentrations of cortisol. Burton et al., 2005 also observed a decrease in CD62L and CD18 in correlation with an increase in blood cortisol during parturition in dairy cows.

Injections with dexamethasone cause both neutrophilia and lymphopenia and the cells showed a decreased response to antigenic stimulation (Oldham and Howard 1992). Dexamethasone treatment also selectively depleted specific lymphocyte populations by reducing CD3+ and WC1+ but not CD2+, CD4+ and CD8+ lymphocytes. Workshop cluster 1 (WC1+) is expressed on γδ T cells and CD4+ or CD8+ is expressed on αβ T cells, which suggests that DEX treatment influences the γδ T cell population more than the αβ T cell population (Burton and Kehrli 1996). In accordance, Menge et al., 2008 observed a decrease in the γδ T cell population following DEX treatment, but in contrast found an increase in CD4+ cells. Nonnecke et al.,

1997 injected DEX and a ≥97% decrease in IFNγ cytokine response to antigen stimulation was cited. Nonnecke et al., 1997 also saw a decrease in IgM secretion, MHC I and II receptor expression, the B cell population, CD3+ cells, the γδ T cell population, CD4+ cells, and CD8+ cells following DEX injection. The amount of DEX administered and timing of doses affect the immune response differently, which is similar to differences in response to stressors. Different stressors will affect the amount of stress related hormones released. Therefore, when studying stress-related influence on immunological parameters, timing of sample collection is very important.

Conclusion

Weaning causes physiologic changes in the calf that increases stress-related behaviors and stress-related hormones. The hormones released during psychological stress, like weaning, cause changes in immune function and alter responses to pathogens that could ultimately increase susceptibility to disease. Management practices directed to reduce the weaning-related stress response in their calves may decrease the consequences of a suppressed immune system. These consequences are decreased weight gain, increased incidence of disease and sometimes mortality. All three outcomes result in financial loss to the farmer. In order to decrease these negative effects, researchers need to investigate the alterations to the immune systems that occur during weaning stress. Once these alterations are understood, methods to decrease the changes can be implemented.

Chapter 3: Implementation of Fenceline Contact between the Cow-Calf Pair Decreases Weaning Stress as Indicated by Cortisol Metabolites in the Feces (Fall 2008)

Abstract

Weaning is considered a stressful event in beef calves and stress can cause an increase in glucocorticoids (GCs). Glucocorticoids, like cortisol, induce a shift in T cell differentiation from a T helper 1 to a T helper 2 predominant response in mice. Therefore, an experiment was conducted to test the hypotheses that 1) increases in cortisol after abrupt weaning in cattle are associated with alterations from T helper 1 to T helper 2 phenotype by increasing IL-4 and antigen specific IgG_1 and decreasing IFNγ and antigen specific IgG_2 and that 2) fenceline weaning alleviates the increase in cortisol and changes to immune function. Angus heifers (n=12) were blocked by age and weight and randomly allotted into two weaning groups, fence line (FL; n=6) and abrupt (AB; n=6), on day -7. Blood samples were taken on day -7, 0, 7, 14, 21, and 42; fecal samples were taken on day -7, 0, and 3. On day -1, all calves were separated from their dam and transported for 2 hours. On day 0 all calves were vaccinated with RB51. Serum was analyzed for IFNγ, IL-4, and IgG_1 and IgG_2 antibodies to RB51. Fecal samples were analyzed for the cortisol metabolite 11, 17-dioxoandrostane or 11,-17 DOA. Both IgG_1 and IgG_2 antibodies to RB51 increased from day 0 to day 14 ($P<0.05$), however no differences were detected between treatment groups. Fecal cortisol was higher on day 0 in abruptly weaned calves ($P< 0.001$) and did not differ between groups on day -7 or day 3. Fenceline calves had higher IFNγ serum concentrations on day -7 and day 0 than in abrupt calves ($P<0.04$). Transportation did not affect stress hormones and RB51 did not elicit a strong T helper 1 response.

Introduction

Weaning is the separation of the cow-calf pair and is normally associated with dietary, social, environmental, and immunological changes in the calf (Arthington, Eichert et al. 2003). Abruptly weaning a calf from a cow leads to behavioral changes in calves, such as decreased eating and increased walking and vocalizing, decreased weight gain and increased concentrations of stress-associated hormones (Lefcourt and Elsasser 1995; Hickey, Drennan et al. 2003; Price, Harris et al. 2003; Haley, Bailey et al. 2005). During stress, changes to the hypothalamo-pituitary adrenal (HPA) axis and the sympathetic nervous system elicit the release of cortisol and norepinephrine, respectively (Carroll and Forsberg 2007). These hormones negatively influence weight gain and immune function (Hickey, Drennan et al. 2003). Due to the stressful results of abrupt weaning, alternative weaning methods are used to decrease the calves' distress and normally occur in two stages. Fenceline weaning occurs when the calf is moved into an adjacent paddock removing physical contact and ending suckling, but is allowed to maintain social contact with the dam (Boland, Scaglia et al. 2008). Fenceline weaning reduces the time calves spend walking and vocalizing, and increases the time calves spend eating and lying down; fenceline weaning also has a positive effect on weight gain (Stookey 1997; Price, Harris et al. 2003; Haley, Bailey et al. 2005). Cortisol production also influences the immune system by affecting lymphocyte and neutrophil populations; more specifically cortisol alters T cell subpopulations by shifting them from a Th1 to a Th2 predominant response. The Th1 response is characterized by production of IFNγ and IgG_2, while the Th2 response is characterized by IL-4 and IgG_1 (Estes and Brown 2002; Elenkov 2004). Thus to further investigate the alterations to the immune system in response to weaning stress, a study was conducted to determine the influence of weaning on stress hormone production and markers of Th1/Th2 responses.

Materials and Methods

Animals

Twelve Angus and Angus cross heifers (186±21 kgs, 174±16 days of age) located at the Shenandoah Valley Agriculture Research and Extension Center in Steeles Tavern, VA 24472 were blocked by age and weight and then randomly allotted within blocks into two treatment groups, fenceline (FL) or abrupt (AB). All calves were weighed at day -7, 0, 7, 14, and 42. Fenceline calves were separated from their dam at day -7 by a single fence while remaining on pasture. Abruptly weaned calves remained with their dam on pasture until day -1 when all calves were loaded onto a trailer and shipped for 2 hours from Steeles Tavern, VA to Virginia Tech's Kentland Farm in Blacksburg, VA 24060 and housed on pasture. All calves were vaccinated with Pyramid 5 + Presponse SQ (Fort Dodge, Overland Park, KS) and Vision 7 (Intervet Inc., Millsboro, DE) and dewormed with Cydectin (Fort Dodge, Overland Park, KS) 16 days prior to weaning and 17 days prior to RB51 vaccination.

Experimental Procedure

Blood was collected by jugular venipuncture on days -7, 0, 7, 14, 21, 42 into vacutainers (10mL) containing no anticoagulant (Becton Dickson Inc., Franklin Lakes, NJ). Fecal samples were collected from the rectum of the calf while the calf was in the head gate on days -7, 0, and 3. A commercially available vaccine, RB51 (Professional Biological Company, Denver, CO), against *Brucella abortus* was administered (2mL) subcutaneously on day 0.

Fecal Extraction

The extraction was based on the procedure from Mostl et al (2002). Fresh fecal samples were collected into 50mL conical tubes and kept on ice until storage at -80°C. Samples were thawed at room temperature for 4 hours and 0.5g of thawed wet feces was suspended in 5mL of 80% methanol (4mL 100% methanol in 1mL ddHOH). Samples were vortexed for 1-2 minutes and then centrifuged at 2500g for 15 minutes. A 1mL aliquot of supernatant was diluted 1:10 in assay buffer (2.42g trizma base, 17.9g NaCl, 1g BSA, 1ml tween 80 in 1L ddHOH, pH 7.5). Diluted samples were stored at -20°C until analysis.

Cortisol Metabolite Immunoassay

This assay was based on the procedure from Mostl et al (2002). Briefly, 96-well ELISA plates (Pierce Biotechnology Inc., Rockford, IL) were coated with 250µL/well of 2µg/mL protein A (Sigma-Aldrich, St. Louis, MO) in coating buffer (1.59g Na_2CO_3, 2.39g $NaHCO_3$ in 1L ddHOH, pH 9.6), plates were sealed with a plate sealer (Thermo Fisher Scientific Inc., Waltham, MA) and incubated overnight at room temperature. After the coating solution was decanted, plates were subsequently blocked with 300µL/well blocking buffer (3.146g trizma base, 23.3g NaCl, 13g BSA, 1.3g sodium azide in 1.3L ddHOH, pH 7.5) and incubated for at least 3 hours at room temperature. Plates were then washed 3x with 300µL/well wash buffer (0.5ml tween 20 in 2.5L ddHOH). After that, plates were loaded with 10µL/well of sample or standard in duplicate (11-oxoaetiocholanolone), 40µL assay buffer, 100µL/well biotin-labeled steroid (11-oxoaetiocholanolone-17-CMO-biotinyl-3,6,9-trioxaundecanediamin), and 100µL/well antibody (rabbit anti-11-oxoaetiocholanolone-17-CMO:BSA). Blank and non-specific binding (NSB) wells received 50µL assay buffer (no standard or sample), and NSB wells received no antibody.

After loading, the plates were incubated overnight at 4°C with gentle shaking. After incubation, plates were washed 4x with cold (4°C) wash buffer and 250µL/well streptavidin-POD enzyme solution (Roche Diagnostics, Mannheim, Germany) (1µL streptavidin-POD in 30mL assay buffer) was added. The plate was covered and incubated for 45 minutes at 4°C with gentle shaking. Plates were washed 4x with cold (4°C) wash buffer and then 250µL/well TMB substrate (Thermo Fisher Scientific Inc., Waltham, MA) was added. Plates were incubated at 4°C for 45 minutes with gentle shaking and then of 50µL/well stop solution (2M H_2SO_4; Ricca Chemical Company, Arlington, TX) was added. Absorbance was read at 450nm on a plate reader (SpectraMax M5 Multi-Mode Microplate Reader, Molecular Devises, Downington, PA). Sample concentration was determined using the equation of a four parameter logarithmic curve ($y = d + \frac{a-d}{[1+\left(\frac{x}{c}\right)]}$; a=estimated response at zero concentration at, b=slope factor, c=mid-range concentration; d=estimated response at infinite concentration, y=mean optical density value, x= standard concentration) for the standards (standard range was from 2.048-500 pg/10µL). Antibodies, standards, and steroids were provided by E. Mostl from the Institut fur Biochemie and Ludwig Boltzmann Institut fur Veterinarmedizinische Endokrinologie, Vienna, Austria (Erich.moestl@vu-wien.ac.at) for this competitive enzyme immunoassay. All buffer components came from Sigma-Aldrich, St. Louis, MO and were filtered through a Sep-Pak C18 from Waters Division, Milford, MA.

RB51 IgG_1 and IgG_2 Specific ELISA

The assay was based on a procedure described by Cobly et al. (2002). Briefly, 96-well ELISA plates were coated with 200µL/well lyophilized RB51 (10^{10} bacteria pelleted and supernatant removed) diluted 1:20 in carbonate/ bicarbonate buffer (pH 9.6) and incubated overnight at 4°C.

Plates were washed with 250µL PBST-20 (PBS+0.05% tween 20) and 200µL/well diluted serum samples (1:50 in PBST-20) were added in duplicate. Next, plates were incubated for 30 minutes at 37°C. After incubation, plates were washed and then 200µL/well of a 1:6000 dilution of mouse anti-bovine IgG_1 (VMRD, Pullman, WA) or a 1:6000 dilution of mouse anti-bovine IgG_2 (Serotec, Raliegh, NC) in PBST-20 was added. Plates were incubated for 30 minutes at 37°C, washed and 200µL/well goat anti-mouse IgG:HRP (Serotec, Raleigh, NC) diluted 1:10,000 in PBST-20 was added and incubated for 30 minutes at 37°C. A final wash step was performed and then 200µL/well of TMB substrate (Thermo Fisher Scientific Inc., Waltham, MA) was added and incubated for 30 minutes at room temperature in the dark with gentle rocking. The TMB reaction was stopped with 100µL/well 2N H_2SO_4 (Ricca Chemical Company, Arlington, TX) and the absorbance was read at 450nm (SpectraMax M5 Multi-Mode Microplate Reader, Molecular Devises, Downington, PA). Samples were analyzed with a percent positivity of the positive control (a cow that had been previously vaccinated with RB51 and had a higher antibody titer to RB51 as compared to the calves; IgG_1 OD: 3.23±0.07; IgG_2 OD: 2.06±0.14).

Serum Cytokine ELISA

The procedure was modified from Souza et al. (2008) using the IFNγ or IL-4 Bovine Screening Set (Thermo Fisher Scientific Inc., Waltham, MA). Briefly, 96-well plates were coated with 100µL antibody diluted 1:100 in carbonate/bicarbonate buffer and incubated at room temperature overnight. Next, 300µL/well blocking buffer (4% BSA 5% Sucrose in D-PBS; 0.2µm filtered) was added and incubated for 3 hours at room temperature. Plates were then washed three times with 300µL D-PBS+0.05% tween 20 and samples (undiluted) and standards were added in duplicate. After an overnight incubation at room temperature with gentle shaking, plates were

washed four times and 100µL/well biotin labeled detection antibody diluted (1:100 for IFNγ; 1:200 for IL-4) in reagent diluent (4% BSA in D-PBS; 0.2µm filtered) was added. After a 2 hour incubation at room temperature with gentle shaking, plates were washed and 100µL per well Streptavidin-HRP (diluted 1:400 in reagent diluent) was added. Following a 1 hour incubation at room temperature with gentle shaking, plates were washed and 100µL per well TMB substrate was added. Plates were incubated in the dark for 30 minutes with gentle shaking and the reaction was stopped with 100µL stop solution (2M H_2SO_4). The plate absorbance was read at A_{450} minus A_{550} (SpectraMax M5 Multi-Mode Microplate Reader, Molecular Devises, Downington, PA). The blank was subtracted out and the sample concentration was determined using the equation of a four parameter logarithmic standard curve.

Statistical Procedure

All data was evaluated graphically for normality before statistical analysis was implemented. Data was analyzed using repeated measure in the PROC MIXED procedure in SAS (SAS Institute, Inc., Cary, NC). The model included the variables weaning treatment, day and weaning treatment*day interaction; calf (weaning treatment) was the subject and day was the repeated measure. The SLICE option was used to compare treatments within days. The Tukey adjustment was applied to test the significance between days. Significance was determined at $P \leq 0.05$.

Results

Fecal Cortisol Metabolites

Fecal cortisol metabolite concentration was influenced by treatment ($P<0.0047$), date ($P=0.0001$) and treatment*date ($P=0.0006$). Abruptly weaned calves had a higher fecal cortisol concentration ($P<0.0001$) on day 0 as compared to fenceline weaned calves (Figure 3.1).

Weights

Body weight for all calves increased over time ($P<0.0001$) from day -7 to day 42. No treatment effect or treatment*date interaction was detected for body weight or average daily gain (ADG) for the course of the experiment (Table 3.1).

Serum IFNγ

Serum IFNγ ELISA concentrations were higher in fenceline weaned calves at day -7 and day 0 ($P<0.05$) as compared to abruptly weaned calves (Figure 3.2). The difference between treatment groups at the base line time points makes comparing treatment effect difficult. Serum IL-4 was not detected with ELISA in this study.

RB51 Antibodies

A difference in IgG_1 and IgG_2 antibody response to the RB51 vaccine given at day 0 between treatment groups was not detected (Figure 3.3). However, there was a time effect involved in the antibody response with the titers for both IgG_1 and IgG_2 increasing over time from day 0 to day 42 ($P<0.0001$).

Discussion

Weaning is a stressor that increases the secretions of hormones from the adrenal cortex, most notably cortisol. Investigators over the years have observed an increase in "stress" related hormones such as cortisol and noradrenaline during the weaning period. Hickey et al. (2003) observed an increase in plasma, cortisol and noradrenaline after weaning, which supports the results from our examination of cortisol metabolites in the feces. Higher concentrations of cortisol metabolites in the feces of abruptly weaned calves suggests that abrupt weaning causes an increase in adrenal cortex secretions associated with psychological stress. Fenceline weaned calves had little increase in cortisol metabolites after permanent separation from their dam indicating that this two-stage process decreases the stress involved in weaning.

The calves in this study were transported after separation from their dam. Buckham Sporer et al. 2007 confirmed an increase in plasma cortisol levels during transportation which contrasts with our data since fenceline weaned calves had little to no increase in fecal cortisol metabolites. This disparity may be explained by shipping duration. In our study, calves were transported for approximately 2 hours, where as calves in the Buckham Sporer (2007) study were transported for 9 hours and their first sampling time was 4.5 hours; there was an increase in plasma cortisol by 4.5 hours, but plasma cortisol decreased by 9.25 hours. In contrast, Arthington et al. 2003 found elevated plasma cortisol concentrations post weaning, but no increase post shipping for 3 hours. Therefore, there may be a length of transportation interaction in association with cortisol secretion; lower transportation times ($\leq$3 hours) do not cause increases in cortisol, while longer transportation times (>3 hours) cause increases in cortisol. Another factor that may contribute to the differences in cortisol post shipping may be the gender

of the calves. The present study is on heifers and Arthington (2003) used steers and heifers, but Buckham Sporer (2007) used bulls. Buckham Sporer et al., 2007 observed a decrease in plasma testosterone and an increase in plasma progesterone associated with an increase in cortisol 4.5 hours into transportation. The alterations in these other hormones may contribute to the increase in cortisol post shipping. However, this study and Arthington et al., 2003 did not measure testosterone or progesterone so this assumption cannot be verified. Buckham Sporer et al., 2007 also observed an effect of breed on plasma cortisol and progesterone. The present study used Angus and Angus X calves, Arthington (2003) used Brahman x Angus calves, and Buckham Sporer (2007) used Angus, Friesian and Belgian Blue × Friesian. These gender, breed, or length of transportation variations may contribute to the differences in transportation stress, but a conclusion based on these effects cannot be declared at this time. In order to increase the stress response due to transportation, calves should be transported for a minimum of 5 hours not exceeding 8 hours.

Weaning methods have an influence on weight gain (Price, Harris et al. 2003). Fenceline weaned calves tend to gain more weight two and ten weeks post weaning than their abruptly weaned counterparts (Price, Harris et al. 2003; Haley, Bailey et al. 2005; Boland, Scaglia et al. 2008). Boland et al., 2008 also observed higher average daily gain in fenceline weaned calves than abruptly weaned calves. In the present study, we compared the average daily gain of the treatment groups and total body weight over the course of the experiment. Our results disagreed with previous studies. Average daily gain did not differ among treatment groups. In both Price et al., 2003 and Boland et al., 2008 calves were older (~213 and 224 day of age respectively) and heavier (~206 and 241 kgs respectively) than the calves in this study. Arthington et al., 2005 observed that weaning calves at an earlier age increased their average daily gain and feed to gain

ratio. Therefore, since calves in this study were weaned at ~174 days of age the stress associated with weaning may have been alleviated as compared to older weaned calves. Also previous studies record higher cortisol concentrations in association with suppressed weight gain post weaning; however, a significant difference between our treatment groups in average daily gain or body weight was not detected. Another reason average daily gain did not differ among treatments may be gut fill. Gut fill biases live weight resulting in an unreliable indicator of body mass (Drouillard and Kuhl 1999). Gut fill can compose 9-17% of live weight; therefore measurements of live weight become variable depending on gut fill. Gut fill can be influenced by water intake, feed intake, defecation, and the type of feedstuff (Drouillard and Kuhl 1999). To overcome the variation due to gut fill, measurements should be taken as an average of three live weights over the course of one day. This method will reduce the variation of water and feed intake and defecation. Weight measurements should also be extended to 10 weeks post weaning to observe long term effects of weaning on the weight of the calf.

Hickey et al. 2003 tested the IFNγ response of cultured lymphocytes to Con-A in vitro and found that weaning caused an attenuation of IFNγ production following stimulation of samples taken at 24, 28, and 168 hours post weaning. Similarly, Gupta et al., 2007 observed a decrease IFNγ response in stimulated lymphocytes following transportation in association with an increase in plasma cortisol. In this study, IFNγ, a Th1 cytokine, was measured in the serum without stimulation. Due to the difference in IFNγ concentration between treatment groups at day -7 and day 0, changes due to weaning could not be detected. Since these time points were base line measurements, it makes analysis of treatment effect difficult. Also, other studies did not analyze IFNγ concentrations in serum; in future studies analysis of stimulated lymphocytes or gene expression of IFNγ in lymphocytes may provide better analysis. Furthermore, measuring

IFNγ in the serum is dilute and evaluates IFNγ produced by all immune cells, which is a different measurement than Hickey (2003) and Gupta (2007) who measured the IFNγ production of lymphocytes only following the same experimental procedure. Another method to measure IFNγ would be flow cytometry. Utilizing whole blood samples, flow cytometry can analyze the type of cell and if the cell is producing IFNγ. Using this technique, increases or decreases in IFNγ could be detected in specific cell types (dendritic cells, NK cells, T cells).

The RB51 vaccine against *Brucella abortus* induces a Th1 response in mice (Sanakkayala et al., 2005). According to Estes et al. 2002, a Th1 response should result in a predominance of the IgG_2 isotype in cattle, and the Th2 response, an end point of stress hormone stimulation, should result in a predominance of the IgG_1 isotype. The results from our study showed an increase in both IgG_1 and IgG_2 titers over time ($P<0.05$) by comparing the least squares means of each date to one another. However there was no difference between treatment groups for either antibody isotype. Administration of the Pyramid 5 vaccine prior to RB51 vaccination could have biased the immune response especially if the Pyramid 5 vaccine induces a Th2 bias. Eliminating vaccination with Pyramid 5 in future studies or evaluation into the immune response to this vaccine is warranted to determine its effects on RB51 vaccination. Our results suggest that either the vaccine or the stress response was not strong enough to bring on a change in the antibody repertoire and future studies should increase the sample size (number of calves), incorporate an unweaned control group, and analyze fecal cortisol concentrations one day post fenceline separation. Measurement of antibody and cytokine production of isolated lymphocytes stimulated with antigen may be more beneficial. Arthington et al., 2003 and Buckham Sporer et al., 2007 observed differences in acute phase proteins and Boland et al., 2008 observed differences in blood metabolites. Measurements of these parameters may help correlate our next

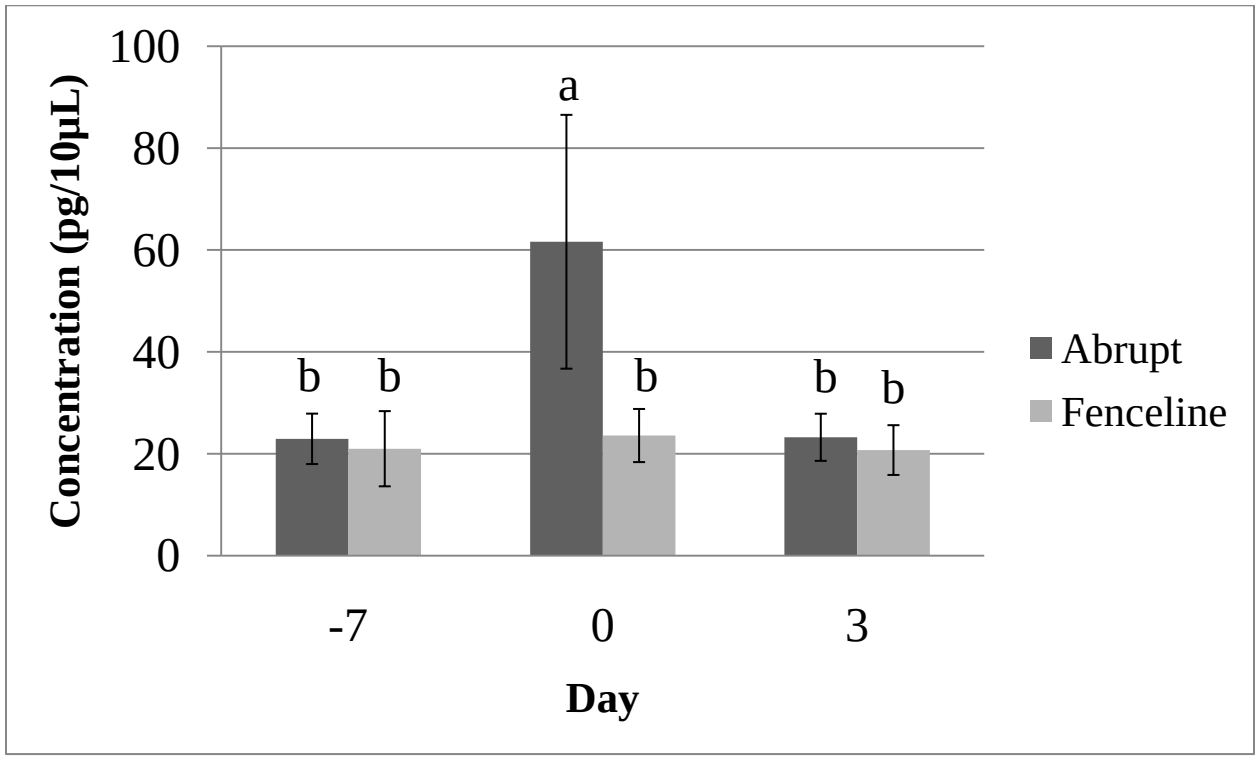
study with the experiments discussed here. Moreover, using calves that are older and heavier (similar to other published experiments) may increase the stress associated with weaning and effect the immunological parameters differently.

Utilizing different analytical methods, altering the timing of collection, or looking at different samples could provide more sensitivity. The RB51 vaccines did not produce a strong Th1 associated antibody response and RB51 in particular does relies more on cell-mediated immunity, and thus does not induce a strong antibody response. The vaccine RB51 does not rely on humoral immunity because it is unable to induce anti-LPS associated O-side chain antibodies. Therefore, the presence of anti-RB51 antibodies is not a good indicator of immunity, just exposure (Colby, Schurig et al. 2002). To assess immunity to RB51 in future studies CD4, CD8, and NK cell populations and their ability to produce IFNγ should be measured via flow cytometry. Also for future experiments, a vaccine with an established quantitative procedure that produces a strong Th1 response should be used. The addition of an unweaned control group would enhance the conclusions of the future studies would answer questions like: are the changes in immune function due to weaning or age; adult cattle have 10-15% γδ T cells while young cattle have 40% γδ T cells (decreasing until 6 months of age). The experiments conducted in this thesis used serum, whole, blood, and white blood cells for analysis of cytokines. Isolation of specific cell populations for gene expression would aid in evaluation of changes in cytokine production by T cells.

In conclusion, fenceline weaning results in lower levels of the stress associated hormone cortisol post permanent separation but immunological differences between treatment groups were not observed. Our results corresponded with previous studies in adrenal hormone secretion

but not weight gain. Further investigation into cytokine expression and lymphocyte profiles would provide insight into the immunological alterations associated with weaning methods and may help explain the increased disease susceptibility of the calf during weaning.

Figure 3.1 Cortisol metabolite concentrations[1] from fecal samples collected[2] from abruptly (n=6) and fenceline (n=6) weaned calves at specific days prior to and following vaccination. Weaning occurred at day -1. Vaccination with RB51 occurred at day 0.



[a,b] Means with unlike letters are different (P<0.0001)

[1] Values expressed as least squares means ± standard deviation

[2] Metabolites show up in the fecal matter 12-18 hours after the induction of stress

Table 3.1 Live weights[1] and average daily gain[1] from abruptly (n=6) and fenceline (n=6) weaned calves at specific days prior to and following weaning and vaccination. Weaning occurred at day -1. Vaccination with RB51 occurred at day 0.

	Live Weight (kgs) and Average Daily Gain (kgs/day)						
	Day[3]						
Treatment[4]	-7[a]	0[a]	7[b]	14[c]	42[d]	SE	ADG
Abrupt	188	185	197	205	218	9.41	0.61
Fenceline	184	187	193	202	215	9.40	0.62

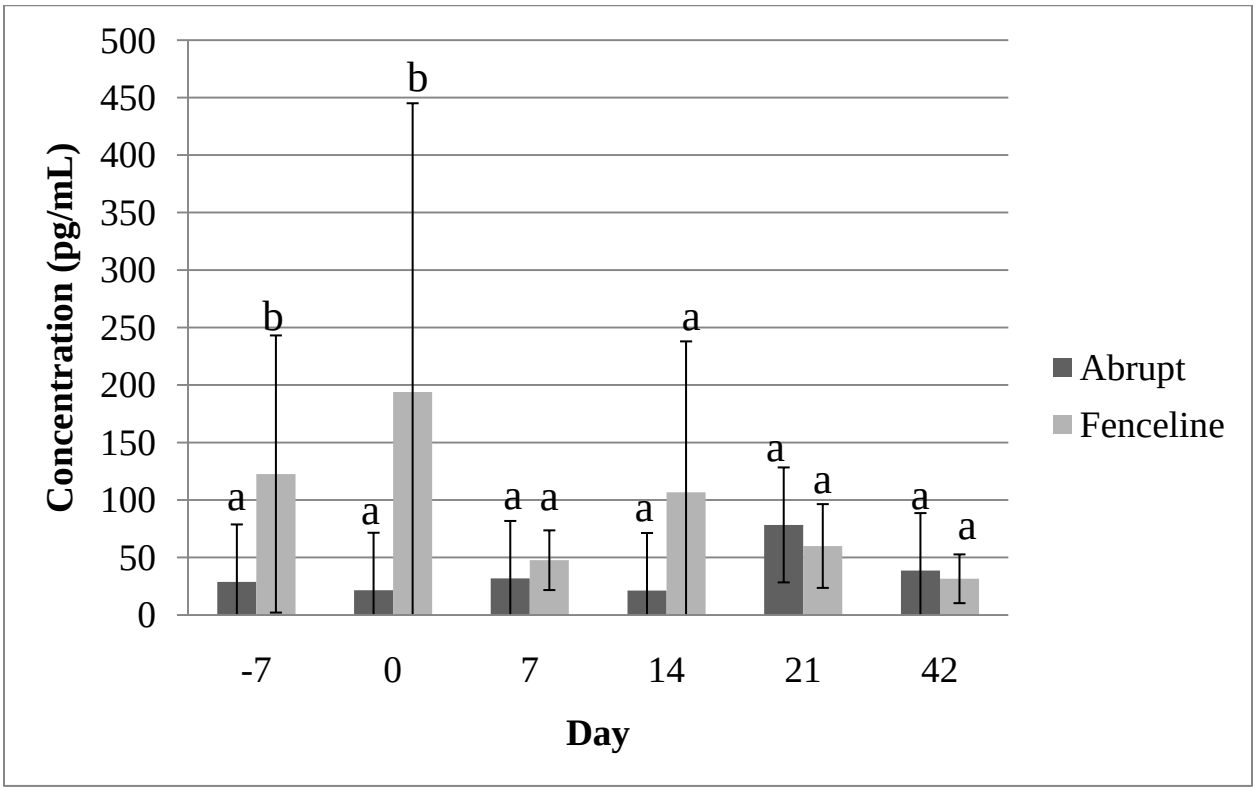
[1]Values expressed as least squares means with pooled standard error (SE)

[2]Body weights for calves increase over time $P < 0.0001$

[3]Treatments did not differ for weight or ADG

[a,b,c,d]Day with no common superscript differ ($P < 0.05$)

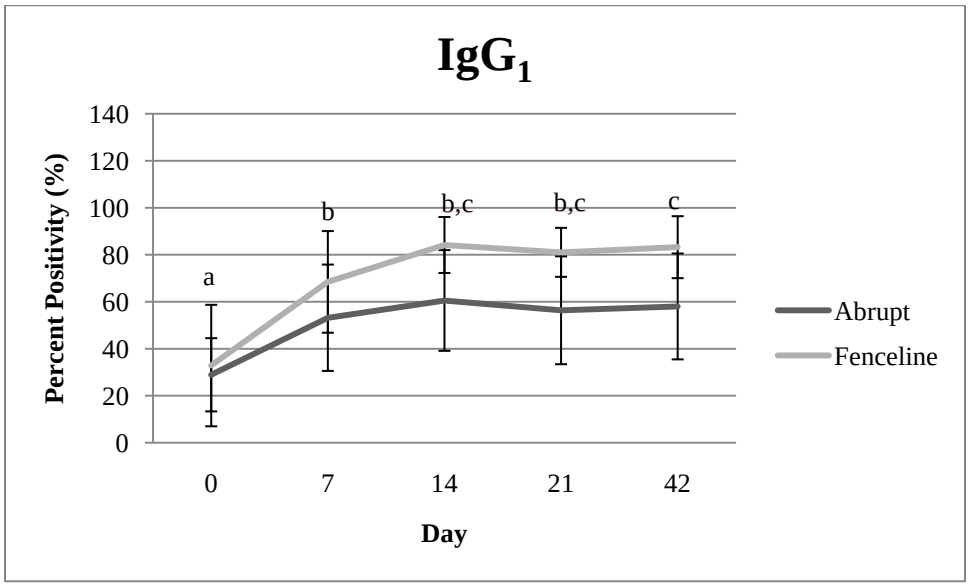
Figure 3.2 Serum IFNγ concentration[1] for abruptly (n=6) and fenceline (n=6) at specific days prior to and following vaccination. Weaning occurred at day -1. Vaccination with RB51 occurred at day 0.

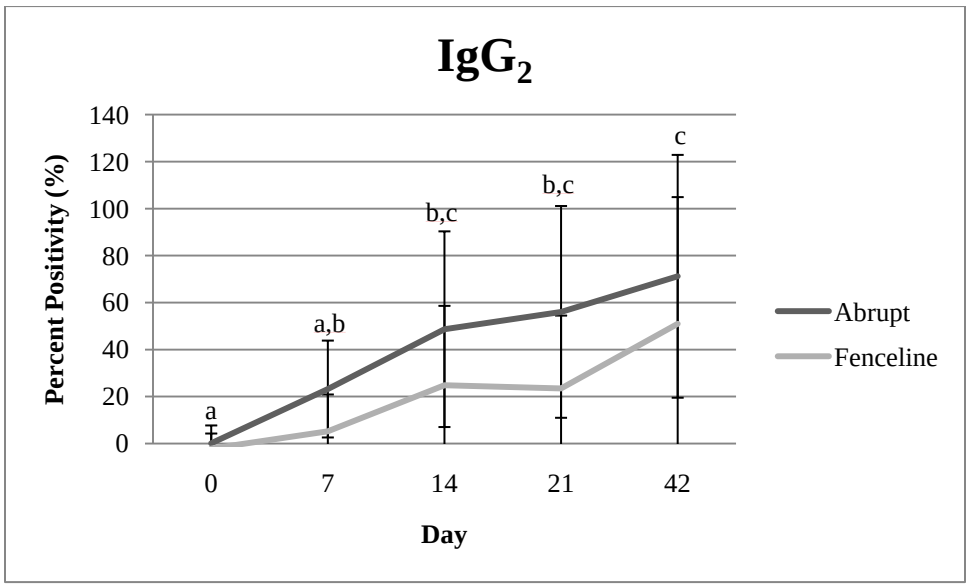


[a,b] Means with unlike letters are significantly different ($P<0.05$)

[1] Values expressed as least squares means ± standard deviation

Figure 3.3 Percentage[1] of RB51 specific antibodies[2] (IgG_1 and IgG_2) from serum samples for abruptly (n=6) and fenceline (N=6) weaned calves at specific days prior to and following vaccination. Weaning occurred at day -1. Vaccination with RB51 occurred at day 0.





[1] Percentage was determined using a positive control (sample OD value/positive control OD value x 100%)

[2] Values expressed are least squares means ± standard deviation

[a,b,c] Means within day with no common superscript differ ($P<0.05$)

Chapter 4: Increases in Cortisol during Weaning Cause Alterations in Lymphocyte Phenotype and Gene Expression (Fall 2009)

Abstract

Weaning in the beef cattle industry is associated with increased levels of stress-related hormones and alterations to the immune system. Immunological changes occur in both the innate and adaptive immune system. Therefore, an experiment was conducted to test the hypotheses that 1) higher concentrations of fecal cortisol metabolites in abruptly weaned calves affect antibody production by decreasing antigen specific IgG_2 and increasing antigen specific IgG_1, by increasing αβ T cells and decreasing γδ T cells, and by increasing IL-4 and IL-10 gene expression and decreasing IFNγ and CD62L gene expression and 2) that fenceline weaning alleviates those changes. Forty-four Angus and Angus cross calves (19 heifers and 25 steers; 181±27 kgs; 148±17 days old) were blocked for age and gender and allotted into two weaning treatment groups, fenceline (FL) and abrupt (AB). Half of the fenceline calves were separated from their dams by a single fence at day -7 and the other half on day -6, all calves were permanently separated from their dam at day 0, and all calves were vaccinated with a killed *Histophilus somni* vaccine, Somubac (Pfizer, Exton, PA), at day 1. Serum samples were taken on all calves at day -7 or -6, 1, 3, 8, 15, and 22. Blood was taken for white blood cell isolation and gene expression at day -6, 1 and 3 on only 21 calves (the fenceline treatment of these 21 calves occurred at day -6). Fecal samples were collected at day -6, 1 and 3. All calves were weighed on day -7, 0, 8 and 22. Serum samples were analyzed for IgG_1 and IgG_2 antibodies against the *H. somni* vaccine. White blood cells were analyzed for lymphocyte phenotypes and whole blood samples were analyzed for gene expression. Fecal samples were analyzed for cortisol metabolites. Abruptly weaned (AB) calves had a higher concentration of cortisol metabolites than fenceline (FL) calves at day 1 ($P<0.0001$). No difference in average daily gain

or *H. somni* specific antibodies between treatment groups was detected. There was a treatment*date interaction in lymphocyte and neutrophil populations ($P<0.05$). FL lymphocyte percentages increased from day -6 to day 1 and then decreased from day 1 to day 3, while AB lymphocyte percentages increased from day -6 to day 3. Neutrophil percentages of the total leukocyte population from FL calves decreased from day -6 to day 1 and increased from day 1 to day 3, while neutrophil percentages from AB calves decreased from day -6 to day 3. No difference in treatment groups was detected for lymphocyte phenotypes or gene expression; however, a date effect was detected: CD4 and CD8 increased over time ($P<0.0001$), WC1 and TcR1 decreased over time ($P=0.0243$ and $P=0.0027$ respectively), and GAPDH and CD62L decreased over time ($P<0.0001$). Expression of IFNγ, IL-4, and IL-10 did not change over time. Two-stage weaning had no effect on immune function based on the parameters assessed within this study.

Introduction

Weaning in the beef cattle industry is the abrupt separation of the cow-calf pair and the termination of milk feeding (Price, Harris et al. 2003; Boland, Scaglia et al. 2008). Removal of the calf from its dam is known to be stressful to the calf as indicated by increases in stress-related hormones, cortisol and norepinephrine, and changes in behavior (Pollock, Rowan et al. 1992; Lefcourt and Elsasser 1995; Hickey, Drennan et al. 2003; Price, Harris et al. 2003; Haley, Bailey et al. 2005). Alternative weaning methods have been developed to decrease the stress associated with weaning, and normally occurs in two-stages. One alternative weaning method is fenceline weaning; the calf is separated from its dam by a single fence that restricts suckling but allows the calf to maintain contact with its dam. These alternatives decrease the behavioral changes associated with weaning and increase weight gain as compared to abruptly weaned calves (Price, Harris et al. 2003; Boland, Scaglia et al. 2008; Burke, Scaglia et al. 2009).

The stress-related hormones are glucocorticoids and catecholamines. When released after psychological stress, these hormones cause changes to the immune system (Elenkov 2004). Glucocorticoids affect both the innate response through neutrophils and the adaptive response through lymphocytes. Helper T (Th) lymphocytes can be either Th1 cells that produce IL-2, IL-12, IFN-γ and TNF-α, or Th2 cells that produce IL-4, IL-10 and IL-13. Cytokine production from Th1 and Th2 cells inhibit one another; therefore the Th1 cytokines, IFN-γ and IL-12, inhibit Th2 cell activity, while the Th2 cytokines, IL-4 and IL-10, inhibit Th1 cell activity (Brown, Rice-Ficht et al. 1998; Elenkov 2004).

Transportation stress, castration stress, periparturient stress, and synthetically induced stress alter neutrophil function, change lymphocyte phenotypes, and alter cytokine production. Burton et al. 2005 observed reduced neutrophil migration and apoptosis through decreased gene

expression of CD62L and Fas in association with an increase in serum cortisol concentrations in periparturient cows. Hickey et al. 2003 observed decreased IFNγ production in stimulated lymphocytes and an increased neutrophil:lymphocyte ratio in accordance with elevated cortisol in the serum of abruptly weaned calves. Burton et al. 1996 concluded that injection with synthetic glucocorticoids altered lymphocyte phenotypes by decreasing the γδ T cell population but not the αβ T cell population by looking at the cell markers CD4, CD8 and WC1. Therefore, an experiment was conducted to investigate the hypothesis that abrupt weaning increases the release of cortisol associated with a bias to antigen specific IgG_1 over IgG_2, an increase in the αβ cell markers CD4 and CD8, a decrease in the γδ cell markers TcR1 and WC1, an increase in the gene expression of IL-4 and IL-10, and a decrease in the gene expression of IFNγ and CD62L and that fenceline weaning assuages the increase in cortisol and the changes to immune function. These immunological parameters were chosen because previous literature indicates that stress and subsequent increases in cortisol cause alterations to these markers. Therefore, an experiment was conducted to test the hypothesis that 1) abrupt weaning increases the cortisol release which is associated with alterations to antibody production, lymphocyte phenotypes and gene expression and that 2) fenceline weaning assuages the increase in cortisol and the changes to immune function.

Materials and Methods

Animals

Forty-four Angus and Angus cross calves (19 heifers and 25 steers) born and located at the Shenandoah Valley Agriculture Research and Extension Center in Steeles Tavern, VA 24472 with a weight of 181±27 kilograms and an age of 148±17 days were blocked by age and gender and were then randomly allotted into two treatment groups, fenceline (FL; n=23) and abrupt (AB; n-22). Calves for this study came from two herds (A, n=24, 147±16 days of age, 179±27 kgs, 12 heifers, 12 steers; B, n=21, 149±19 days of age, 182±28 kgs, 7 heifers, 14 steers). All calves were utilized for weight, fecal cortisol metabolites and vaccine response measurements. Only, calves in group B were utilized for gene expression and lymphocyte phenotype. Fenceline weaned calves in group A were separated from dams on day -7 and fenceline weaned calves from group B were separated from dams on day -6. All calves were physically separated from dams on day 0 and remained in original groups from day 0 to day 7. All calves were then comingled from day 8 to day 22. All calves were vaccinated with Pyramid 5 + Presponse SQ (Fort Dodge, Overland Park, KS) and Vision 7 (Intervet Inc., Millsboro, DE) and dewormed with Cydectin (Fort Dodge, Overland Park, KS) during fenceline weaning (day -7 and day -6 for groups A and B respectively).

Experimental Procedure

Blood was collected from half the calves (n=21) on day -6, 1, 3 into 7mL evacuated tubes containing EDTA (Becton Dickson Inc., Franklin Lakes, NJ) and 2.5mL of blood was collected into PAXgene Blood RNA Tubes (Becton Dickson Inc., Franklin Lakes, NJ). Blood was collected on day -7, -6, 1, 8, 15, 22 into 10mL evacuated tubes containing no anticoagulant

(Becton Dickson Inc., Franklin Lakes, NJ) from all calves (n=44). Fecal samples were taken from the rectum of the calf while in the head gate on day -6, 1, and 3 from all calves (n=44). All calves were weighed on day -7, 0, 8 and 22. A commercially available vaccine (Somubac, Pfizer Animal Health, Exton, PA) consisting of inactivated, standardized cultures of *Histophilus somni*, with an aluminum hydroxide adjuvant was administered subcutaneously according to the manufacturer's instructions at day 1 to all calves (n=44).

Fecal Extraction

The extraction was based on the procedure from Mostl et al (2002). Fresh fecal samples were collected into 50mL conical tubes and kept on ice until storage at -80°C. Samples were thawed at room temperature for 4 hours and 0.5g of thawed wet feces was suspended in 5mL of 80% methanol (4mL 100% methanol in 1mL ddHOH). Samples were vortexed for 1-2 minutes and then centrifuged at 2500g for 15 minutes. A 1mL aliquot of supernatant was diluted 1:10 in assay buffer (2.42g trizma base, 17.9g NaCl, 1g BSA, 1ml tween 80 in 1L ddHOH, pH 7.5). Diluted samples were stored at -20°C until analysis.

Cortisol Metabolite Immunoassay

This assay was based on the procedure from Mostl et al (2002). Briefly, 96-well ELISA plates (Pierce Biotechnology Inc., Rockford, IL) were coated with 250µL/well of 2µg/mL protein A (Sigma-Aldrich, St. Louis, MO) in coating buffer (1.59g Na_2CO_3, 2.39g $NaHCO_3$ in 1L ddHOH, pH 9.6), plates were sealed with a plate sealer (Thermo Fisher Scientific Inc., Waltham, MA) and incubated overnight at room temperature. After the coating solution was decanted, plates were subsequently blocked with 300µL/well blocking buffer (3.146g trizma base, 23.3g NaCl, 13g

BSA, 1.3g sodium azide in 1.3L ddHOH, pH 7.5) and incubated for at least 3 hours at room temperature. Plates were then washed 3x with 300µL/well wash buffer (0.5ml tween 20 in 2.5L ddHOH). After that, plates were loaded with 10µL/well of sample or standard in duplicate (11-oxoaetiocholanolone), 40µL assay buffer, 100µL/well biotin-labeled steroid (11-oxoaetiocholanolone-17-CMO-biotinyl-3,6,9-trioxaundecanediamin), and 100µL/well antibody (rabbit anti-11-oxoaetiocholanolone-17-CMO:BSA). Blank and non-specific binding (NSB) wells received 50µL assay buffer (no standard or sample), and NSB wells received no antibody. After loading, the plates were incubated overnight at 4°C with gentle shaking. After incubation, plates were washed 4x with cold (4°C) wash buffer and 250µL/well streptavidin-POD enzyme solution (Roche Diagnostics, Mannheim, Germany) (1µL streptavidin-POD in 30mL assay buffer) was added. The plate was covered and incubated for 45 minutes at 4°C with gentle shaking. Plates were washed 4x with cold (4°C) wash buffer and then 250µL/well TMB substrate (Thermo Fisher Scientific Inc., Waltham, MA) was added. Plates were incubated at 4°C for 45 minutes with gentle shaking and then of 50µL/well stop solution (2M H_2SO_4; Ricca Chemical Company, Arlington, TX) was added. Absorbance was read at 450nm on a plate reader (SpectraMax M5 Multi-Mode Microplate Reader, Molecular Devises, Downington, PA). Sample concentration was determined using the equation of a four parameter logarithmic curve ($y = d + \frac{a-d}{[1+\left(\frac{x}{c}\right)]}$; a=estimated response at zero concentration at, b=slope factor, c=mid-range concentration; d=estimated response at infinite concentration, y=mean optical density value, x= standard concentration) for the standards (standard range was from 2.048-500 pg/10µL). Antibodies, standards, and steroids were provided by E. Mostl from the Institut fur Biochemie and Ludwig Boltzmann Institut fur Veterinarmedizinische Endokrinologie, Vienna, Austria (Erich.moestl@vu-wien.ac.at) for this competitive enzyme immunoassay. All buffer

components came from Sigma-Aldrich, St. Louis, MO and were filtered through a Sep-Pak C18 from Waters Division, Milford, MA.

Flow Cytometry

Whole blood (3mL) from the EDTA vacutainer tubes (Becton Dickson Inc., Franklin Lakes, NJ) was added to 12mL ACK Lysis Buffer (8.3g NH_4Cl, 1.0g $KHCO_3$, 0.0327g EDTA in 1L ddHOH, pH 7.2-7.4; Sigma-Aldrich, St. Louis, MO) in 15mL centrifuge tubes and samples were gently mixed by manual rotation for 3 minutes. Tubes were then centrifuged at 300g at 4°C for 5 minutes. Supernatant was discarded and 1mL of PBS+1%BSA was added to the remaining pellet. The pellet was washed three times with 1mL of PBS+1%BSA and finally resuspended with 1mL of PBS+1%BSA. Cells were counted and assessed for viability on a hemocytometer using a Trypan Blue Stain (Invitrogen, Carlsbad, CA) which dyed the dead cells blue (on average ~93% viable cells); the remaining unstained cells were counted. Cell counts were used to adjust cell suspension to $1x10^7$ live cells per mL, then 100µL ($1x10^6$ live cells) was added to each well of a 96 well v-bottom plate (Fisher Scientific Company, Middleton, VA) along with 50µL of primary antibody at the following dilutions: mouse anti-bovine CD4 (VMRD, Pullman, WA IL-A11, IgG_{2a} isotype) 1:400, mouse anti-bovine CD8 (VMRD BAQ 111A, IgM isotype) 1:400, mouse anti-bovine WC1-N3 γδ (VMRD CACTB32A, IgG_1 isotype) mouse anti-bovine 1:400, CD21 (VMRD BAQ15A, IgM isotype) 1:200, and mouse anti-bovine TcR1-N24 (VMRD GB21A, IgG_{2b} isotype) 1:100. All the primaries were added to separate wells except CD4 and CD8 which were added to the same well. Plates were then incubated for 15 minutes in the dark on ice. After a centrifugation at 300g for 5 minutes at 4°C, the primary antibody was decanted and the plates were washed two times with 200µL PBS+1%BSA at 4°C with a 5 minute

centrifugation at 300g at 4°C in between washes. Following, 50µL secondary antibody was added at these dilutions: goat anti-mouse IgG_{2a} FITC (Invitrogen, Carlsbad, CA M32201) 1:400, rat anti-mouse IgM APC (SouthernBiotech, Birmingham, AL 1140-11) 1:150, rat anti-mouse IgG_1 R-PE (SouthernBiotech, Birmingham, AL 1144-09) 1:200, rat anti-mouse IgG_{2b} R-PE (SouthernBiotech, Birmingham, AL 1185-09) 1:200. The plate was subsequently incubated on ice for 15 minutes in the dark and washed two times with cold PBS+1%BSA. Remaining pellets were resuspended in 100µL cold PBS+1%BSA and 100µL 4% paraformaldehye (Fisher Scientific Company, Middleton, VA) was added to each well. After incubation in the dark for 15 minutes on ice, cells were resuspended in cold 100µL PBS, transferred to microtiter tubes (Fisher Scientific Company, Middleton, VA), and stored overnight until analysis with a flow cytometer (BD FACSAria, BD Biosciences, San Jose, CA). For each sample, 10,000 total cells were collected and analysis was done by FlowJo software (Tree Star, Inc., Ashland, OR).

H. somni-specific ELISA

Antibody isotypes IgG_1 and IgG_2 were evaluated in serum against *H. somni* with an ELISA method after vaccination of cattle with 2mL Somubac (killed *H. somni* vaccine). All buffers used in this ELISA were the same as previously described in the fecal cortisol immunoassay. Briefly, half of a 96-well plate was coated with the 100µL/well Somubac vaccine (Pfizer Animal Health, Exton, PA), diluted 1:10 in a carbonate/bicarbonate buffer, and the other half coated with just carbonate/bicarbonate buffer and incubated overnight at room temperature. Coating solution was decanted and 300µL/well of blocking buffer was added. After a 3-hour incubation, plates were washed and 100µL undiluted serum samples were added to the plate along with an undiluted positive control and a 1:5 dilution in assay buffer thereof (5 times). The positive

control was a steer, hyperimmunized to the Somubac vaccine (2mL administered subcutaneously every two weeks for 10 weeks), located at the Virginia Tech Beef Center (Blacksburg, VA 24061); IgG_1 OD: 1.84±0.11; IgG_2 OD: 1.13±0.04. A negative control (FBS) and a blank well (assay buffer) were added as well. All samples and controls were added in duplicate on both the coated and non-coated side of the plate. Plates were incubated overnight at 4°C with gentle shaking. Plates were washed and a 100µL/well 1:1000 dilution of antibody was added (IgG_1-HRP, Serotec, Raleigh, NC MCA2440P; IgG_2-HRP, Serotec, Raleigh, NC MCA2441P). After a 2 hour incubation at 4°C with gentle shaking, plates were washed and 100µL of TMB substrate (Thermo Fisher Scientific Inc., Waltham, MA) was added to each well. Plates were incubated for 15 minutes before 100µL/well 2N H_2SO_4 (Ricca Chemical Company, Arlington, TX) was added. Absorbance was read at 450nm. The blank well was subtracted from all other wells. Any response on the non-coated side of the plate was subtracted from the corresponding "coated" well. Percent positivity was calculated for each sample by using the equation of a logarithmic curve generated (Microsoft Excel 2007) from dilutions of the positive control.

RNA Extraction

PAXgene Blood RNA tubes (Becton Dickson, Franklin Lakes, NJ) were stored at 4°C for 3 days and equilibrated to room temperature overnight. RNA was extracted from the blood tubes with the PAXgene Blood RNA Kit (Qiagen Inc., Valencia, CA) following the manufacturer's instructions. The blood tubes were centrifuged at 3220g for 10 minutes and the supernatant was decanted. Four mL of RNase-free water was added to the pellet and the tube was vortexed. The tubes were then centrifuged again at 3220g for 10 minutes. The supernatant was again decanted and 350µL resuspension buffer was added to the pellet and vortexed. The remaining sample was

transferred into a 1.5 microcentrifuge tube and 300µL binding buffer and 40µL proteinase K was added. Following a brief vortex, samples were incubated for 10 minutes in a shaker-incubator (Excella E24 Incubator Shaker Series, New Brunswick Scientific, Edison, NJ) at 350rpm and 55°C. The lysate was then pipetted into a PAXgene shredder spin column and centrifuged for 3 minutes at 20,000 g. Next, the supernatant of the flow-through fraction was transferred to a fresh 1.5mL microcentrifuge tube, 350µL ethanol (96-100% purity grade) was added and then briefly mixed. Subsequently, 700µL of that sample was added to the PAXgene RNA spin column and centrifuged for 1 minute at 14,000g. The flow-through was discarded and the previous step was repeated with the remaining amount of sample. Then, 350µL wash buffer was added to the PAXgene RNA spin column, centrifuged for 1 minute at 14,000g and the flow-through fraction was discarded. DNA was then digested with the addition of DNase. After a 15 minute incubation with DNase, 350µL wash buffer was pipetted into the PAXgene RNA spin column and centrifuged for 1 minute at 14,000g. Flow-through was once again discarded and 500µL wash buffer was added to the PAXgene RNA spin column followed by another centrifugation at 14,000g for 1 minute. The previous step was repeated with a centrifugation at 14,000 for 3 minutes. The PAXgene RNA spin column was then transferred into a fresh 1.5mL microcentrifuge tube, 40µL Buffer elution buffer was added, and centrifuged for 1 minute at 14,000g to elute the RNA. That step was repeated and the eluate was incubated for 5 minutes at 65°C and chilled on ice. RNA samples were stored at -80°C until analysis.

RNA Quantification

Samples from the PAXgene RNA isolation were evaluated for total RNA with the fluorescence-based Quant-iT RiboGreen Kit (Invitrogen Corporation, Carlsbad, CA) following the

manufacturer's directions. Briefly, 2µg/mL RNA standard was prepared by diluting 20µL of 100µg/mL RNA standard with 980µL nuclease-free 1X TE buffer (1:50 dilution). Standards were added to the plate diluted 1:2 with the range of (2µg/mL to 0.03125µg/mL). Samples were then diluted 1:100 in 1X TE buffer and 100µL of diluted sample was added to the plate. A blank well of 1X TE Buffer was used. Lastly, 100µL of diluted ribogreen (1:200 in 1X TE buffer) was added to each well. Ribogreen is light sensitive; therefore the plate was kept in aluminum foil until it was loaded into the plate reader. Plates were loaded into the plate reader and the fluorescence was read at 480nm emission and 520nm excitation. The blank well was subtracted from all other wells. RNA concentration for the samples was determined using a linear standard curve.

cDNA Production

Total RNA was reverse transcribed to complementary DNA (cDNA) using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Each RNA sample (volume required of each RNA sample to achieve 200ng concentration + RNase free water = 10µL) was added to a master mix containing 2µL 10x RT Buffer, 0.8µL 25x dNTP Mix, 2µL 10x RT Random Primers, 1µL MultiScribe Reverse Transcriptase and 4.2µL Nuclease-Free Water. Samples were mixed and briefly centrifuged before thermal cycling. Thermal cycler conditions were as follows: 10 minutes at 25°C, 120 minutes at 37°C, 8 seconds at 85°C. Samples were stored at -20°C until analysis by qPCR.

qPCR

Gene expression for GAPDH, 18S, IFNγ, IL-4, IL-10, and CD62L were analyzed with real-time PCR using the TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA). Each sample was run in duplicate for each gene. Reactions took place on MicroAmp Optical 96-Well Reaction Plates (Applied Biosystems, Foster City, CA). Each well had 1µL 20x TaqMan Gene Expression Assay (for the respective gene of interest), 10µL 2x TaqMan Universal PCR Master Mix, 1µL of the cDNA template (diluted 1:2 in RNase free water), and 8µL RNase-free water. A blank for each gene was used for each run by substituting RNase-free water for the cDNA template. 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 were: 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 Ct of the endogenous control (18S) resulting in a ΔCt. The ΔΔCt was calculated by subtracted the ΔCt of the day -6 (for each gene for each sample) from the ΔCt at day 1 or day 3. A fold increase was determined using $2^{-\Delta\Delta Ct}$ for each sample.

Statistical Procedure

All data was graphed before statistical analysis to look at distribution and assess normality. Groups (A, B) were analyzed separately before combining the data together from each group with group as a variable. Data was analyzed using repeated measure in the PROC MIXED procedure in SAS (SAS Institute, Inc., Cary, NC). The model included the variables weaning treatment, day and weaning treatment*day interaction; calf (weaning treatment) was the subject

and day was the repeated measure. Treatments within day were analyzed using the SLICE option. The Tukey adjustment was applied to test the significance between days. Significance was determined at $P \leq 0.05$.

Results

Fecal Cortisol

The stress related hormone cortisol measured in the feces was used as an indicator of stress in the calf around weaning. A treatment effect (P=0.0006), a date effect (P<0.0001), and a treatment*date interaction (P<0.0001) were detected (Figure 4.1). No significant differences were found at day -6, or day 3 between treatment groups. On day 1, abruptly weaned calves had significantly higher concentrations of the cortisol metabolite in the feces than fenceline weaned counterparts.

Weights

The body weights and average daily gain (ADG) for each treatment group over time are presented in Table 4.1. Body weights for both treatment groups increased over time (P<0.0001). Body weight differed between treatment groups (P<0.01) from the initiation of the study. Body weight at the start of the experiment was higher in abruptly weaned calves (P=0.0085). A difference in average daily gain was not detected between treatments.

Anti H. somni Antibodies

A difference in antibody titers (IgG_1 or IgG_2) against the *H. somni* vaccine was not detected between weaning groups. There was a date effect (P=0.0119) for IgG_1, but no date effect was detected for IgG_2 (Figure 4.2).

Flow Cytometry

No difference in treatment was detected for percentages of neutrophils or lymphocytes derived from lysed whole blood, but there was a treatment*date interaction (P=0.0107) for neutrophil percentages and a date (P=0.0379) and treatment*date interaction (P=0.0231) for lymphocyte percentages (Table 4.2). Lymphocytes (%) increased from day -6 to day 1 (P<0.05) and then decreased from day 1 to day 3 for fenceline calves (P<0.05), while lymphocytes (%) did not differ for abruptly weaned calves. Neutrophils (%) increased from day 1 to day 3 for fenceline calves (P<0.05), while neutrophils (%) decreased from day 1 to day 3 for abruptly weaned calves (P<0.05); neutrophils (%) did not change from day -6 to day 1 for either group. The cell markers CD4 and CD8 increased in both treatments over time (P<0.0001) from day -6 to day 3. While WC1 decreased in both treatment groups over time (P=0.0243) from day -6 to day 3 and TcR1 decreased in both treatment groups over time (P=0.0027) from day -6 to day 3. No date effect was found for CD21 (both treatment groups remained relatively constant for CD21 over time). None of the cell markers showed a significant difference in treatment or treatment*date interaction.

qPCR

Results from qPCR were analyzed using a relative quantification (ΔCt) against a endogenous control gene (18S) and are shown in Table 4.3 as the least squares means and standard error of the (ΔCt) for each gene in each treatment at a specific time point. No treatment effects were detected for any of the target genes analyzed via qPCR. However, a date effect was detected for GAPDH where the gene expression decreased over time for both treatment groups (P<0.0001) from day -6 to day 3. A date effect was also detected for CD62L; this gene decreased over time

for both treatment groups (P=0.0006) from day -6 to day 3. However, no treatment*date interaction was found for GAPDH or CD62L. No date effect or treatment*date interaction was found for IFNγ, IL-4, or IL-10.

Discussion

Stressful situations, like weaning, cause increases in hormones like cortisol which can have a detrimental effect on the immune system. Researchers have observed subsequent alterations to immune function associated with increases in stress hormones. Those changes include cytokine expression (Fisher, Crowe et al. 1997; Hickey, Drennan et al. 2003), neutrophil function (Burton, Madsen et al. 2005), lymphocyte populations (Burton and Kehrli 1996; Anderson, Watson et al. 1999), and antibody responses (Estes and Brown 2002). Cortisol metabolites should peak in the feces 14-18 hours after the stressful event (Morrow, Kolver et al. 2002). Our calves were sampled six days prior to weaning (day -6) the day after weaning (day 1) and three days after weaning (day 3). Abruptly weaned calves in the present study had a peak in fecal cortisol post weaning which concurs with the literature with peaks in blood cortisol post weaning (Lefcourt and Elsasser 1995; Hickey, Drennan et al. 2003).

Weaning method is associated with weight gain; fenceline weaned calves on average gained more weight than abruptly weaned calves indicated by average daily gain (Price, Harris et al. 2003; Haley, Bailey et al. 2005; Boland, Scaglia et al. 2008). However, in our study there was no difference between treatment groups in average daily gain. As previously discussed in Chapter 3, the calves in this study were younger (~148 days old) and weighed less (~181 kgs) as compared to Price et al., 2003 and Boland et al., 2008. Arthington et al. 2005., observes that abruptly weaning calves at an earlier age decreases the stress involved with weaning and increases average daily gain and the feed to gain ratio. Thus, since our calves are younger the stress response may be less and consequently the abruptly weaned calves have better average daily gain in association with the weaning period. In the future, using older calves (closer to ~205 days of age) would help in comparison of our experiment to already published literature.

Blocking by weight before randomly allotting calves into treatments (so that initial body weights for each group are approximately the same) would be helpful in determining differences in weight gain over the course of the experiment. Also, measuring metabolic parameters like glucose, blood urea nitrogen (BUN) and non-esterified fatty acid (NEFA) similar to Boland (2008) (decreased feed intake causes decreases in glucose and increases in BUN and NEFA) would be helpful in determining feed intake. Another group could be added to the experimental design by using a dexamethasone injection; this group could be used as a positive control (i.e. dexamethasone is known to induce a Th2 response in cattle).

Antibody response in cattle after the pathogen challenge or vaccination can be biased to either a Th1 (IgG_2) or Th2 response (IgG_1) (Estes and Brown 2002). *Histophilius somni*, previously known as *Haemophilus somnus*, induces a predominantly Th1 response (Berghaus, Corbeil et al. 2006). The calves in this study were administered a killed vaccine of *H. somni* the day after weaning (day 1). Antibody titers to the vaccine were measured in the serum on day 1, day 8, day 15, and day 22; there was no difference between treatment groups for antibody titers to *H. somni*. The results agree with our last study where antibodies to RB51, another Th1 inducing vaccine, were not different between treatment groups. Gershwin et al., 2005 observed increased IgG_1 and IgG_2 after infection with H. somni, but titers increased from day 13 to 28 post infection. Since IgG titers were measured from day 1 and 22 and no significant response was observed, measurements may need to be extended. The vaccine in this study includes an adjuvant aluminum hydroxide which has been implicated in producing a Th2 response (Estes and Brown 2002). Therefore, use of another vaccine for analysis of antigen-specific antibodies know to induce a strong Th1 response may be helpful. Also, vaccination with Pyramid 5 prior to vaccination with *H. somni* may influence the response to the vaccine especially if Pyramid 5

induces a Th2 response. Platt et al., 2006 reported a Th1 bias while using a similar vaccine to Pyramid 5. But, Berghaus et al., 2006 reported a Th2 response to BRSV (one of the pathogens in the pyramid 5 vaccine). In future studies, vaccination with Pyramid 5 prior to the start of the study should be eliminated or the vaccine response and potential bias to a Th1 inducing vaccine like RB51 or *H. somni* should be determined. The vaccine in this study includes an adjuvant aluminum hydroxide which has been implicated in producing a Th2 response (Estes and Brown 2002). Therefore, use of another vaccine or antigen that already has an established protocol or kit yielding quantitative results versus qualitative results may aid in the analysis of antigen-specific antibody concentrations. For future studies isolation and stimulation of B cells *ex vivo* with the vaccine may be more beneficial because the procedure would be more controlled and sensitive.

Lymphocyte phenotypes were determined via flow cytometry at day -6, day 1, and day 3. The cell markers CD4 (T helper cells), CD8 (T cytotoxic cells), WC1 (a subset of γδ T cells), TcR1 (γδ T cells), and CD21 (B cells) were analyzed. Neutrophil and lymphocyte populations were also measured. Weaning treatment did not have an effect on the lymphocyte phenotypes, but date did. Dexamethasone, used as a synthetic inducer of stress, decreases γδ T cells using the cell marker WC1 and an increase in αβ T cells using CD4 (Burton and Kehrli 1996; Menge and Dean-Nystrom 2008). The results from the current study agreed with previous studies in that the αβ T cell markers CD4 and CD8 increased over time and the γδ T cell markers decreased over time. However, we did not see a change in the B cell population while Nonnecke et al., 1997 recorded a decrease in B cells following a stressful event. Also, Hickey et al., 2003 observed increases in stress hormones associated with neutrophilia and lymphopenia, which we did not observe in this study. Burton (1996), as well as the present study, used percentages of cell

markers by counting the number of cells that express the cell specific cell marker in 5,000 and 10,000 events respectively. Using percentages to measure cell populations can be problematic in that increases or decreases in cell percentages could be compensatory. Therefore, obtaining accurate total leukocyte counts prior to analysis with flow cytometry would aid in converting percentages to actual values. In future studies, the lysis method used for this method should be validated; because the buffer used for lysis was not validated before use it could negatively affect our results by altering cell populations. Flow cytometry could also be used to assess neutrophil function by looking for the CD62L cell marker on neutrophils.

Lymphocyte phenotypes also change as the calf grows older. With increasing age αβ T cell increase and γδ decrease up until approximately 6 months of age (Barrington and Parish 2001). In our study calves' immune systems were still maturing. Therefore, the changes in lymphocyte phenotypes could be due to age and not weaning. The use of older (>6 months) calves and/or the addition of an unweaned control group to the experimental procedure will help to eliminate the confounding factor of age on immune response.

Gene expression of GAPDH, CD62L, IFNγ, IL-4 and IL-10 was determined via real time PCR analysis. The leukocyte migration cell marker, CD62L or L-selectin, decreases in neutrophils during stress or increases in cortisol (Weber, Madsen et al. 2001; Weber, Toelboell et al. 2004; Burton, Madsen et al. 2005). The gene expression for CD62L in both the fenceline and abrupt group decreased after weaning, which is in accordance with the literature cited. Interestingly, CD62L down regulation is normally associated with an increase in neutrophils in the blood. However, neutrophilia was not observed. However, measurements of gene expression in this study were on whole blood samples versus neutrophils populations only; therefore, CD62L down regulation may be on leukocytes other than neutrophils. In future

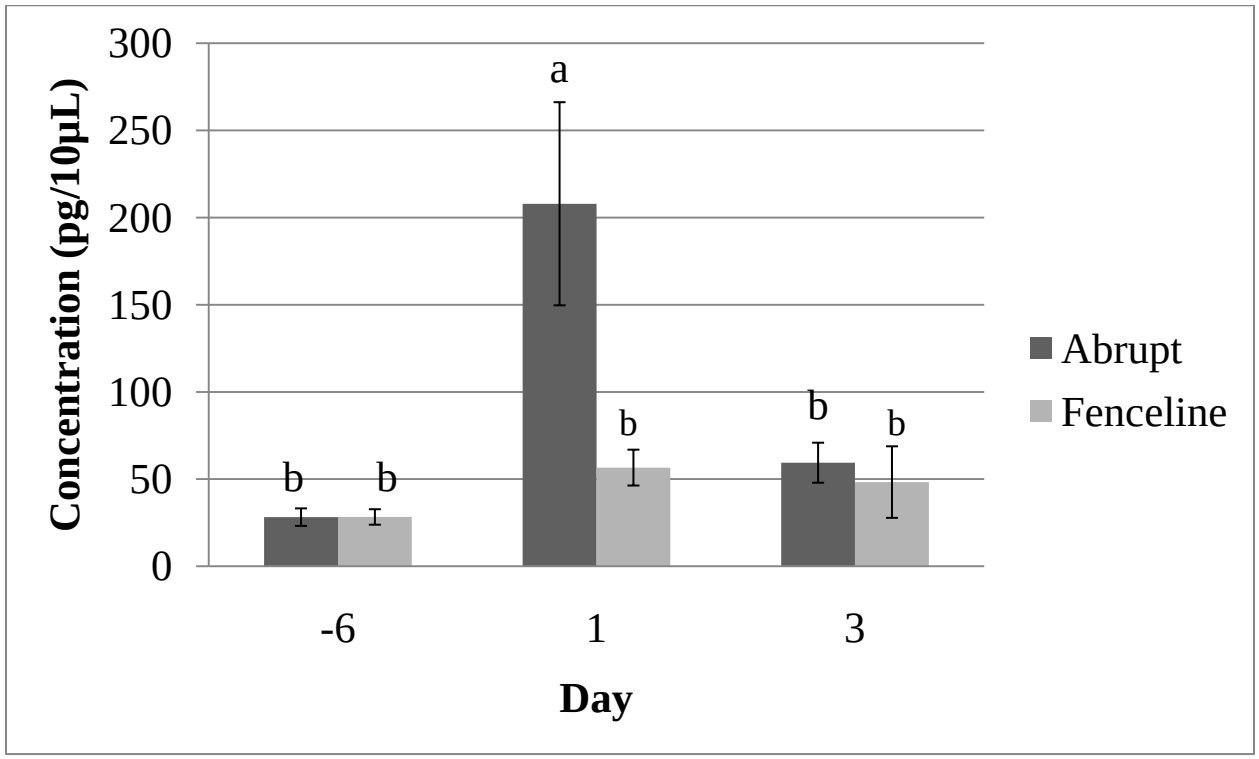
studies, isolation of specific cell types, both neutrophils and lymphocytes would help determine if CD62L is decreasing in neutrophils or another cell population. Glyceraldehyde 3-phosphate dehydrogenase or GAPDH is usually used as an endogenous control for qPCR. However, after trial runs we noted that GAPDH expression did not remain constant. So we used 18S as a control instead, and switched GAPDH to a target gene. We noted a decrease in GAPDH gene expression after weaning. The gene GAPDH is an enzyme that aids in the breakdown of glucose, but has also been implicated in the initiation of apoptosis (Dastoor and Dreyer 2001). Stress or elevated cortisol downregulates genes that code for apoptosis (Chang, Madsen et al. 2004; Burton, Madsen et al. 2005), therefore if GAPDH codes for apoptosis it may be downregulated as well; further analysis on both pro-apoptotic (Fas, FADD, Daxx, etc.) and anti-apoptotic genes (BAFF, TANK, TRAF-6, etc.) in isolated neutrophil populations would provide further insight into the status of programmed cell death during the weaning period. The cytokines IFNγ, IL-4 and IL-10 did not change over time and there was no difference for weaning method. We only analyzed the cytokines for three days post weaning; the analysis might need to be extended to seven days post weaning to observe the change in cytokine expression. The graphs of gene expression indicate a downward trend in the cytokines IFNγ, IL-4, and IL-10, but the change is not significant suggesting that the Th1/Th2 paradigm may not hold true for cattle as it does for mice. Analyzing additional cytokines like IL-12 and IL-2 (Th1) and IL-13 and IL-5 (Th2) would help in identifying a Th1 or Th2 bias associated with weaning. Furthermore, stimulating T cells in vitro with Con-A, KLH, or another Th1 inducing antigen for analysis of cytokine production may be more beneficial then gene expression of IFNγ in whole blood samples. This procedure may be more effective because it would assess cytokine gene expression in T cells alone, while using whole blood analyzes cytokine production in all

immune cells, like dendritic cells that produce high concentrations of IFNγ. By isolating the specific cell type of interest, in this case T cells, the analysis would be more specific.

In conclusion, weaning treatment altered the concentration of cortisol secreted in response to stress, but did not significantly affect particular immune responses which rejects the hypothesis generated before the experiment. Since abrupt weaning did not induce a strong Th2 response, the effects of fenceline weaning could not be assessed in comparison to abrupt weaning. Changes in analysis method, timing of collection, or sample evaluated could provide more sensitivity. Results support the idea that two-stage weaning decreases the stress response, but does not show a significant difference in immune function; however fenceline weaning may differ from abrupt weaning and this experiment did not detect those differences. Therefore, utilizing fenceline weaning may not benefit mortality or morbidity, but further investigation is needed to verify these results. The *H. somni* vaccine did not produce a strong Th1 response in the calves from this study; therefore it should not be used again in an experiment like this. The absence of an unweaned control group limited the conclusions of the data from this study. In future studies incorporation of an unweaned control group would help to determine if the changes to immune function are due to weaning or age. Abruptly weaned calves could also be stressed more by comingling them. The reorganization of social structure is an important stressor involved with weaning in the industry; therefore, adding a comingling stage to the experimental design would be beneficial. Also, isolating specific cell populations for analysis of gene expression would aid in evaluation of changes in cytokine production by T cells and migration and apoptosis of neutrophils. Lastly, the timing of sample collection is important to measure of immunological changes successfully; sample collection for gene expression should be extended to 7 days post weaning and sample collection for antibody production should be

extended to more than 28 days post vaccination. Calves for future studies should be older than 6 months to help decrease the effect of age on the immune system. In upcoming experiments, the experimental procedure and vaccine utilized in this study should not be applied again; changes should be made to the type of sample analyzed, the methods of sample collection and timing of sample collection. Also addition of an unweaned control group and/or a positive control group (dexamethasone injection) would be helpful in the analysis of the immune response to abrupt and fenceline weaning.

Figure 4.1 Cortisol metabolite concentrations[1] from fecal samples collected[2] from abruptly (n=22) and fenceline (n=23) weaned calves at specific days prior to and following vaccination. Weaning occurred at day 0. Vaccination with Somubac occurred at day 1.



[a,b] Means with unlike letters are different (P<0.05)

[1] Values expressed as least squares means ± standard deviation

[2] Metabolites show up in the fecal matter 12-18 hours after the induction of stress

Table 4.1 Live weights[1] and average daily gain[1] from abruptly (n=22) and fenceline (n=23) weaned calves at specific days prior to and following weaning and vaccination. Weaning occurred at day 0. Vaccination with Somubac occurred at day 1.

	Live Weight (kgs) and Average Daily Gain (kgs/day)					
	Day[2,4]					
Treatment[3]	-7[a]	0[b]	8[b]	22[b,c]	SE	ADG
Abrupt	192	197	196	201	5.92	0.32
Fenceline	170	174	176	177	5.74	0.25

[1]Values expressed as least squares means with pooled standard error (SE)

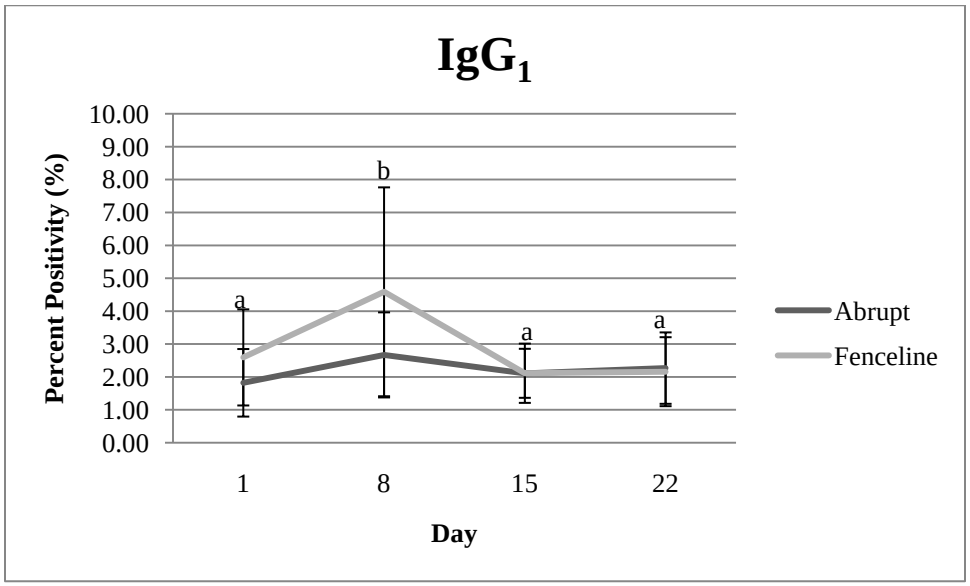
[2]Body weights for calves increase over time $P < 0.0001$

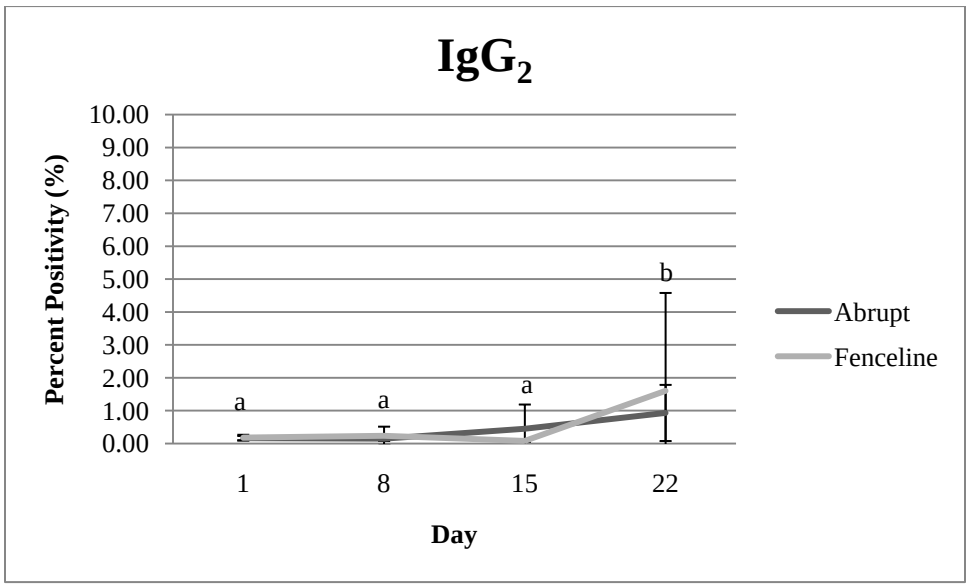
[3]Treatments differed by $P < 0.01$

[4]Treatments significantly differed at day -7 ($P = 0.0085$), day 0 ($P = 0.0067$), day 8 ($P = 0.016$), and day 22 ($P = 0.0044$)

[a,b,c]Day with no common superscript differ ($P < 0.05$)

Figure 4.2 Percentage[1] of *H. somni* specific antibodies[2] (IgG_1 and IgG_2) from serum samples for abruptly (n=6) and fenceline (N=6) weaned calves at specific days prior to and following vaccination. Weaning occurred at day 0. Vaccination with Somubac occurred at day 1.





[1] Percentage was determined using a positive control (sample OD value/positive control OD value x 100%)

[2] Values expressed are least squares means ± standard deviation

[a,b,c] Means within day with no common superscript differ ($P<0.05$)

Table 4.2 Least squares means and pooled standard error for cell surface markers within treatment and time point: CD4 (T helper cells), CD8 (T cytotoxic cells), WC1 (γδ T cells), TcR1 (γδ T cells), and CD21 (B cells)

Cell Type	Percentage of Cells Expressing Specific Marker via Flow Cytometry (%)						
	Day -6		Day 1		Day 3		
	Abrupt[2]	Fenceline[2]	Abrupt[2]	Fenceline[2]	Abrupt[2]	Fenceline[2]	SE
Neutrophils [3]	27.2	26.1	26.4	22.5	22.5	27.0	2.12
Lymphocytes[1,3]	57.0	58.3	59.5	64.0	61.6	56.5	2.66
CD4+[1]	10.6	9.2	14.6	15.1	15.1	15.5	1.30
CD8+[1]	15.9	17.1	18.7	21.3	23.5	25.2	1.17
WC1+[1]	15.0	19.8	13.2	17.8	13.1	16.9	2.03
TcR1+[1]	34.6	38.0	30.5	34.1	32.0	33.3	2.51
CD21+	39.1	37.8	39.3	34.3	37.3	35.4	2.33

[1]Date effect of $P<0.05$

[2]No significant difference in treatment was detected

[3]Treatment*date interaction of $P<0.05$

Table 4.3 Gene expression in table represented with the least squares means and pooled standard error of the ΔCt of each treatment. The genes in this table are for GAPDH (enzyme involved in glycolysis and apoptosis), CD62L (leukocyte migration), IFNγ (Th1 cytokine), IL-4 (Th2 cytokine), and IL-10 (Th2 cytokine)

Gene	ΔCt of Target Gene Against Endogenous Contol (18S)						
	Day -6		Day 1		Day 3		
	Abrupt[2]	Fenceline[2]	Abrupt[2]	Fenceline[2]	Abrupt[2]	Fenceline[2]	SE
GAPDH[1]	2.1	2.4	1.5	1.7	1.7	1.7	0.1
CD62L[1]	1.4	1.6	1.0	1.1	1.0	1.1	0.2
IFNγ	16.0	16.2	15.6	16.0	15.6	15.7	0.2
IL-4	13.4	13.9	13.6	13.7	13.7	13.2	0.2
IL-10	11.3	11.6	10.9	11.1	11.2	11.3	0.3

[1]Date effect of $P < 0.001$

[2]No significant difference in treatment was detected

Chapter 5
Summary and Conclusions

The author concludes that alternative weaning methods decrease the transient cortisol increase from cow-calf separation but does not influence the immune response post weaning as detected based on the assays performed. Abruptly weaned calves had significantly higher concentrations of cortisol metabolites the day after weaning as compared to their fenceline counterparts. Weaning methods had no affect on the vaccine-specific antibody response for *B. abortus* or *H. somni*. Calves in both weaning treatments exhibited increases in percentage of CD4 and CD8 cell markers and decreases in the percentages of WC1 and TcR1 cell markers via flow cytometry and decreased the gene expression for GAPDH and CD62L via qPCR; however, no treatment effect was detected for any of these parameters. Increases in CD4 and CD8 percentages and decreases in WC1 and TcR1 percentages may indicate that the stress response induces a decrease in γδ T and increase αβ T cells. Decreased gene expression for GAPDH and CD62L may result in alteration to neutrophil function post weaning by decreasing neutrophil cell migration and apoptosis. Decreased migration of neutrophils out of the circulation and elongation of the cell's lifespan should cause an increase in the neutrophil population in the blood; however there was no elevation in neutrophil percentages. Further investigation using other genes that code for neutrophil migration and apoptosis and assessment of those genes on isolated neutrophil populations will provide insight into neutrophil function post weaning. Cytokine expression in whole blood did not differ between treatments or days for IFNγ, IL-4 and IL-10; in this case gene expression may need to be analyzed up to day 7 post weaning. Isolation of specific cell populations, like lymphocytes, for assessment of cytokine gene expression and antibody production will aid in the assessment of immunological changes post weaning.

Addition of an unweaned control group in future studies will aid in determining if the changes to immune function are induced by weaning or age.

Based off the results from the last two years abrupt weaning is associated with higher concentrations of fecal cortisol metabolites than fenceline weaned calves. However, these differences in cortisol do not lead to differences in immunological parameters. Therefore, fenceline weaning may not alleviate changes in immune function post weaning. Adjusting the analysis method, timing of collection, or sample could provide more insight for future studies. The *H. somni* and RB51 vaccine did not elicit a strong Th1 response in the experiments over the past two years; therefore these antigens should not be used again in an experiment like this. The addition of an unweaned control group may enhance the conclusions for similar experiments and would help determine if the alterations to immunological parameters are due to weaning or age. Because cytokine expression in this thesis was analyzed in whole blood, subsequent changes may be in cells other than T cells. Therefore, isolation of lymphocytes for cytokine measurements would aid in evaluation of alterations to T cells. Also, the timing of sample collection is essential in the ability to measure immunological changes successfully; thus, gene expression analysis should be extended to 7 days post weaning and antibody production analysis should be extended to >28 days. In the future, the experimental procedure and vaccine utilized in these studies should not be applied again; changes should be made to the type of sample analyzed, the methods of sample collection and timing of sample collection.

APPENDIX A

ELISpot for bovine IFNγ from PBMCs stimulated with RB51

Figure 1

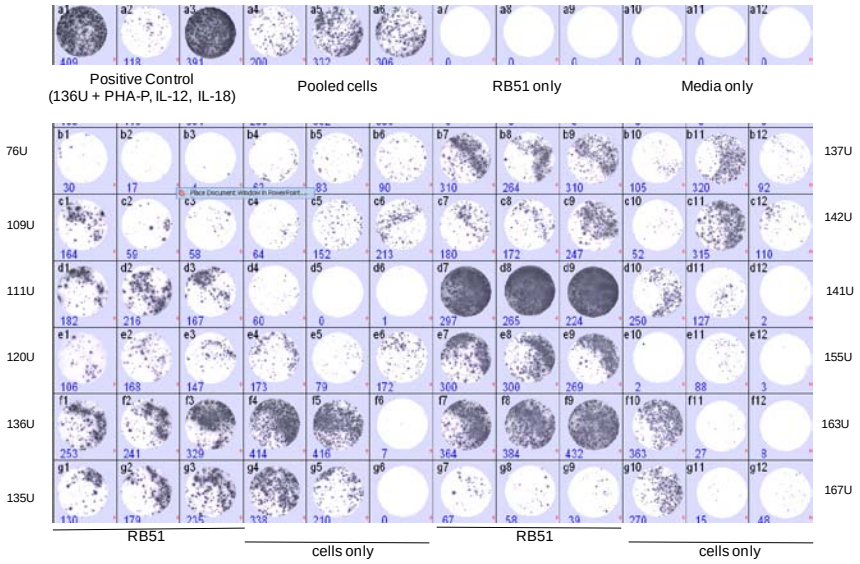
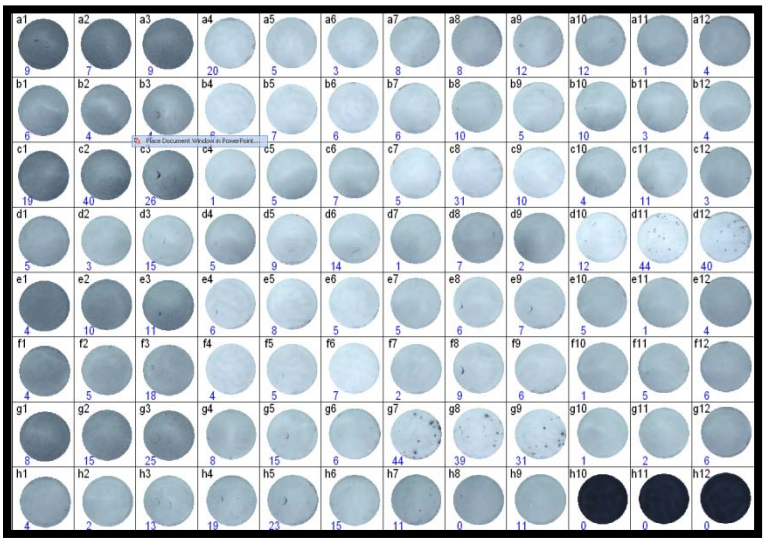


Figure 2



Materials and Methods: Blood collected into four evacuated tubes containing lithium heparin (Becton Dickson Inc., Franklin Lakes, NJ) was combine for each animal in a sterile 50mL conical tube (Becton Dickson Inc., Franklin Lakes, NJ); amount of blood collected, read as an

approximate value on the conical tube, was recorded for later use. The blood was then diluted 1:2 in sterile PBS, pH 7.4 (Invitrogen, Carlsbad, CA) at room temperature. 8-10mL of the diluted blood was layered onto 5mL of Lymphoprep (Greiner Bio-One, Monroe, NC) in a 15mL tube (Becton Dickson Inc., Franklin Lakes, NJ). Next, layered blood was centrifuged for 30 min at 1900rpm (800 g) at room temperature with no brake or acceleration. After centrifugation, the plasma (top layer) was removed and discarded and the layer containing the lymphocyte band was pipette into 10mL cold RPMI-1640 (Invitrogen, Carlsbad, CA) in a fresh 15mL tube. Then, the lymphocytes were centrifuged for 7 minutes at 1400rpm at 7°C with the brake and acceleration at 7. Supernatant was discarded and pellet was resuspended in a fresh 10mL of RPMI-1640. An aliquot was used to determine the cell count on a Multisizer 3 Coulter-Counter (Beckman Coulter, Brea, CA).

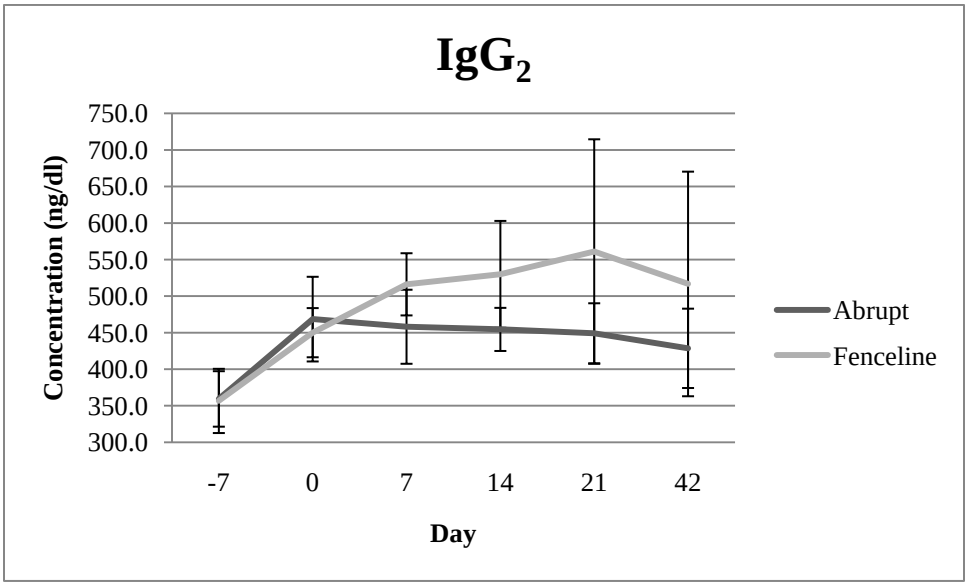
Lymphocyte suspensions were adjusted to a concentration of $1x10^6$ cells/mL using the cell counts from the Multisizer and used for the ELISpot assay according to the manufacturer's instructions (ELISpot for bovine IFNγ, Mabtech, Nacha Strand, Sweden). Briefly, PVDF type EIIP (Millpore Corp., Billerica, MA) plates were coated the night before lymphocyte isolation with 70% EtOH for 2 minutes maximum, washed five times with 200µL/well sterile HOH (Invitrogen, Carlsbad, CA), 100µL/well of capture antibody was added, and incubated overnight at 4°C. After lymphocyte isolation, plates were washed five times with 200µL/well sterile H_2O and 200µl RPMI+10%FBS (Sigma-Aldrich, St. Louis, MO) was added to each well. Following an incubation for 30-120 minutes at room temperature on a rocker, media was discarded and $1x10^6$ cells/well in 50µL was immediately added in to six wells and then 50µL irradiated RB51 strain (stimulant) were added to three wells and 50µL media (non-stimulant) was added to the

remaining three wells. A positive control well was on each plate using a randomly chosen calf stimulated with phytohemagglutinin (Sigma-Aldrich, St. Louis, MO), recombinant human IL-12 (Serotec, Raleigh, NC) and recombinant human IL-18 (Thermo Fisher Scientific Inc., Waltham, MA). A negative control well with the same random calf with no stimulation and a blank well that had no cells only media was run on each plate. After the addition of samples and stimulants plates were incubated at 37°C with 5% CO_2 for 18 hours. Following the incubation, plates were washed five times with 200µL/well PBS and 100µL/well detection antibody was added. After a 2 hour incubation at room temperature plates were washed five times with 200µL/well PBS. Then 100µL/well Streptavidin-ALP (diluted 1:1000 in sterile PBS-0.05% FBS) was added and plates were incubated for 1 hour at room temperature. After another wash 100µL substrate solution (BCIP/NBT-plus filtered through a 0.45um filter) was added to each well. Plates were developed until distinct spots emerged. Color development was stopped by washing extensively with tap water. After allowing the plate to dry analysis was performed on an ELISpot Reader (AID Autoimmun Diagnostika GmbH, Strassberg, Germany).

Results: ELISpot results for fresh lymphocytes at each time period could not be compared because different positive and negative controls were run at each time point (Figure 1). Therefore, cryopreserved lymphocytes were thawed in a 37°C water bath and resuspended in pre-warmed complete media. Cell counts were reanalyzed on a multiziser. ELISpot results with cryopreserved cells yielded no cytokine response in the lymphocytes (Figure 2). For the future assessing cell viability with a hemocytometer in addition to cell counts on the multisizer will aid in the addition of $1x10^6$ cells that are live and therefore able to produce cytokines.

APPENDIX B

Mean (±SD) for total serum IgG_2 antibodies SRID

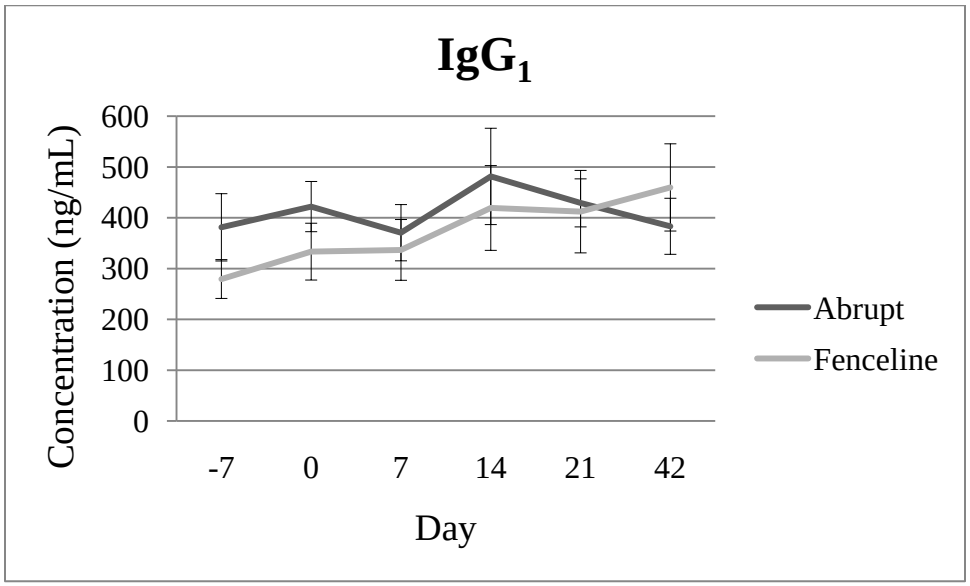


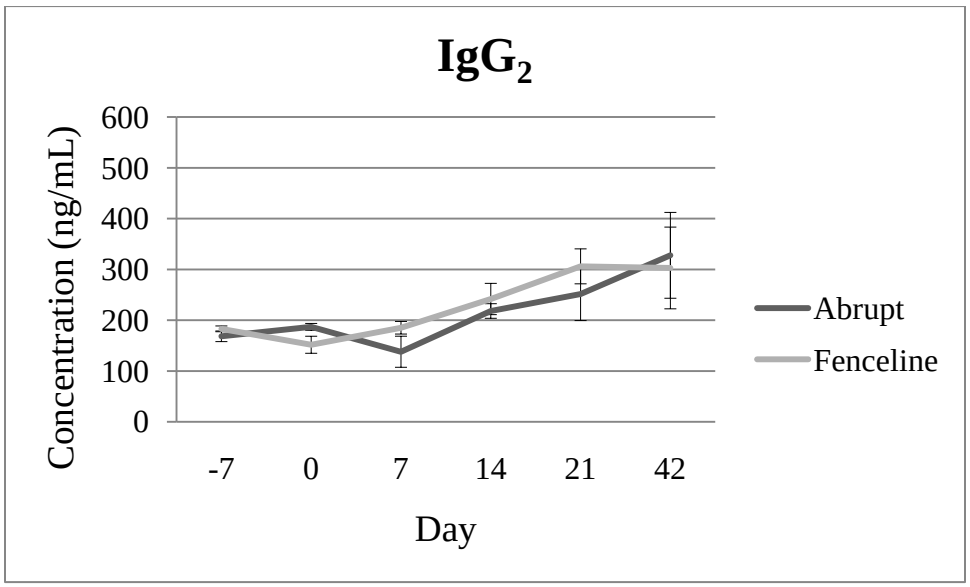
Material and Methods: Assay was performed as per the manufacturer's instructions (VMRD, Pullman, WA). Briefly, 3µL of standard or undiluted serum sample was added to each well without disturbing the agar (standards only needed run on one plate). Next, plates were covered and incubated at room temperature for 18-24 hours. After the incubation, the diameter was measured in millimeters either by using the graduated scale on the plate or by using a comparator (available from VMRD, catalog number 011). Then the standards were used to generate a linear curve in Microsoft Excel 2007 with the log concentration on the y-axis and the diameter on the x-axis). Afterwards, the log concentration of each sample was found using the equation of the standard curve. Finally, the concentration was calculated using the inverse of the log concentration.

Results: No treatment effect, date effect, or treatment*date interaction was detected for the IgG_2 total antibody concentration. However, date (P=0.0575) was approaching significance.

APPENDIX C

Mean (±SD) Total Serum IgG_1 and IgG_2 ELISA





Materials and Methods: The assay was performed according to the manufacturer's instructions (Bethyl Laboratories, Inc., Montgomery, TX). Briefly, 96-well ELISA plates were coated with 100µL/well capture antibody (Sheep Anti-Bovine IgG_1 Antibody Affinity Purified or Sheep Anti-Bovine IgG_2 Antibody Affinity Purified) diluted 1:100 in coating buffer (0.05 M

Carbonate-Bicarbonate, pH 9.6). After a 60 minute incubation at room temperature, plates were washed 3 times with 300µL/well wash buffer (50 mM tris, 0.14 M NaCl, 0.05% tween 20, pH 8.0). Then the plate was blocked with 300µL/well blocking buffer (50 mM tris, 0.14 M NaCl, 0.05% tween 20, pH 8.0) and incubated for 30 minutes at room temperature. After another wash, 100µL/well standards (range 10,000-7.8 ng/mL) and diluted samples (1:33,600 for the IgG_1 ELISA; 1:60,000 for the IgG_2 ELISA) were added and incubated for 60 minutes at room temperature. Then, plates were washed 5 times and 100µL/well diluted HRP conjugate (Sheep anti-Bovine IgG_1 Antibody HRP Conjugated, diluted 1:85,000; Sheep anti-Bovine IgG_1 Antibody HRP Conjugated, diluted1:10,000) in conjugate diluent (50 mM tris, 0.14 M NaCl, 0.05% tween 20, pH 8.0). Following a 60 minute incubation, plates were washed 5 times and 100µL/well development buffer (1 volume TMB substrate to 1 volume Solution B) was added. Plates were incubated for 15 minutes and then the reaction was stopped with 100µL/well 2M H_2SO_4. Plates were read at 450nm on a plate reader. Sample concentration was generated from the equation of the standard curve after the blank well OD value was subtracted from all other wells.

Results: No treatment effect, date effect, or treatment*date interaction was detected for the IgG_1 total antibody concentration. However, date for IgG_1 (P=0.0577) was approaching significance. No treatment or treatment*date interaction was detected for IgG_2 total antibody concentration. However, a date effect was detected (P<0.0001).

APPENDIX D

Average enviornmental conditions recorder at Kentland Farm (Blacksburg, VA, 37°11´ N, 80°35´ W) over the collection period[5] of September, October, and November, 2008

	Daytime[1]			Nighttime[2]		
Condition	September	October	November	September	October	November
Temperature (°C)	20.8	13.5	6.8	15.3	6.8	2.0
Relative Humidity (%)	72.6	63.8	64.0	92.7	88.6	82.2
Wind Speed (km/hr)	5.3	6.0	7.3	1.3	2.1	4.3
Soil Temperature (°C)	19.2	13.4	7.4	19.6	13.8	7.7
Sunlight (kW/m^2)	0.4	0.3	0.2	0.0	0.0	0.0
THI[4]	67.6	56.7	47.0	59.5	45.2	37.8
Precipitation (0.01 in)[3]	0.2	0.1	0.2			

[1]Daytime condition averages calculated from 8:00am to 7:59pm

[2]Nighttime condition averages calculated from 8:00pm to 7:59am

[3]Precipitation calculated as average rainfall per day

[4]Temperature Humidity Index = (0.8 x Temperature) + [(% Relative Humidity/100) x (Temperature – 14.4)] + 46.4

[5]Samples were collected on 9/30/08, 10/7/08, 10/14/08, 10/28/08, and 11/18/08

APPENDIX E

Example SAS Code

```
dm 'clear log';  dm 'clear output';
options ls=90;

data 2008; set work.2008jmpfile;

ods rtf;
ods graphics on;

proc mixed data=2008;
class weaning_treatment calf_id date;
model fecal_cortisol=weaning_treatment|date;
repeated date / subject=calf_id(weaning_treatment) type=ar(1);

lsmeans weaning_treatment date / adjust=tukey;
lsmeans weaning_treatment*date / slice=date;

title 'Fecal Cortisol';

proc mixed data=2008;
class weaning_treatment calf_id date;
model weight=weaning_treatment|date;
repeated date / subject=calf_id(weaning_treatment) type=ar(1);

lsmeans weaning_treatment date / adjust=tukey;
lsmeans weaning_treatment*date / slice=date;

title 'Weight';

run;

ods rtf close;
ods graphics off;
```

APPENDIX F

Average enviornmental conditions recorded at Shenandoah Valley Agricultural Research and Extension Center (Steeles Tavern VA, 37°92´ N, 79° 0´ W) over the collection period[5] of September and October, 2009

	Daytime[1]		Nighttime[2]	
Condition	September	October	September	October
Temperature (°C)	18.7	13.2	13.5	8.3
Relative Humidity (%)	66.4	65.0	87.3	84.4
Wind Speed (km/hr)	7.5	8.7	3.6	5.1
Soil Temperature (°C)	20.0	14.7	19.6	13.7
Sunlight (kW/m^2)	0.3	0.3	0.0	0.0
THI[4]	64.3	56.2	56.4	47.9
Precipitation (0.01 in)[3]	0.4	0.3		

[1]Daytime condition averages calculated from 8:00am to 7:59pm

[2]Nighttime condition averages calculated from 8:00pm to 7:59am

[3]Precipitation calculated as average rainfall per day

[4]Temperature Humidity Index = (0.8 x Temperature) + [(% Relative Humidity/100) x (Temperature – 14.4)] + 46.4

[5]Samples were collected on 9/9/09, 9/16/09, 9/18/09, 9/23/09, 9/30/09 and 10/7/09

APPENDIX G

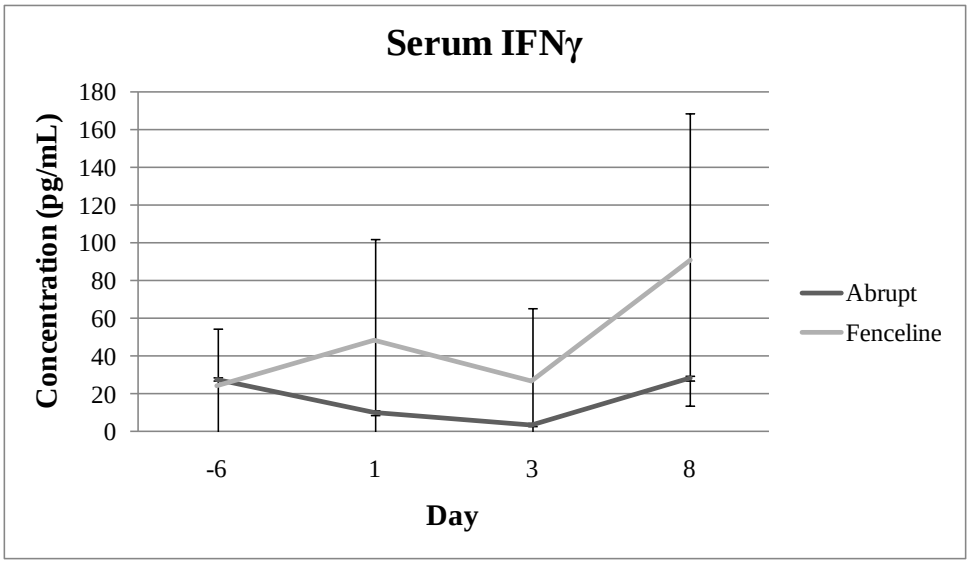
Least squares means and pooled standard error for IFNγ (pg/mL) for each treatment at different days

	Day[2]				
Treatment[1]	-6	0	3	8	SE
Abrupt	28.2	10.1	3.9	28.6	19.42
Fenceline	24.0	48.9	26.6	91.4	18.99

[1]No differences in treatments were detected

[2]Date effect of $P<0.05$

Least squares means (±SD) for serum IFNγ ELISA

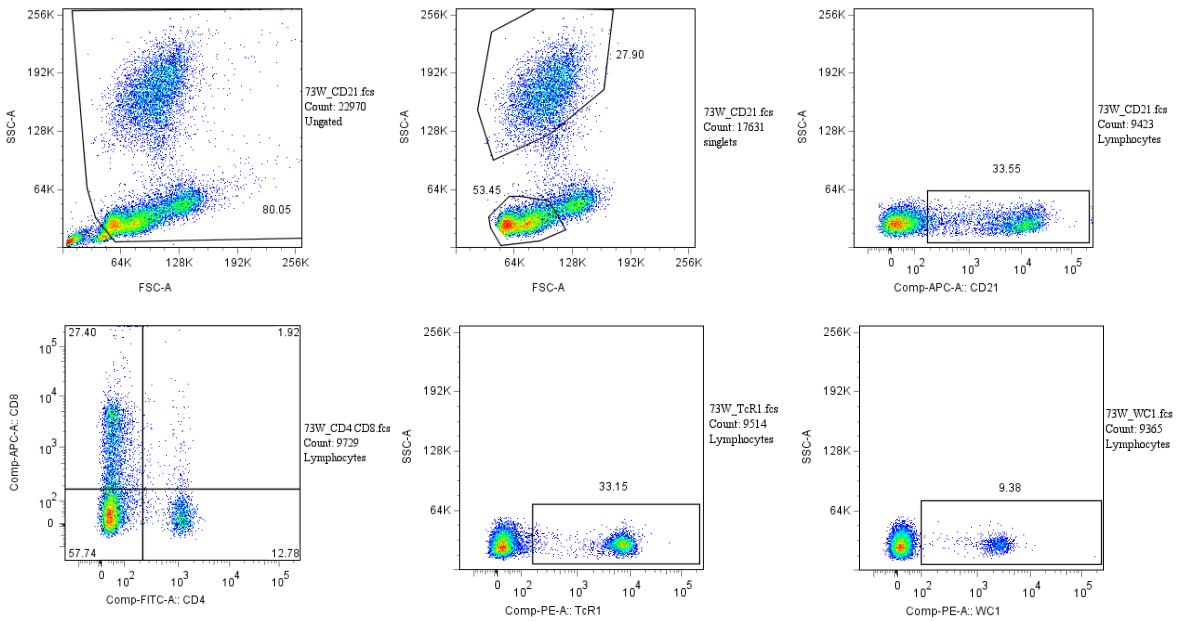


Material and Methods: protocol shown in Chapter 3

Results: 75% of the samples that had measurable IFNγ concentrations were from calves that were fenceline weaned.

APPENDIX H

Representative Flow Cytometry Diagram
Generated with FlowJo Software and BD FACSAria



Calf 73W (Group B Fenceline Weaning)
From left to right, top to bottom:
Graph 1: RBCs and dead cells were gated out
Graph 2: Neutrophil and lymphocyte populations
Graph 3: Gated for the CD21 marker (APC)
Graph 4: Gated for CD4 and CD8 markers (FITC and APC respectively)
Graph 5: Gated for the TcR1 marker (R-PE)
Graph 6: Gated for the WC1 marker (R-PE)
The cell markers were evaluated in the lymphocyte population only
SSC: side scatter
FSC: forward scatter

APPENDIX I

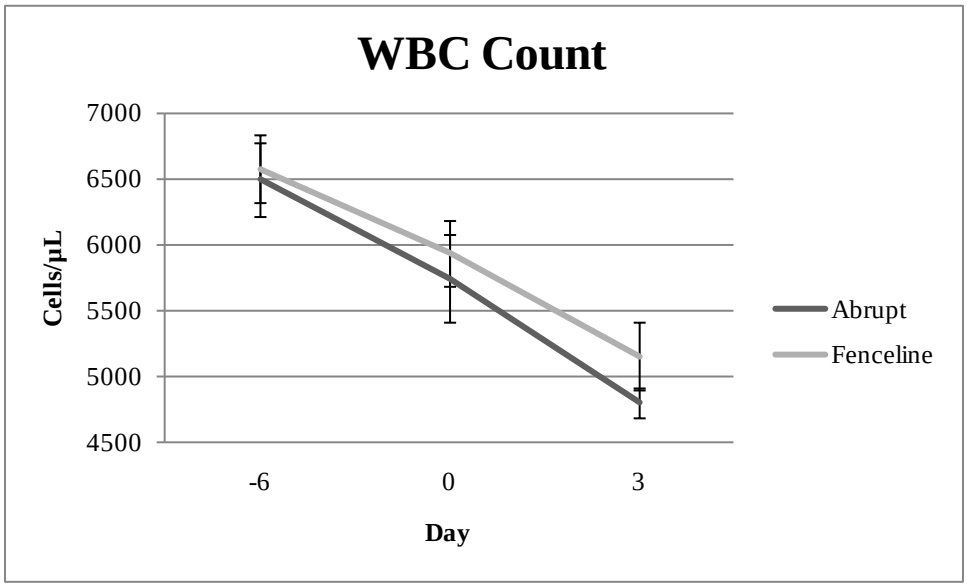
Least squares means and standard error for white blood cell count (cells/µL) for each treatment over time

	Day[2]			
Treatment[1]	-6	0	3	SE
Abrupt	6499	5748	4798	162.2
Fenceline	6575	5938	5159	154.6

[1]No differences in treatments were detected

[2]Date effect of $P < 0.0001$

Least squares means (±SD) for white blood cell counts



Materials and Methods: The total white blood cell count was generated from 3mL of a whole blood samples using a Multisizer 3 Coulter-Counter (Beckman Coulter, Brea, CA) and adjusted to a cell per microliter value from 10^6 cells per milliliter. Normal range for healthy cattle is 4000-11000 cells per microliter.

APPENDIX J

Example SAS Code

```
dm 'clear log';  dm 'clear output';
options ls=90;

data flow; set work.2009jmpfile;

ods rtf;
ods graphics on;

proc mixed data=flow;
class weaning_treatment calf_id date;
model neutrophils=weaning_treatment|date;
repeated date / subject=calf_id(weaning_treatment) type=ar(1);

lsmeans weaning_treatment date / adjust=tukey;
lsmeans weaning_treatment*date / slice=date;

title 'Neutrophils';

proc mixed data=flow;
class weaning_treatment calf_id date;
model lymphocytes=weaning_treatment|date;
repeated date / subject=calf_id(weaning_treatment) type=ar(1);

lsmeans weaning_treatment date / adjust=tukey;
lsmeans weaning_treatment*date / slice=date;

title 'Lymphocytes';

proc mixed data=flow;
class weaning_treatment calf_id date;
model cd4_=weaning_treatment|date;
repeated date / subject=calf_id(weaning_treatment) type=ar(1);

lsmeans weaning_treatment date / adjust=tukey;
lsmeans weaning_treatment*date / slice=date;

title 'CD4+';

proc mixed data=flow;
class weaning_treatment calf_id date;
model cd8_=weaning_treatment|date;
repeated date / subject=calf_id(weaning_treatment) type=ar(1);
```

```
lsmeans weaning_treatment date / adjust=tukey;
lsmeans weaning_treatment*date / slice=date;

title 'CD8+';

proc mixed data=flow;
class weaning_treatment calf_id date;
model wc1_=weaning_treatment|date;
repeated date / subject=calf_id(weaning_treatment) type=ar(1);

lsmeans weaning_treatment date / adjust=tukey;
lsmeans weaning_treatment*date / slice=date;

title 'WC1+';

proc mixed data=flow;
class weaning_treatment calf_id date;
model tcr1_=weaning_treatment|date;
repeated date / subject=calf_id(weaning_treatment) type=ar(1);

lsmeans weaning_treatment date / adjust=tukey;
lsmeans weaning_treatment*date / slice=date;

title 'TcR1+';

proc mixed data=flow;
class weaning_treatment calf_id date;
model cd21_=weaning_treatment|date;
repeated date / subject=calf_id(weaning_treatment) type=ar(1);

lsmeans weaning_treatment date / adjust=tukey;
lsmeans weaning_treatment*date / slice=date;

title 'CD21+';

run;

ods rtf close;
ods graphics off;
```

Literature Cited

Anderson, B. H., D. L. Watson, et al. (1999). "The effect of dexamethasone on some immunological parameters in cattle." Vet Res Commun **23**(7): 399-413.

Appelberg, R. (2007). "Neutrophils and intracellular pathogens: beyond phagocytosis and killing." Trends Microbiol **15**(2): 87-92.

Arthington, J. D., S. D. Eichert, et al. (2003). "Effect of transportation and commingling on the acute-phase protein response, growth, and feed intake of newly weaned beef calves." J Anim Sci **81**(5): 1120-1125.

Arthington, J. D., X. Qiu, et al. (2008). "Effects of preshipping management on measures of stress and performance of beef steers during feedlot receiving." J Anim Sci **86**(8): 2016-2023.

Arthington, J. D., J. W. Spears, et al. (2005). "The effect of early weaning on feedlot performance and measures of stress in beef calves." J Anim Sci **83**(4): 933-939.

Banchereau, J. and R. M. Steinman (1998). "Dendritic cells and the control of immunity." Nature **392**(6673): 245-252.

Barrington, G. M. and S. M. Parish (2001). "Bovine neonatal immunology." Vet Clin North Am Food Anim Pract **17**(3): 463-476.

Berghaus, L. J., L. B. Corbeil, et al. (2006). "Effects of dual vaccination for bovine respiratory syncytial virus and Haemophilus somnus on immune responses." Vaccine **24**(33-34): 6018-6027.

Boland, H. T., G. Scaglia, et al. (2008). "Effects of Alternate Weaning Methods on Behavior, Blood Metabolites, and Performance of Beef Calves." The Professional Animal Scientist **24**(6): 539-551.

Brown, W. C., A. C. Rice-Ficht, et al. (1998). "Bovine type 1 and type 2 responses." Vet Immunol Immunopathol **63**(1-2): 45-55.

Buckham Sporer, K. R., J. L. Burton, et al. (2007). "Transportation stress in young bulls alters expression of neutrophil genes important for the regulation of apoptosis, tissue remodeling, margination, and anti-bacterial function." Vet Immunol Immunopathol **118**(1-2): 19-29.

Burke, N. C., G. Scaglia, et al. (2009). "Influence of two-stage weaning with subsequent transport on body weight, plasma lipid peroxidation, plasma selenium, and on leukocyte glutathione peroxidase and glutathione reductase activity in beef calves." Vet Immunol Immunopathol **127**(3-4): 365-370.

Burton, J. L. and M. E. Kehrli, Jr. (1996). "Effects of dexamethasone on bovine circulating T lymphocyte populations." J Leukoc Biol **59**(1): 90-99.

Burton, J. L., M. E. Kehrli, Jr., et al. (1995). "Regulation of L-selectin and CD18 on bovine neutrophils by glucocorticoids: effects of cortisol and dexamethasone." J Leukoc Biol **57**(2): 317-325.

Burton, J. L., S. A. Madsen, et al. (2005). "Gene expression signatures in neutrophils exposed to glucocorticoids: a new paradigm to help explain "neutrophil dysfunction" in parturient dairy cows." Vet Immunol Immunopathol **105**(3-4): 197-219.

Carroll, J. A. and N. E. Forsberg (2007). "Influence of Stress and Nutrition on Cattle Immunity." Veterinary Clinics of North America: Food Animal Practice **23**(1): 105-149.

Carroll, M. C. (2004). "The complement system in regulation of adaptive immunity." Nature Immunology **5**(10): 981-986.

Chang, L. C., S. A. Madsen, et al. (2004). "Effects of glucocorticoids on Fas gene expression in bovine blood neutrophils." J Endocrinol **183**(3): 569-583.

Charmandari, E., C. Tsigos, et al. (2005). "Endocrinology of the stress response." Annu Rev Physiol **67**: 259-284.

Chase, C. C. L., D. J. Hurley, et al. (2008). "Neonatal immune development in the calf and its impact on vaccine response." Veterinary Clinics of North America-Food Animal Practice **24**(1): 87-+.

Church, J. S. and R. J. Hudson (1999). "Comparison of the stress of abrupt and interval weaning of farmed wapiti calves (Cervus elaphus)." Small Ruminant Research **32**(2): 119-124.

Colby, L. A., G. G. Schurig, et al. (2002). "An indirect ELISA to detect the serologic response of elk (Cervus elaphus nelsoni) inoculated with Brucella abortus strain RB51." J Wildl Dis **38**(4): 752-759.

Colditz, I. G., M. A. Schneider, et al. (2007). "Chemokines at large: In-vivo mechanisms of their transport, presentation and clearance." Thrombosis and Haemostasis **97**(5): 688-693.

Cortese, V. S. (2009). "Neonatal immunology." Vet Clin North Am Food Anim Pract **25**(1): 221-227.

Crawley, A., C. Raymond, et al. (2003). "Control of immunoglobulin isotype production by porcine B-cells cultured with cytokines." Vet Immunol Immunopathol **91**(2): 141-154.

Dastoor, Z. and J. Dreyer (2001). "Potential role of nuclear translocation of glyceraldehyde-3-phosphate dehydrogenase in apoptosis and oxidative stress." J Cell Sci **114**(9): 1643-1653.

Drouillard, J. S. and G. L. Kuhl (1999). "Effects of previous grazing nutrition and management on feedlot performance of cattle." J. Anim Sci. **77**(suppl_2): 136-146.

Elenkov, I. J. (2004). "Glucocorticoids and the Th1/Th2 balance." Ann N Y Acad Sci **1024**: 138-146.

Elenkov, I. J. (2008). "Neurohormonal-cytokine interactions: implications for inflammation, common human diseases and well-being." Neurochem Int **52**(1-2): 40-51.

Elenkov, I. J. and G. P. Chrousos (1999). "Stress Hormones, Th1/Th2 patterns, Pro/Anti-inflammatory Cytokines and Susceptibility to Disease." Trends Endocrinol Metab **10**(9): 359-368.

Elenkov, I. J. and G. P. Chrousos (2002). "Stress hormones, proinflammatory and antiinflammatory cytokines, and autoimmunity." Ann N Y Acad Sci **966**: 290-303.

Enríquez, D. H., R. Ungerfeld, et al. (2009). "The effects of alternative weaning methods on behaviour in beef calves." Livestock Science **In Press, Corrected Proof**.

Estes, D. M. (1996). "Differentiation of B cells in the bovine. Role of cytokines in immunoglobulin isotype expression." Vet Immunol Immunopathol **54**(1-4): 61-67.

Estes, D. M. and W. C. Brown (2002). "Type 1 and type 2 responses in regulation of Ig isotype expression in cattle." Vet Immunol Immunopathol **90**(1-2): 1-10.

Fisher, A. D., M. A. Crowe, et al. (1996). "Effect of castration method and the provision of local anesthesia on plasma cortisol, scrotal circumference, growth, and feed intake of bull calves." Journal of Animal Science **74**(10): 2336-2343.

Fisher, A. D., M. A. Crowe, et al. (1997). "Effects of cortisol on in vitro interferon-gamma production, acute-phase proteins, growth, and feed intake in a calf castration model." J Anim Sci **75**(4): 1041-1047.

Fluharty, F. L., S. C. Loerch, et al. (2000). "Effects of weaning age and diet on growth and carcass characteristics in steers." J. Anim Sci. **78**(7): 1759-1767.

Gershwin, L. J., L. J. Berghaus, et al. (2005). "Immune mechanisms of pathogenetic synergy in concurrent bovine pulmonary infection with Haemophilus somnus and bovine respiratory syncytial virus." Vet Immunol Immunopathol **107**(1-2): 119-130.

Gordon, S. (1998). "The role of the macrophage in immune regulation." Research in Immunology **149**(7-8): 685-688.

Haley, D. B., D. W. Bailey, et al. (2005). "The effects of weaning beef calves in two stages on their behavior and growth rate." J Anim Sci **83**(9): 2205-2214.

Hickey, M. C., M. Drennan, et al. (2003). "The effect of abrupt weaning of suckler calves on the plasma concentrations of cortisol, catecholamines, leukocytes, acute-phase proteins and in vitro interferon-gamma production." J Anim Sci **81**(11): 2847-2855.

Hodgson, P. D., P. Aich, et al. (2005). "Effect of stress on viral-bacterial synergy in bovine respiratory disease: novel mechanisms to regulate inflammation." Comp Funct Genomics **6**(4): 244-250.

Janeway, C., K. P. Murphy, et al. (2008). Janeway's Immuno Biology. New York, Garland Science.

Janeway, C. A., Jr. and R. Medzhitov (2002). "Innate immune recognition." Annu Rev Immunol **20**: 197-216.

Kaiko, G. E., J. C. Horvat, et al. (2008). "Immunological decision-making: how does the immune system decide to mount a helper T-cell response?" Immunology **123**(3): 326-338.

Kannan, G., T. H. Terrill, et al. (2000). "Transportation of goats: effects on physiological stress responses and live weight loss." J Anim Sci **78**(6): 1450-1457.

Kelly, M., J. M. Hwang, et al. (2007). "Modulating leukocyte recruitment in inflammation." J Allergy Clin Immunol **120**(1): 3-10.

Kobayashi, S. D., J. M. Voyich, et al. (2003). "Regulation of the neutrophil-mediated inflammatory response to infection." Microbes Infect **5**(14): 1337-1344.

Kojima, C. J., H. G. Kattesh, et al. (2008). "Physiological and immunological responses to weaning and transport in the young pig: Modulation by administration of porcine somatotropin." Journal of Animal Science **86**(11): 2913-2919.

Lefcourt, A. M. and T. H. Elsasser (1995). "Adrenal Responses of Angus X Hereford Cattle to the Stress of Weaning." Journal of Animal Science **73**(9): 2669-2676.

Lim, M. M. and L. J. Young (2006). "Neuropeptidergic regulation of affiliative behavior and social bonding in animals." Hormones and Behavior **50**(4): 506-517.

Lippolis, J. D. (2008). "Immunological signaling networks: integrating the body's immune response." J Anim Sci **86**(14 Suppl): E53-63.

Lo, D. Y., W. M. Lee, et al. (2005). "Effects of dexamethasone on peripheral blood mononuclear cell phenotype in weanling piglets." Comparative Immunology, Microbiology and Infectious Diseases **28**(4): 251-258.

Lupoli, B., B. Johansson, et al. (2001). "Effect of suckling on the release of oxytocin, prolactin, cortisol, gastrin, cholecystokinin, somatostatin and insulin in dairy cows and their calves." Journal of Dairy Research **68**(2): 175-187.

MacKenzie, A. M., M. Drennan, et al. (1997). "Effect of transportation and weaning on humoral immune responses of calves." Research in Veterinary Science **63**(3): 227-230.

Menge, C. and E. A. Dean-Nystrom (2008). "Dexamethasone depletes gammadelta T cells and alters the activation state and responsiveness of bovine peripheral blood lymphocyte subpopulations." J Dairy Sci **91**(6): 2284-2298.

Morrow, C. J., E. S. Kolver, et al. (2002). "Fecal glucocorticoid metabolites as a measure of adrenal activity in dairy cattle." Gen Comp Endocrinol **126**(2): 229-241.

Mostl, E., J. L. Maggs, et al. (2002). "Measurement of cortisol metabolites in faeces of ruminants." Vet Res Commun **26**(2): 127-139.

Narayanan, S. K., T. G. Nagaraja, et al. (2002). "Leukotoxins of gram-negative bacteria." Veterinary Microbiology **84**(4): 337-356.

Newberry, R. C. and J. C. Swanson (2008). "Implications of breaking mother-young social bonds." Applied Animal Behaviour Science **110**(1-2): 3-23.

Nonnecke, B. J., J. L. Burton, et al. (1997). "Associations between function and composition of blood mononuclear leukocyte populations from Holstein bulls treated with dexamethasone." J Dairy Sci **80**(10): 2403-2410.

Oldham, G. and C. J. Howard (1992). "Suppression of bovine lymphocyte responses to mitogens following in vivo and in vitro treatment with dexamethasone." Veterinary Immunology and Immunopathology **30**(2-3): 161-177.

Osburn, B. I. and J. L. Stott (1989). "Immune response to vaccination." Adv Vet Sci Comp Med **33**: 93-108.

Paape, M. J., D. D. Bannerman, et al. (2003). "The bovine neutrophil: Structure and function in blood and milk." Vet Res **34**(5): 597-627.

Parkin, J. and B. Cohen (2001). "An overview of the immune system." Lancet **357**(9270): 1777-1789.

Petersen, H. H., J. P. Nielsen, et al. (2004). "Application of acute phase protein measurements in veterinary clinical chemistry." Vet Res **35**(2): 163-187.

Platt, R., W. Burdett, et al. (2006). "Induction of antigen-specific T-cell subset activation to bovine respiratory disease viruses by a modified-live virus vaccine." Am J Vet Res **67**(7): 1179-1184.

Pollock, J. M., T. G. Rowan, et al. (1992). "Effects of Weaning on Antibody-Responses in Young Calves." Veterinary Immunology and Immunopathology **33**(1-2): 25-36.

Preisler, M. T., P. S. Weber, et al. (2000). "Glucocorticoid receptor expression profiles in mononuclear leukocytes of periparturient Holstein cows." J Dairy Sci **83**(1): 38-47.

Price, E. O., J. E. Harris, et al. (2003). "Fenceline contact of beef calves with their dams at weaning reduces the negative effects of separation on behavior and growth rate." J Anim Sci **81**(1): 116-121.

Randolph, G. J., C. Jakubzick, et al. (2008). "Antigen presentation by monocytes and monocyte-derived cells." Current Opinion in Immunology **20**(1): 52-60.

Rasby, R. (2007). "Early weaning beef calves." Vet Clin North Am Food Anim Pract **23**(1): 29-40.

Rautajoki, K. J., M. K. Kylaniemi, et al. (2008). "An insight into molecular mechanisms of human T helper cell differentiation." Ann Med **40**(5): 322-335.

Roth, J. A. and K. P. Flaming (1990). "Model systems to study immunomodulation in domestic food animals." Adv Vet Sci Comp Med **35**: 21-41.

Saeb, M., H. Baghshani, et al. (2010). "Physiological response of dromedary camels to road transportation in relation to circulating levels of cortisol, thyroid hormones and some serum biochemical parameters." Trop Anim Health Prod **42**(1): 55-63.

Saker, K. E. (2006). "Nutrition and immune function." Veterinary Clinics of North America-Small Animal Practice **36**(6): 1199-+.

Salak-Johnson, J. L. and J. J. McGlone (2007). "Making sense of apparently conflicting data: stress and immunity in swine and cattle." J Anim Sci **85**(13 Suppl): E81-88.

Sanakkayala, N., A. Sokolovska, et al. (2005). "Induction of antigen-specific Th1-type immune responses by gamma-irradiated recombinant Brucella abortus RB51." Clin Diagn Lab Immunol **12**(12): 1429-1436.

Savina, A. and S. Amigorena (2007). "Phagocytosis and antigen presentation in dendritic cells." Immunol Rev **219**: 143-156.

Schichowski, C., E. Moors, et al. (2008). "Effects of weaning lambs in two stages or by abrupt separation on their behavior and growth rate." J Anim Sci **86**(1): 220-225.

Sporer, K. R., L. Xiao, et al. (2008). "Transportation stress alters the circulating steroid environment and neutrophil gene expression in beef bulls." Vet Immunol Immunopathol **121**(3-4): 300-320.

Stookey, J. M., K. S. Schwartzkopf-Fenswein, C. S. Waltz, and J. M. Watts (1997). "Effects of remote and contact weaning on behaviour and weight gain of beef calves." J. Anim. Sci. **75(Suppl. 1)**(157 (Abstr.).).

Sumner, B. E. H., R. B. D'Eath, et al. (2008). "Early weaning results in less active behaviour, accompanied by lower 5-HT1A and higher 5-HT2A receptor mRNA expression in specific brain regions of female pigs." Psychoneuroendocrinology **33**(8): 1077-1092.

Takeda, K., T. Kaisho, et al. (2003). "Toll-like receptors." Annu Rev Immunol **21**: 335-376.

Tausk, F., I. Elenkov, et al. (2008). "Psychoneuroimmunology." Dermatol Ther **21**(1): 22-31.

Tilbrook, A. J. and I. J. Clarke (2006). "Neuroendocrine mechanisms of innate states of attenuated responsiveness of the hypothalamo-pituitary adrenal axis to stress." Frontiers in Neuroendocrinology **27**(3): 285-307.

von Keyserlingk, M. A. and D. M. Weary (2007). "Maternal behavior in cattle." Horm Behav **52**(1): 106-113.

Weary, D. M., J. Jasper, et al. (2008). "Understanding weaning distress." Applied Animal Behaviour Science **110**(1-2): 24-41.

Weber, P. S., S. A. Madsen, et al. (2001). "Pre-translational regulation of neutrophil L-selectin in glucocorticoid-challenged cattle." Vet Immunol Immunopathol **83**(3-4): 213-240.

Weber, P. S., T. Toelboell, et al. (2004). "Mechanisms of glucocorticoid-induced down-regulation of neutrophil L-selectin in cattle: evidence for effects at the gene-expression level and primarily on blood neutrophils." J Leukoc Biol **75**(5): 815-827.

Yagi, Y., H. Shiono, et al. (2004). "Transport stress increases somatic cell counts in milk, and enhances the migration capacity of peripheral blood neutrophils of dairy cows." J Vet Med Sci **66**(4): 381-387.