

**Bovine Coccidiosis: Dynamics of infection in grazing cattle and the potential role of stress and immunity**

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**Abstract**

Eimerian parasites infect cattle worldwide. Information on the infection dynamics of these parasites is lacking in the central Appalachian region of the United States. Studies aimed at characterizing the seasonal dynamics of eimerian parasites in this region were carried out in order to assess the impact of these organisms in grazing systems. In these studies the prevalence of *Eimeria* spp. infection was highest in calves less than one year of age and subsequently decreased to stable levels in older animals. Although *E. bovis* was the most common species identified in calves, heifers and cows, mixed species infections dominated. Additional studies were carried out to investigate the effect of stress on *Eimeria* spp. infection in beef calves. Lower stress, two-stage, weaning methods had no effect on *Eimeria* spp. infection dynamics in beef calves. These findings must be interpreted in light of the fact that calves used in this study were not managed in a way typical of many calves in the U.S.A. The fact that they were only transported short distances, never commingled, or exposed to a livestock market may explain why a rise in post weaning FOC was not observed. A model of stress-induced coccidiosis was developed using dexamethasone and *E. bovis* challenge. In this model, an oral challenge of at least 500,000 sporulated *E. bovis* oocysts in addition to dexamethasone injection at 7 days post challenge increased subsequent FOC. Further investigation of the immune response to *E. bovis* challenge during times of stress indicates that stress-induced suppression of cell mediated immunity and *E. bovis* challenge are required to increase subsequent oocyst shedding. These

findings may represent the mechanism associated with stress-induced outbreaks of coccidiosis reported to occur in beef cattle in the United States.

## **Dedication**

I dedicate this work to my parents Janet and Scotty who have provided me with undying love and support through all of my life and to my friends who always encouraged me to chase my dreams of a career in veterinary medicine.

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## Attributions

Chapter 3: Variation in *Eimeria* oocyst count and species composition in weanling beef heifers

Williams S. Swecker Jr. DVM, PhD: Dr. Swecker provided guidance on study design and cattle handling and sampling.

David S. Lindsay, PhD: Dr. Lindsay provided guidance on parasite identification and techniques.

Guillermo Scaglia, PhD: Dr. Scaglia provided guidance on study design and sampling strategy.

Anne M. Zajac DVM, PhD: Dr. Zajac provided guidance on parasitological techniques and study design.

Chapter 5: The effect of weaning method on coccidial infections in beef calves.

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## Chapter 1

### Introduction

Respiratory infections and gastrointestinal parasitism constitute the most important diseases in calves worldwide (Svensson, 1994). Along with trichostrongyle nematodes, apicomplexan parasites in the genus *Eimeria* are the most important parasites of cattle in North America. Transmission of *Eimeria* spp. is dependent upon the development of an environmental life stage, thus effective transmission requires favorable environmental conditions. Over the past century, limited research investigating the prevalence of these parasites in cattle of various ages has been conducted worldwide. Due to regional differences in climate and cattle production practices, further research is warranted to investigate the prevalence and seasonal dynamics of these organisms in specific regions.

No available research addresses the seasonal infection dynamics of these parasites in grazing cattle in the central Appalachian region of the United States. Cattle production in this region consists largely of cow-calf production and most producers continually utilize the same pastures to meet the nutritional requirements of cow and calf. Thus, producers in this area are at risk of suffering production losses from *Eimeria* spp. infections in their calf crop during the grazing season. Without knowledge of the seasonal dynamics of these parasites in cattle populations in this region it is difficult for veterinarians to make cost effective control recommendations to producers. Additionally, producers in this area garner most of their profit from selling weaned calves to stocker and feeder cattle operations concentrated in areas west of the Mississippi river. It has long been recognized that the stress associated with weaning and transport of these calves

increases their susceptibility to infectious diseases including *Eimeria* spp., but little information is available on the dynamics of these parasites in grazing calves prior to weaning. Although, millions of dollars are spent by the United States cattle industry to control this group of parasites there has been little research conducted on these organisms over the past 20 years.

Interest in the effect of weaning and transport stress on immune function in calves has however, remained strong during this time period. In fact, weaning strategies aimed at decreasing the stress of weaning have been investigated and show some promise as a lower stress alternative to abrupt weaning practices. Even with the reported outbreaks of stress associated coccidiosis in recently weaned and shipped calves little research has been carried out to investigate the effect of stress-induced immune suppression on *Eimeria* spp. infection. This dissertation compiles work aimed at investigating the seasonal dynamics of eimerian parasites in grazing cattle of various age groups in the central Appalachian region. Furthermore, this dissertation provides information on the immune response to *Eimeria* spp. infection during times of dexamethasone induced stress in cattle and provides a model of stress associated coccidiosis in weaned beef calves.

In study 1, rectal fecal samples from 11 recently weaned replacement beef heifers were collected for 10 consecutive days to assess the daily variation in fecal oocyst count and species composition. Additional samples were collected from the same animals 4 and 7 months later in order assess changes in these same parameters over a longer period of time. Soil samples were also collected from the pasture to investigate the *Eimera* spp. composition available in the heifer's environment.

The second study was aimed at characterizing both the level of infection and species dynamics of *Eimeria* spp. in naturally infected, grazing cattle at various stages of production in the central Appalachian region of the United States. In this study, rectal fecal samples were collected from 72 spring (February-March) born calves monthly from May through weaning in October. Fecal samples were also collected from the dams of the calves in May, July, and September. Rectal fecal samples were also collected from a subset (n=36) of replacement heifer calves monthly from November through April. These samples were analyzed and the seasonal dynamics of *Eimeria* spp. were described.

In study 3, the effect of weaning method on fecal oocyst count (FOC) and *Eimeria* spp. composition was assessed. A group of 108 beef calves (190-240 days of age) were allocated to 3 treatments on day -7: a fenceline weaned group where calves could see and hear their dams, but were physically separated from them by a fence (FL), a noseclipped group where suckling was prevented (NC), and a group left with their dams to be traditionally weaned (TW) by abrupt separation on day 0. On day 0, noseclips were removed from the NC calves and all groups were completely separated from their dams. The steers (n=50) were transported 172 km to another farm, and heifers (n=58) were taken to distant fields on the same farm. Rectal fecal samples were collected from all calves on days -7, 0, and 14. Steers were also sampled on days 7, 28, and 42. A group of steers (n=24) from each of the treatments previously described were fitted with pedometers and intensively observed on days 1-4. The Modified McMaster test and Wisconsin sugar flotation test were used for quantification and identification of *Eimeria* spp. oocysts.

The results of our initial studies indicated that all grazing calves are infected with and shed pathogenic *E. bovis* oocysts prior to weaning. Reports indicate that immunity to development of disease is established experimentally following primary *E. bovis* infection in calves. Given these two facts, it seems likely that all calves should be immune to development of coccidiosis prior to the stress of weaning and transport, yet information from feedlots suggests that recently weaned and shipped calves often exhibit signs of clinical coccidiosis. Thus, the objectives of the remaining two studies were to develop a model of stress associated coccidiosis in cattle and employing this model to investigate the immune response to *E. bovis* challenge in calves during times of stress.

The main objectives of study 4 were to first identify a pharmacologic agent that would induce changes in immune parameters in beef calves similar to those seen following weaning and transport and, secondly, to use this agent in combination with varying doses of *E. bovis* to identify the number of oocysts required to increase subsequent fecal oocyst count. The first objective was accomplished by investigating the effects of dexamethasone and ACTH/Epinephrine injection on plasma cortisol concentration, neutrophil:lymphocyte (N:L) ratio, lymphoproliferative responses and IFN- $\gamma$  production in recently weaned beef calves. Due to the observed alterations in N:L ratio, lymphoproliferative responses and IFN- $\gamma$  production following dexamethasone administration this agent was selected for use in subsequent studies. The second objective was carried out by investigating the effects of varying levels of *E. bovis* challenge in conjunction with dexamethasone administration on subsequent fecal oocyst count in recently weaned beef calves.

In study 5, the newly designed stress model was employed to investigate the immune response to *E. bovis* challenge during times of stress. Thirty-two Angus-cross calves (6-7 mo.) were blocked by FOC and randomly assigned within blocks to one of 4 treatment groups: non-stressed non-challenged (NSNC), non-stressed challenged (NSC), stressed non-challenged (SNC), and stressed challenged (SC). Challenged calves were gavaged with 1.5 million oocysts on day 0 and stressed calves were given dexamethasone (0.35 mg/kg) on day 7 PC. Blood was collected from 4 calves per treatment on days -1, 8, 21 PC. Feces were collected from all calves on day -60, -1, 8 and daily 15-28 post challenge. Complete blood counts and lymphocyte separations were carried out on blood samples. Lymphocytes were stained for cell surface markers for flow cytometric analysis, and stimulated with *E. bovis* antigen and mitogen for lymphocyte proliferation, IFN- $\gamma$  and IL-4 production. Modified McMaster tests and fecal dry matter analysis were conducted on all fecal samples. The same four calves used for blood collection from the NSNC and SC group were euthanized on day 24 post challenge. Cecae were grossly scored for the presence of lesions consistent with *Eimeria* infection. Additionally, ileocecal and colic lymph nodes were collected. Mass of the ileocolic lymph nodes was recorded and lymphocytes were isolated from the ileocolic and cecal lymph node for cell surface marker staining and subsequent flow cytometric analysis. Additionally, tissue sections from the ileum and cecum were preserved for H&E staining and immunohistochemical staining. Pathological descriptions of stained sections were used to assign histopathologic lesion scores. Immunohistochemical staining was employed to quantify the number of CD4<sup>+</sup>, CD8<sup>+</sup>, WC1<sup>+</sup>, and CD21<sup>+</sup> lymphocytes present in each tissue.



## Chapter 2

### Literature Review

#### Introduction

Coccidia are single celled protozoan parasites that undergo some part of their development inside the intestinal cells of their animal host (Levine and Ivens 1986; Jolly and Bardsley, 2006). Thousands of coccidian species have been described in wild and domestic animals and some are known to cause serious disease while others are of little clinical significance. All members of this large group belong to the phylum Apicomplexa, so named by the presence of an apical complex in at least one life stage. According to the taxonomy proposed by Long (1982), all coccidia of veterinary importance fall within the class Sporozasida, subclass Coccidiasina, and order Eucoccidiorina. There are 3 suborders within the Eucoccidiorina, but the largest and most relevant to domestic animals is suborder Eimeriorina (Fitzgerald, 1980). This suborder encompasses 9 diverse families, including the Eimeriidae with 16 genera, of which the genus *Eimeria* is most important to cattle.

#### Bovine *Eimeria*

#### Impact

Eimerian parasites are the most prevalent gastrointestinal parasites of cattle worldwide (Dauguschies and Najdrowski, 2005). Cattle production units have increased in size in recent decades in order to improve efficiency and transmission of infectious disease is frequently enhanced in these larger units (Step et al., 2002). *Eimeria* spp. infection becomes more economically important as these systems become more intensive and the costs associated with treatment, prevention and impaired performance result in substantial economic losses to the cattle industry (Fitzgerald, 1980; Burger, 1983).

According to Fitzgerald (1980) the cost associated with subclinical *Eimeria* spp. infections exceed those associated with clinical disease because the latter occurs much more infrequently. Additionally subclinical infection is known to impair feed conversion, intestinal physiology and growth of cattle (Fox, 1985; Cornelison et al., 1995).

#### Basic biology

According to Levine and Ivens (1986) there are 17 described species of *Eimeria* infecting cattle worldwide. Only 13 of these species, however, infect cattle in North America (Christensen, 1941; Levine and Ivens, 1986). All *Eimeria* spp. share a similar monoxenous life cycle, which includes a parasitic period within the host known as the endogenous phase and a non-parasitic period outside the host known as the exogenous phase (Hammond, 1964; Long, 1982; Dauschies and Najdrowski, 2005, Jolley and Bardsley, 2006). The passage of an unsporulated oocyst stage in the feces of an infected host begins the exogenous phase of development. The unsporulated oocyst contains a single cell and is not immediately infective, but requires time and correct environmental conditions to sporulate to the infective form. Although the optimal conditions for sporulation of all *Eimeria* spp. are not fully described, typically oxygen, moisture and temperatures between 13 and 32°C are required (Marquardt et al., 1960). The time required for sporulation is dependent on environmental conditions and species, but is generally complete for all species in 1 to 21 days (Long, 1982.) As sporulation takes place the single cell first divides to form four sporoblasts, which eventually develop into four sporocysts, within each sporocyst two sporozoites will develop. Thus at maturity, each *Eimeria* spp oocyst contains 8 infective sporozoites.

Oocysts of *Eimeria* are known to be considerably resistant to many environmental conditions and can survive for up to 2 years outside the host (Schneider et al. 1972).

Oocysts of multiple *Eimeria* spp. known to infect cattle can survive temperatures as low as  $-7^{\circ}\text{C}$  for several months (Marquardt et al. 1960). Although these cold temperatures may not kill the oocysts, sporulation of *E. zuernii* is known to be extremely long below  $12^{\circ}\text{C}$  and morphologically abnormal at temperatures above  $40^{\circ}\text{C}$ . Many common disinfectants are not harmful to *Eimeria* spp oocysts (Senger et al., 1959), but desiccation of oocysts below 25% relative humidity for several days and direct sunlight for as little as 4 hours are lethal to developing oocysts (Marquardt et al. 1960).

Once a fully sporulated *Eimeria* spp. oocyst is ingested by an appropriate host the endogenous phase of development begins. In order for sporozoites to penetrate intestinal cells they must first be liberated from the oocyst. This process is referred to as excystation and the mechanism by which it is accomplished is complex and dependent upon several host-parasite interactions. The initial phase of excystation in cattle is believed to take place in the rumen because experimental excystation of these species is more efficient when they are exposed to high levels (15%) of atmospheric  $\text{CO}_2$  (Hammond, 1973, Long, 1982). Furthermore, microscopic evaluation of the oocyst after exposure to  $\text{CO}_2$  reveals thinning or opening of the micropyle and allows for methylene blue staining of internal oocysts structures (Long, 1982). Interestingly, similar changes are noted in *Eimeria* spp. that infect poultry following mechanical grinding as would take place in the gizzards of these host species (Pellerdy, 1974). The second phase of excystation in which sporozoites are completely liberated from the oocyst, requires a combination of bile and trypsin. These compounds are responsible for enzymatic

breakdown of the morphological plug located at one end of the sporocysts, called a Steidia body. In addition to assisting in degradation of the oocyst, bile also makes sporozoites more motile resulting in their final liberation from the oocyst (Long, 1982, Svensson, 1994).

After excystation, cellular penetration by the liberated sporozoites occurs. The cell type and location within the intestine is highly specific among *Eimeria* spp. (Pellerdy, 1974, Long, 1982; Svensson, 1994). The underlying mechanism for this specificity has been attributed to the parasite's ability to recognize host cell surface molecules, although the exact nature of these molecules is not known (Hermosilla et al., 2002; Dauschies and Najdrowski, 2005). It does appear, however, that sporozoites are able to penetrate host cells with minimal damage (Behrendt et al., 2004) and compounds released from the apical complex may be responsible for entry and parasitophorous vacuole formation (Heise et al., 1999a; Heise et al., 1999b). Once sporozoites are inside the appropriate host cell, they take the form of round trophozoites and begin the process of asexual multiple fission known as schizogony (Pellerdy, 1974, Long, 1982, Svensson, 1994; Dauschies and Najdrowski, 2005). Trophozoites divide numerous times and take the form of first generation schizonts (synonymous with meronts). These stages become particularly large and grossly visible within the host's intestine in the case of *E. bovis*, *E. zuernii*, and *E. auburnensis*, which is a reflection of the large number of divisions in these species (Boughton, 1942, Hammond et al., 1964). Once the first generation merozont is mature, the host cell is ruptured, releasing first generation merozoites into the lumen of the intestine. Depending on the species of *Eimeria* the first generation merozoites may infect adjacent intestinal cells or cell types in different parts of the intestinal tract, but

ultimately round up and undergo a second schizogony, giving rise to second generation merozoites (Hammond, 1964, Levine and Ivens, 1986, Svensson, 1994). Although two rounds of schizogony are most common in bovine *Eimeria* spp., subsequent schizogonies do occur in some species (Hammond, 1964, Levine and Ivens, 1986). After the last generation of schizogony, a phase of sexual reproduction known as gamogony begins. The majority of the last generation merozoites that penetrate intestinal cells develop into macrogametocytes, which grow to form large macrogametes. A smaller number become flagellated microgametes by a process similar to schizogony. Microgametes rupture their host cell, penetrate host cells with macrogametes and ultimately form diploid zygotes. A wall is laid down around the developing zygote, which at completion ruptures out of the host cell and is passed in the feces as an unsporulated oocyst (Hammond, 1964; Pellerdy, 1974; Long, 1982).

#### Pathobiology

All species of *Eimeria* that infect cattle result in some level of damage to the intestine as a result of their intracellular life cycle (Dauguschies and Najdrowski, 2005), but most infections are subclinical or result in only mild diarrhea (Cornelissen et al., 1995; Busato et al., 1998). In fact, of 17 *Eimeria* spp. that parasitize cattle worldwide only a few species are considered pathogenic (Hammond, 1964; Levine and Ivens, 1986; Svensson, 1994; Daugschies and Najdrowski, 2005, Jolley and Bardsley, 2006). Heavy infections with *E. bovis* or *E. zuernii* can cause severe life-threatening disease known as coccidiosis. Calves suffering from this disease may have severe diarrhea, hematochezia, tenesmus, anemia, anorexia, dehydration, weight loss, and may eventually die (Hammond, 1964, Stockdale et al. 1981, Burger, 1983; Daugschies et al., 1986; Ernst and

Benz, 1986). The disease is self limiting and signs begin to improve when intestinal replication is complete, but subsequent growth in calves that recover from severe disease is often diminished (Stockdale et al., 1981). *Eimeria alabamensis* is also a reported cause of diarrhea associated with weight loss when present at high levels (Svensson, 1994).

Most information regarding the pathogenesis of *Eimeria* spp. infection in cattle is drawn from detailed gross and histopathologic descriptions of intestinal lesions following experimental infection. These lesions are most extensive for *E. bovis* and *E. zuernii* infection. Friend and Stockdale (1980), provide a thorough description of changes in naïve Holstein calves following an experimental infection with 100,000 *E. bovis* oocysts and serial euthanasia. Calves were also given 40 mg of dexamethasone on 13, 14, and 15 days post infection in an effort to increase the severity of infection. Gross lesions were confined to the proximal spiral colon, cecum, and distal two meters of the ileum in all calves euthanized from day 16-26. The gross lesions progressed rapidly from mild mucosal edema of the cecum and colon on day 16 to congestion and ulceration of the ileal, cecal, and colonic mucosa on day 19-20. Additionally, a diphtheritic membrane and fibrin strands covered the cecal and colonic mucosa during this period and persisted in calves euthanized on days 22, 24 and 26. The histopathologic lesions evident from day 19-26 were also marked. Large numbers of mature schizonts were present in the central lacteal endothelial cells of the ileum in calves euthanized on day 16-18. The adjacent lamina propria was heavily infiltrated by lymphocytes, macrophages and plasma cells, while only a few polymorphonuclear (PMN) cells were present. Ileal blood vessel congestion and central lacteal dilation were also persistent lesions. Later in the course of infection (day 22-26), cryptal “abscesses” were common in the ileum along with edema

of the lamina propria and marked blunting and erosion of the villi. Histopathologic lesions of the large intestine were consistently more severe in the cecum and greatly diminished distal to the cecocolic junction. On days 16 and 18 post infection the cecal glands were markedly dilated and every epithelial cell lining the lower quarter of the gland was infected with second generation schizonts or sexual stages of *E. bovis*. Lesions worsened on day 19-21, and were characterized as a necrotizing diphtheritic typhlitis. Nearly all cecal epithelial cells were sloughed and the exposed lamina propria was covered with a diphtheritic membrane. Complete crypt collapse was noted on days 24-26 post infection and cellular infiltration consisting of lymphocytes, PMN, and macrophages was noted. These findings led the authors to conclude that oocyst excretion occurs at the height of gross and histopathologic changes within the cecum (day 19-22 post infection). They also speculated that calves likely exhibit the most severe clinical signs at the height of oocyst excretion. Furthermore, the immune cell infiltration associated with second generation schizonts was more marked than the infiltration associated with sexual stages.

Similar gross and histopathologic changes associated with *E. zuernii* infection of naïve calves were noted by Stockdale (1987). Calves in this study were given an oral dose of 600,000 sporulated *E. zuernii* oocysts, 20 mg of dexamethasone on day 13, 14, and 15, and euthanized on day 10, 12, 14-24, 26, 28, 30 and 32. Gross lesions on day 18-26 post infection were similar to those seen following *E. bovis* infection, but lesions extended more distally into the spiral colon than did those of *E. bovis*. Histopathological changes were also similar to those described for *E. bovis* infection with a few exceptions. First generation schizonts were detected within connective tissue cells in the last 3 meters of the ilea of infected calves as opposed to epithelial cells of the central lacteals in *E.*

*bovis* infection. Schizonts were surrounded by large numbers of lymphocytes similar to those seen with *E. bovis* infection. Histopathologic lesions followed a similar course from edema noted on day 16 to complete destruction and collapse of the cecal mucosa on day 22. Unlike the lesions seen with *E. bovis*, which were confined to the cecum, the lesions detected in this study extended distally past the spiral colon. Additionally, gross and histopathologic description continued through day 30 post infection in this study. Both gross and histopathologic descriptions show a period of sequential resolution of lesions from day 22-32 post infection. Gross mucosal ulceration, hemorrhage, and diphtheritic membranes gradually diminished and histologic regeneration of mucosal epithelium was described during this time period. The author concluded that although gross and histologic lesions associated with *E. zuernii* infection are similar to those induced by *E. bovis*, specific differences in the site of the lesions should allow accurate diagnosis of the species causing disease. If lesions occur distally in the ileum and colon then *E. zuernii* is the causative organism, while if lesions occur primarily in the proximal ileum and cecum then *E. bovis* is more likely. Additionally, nearly all calves euthanized on day 18-28 post infection were clinically affected. Since these clinical findings occurred at the height of gross and histopathologic lesions the author concluded that accurate diagnosis of coccidiosis could be made by performing field necropsies. The association of second generation *E. zuernii* merozoites and lymphocytic infiltration in the ileum was similar to that observed following *E. bovis* infection, allowing the author to speculate that the immune response may be primarily directed toward these stages.

The gross and histopathologic lesions following experimental infection of a single calf with  $1.5 \times 10^7$  *E. alabamensis* oocysts for three consecutive days have been described



by Svensson (1994). The calf was euthanized on day 6 after the initial infection. Catarrhal enteritis was described grossly throughout the length of the jejunum, ileum, and cecum. The severity of the lesions was greatest in the lower jejunum and ileum where patchial hemorrhages, diffuse necrotic inflammation and epithelial destruction were also noted. Inflammation associated with the coccidial stages was characterized as mononuclear in nature with very few neutrophils and eosinophils. The typical intranuclear location of this *Eimeria* spp. was verified by both light and electron microscopy. The author concluded that the gross and histopathologic lesions present following this infection were sufficient to result in the watery diarrhea observed in calves following high doses of sporulated *E. alabamensis* oocysts.

It is believed that several host and parasite factors are responsible for the pathogenicity of these species. *Eimeria* spp. with life cycles confined to the small intestine are generally less pathogenic than those which have some part of their life cycle in the large intestine, such as *E. bovis* and *E. zuernii* (Levine and Ivens, 1986). This may be due to the large intestine's ability to compensate for the fluid lost by a damaged small intestine (Long, 1982; Svensson, 1994). Furthermore, the majority of the intestinal damage associated with *Eimeria* infection is a result of cell lysis from sexual replication (Friend and Stockdale, 1981; Mundt et al., 2005; Jubb et al., 2007). Given that sexual replication of *E. bovis* and *E. zuernii* occur in the large intestine it is not surprising that signs of large bowel diarrhea result from heavy infections with these species (Hammond, 1964; Friend and Stockdale, 1981; Stockdale, 1981; Dauschies et al., 1986; Mundt et al., 2005). The reproductive capacity of each *Eimeria* sp. also contributes to its pathogenic potential (Jolley and Bardsley, 2006). For example, each oocyst of *Eimeria bovis*, *E.*

*zuernii*, and *E. auburnensis* has the capacity to give rise to millions of second-generation merozoites by asexual multiplication (Hammond, 1964, Long, 1982) and each of these merozoites has the potential to destroy an intestinal cell during sexual replication. Thus, the sheer number of intestinal cells destroyed by these species likely contributes to their pathogenicity (Hammond, 1964; Friend and Stockdale, 1981). Lastly, the function of the cell type affected is also reported as a contributing factor to the pathogenicity of various *Eimeria* spp. In general, *Eimeria* spp. that replicate in cells within the intestinal crypts have a higher pathogenic potential than those that replicate in cells near the apex of the intestinal villus because destruction of stem cells in the crypt requires more time for regeneration of intestinal epithelium (Pellerdy, 1974; Long, 1982).

The pathophysiologic effects of *Eimeria* spp. infection on susceptible calves has been described by several authors. Inappetence is a common clinical finding in calves suffering from coccidiosis (Stockdale et al., 1981; Dauschies et al., 1986) and a 60% reduction in feed intake at 20-24 days after *E. bovis* infection has been reported (Sartin et al., 2000). This reduction in feed intake puts calves in a catabolic state, forcing them to utilize their own fat and protein stores for energy. It has been hypothesized that the weight loss associated with *E. bovis* infection may be ameliorated by the administration of anabolic agents. A study conducted by Heath et al. (1997) confirmed this hypothesis and found that calves implanted with an estrodadiol-progesterone growth promotant and infected with *E. bovis* gained more weight than non-implanted calves infected with *E. bovis*. In addition to increased weight gains, implanted calves also exhibited diarrhea and inappetence for fewer days than did their non-implanted counterparts. Also healthy pair-fed control calves gained more weight than did their *E. bovis* infected counterparts

leading the researchers to conclude that all weight loss associated with *E. bovis* is not explained by inappetence alone. Based on the fact that sublethal *E. bovis* infection does not reduce apparent digestibility of nutrients (Dauguschies et al., 1998), these authors speculated that the additional resources allocated to immune function in the *E. bovis* infected calves may explain the increased weight loss in this group.

Alterations in fluid, plasma proteins, and electrolytes are an additional sequella to the severe intestinal damage imparted by pathogenic *Eimeria* spp. Calves infected with *E. zuernii* had decreased plasma sodium, chlorine, and protein compared to non-infected controls (Stockdale et al., 1981). According to the author, these findings were a result of increased fluid loss and decreased absorption across a diseased intestine. Additionally, infected calves had decreased packed cell volume, erythrocyte count and hemoglobin, which were attributed to hemorrhage from the damaged intestine.

#### Diagnosis

In cases where calves present with hematochezia containing tissue and fibrin, coccidiosis due to *E. bovis* or *E. zuernii* should be considered (Step et al. 2002; Daugschies and Najdrowski, 2005). Oocysts of *Eimeria* spp. are easily identified by light microscopy following concentration with conventional flotation techniques. The mere identification of *Eimeria* spp. oocysts in the feces is not diagnostic, however, because many species are considered non-pathogenic (Levine and Ivens, 1986) and clinically normal calves regularly shed *Eimeria* spp. oocysts in their feces (Ernst et al., 1984; Step et al., 2002). Thus, oocysts should be identified to species and a quantitative fecal examination should be carried out in order to make an accurate diagnosis of coccidiosis (Step et al., 2002). Even if these steps are taken, diagnosis of clinical coccidiosis is

difficult based on one oocyst count and it has been suggested that several animals within the group be sampled to obtain a true estimate of herd infection level (Dauguschies and Najdrowski, 2005). Post mortem examination of expired calves provides the most accurate diagnosis of coccidiosis (Step et al., 2002). The presence of gross lesions consistent with *E. bovis* or *E. zuernii* infection at necropsy provide a presumptive diagnosis. Sections of diseased intestine should be preserved for histopathologic examination and the presence of histologic lesions and/or coccidial stages provide a definitive diagnosis (Step et al., 2002 Daugschies and Najdrowski, 2005).

#### Treatment and Prevention

Calves suffering from severe clinical coccidiosis require supportive care. As discussed previously, fluid, electrolyte, and acid/base imbalances pose the most immediate threat to the survival of the calf (Step et al., 2002; Jolley and Bardsley, 2006; Radostits et al., 2007), thus administration of fluids and electrolytes is of utmost importance. Likewise, anemia may be severe in calves afflicted by coccidiosis and blood transfusion should be considered when the PCV is below 15% (Radostits et al., 2007). Chemotherapeutic agents specifically approved for the treatment of coccidiosis mostly target gamonts developing in the large intestine (Dauguschies and Najdrowski, 2005). Although therapy should be implemented in acute outbreaks, its usefulness is limited as the organism has nearly completed its life cycle (Fox, 1985; Step et al., 2002; Mundt et al., 2005). Amprolium is a thiamine antagonist that is approved for the treatment of *E. bovis* and *E. zuernii* coccidiosis in calves. Its efficacy and safety have been tested in several studies of calves of various age groups (Peardon et al., 1965; Hammond et al., 1966; Norcross et al., 1974). Additionally, sulfonamides are often used for the treatment

of coccidiosis in cattle (Radostits et al., 2007). Although only sulfaquinoxalone is approved for the treatment of this condition, efficacy of other sulfonamides in treating clinical coccidiosis in calves has been shown (Hammond et al., 1956). The coccidiocidal agent toltrazuril has recently been approved for the treatment of bovine coccidiosis in Canada and parts of Europe and has demonstrated superb efficacy in treating this condition under field conditions (Mundt et al., 2007)

Preventive measures should be taken any time a clinical case of coccidiosis is confirmed in a herd of cattle. Management factors that improve sanitation and reduce stress in calves should be implemented (Step et al., 2002). Additionally, drugs known as coccidiostats are marketed for the control of *E. bovis* and *E. zuernii* coccidiosis in cattle. Ionophore antimicrobials are commonly used in cattle production (Step et al., 2002; Radostits et al., 2007). In addition to their coccidiostatic activity they also increase feed efficiency. The efficacy and safety of these products has been tested in cattle of various age groups (Fitzgerald and Mansfield, 1972; Fitzgerald and Mansfield, 1979; Fitzgerald and Mansfield, 1984). Decoquinate is another compound approved for the prevention of *E. bovis* and *E. zuernii* coccidiosis. It interferes with mitochondrial electron transport of the coccidian organism (Lindsay and Blagburn, 2001) and its efficacy and safety has been confirmed in cattle of various age groups (Miner and Jenson, 1976; Fitzgerald and Mansfield, 1989a; Fitzgerald and Mansfield; 1989b). Overall, prevention of coccidiosis by a combination of changes in management that improve sanitation and administration of coccidiostatic agents is usually sufficient to halt outbreaks of coccidiosis in herds (Step et al., 2002).

Epidemiology

Infective oocysts of *Eimeria* spp. are ubiquitous in all areas where cattle are kept (Hammond, 1964; Cornilissen et al., 1995; Dauschies and Najdrowski, 2005, Jolley and Bardsley, 2006). Studies on the prevalence of *Eimeria* spp. infection in cattle are numerous and it is generally accepted that calves have the highest prevalence of infection and shed high numbers of oocysts during their first year of life (Fitzgerald, 1962; Ernst et al., 1984, Ernst et al., 1987; Cornilissen et al., 1995; Diaz de Ramirez et al., 2001). Further investigation revealed that calves are naturally infected shortly after birth with a diverse population of *Eimeria* spp. Diaz de Ramirez et al. (2001) found that all (n=55) calves separated from their dams at 3 days of age and subsequently housed individually shed *Eimeria* spp. oocysts by 3 months of age. Well over half of these calves were shedding *E. bovis* oocysts by 28 days of age, leading them to conclude that calves are naturally infected with pathogenic *Eimeria* species within the first week of life. Similar results were obtained by Sanchez et al. (2008), who observed the highest prevalence of oocyst shedding in individually housed dairy calves at 26-30 days of age. In both studies, however, no signs of coccidiosis were observed.

Studies conducted with calves 3-12 months of age also indicate a high prevalence of *Eimeria* spp. infection. A four year, longitudinal study conducted by Ernst et al. (1984) on pastured beef cattle in northern Georgia found that 90% of fecal samples from calves were positive for *Eimeria* spp. oocysts. Although *E. bovis* was the most prevalent species identified from these calves no signs of coccidiosis were observed. Twelve *Eimeria* spp. were recovered over the course of the study and mixed *Eimeria* spp. infections were most common. These results are in accordance with many other surveys reporting the prevalence of *Eimeria* spp. infection in calves from the U.S.A (Szanto et al.,

1964; Nyberg et al., 1967; Jacobson and Worely, 1969) and worldwide (Lee and Armour, 1959; Hasbullah et al, 1990; Cornelisson et al., 1995; Matjila and Penzhorn, 2001).

Seasonal patterns in *Eimeria* spp. oocyst shedding were also evident in the Georgia calves. The highest number of oocysts and highest prevalence of samples containing *E. bovis* oocysts were detected in May when the calves were approximately 4 months old. Interestingly, the oocyst counts decreased sharply through mid-summer and remained stable through the last sampling prior to weaning. The authors concluded that calves shed high numbers of oocysts early in life and then gained immunity that kept oocyst counts low. Unfortunately, these calves were not sampled beyond weaning so no information is available on how weaning affected the oocyst output in these naturally infected calves.

Few studies are available on the prevalence of *Eimeria* spp. oocyst shedding in yearling cattle. One study conducted by Cornelisson et al. (1995) on housed dairy cattle found that 43% of yearling cattle shed *Eimeria* spp. oocysts. The numbers of oocysts shed by these yearling cattle were significantly lower than the numbers shed by calves, but mixed infections still dominated. Studies investigating the prevalence of *Eimeria* spp. infection in adult cows have observed a lower prevalence than that reported for calves and yearlings (Grisi and Todd, 1978; Ernst et al 1984, Cornelisson et al. 1995). The level of *Eimeria* spp. shedding was greatly reduced in cows as compared to calves and yearlings, but mixed infections were still common.

The results of all the studies investigating the prevalence of bovine *Eimeria* infection indicate that all cattle are infected with *Eimeria* spp. in all conventional management systems. Although subclinical infection is quite common, clinical disease is

not. Herd level outbreaks of clinical coccidiosis in cattle do, however, occur and are most common in calves less than one year of age in association with stressful events such as weaning, transport and harsh weather.

In Australia, outbreaks of diarrhea in recently weaned beef calves have been attributed to *E. zuernii* infection (Parker et al., 1984; Parker et al., 1986). Clinical signs occurred in these calves at 3-4 weeks after weaning and coincided with highest fecal *E. zuernii* output. Likewise necropsy conducted on expired calves confirmed the presence of large numbers of *E. zuernii* sexual stages within the large intestine. Similar outbreaks of coccidiosis have been reported in recently weaned calves from western North America (Fitzgerald, 1962, Niilo, 1970a, Niilo, 1970b). Unlike the outbreaks in Australia that occurred during hot, dusty times of the year, these occurred during periods of cold harsh weather and were termed ‘winter coccidiosis’. In both circumstances the weather was not conducive to *Eimeria* sporulation around the time that outbreaks were observed. These observations have led to speculation about the source of the oocysts that resulted in these outbreaks.

Two hypotheses have been proposed concerning the potential sources of infective *Eimeria* spp. oocysts in herd outbreaks of disease. The first of these (Marsh, 1938) is termed the exacerbation hypothesis because it states that stress factors such as change in diet, cold weather, and weaning make calves more susceptible to infection by *Eimeria* already present in their environment and that certain *Eimeria* spp. present in the gut at the time of stress have the capacity to increase their reproductive potential. The second hypothesis (Boughten, 1945) is termed the concentration hypothesis because it centers on the premise that coccidia normally present in the environment serve as the ultimate



source of infection and these infective stages are concentrated in areas of high animal density resulting in high infection pressure and outbreaks of disease.

According to Marquardt et al. (1960) conditions in Australia (Parker et al., 1984; Parker et al., 1986) and Canada (Niilo, 1970a) would not have been suitable for *Eimeria* sporulation because in Australia temperatures were greater than 40°C and below -7°C in Canada. Furthermore, sampling of the soil in the calves' environment by Fitzgerald (1962) in a similar outbreak of coccidiosis in recently weaned calves did not reveal the presence of a substantial number of infective oocysts. These studies provide some support of the exacerbation hypothesis for stress associated outbreaks of coccidiosis in cattle (Marquardt, 1962, Niilo, 1970a, Parker et al., 1986, Step et al., 2002). It has been suggested that developmental stages of pathogenic *Eimeria* spp. become inhibited in the tissues of calves and stress-induced immunosuppression allows them to reactivate and cause clinical disease (Marquardt, 1976; Speer et al., 1985). Lindsay and colleagues (1990) attempted to both identify extraintestinal stages of *E. bovis* as well as cause relapse of clinical coccidiosis using immunosuppressants in experimentally infected calves. The calves developed signs consistent with clinical coccidiosis at 18-20 days post infection, but *Eimeria* spp. oocyst shedding could not be induced by repeated high doses of corticosteroids in the recovered calves beginning 64 days after the initial infection. Immunohistochemical staining did reveal the presence of immature first generation schizonts (meronts) in the mesenteric lymph node of one calf euthanized at 9 days post infection. Although a relapse of disease could not be induced in this study, the authors concluded that extraintestinal stages of *E. bovis* may play a role in the development of stress associated outbreaks of coccidiosis.

Many authors still support the concentration hypothesis (Jolley and Bergstrom, 1977; Svensson, 1994, Dauschies and Najdrowski, 2005, Jolley and Bardsley, 2006), mostly because outbreaks of disease occur in calves crowded into small areas where large numbers of oocysts can be recovered (Svensson, 1997; Svensson et al. 2000). Summer outbreaks of coccidiosis were observed in calves from Wyoming and were attributed to drying of pastures and congregation of animals around highly contaminated areas such as creek bottoms and water troughs (Jolley and Bergstrom, 1997). The most recent evidence in support of the concentration hypothesis, however, comes from research conducted in Sweden (Svensson et al., 1993, Svensson et al., 1994, Svensson, 2000). In these studies, young calves consistently develop diarrhea and lose weight 2-3 weeks after winter turnout onto pasture. These clinical signs occur in association with fecal shedding of high numbers *E. alabamensis* oocysts and heavy environmental contamination with *E. alabamensis* oocysts. Additionally, hay harvested from these areas induced diarrhea and weight loss when fed to other housed calves (Svensson, 1997). These findings led the authors to conclude that exposure to high levels of infective *Eimeria* spp. oocysts in the environment is sufficient to induce clinical disease in the absence of stress.

It is possible that outbreaks of coccidiosis in recently stressed calves are a result of a combination of factors outlined in each hypothesis. It is true that ambient weather conditions did not support sporulation of *Eimeria* spp. oocysts in the outbreaks observed in Australia and Canada, but no attempt was made to quantify the number of previously sporulated oocysts in the environment that may have been present at high levels. Fitzgerald (1962) did attempt to recover oocysts from the environment of calves suffering from clinical coccidiosis, but was unsuccessful. No attention was given, however, to the

level of oocyst contamination in the holding pens that these calves were housed in 10 days prior to weaning. Similarly, Niilo (1970a) and Parker et al. (1984; 1986) made no attempt to ascertain whether or not the calves could have been exposed to high numbers of oocysts prior to the stress of weaning and harsh weather. Coccidial stages may have already been replicating in calf intestines at the time of exposure to stress. Likewise, it is impossible to say that the calves in the study by Jolly and Bergstrom (1997) were not stressed by heat and crowding when they were exposed to high levels of *Eimeria* spp. oocysts apparently present in the areas of animal congregation. The available evidence does not completely elucidate the mechanism for stress associated outbreaks of coccidiosis in cattle, because the temporal relationship of oocyst exposure and stress required to produce disease has not been thoroughly investigated. It may be that a combination of exposure to high numbers of pathogenic oocysts prior to or at the same time as a stressful event is required to induce clinical disease. Indirect support of this temporal relationship of *Eimeria* spp. oocyst exposure and stress is available in other studies in which clinical disease was greatly exacerbated when the immunosuppressant, dexamethasone was administered to calves 13-15 days after an oral inoculation with *E. zuernii* (Niilo, 1970c; Stockdale 1987) and *E. bovis* (Friend and Stockdale, 1981). Interestingly, if dexamethasone was administered at the same time as *E. zuernii* oocysts an exacerbation of clinical signs was not observed (Niilo, 1970c). These findings led the author to conclude that the accumulation of coccidial stages in the intestine was necessary if immune suppression is to result in clinical coccidiosis. When considered together, all of these findings suggest more thorough investigation of the temporal

relationship of exposure to *Eimeria* spp. and induction of stress is necessary to more fully appreciate the mechanism of stress associated outbreaks of coccidiosis in cattle.

### Immunology

Cattle become less likely to develop clinical coccidiosis as they age (Hammond, 1964). Generally, it is believed that following a primary infection by *Eimeria* spp. cattle are subsequently protected from the development of clinical disease by immunity (Dauguschies et al., 1986; Lindsay et al., 1990; Svensonn et al., 1996). However, immune protection may fail during stressful events such as weaning, transport, and harsh weather. Immunity is specific to each coccidian species and immunity to one species confers no protection to infection by another species (Fayer, 1980). The degree of immunity induced by the primary infection also appears to be somewhat dependent on quantity of oocysts administered. Work by Conlogue and colleagues (1984), revealed that calves primarily infected with 2000 *Eimeria* spp. oocysts displayed the same clinical signs and reduced weight gains after a challenge with 200,000 *Eimeria* spp. oocysts naïve calves. There is also evidence that age alone affords some natural resistance. Calves reared coccidia free require higher doses of *E. bovis* to induce clinical disease as they age (Hammond, 1964). This author concluded that resistance to *E. bovis* induced coccidiosis is mediated by age and immunity. Regardless of the exact mechanism, it appears that as cattle age their susceptibility to both infection and disease decreases.

Cell mediated immunity appears to be most important in the immune response to *Eimeria* infection in several species including cattle (Fitzgerald, 1967, Long, 1982; Speer et al., 1985; Hughes, 1988; Fiege et al., 1992). Humoral immunity is also stimulated by *Eimeria* spp. infection as evidenced by the production of merozoite specific antibodies in

cows shortly after *E. bovis* infection (Fiege et al., 1992). Likewise, these antibodies are transferred in the colostrum to calves, but the level of antibody transfer does not correlate with disease susceptibility following subsequent *E. bovis* challenge (Fiege et al., 1992). Thus, the authors concluded that antibody production reflects exposure but does not confer protection to *E. bovis* infection. According to Faber et al. (2002), however, the level of anti-*E. bovis* merozoite IgG2 present in the serum from naturally infected calves inversely correlated with the level of *E. bovis* oocyst excretion. These authors speculated that *E. bovis* infection may stimulate natural killer cells to release IFN- $\gamma$  and thus lead to the production of increased levels of IgG2.

Most recent studies investigating the immune response to *Eimeria* have been carried out in rodents and poultry, but the results are thought to generally apply to cattle (Dauguschies and Najdrowski, 2005; Lillehoj and Trout, 1996). Support for the importance of cellular immunity is evident in many of these studies. In mice (Rose et al., 1988) and chickens (Rose et al., 1982) immunity can be adoptively transferred between individuals by using spleen cells. Likewise, a study conducted by Rose and colleagues (1987) found that athymic rats and mice display a more severe primary infection with *Eimeria* spp. and have no resistance to reinfection. The role of helper T-cells in immunity to *Eimeria* spp. infection has also been examined in several species. Depletion of cluster of differentiation (CD) 4<sup>+</sup> lymphocytes (T-helper cells) by anti-CD4<sup>+</sup> antibody administration increased the severity of primary *E. vermiformis* infection in mice (Rose et al., 1992). Additionally, immunity to *E. vermiformis* infection was not adoptively transferred to naïve mice by transfer of lymphocytes from immune mice which were depleted in the CD4<sup>+</sup> subsets (Rose et al., 1988). These results led to the conclusion that

CD4<sup>+</sup> lymphocytes are responsible for mediating the protective response to *Eimeria* spp. infection in mice. Additional evidence for the importance of T-cells in resistance to coccidiosis has also been elucidated by administration of pharmacologic agents that are known to suppress cellular immune responses. Administration of the specific T-cell inhibitor, cyclosporin A prior to oral administration of *Eimeria* spp. oocysts increased susceptibility to primary infection in chickens (Lillehoj, 1987). Similarly, dexamethasone treated chickens exhibited increased susceptibility to *Eimeria* spp. infection and had reduced T-cell proliferation, IL-2 and IFN- $\gamma$  production. Likewise, the use of dexamethasone in calves on day 13 -15 after *E. zuernii* infection greatly exacerbated clinical signs of clinical coccidiosis and intestinal lesions compared to calves did not receive dexamethasone treatment (Niilo, 1970c).

Studies investigating the effect of adoptive transfer of immune cells on subsequent resistance to *Eimeria* spp. infection in cattle are not available. Although the exact role that each lymphocyte subtype plays in the immune response to *Eimeria* spp. infection in cattle is not completely understood, evidence suggests that CD4<sup>+</sup> lymphocytes are the major effector cell type. By using fluorescence activated cell sorting techniques (FACS), Hermosilla et al. (1999), showed that peripheral CD4<sup>+</sup>, CD8<sup>+</sup> and CD2<sup>+</sup> lymphocytes were increased during primary infection with *E. bovis*. Additionally, percentages of lymphocyte subtypes along with  $\gamma\delta$ <sup>+</sup> lymphocytes were also increased in intestinal lymph nodes from infected calves. In a similar study by Suhwold et al. (2010), primary infection with *E. bovis* in calves produced the same increase in circulating CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes during prepatency, but no differences in cell types were detected following *E. bovis* challenge 49 days later. Interestingly, the numbers of CD4<sup>+</sup> and CD8<sup>+</sup>

lymphocytes were increased in samples collected from the colonic mucosa of calves 40 days after primary infection. Only CD4<sup>+</sup> lymphocytes were increased in calves 8 days after challenge infection. Although  $\gamma\delta^+$  lymphocytes have been cited as an important component of mucosal immunity in cattle, particularly in response to *Cryptosporidium parvum* infection (Abrahamsen, 1998), *E. bovis* infection did not stimulate an increase in circulating or mucosal  $\gamma\delta^+$  lymphocytes. These results led the authors to conclude that CD4<sup>+</sup> lymphocytes are likely the most important cell type in the immune response to primary and challenge *E. bovis* infection in calves. These results are different from those obtained from challenge *Eimeria* spp. infection in poultry. Two reports indicate that CD8<sup>+</sup> lymphocytes are increased in the intestinal mucosa of chickens challenged with *Eimeria* spp. oocysts. Increases in mucosal CD4<sup>+</sup> lymphocytes were not detected in either study (Lillehoj and Bacon, 1991; Trout and Lillehoj, 1995). This finding led the authors of both studies to conclude that CD8<sup>+</sup> and not CD4<sup>+</sup> lymphocytes are responsible for the immunity to challenge *Eimeria* spp. infection in chickens.

Investigations into activation of antigen specific T-cells have also been carried out in cattle (Hughes et al., 1989, Hermosilla et al., 1999, Suhwold et al., 2010). Although the source of *E. bovis* antigen (EbAg) varied somewhat among studies, an increased lymphoproliferative response to stimulation with EbAg early in prepatency was observed in calves following primary *E. bovis* infection compared to controls. In the studies conducted by Hermosilla et al. (1999) and Suhwold et al. (2010), EbAg stimulation of lymphocytes also resulted in increased IFN- $\gamma$  production in primary *E. bovis* infection. Likewise, in a similar study conducted by Taubert et al. (2008), an increase in IFN- $\gamma$  mRNA was detected in supernatants from lymphocytes stimulated with EbAg.

Additionally, both studies found an increase in IFN- $\gamma$  production in lymphocytes isolated from the ileocecal lymph node at 8 days post infection. The increase in IFN- $\gamma$  production was not, however, sustained and returned to baseline levels before patent infection developed. No increase in IFN- $\gamma$  production was detected in the same calves following *E. bovis* challenge. Given the nature of the short term increase in IFN- $\gamma$  production in response to EbAg stimulation, the researchers speculated that the immune response to primary *E. bovis* infection may be directed against first generation merozoites developing in the small intestine during this time period. This hypothesis is supported by the earlier histopathologic findings of lymphocyte infiltration in close association with first generation schizonts (meronts) in the small intestine of calves following primary *E. bovis* and *E. zuernii* infection (Friend and Stockdale, 1981; Stockdale, 1987). The authors further postulated that primary *E. bovis* infection results in a CD4<sup>+</sup> driven Th1 response leading to increased levels of IFN- $\gamma$  production, which may trigger the production of nitric oxide (NO) at the intestinal level and rejection of primary infection. However, IFN- $\gamma$  mRNA was not increased in EbAg stimulated lymphocytes isolated following *E. bovis* challenge, leading Taubert et al. (2008) to conclude that IFN- $\gamma$  production was not necessary for immunity to challenge infection. They hypothesized that either an early abrogation of parasite development or IFN- $\gamma$  independent cellular mechanisms were at work in protecting calves from *E. bovis* challenge.

Other mechanisms may be active in protection against *E. bovis* challenge infection. Behrendt et al. (2008) examined the phagocytic and oxidative burst capabilities of bovine PMN neutrophils in an in vitro model. They reported a biphasic increase in both the phagocytic and oxidative burst capabilities of PMN isolated from *E.*



*bovis* infected calves compared to naïve calves. These increases were evident on days 1 and 13-18 post infection, which correspond to important time periods in the course of *E. bovis* development within the intestine. The increase seen at day 1 post infection corresponds to sporozoite transmigration through the ileal mucosa and increases at day 13-18 correspond to maturation and release of first generation merozoites.

Polymorphonuclear cells isolated from infected calves also significantly reduced the number of *E. bovis* sporozoites following a 3.5 hr incubation. Phagocytic capabilities of PMN were also assessed in this study by fluorescence staining *E. bovis* sporozoites and incubation with PMN from infected and non-infected calves. Subsequent FACS analysis revealed that a significantly higher proportion of PMN from infected calves contained sporozoites. To differentiate between phagocytosis and sporozoite penetration of the PMN, the experiment was repeated with heat inactivated sporozoites. Although the number of PMN containing sporozoites was decreased, there were still significantly more present in PMN from infected calves than from naïve calves. Additionally, transmission and scanning electron microscopy revealed that PMN from experimentally infected calves actively attacked and phagocytized *E. bovis* sporozoites. Finally, real-time PCR analysis showed a higher level of nitric oxide synthase mRNA in PMN isolated from experimentally infected calves compared to naïve controls. The researchers concluded that PMN are active players in the innate immune response to *E. bovis* infection.

Further investigation of the role of PMN in the immune response to *E. bovis* infection was carried out by Behrendt et al. (2010). The focus of this study was the ability of PMN to form neutrophil extracellular traps (NET) when incubated with viable *E. bovis* sporozoites. These NET structures were first described by Brinkmann et al. (2004), who

showed that NETs formed by human PMN are able to trap and kill gram positive and negative bacteria. Scanning electron microscopy revealed that NETs were formed when PMN were incubated with viable *E. bovis* sporozoites for 4 hours. These long filamentous structures were able to trap and immobilize viable sporozoites. Furthermore, the subsequent infectivity of these sporozoites for bovine endothelial cells was significantly reduced compared to control sporozoites. The researchers concluded that NET structures formed by PMN may play an important role in trapping and immobilizing *E. bovis* sporozoites before they have the opportunity to infect host cells and thus may be an important part of innate clearance of *E. bovis* challenge infection.

## Stress and the Immune System

### Overview of Stress in Domestic Animals

Stress is defined as a biologic response that occurs when a threat to homeostasis is perceived (Kim et al., 2011). This biologic response has been termed a “stress response”, and is characterized by modifications in physiologic processes that allow an animal to respond to stressful stimuli with minimal alterations to homeostasis (Breazile et al., 1988). When an animal perceives a stressful stimulus it generally has three biologic responses at its disposal to either eliminate or help it cope with the stress. The first and likely most common response is behavioral and may involve the animal moving to a more favorable location without the stressor (Moberg, 1987). When such behavioral responses are not possible the animal must alter its biology by activating the neuroendocrine and autonomic systems (Moberg, 1987).

The neuroendocrine response to stressful events involves activation of the hypothalamic-pituitary-adrenal (HPA) axis (Minton, 1994). This system is activated

when a stressor (pain, fear, anxiety) is perceived by higher neural centers in the brain of an animal. These centers subsequently signal parvocellular neurons located in the paraventricular nucleus within the hypothalamus. Subsequent release of the neuropeptide hormones corticotropin releasing hormone (CRH) and vasopressin (VP) into the hypophyseal portal system stimulates the anterior pituitary gland to release the peptide Adrenocorticotrophic hormone (ACTH) into the blood stream (Tilbrook and Clarke, 2006). Adrenocorticotrophic hormone stimulates the release of the glucocorticoid, cortisol from the adrenal gland. A negative feedback loop exists between blood cortisol concentration and the brain and anterior pituitary gland which regulate the subsequent secretion of CRH, VP, and ACTH.

Cortisol is considered the classic stress hormone and increased concentration in the blood has effects on metabolism that are beneficial in coping with stress in the short term. Glucocorticoids produce shifts in carbohydrate metabolism that increase circulating energy substrates at the cost of energy stores (Breazile, 1988). They act upon adipocytes to stimulate increased lipolysis and on skin, connective tissue and muscle to stimulate proteolysis. Additionally, glucose uptake by all of these tissues is decreased in the presence of increased circulating glucocorticoids, making more glucose available to the brain. These physiologic alterations are ultimately characterized by increased blood levels of amino acids, fatty acids and glycerol, which are used by the liver for gluconeogenesis. The end result is increased blood glucose which can be utilized in dealing with the stressful situation (Breazile, 1988). Removal of the stressor results in decreased glucocorticoid secretion and rapid return to the normal homeostatic state. Since the physiologic response to glucocorticoids is largely catabolic, however, excessive

or prolonged secretion of these hormones results in pathologic states. Muscle wasting due to excessive proteolysis and steroid diabetes resulting from prolonged gluconeogenesis are sequella to prolonged increases in circulating glucocorticoids. Importantly in animal production, infertility, immune suppression and reduced growth are also reported outcomes of prolonged or excessive glucocorticoid concentrations (Breazile, 1988).

Although the HPA axis has garnered most of the attention in research models investigating stress, the autonomic nervous system is also an important player in the stress response (Moberg, 1987; Minton, 1994, Tilbrook and Clarke, 2006). Acute stress is known to activate the sympatho-adrenal medullary (SAM) axis by stimulation of higher brain centers that cause release of catecholamines from the adrenal medulla. Catecholamines, most notably epinephrine, stimulate rapid physiological changes to allow an animal to deal with a stressful stimulus. Generally this response results in decreased activity of organ systems that are not essential in coping with the stressor in the short term. For instance, catecholamines decrease intestinal peristalsis in order to conserve energy for other vital processes. Conversely, catecholamines act on the cardiovascular system to increase heart rate and cardiac output in order to deliver blood to vital organs such as muscle, which may be needed to deal with the short term stress (Minton, 1994).

Although the biological mechanisms and compounds produced during the stress response have been described, quantifying the stress response in domestic species is fraught with difficulties (Moberg, 1987). One such difficulty is individual animal variability in the response to an identical stressful stimulus. It has been postulated that

genetic background, previous experiences, sex and physiologic conditions shape the individual animal's behavioral and biological response to stress (Moberg, 1987; Grandin, 1997). In a study conducted by Moberg and Wood (1982) lambs of identical genetic makeup were reared with their mothers or in isolation until 14 days of age. When these lambs were subsequently exposed to a stressful situation, the lambs reared with their mothers exhibited a higher frequency of behaviors (vocalization and increased activity) consistent with stress. Serum cortisol concentration, however, was not different between the two groups. The authors concluded that previous experiences modulate subsequent behavioral responses despite equal elevations in serum cortisol. They additionally cautioned against the use of only one or two measures of stress in domestic farm animals due to individual animal variability in these measures. They hypothesized that a truer picture of the stress response in farm animals could be realized if combinations of several stress parameters including behavioral and biological variables were measured.

Serum or plasma cortisol concentration is often used as an indicator of the level of stress an animal perceives (Breazile, 1988). Although it has proven to be a useful indicator of stress (Broom and Johnson, 1993; Terlouw et al., 1993), caution is still advised. Normal diurnal rhythm in the secretion of ACTH and cortisol is known to occur. It is reported that peak secretion of cortisol occurs in the morning and minimum secretion late at night for most mammals (Breazile, 1998). Thus, sequential comparisons of cortisol levels on the same animals before and after a stressful stimulus may be biased by this natural rhythm. Furthermore, the act of collecting blood is known to result in significant elevations in blood cortisol concentrations. These limitations have led to investigations into measurement of cortisol in bodily fluids which can be collected less

invasively and which are less sensitive to immediate changes. Cortisol concentration in urine (Hultgren, 1998), saliva (Cooper et al., 1989) and feces (Mostl et al., 2002) is reflective of both recent elevations in blood cortisol concentration and less sensitive to immediate changes. Thus, the quantification of cortisol concentration in these fluids has become common in measuring the stress response in domestic and wild animals.

In addition to cortisol, other parameters have also been used to quantify stress in cattle (Breazile, 1998). Many of these compounds measured in blood fluctuate in response to circulating cortisol concentrations, but may not be as sensitive to rapid changes or exhibit diurnal variation. Blood glucose, blood urea nitrogen and nonesterified fatty acid concentrations increase as cortisol concentration increases because of fluctuations in carbohydrate, protein metabolism, and fat metabolism (Uetake et al., 2006). Although quantifying stress in domestic animals is difficult, it is generally believed that an accurate assessment of stress level can be attained in these species by examining a combination of behavioral and biologic markers (Moberg and Wood, 1982; Grandin, 1997).

Weaning is defined as the physical separation of a mother and offspring, which brings an end to suckling and forces a change to solid food (Price et al., 2003). In nature, this process is gradual, but in conventional cattle management this process is abrupt and generally occurs when the calf is approximately 205 days of age (Fluharty et al., 2000). This event is considered stressful to calves and elevations in stress hormones, including cortisol have been documented (Lefcourt and Elsasser 1995; Hickey et al., 2003; Kim et al., 2011). Recognition of the stressful nature of abrupt weaning has resulted in the development of alternative weaning methods aimed at decreasing distress. These

alternative methods usually involve two stages. The first of these stages prevents the calf from suckling its dam, but still allows limited physical contact for a period of time. The second stage completely separates the calf from its dam (Haley et al., 2005).

The most common of two stage weaning strategy involves using a fence to separate a calf from its dam to prevent sucking or installation of an antisuckling device to prevent suckling while the calf remains with its dam (Boland et al. 2008). Fence line weaning has been shown to decrease stress related behaviors in calves compared to calves weaned by abrupt separation (Stookey, 1997; Price et al., 2003). Furthermore, fence line weaned calves also gained significantly more weight than did abruptly separated calves during the 2 weeks following weaning, which resulted in heavier calves at 10 weeks post weaning (Price et al., 2003). Similar results have been reported with the use of antisuckling devices. Haley et al. (2005) reported that calves weaned by antisuckling noseclips vocalized less, spent less time walking and more time eating and resting than abruptly weaned calves. There was, however, no difference in weight gains observed following separation in these this study.

The link between stress and infectious disease has long been recognized (Breazile, 1988) and is observed in calves following the stress of weaning (Roth et al., 1982; Callan and Garry, 2002; Snowden, 2009). This association may be related to the immunomodulatory effects of stress hormones. Glucocorticoids are known anti-inflammatory compounds through their direct action on cell nuclei (Breazile, 1988) causing changes in metabolic enzyme synthesis. These compounds lead to the production of peptide hormones known as lipiocortins that subsequently limit the activation of the enzyme phospholipase A2 (Blackwell et al., 1980). Phospholipase A2 is

responsible for releasing membrane bound arachidonic acid, which is in turn metabolized by cyclooxygenase to form prostaglandins, thromboxane and leukotrienes. Each of these compounds plays an important role in chemotaxis of reactive cells into tissues. Thus, when the production of these compounds is limited the ability to generate an inflammatory response in tissues is greatly reduced.

In addition to their direct anti-inflammatory effect, glucocorticoids also exert a profound effect on circulating leukocytes (Breazile, 1988; Burton et al. 2005). This response is commonly referred to as a stress leukogram and is characterized by a leukocytosis with a mature neutrophilia and lymphocytopenia. The mechanism for lymphopenia is related to increased margination of lymphocytes and translocation to tissue as well as inhibition of re-entry into circulation through high endothelial venules (Breazile, 1998). The mechanism for neutrophilia largely results from demargination of neutrophils from blood vessel walls due to a decrease in surface adhesion molecules (Burton et al., 1995). The end result of high levels of circulating glucocorticoids is increased susceptibility of cattle to various infectious agents including bacteria and viruses (Roth and Kaeberle, 1982; Burton and Erskine, 2003). Recent evidence, however, suggests that the observed increase in disease susceptibility in response to high levels of glucocorticoids may not be a result of broad immune suppression, but more precisely a dysregulation in the functional branches of the immune system (Elenkov and Chrousos; 1999).

#### Stress and Th1/Th2 Balance

The adaptive immune system is often divided into two branches based on the functionality of each (Coico and Sunshine, 2009). The first of these branches is referred



to as cell mediated immunity, because the protective function of this branch is associated with cells. The second branch is referred to as humoral immunity because the protective function is associated with antibodies found in bodily fluids (humours). In both branches, a specialized type of lymphocyte, the thymic (T)-helper cell, is essential in the development of a coordinated response (Mosmann and Sad, 1996). These T-helper lymphocytes all develop in the thymus, where they undergo stringent negative selection against recognition of self antigens (Coico and Sunshine; 2009). In addition, these cells also acquire a T-cell receptor molecule, which has the ability to recognize antigen only when bound to a specific signaling molecule known as the major histocompatibility complex two (MHC II) (Mosmann and Sad, 1996). Within the thymus, the T-helper cell also gains a transmembrane, co-receptor molecule, known as cluster of differentiation (CD) molecule 4. This molecule does not bind antigen, but binds to other portions of the MHC II molecule at antigen presentation thereby lowering the threshold for antigen recognition by the T-helper cell (Coico and Sunshine, 2009). Once released from the thymus the naïve T-helper cells circulate through the blood as well as the peripheral and mucosal lymph nodes in search of foreign antigen.

Naive T-helper cells can only recognize foreign antigen if it is presented to them on the MHC II molecule (Coico and Sunshine, 2009). This molecule is only present on certain cells referred to as professional antigen presenting cells, which include macrophages, dendritic cells, and B-cells. These cells have the ability to internalize and process foreign protein by a process known as exogenous antigen processing. Once processed, the antigen is relocated to the exterior of the cell in the antigen binding region of the MHC II molecule (Coico and Sunshine, 2009). When a naïve CD4<sup>+</sup> T-cell

carrying an appropriate T-cell receptor comes into contact with this antigen presenting cell and all costimulatory molecules align, the naïve T-cell becomes activated.

Activation is carried out by a series of intracellular reactions stimulated by the intracellular portion of the T-cell receptor. A complex signaling cascade begins, which ultimately leads to the production of interleukin (IL) 2. This molecule serves as a potent growth factor for T-lymphocytes and allows for expansion of T-helper cells carrying an identical T-cell receptor in a process known as clonal expansion (Coico and Sunshine, 2009).

At this point the activated T-helper cell differentiates into one of four major subsets (Coico and Sunshine, 2009). These subsets are defined based on the array of cell signaling molecules (cytokines) that each produce. The exact signaling mechanism that results in the T-helper cell's differentiation into one of the 4 subtypes is not known. It is clear that certain cytokines stimulate the development of the T-helper cell into one of the four subtypes, although the source of each of these cytokines is not always clear (Elenkov and Chrousos, 1999; Coico and Sunshine; 2009). It is believed that IL-12 produced by dendritic and natural killer (NK) cells stimulate T-helper cells to differentiate into T-helper one (Th1) subtypes. This subtype subsequently produces IFN- $\gamma$ , which important in activating macrophages, NK cells, and CD8<sup>+</sup> T- lymphocytes. These cell types are the effector cells important for killing intracellular pathogens such as viruses, protozoa and bacteria. Interleukin 4 is believed to be the most important cytokine in the differentiation of T-helper cells into T-helper type two (Th2) cells. Currently the source of the IL-4 responsible for this differentiation is not clear, but it is suggested that it comes from mast cells or natural killer T (NKT) cells (Elenkov and Chrousos, 1999; Coico and Sunshine,

2009). When T-helper cells differentiate into Th2 subsets they produce IL-4, IL-5, and IL-13. These cytokines act on eosinophils and B-cells, which are important in helminth infections and development of allergic reactions (Elenkov and Chrousos, 1999; Coico and Sunshine, 2009). Tumor growth factor (TGF)  $\beta$  and IL-21 stimulate the differentiation of naïve T-helper cells into T-helper 17 (Th17) subsets. This subset produces IL-17 and IL-22, which activate neutrophils and epithelial cells. These cell types are important for responses to fungi and extracellular bacteria. Finally, TGF- $\beta$  stimulates the differentiation of naïve T-cells into T-regulatory (Treg) cells which produce IL-10. This is an inhibitory cytokine that is believed to limit the function and proliferation of other T-cell subsets (Coico and Sunshine, 2009).

Interestingly, Elenkov and Chrousos (1999) reported that Th1 and Th2 cytokines are mutually inhibitory to one another and this finding is also echoed by Coico and Sunshine (2009). The best example of this cross inhibition is observed in mice. Murine IL-12 and IFN- $\gamma$  has been shown to down regulate the Th2 response and polarize the immune response toward a Th1 response (Szabo et al., 2000). Conversely, murine IL-4 and IL-10 are known to down regulate the Th1 response and shift immunity toward a Th2 response (Fiorentino et al., 1989).

The Th1/Th2 paradigm of adaptive immunity and associated cross regulation of the two branches has provided a useful framework for understanding the apparent bias toward cell mediated or humoral immunity induced by pathogens (Brown et al., 1998). Several authors (Kelso, 1995; Brown et al., 1998) now feel that this paradigm is an oversimplification of a much more complex immunoregulatory network. Furthermore, Brown et al., (1998) explains that this paradigm, which effectively explains murine

model results, may not hold true in other species. These authors applied the same system used by Mosmann and Coffman (1989) and Romagnani (1994) to define the human and murine Th1/Th2 paradigm to characterize bovine Th subsets. T-helper cell clones were derived from cattle immune to *Babesia bovis*, *B. bigemina*, or *Fasciola hepatica*. Additionally, purified protein derivative (PPD) specific T-cells were also obtained from cattle with no known exposure to *Mycobacterium bovis*. Over sixty Th clones were compared for mRNA expression following mitogen or antigen stimulation. In these experiments it was determined that the majority of parasite-specific Th cell clones co-expressed Th1 (IFN- $\gamma$ ) and Th2 (IL-4) cytokines. Similarly, IL-10 transcripts were detected from Th1 and Th2 T-cell clones specific for *Babesia* or *Fasciola*. Conversely, PPD-specific T-cell clones has a more restricted cytokine phenotype, with very few expressing both IL-4 and IFN- $\gamma$ . According to these authors, these findings suggest more heterogeneity in cytokine expression of CD4<sup>+</sup> T-lymphocytes in cattle than previously reported in mice. Based on the results of the PPD-specific T-cells, however, the authors confirmed that polarized Th1 or Th2 responses can be induced in cattle. Research by Rodriguez et al. (1996) also supports this finding. T-cell clones from cattle immunized against *B. bigemina* expressed predominant Th1 cytokines while expressing low levels of Th2 type cytokines.

Brown et al. (1998) also reviewed several studies investigating the regulation of bovine derived T-cell proliferation and IFN- $\gamma$  production by various cytokines. Particular emphasis was put on the immunoregulatory potential of IL-10, IL-12 and IL-4. Recombinant IL-10 was shown to be suppressive for bovine T-cell proliferation and IFN- $\gamma$  production (Brown et al., 1994; Chitko-McKown et al., 1995). Addition of

recombinant IL-12 and mitogen to bovine derived T-cell populations induced a moderate increase in cell proliferation and a marked increase in IFN- $\gamma$  production. Lastly, addition of IL-4 had minimal effects of Th cell proliferation and either suppressed or had no effect on subsequent IFN- $\gamma$  production (Brown and Estes, 1997). Taken together, these findings suggest that the cross regulatory pathways between the functional Th1 and Th2 branches of the immune system are more complex than previously described for mice (Brown et al., 1998). However, a general similarity exists between these species and certain antigens are able to polarize the bovine immune response toward a Th1 or Th2 response (Brown et al., 1998).

In recent years, much attention has been paid to the effect of stress on the immune system in humans and other animal species (Yang and Glaser, 2002). As described above, stressful events stimulate the HPA and SMA axis leading to increased levels of stress hormones including glucocorticoids and catecholamines. Glucocorticoids, through their classical action on cytoplasmic/nuclear receptors have been shown to suppress IL-12 production by antigen presenting cells (Elenkov et al., 1996; Blotta et al, 1997). Decreased IL-12 production would limit the differentiation of naïve T-helper into Th1 cells, thus decreasing the subsequent production of IFN- $\gamma$ . Elenkov and Chrousos (1999) propose that a decrease in these two cytokines results in an increase in IL-4 synthesis and a subsequent polarization of the immune system toward a Th2 response. This proposal is supported by Dekruyff et al. (1998), who showed that glucocorticoid treatment of macrophages resulted in less production of IL-12 which led to significantly decreased production of IFN- $\gamma$  by CD4<sup>+</sup> T-cells. The same glucocorticoid treatment resulted in increased production of IL-4 by these cells. Likewise, catecholamine production during

stress is also believed to contribute to polarization of the immune response toward Th2. Elenkov and Chrousos (1999) also reported that norepinephrine and epinephrine inhibited the production of IL-12 and enhanced the production of IL-10 in human whole blood cultures stimulated with lipopolysaccharide.

Research conducted in vivo also supports stress-induced polarization toward a Th2 type response. Viveros-Paredes et al. (2006) demonstrated that mice treated with electric shock produced a higher level of corticosterone than did unshocked mice. Furthermore, splenocytes isolated from the mice that underwent the electric shock produced a higher level of IL-4 and IL-10 and less IFN- $\gamma$  after stimulation with mitogen than did splenocytes from the control mice. Likewise, lymphocytes isolated from blood of shocked mice exhibited a decreased lymphoproliferative response to mitogen stimulation than did control mice. Lastly, the mice that underwent the electric shock exhibited a less intense delayed hypersensitivity reaction to injection with flurobenze compared to controls. In light of these results, the authors concluded that stimulation of the HPA axis results in dysregulation of the immune system and polarization toward a Th2 type response in the mouse.

The effect of stress hormone administration on adaptive immune function in cattle has also been investigated. Nonnecke and colleagues (1997) found decreased production of IFN- $\gamma$  by lymphocytes isolated from bulls two days after dexamethasone injection compared to bulls that did not receive dexamethasone. Interestingly, they also detected a significant reduction in the percentages of circulating CD4<sup>+</sup> and CD8<sup>+</sup> in the bulls that received the dexamethasone injection. A similar study was conducted by Menge and Dean-Nystrom (2008). This study investigated changes in circulating white blood cell

types, proliferative responses of lymphocytes and cytokine mRNA production following injection of dexamethasone for two consecutive days in Jersey bulls. Two days after dexamethasone treatment a marked stress leukogram characterized a mature neutrophilia and lymphocytopenia was detected. A decrease in CD8<sup>+</sup> and  $\gamma\delta^+$  T-cells was also detected. Furthermore, lymphocyte proliferation in response to mitogen stimulation was decreased in the bulls that received dexamethasone. Lastly, mitogen stimulated lymphocytes isolated from the blood of the dexamethasone treated bulls produced fewer mRNA transcripts for the Th1-type cytokines (IFN- $\gamma$  and IL-2) than did those from control bulls. The results of these studies led the authors to suggest that dexamethasone administration in cattle does not generally down regulate the adaptive immune response, but has variable effects on different elements of the immune response. Similar results have been obtained by the administration ACTH to cattle of various age groups (Paape et al., 1977; Gwazdauskas et al., 1980; Roth et al., 1982). In each of these studies a significant increase in plasma cortisol was induced by administration of ACTH. Paape et al. (1977) and Gwazdauskas et al. (1980) determined that a dose of at least 100 IU of purified porcine ACTH by intravenous or intramuscular injection was required to induce a stress leukogram in lactating dairy cows. Roth et al. (1982) employed this dose regimen and found that lymphocyte proliferation to mitogen was diminished in steers and heifers that received ACTH compared to non-treated controls. Results of this study led the researchers to conclude that ACTH administration stimulates significant cortisol release, which in turn results in suppressed cellular immune function. Unfortunately, no studies are available that investigate changes in lymphocyte subtypes in response to ACTH treatment in cattle.

Weaning is known to increase plasma and fecal cortisol levels in calves (Lefcourt and Elsasser 1995; Hickey et al., 2003; Gilbertie et al., 2010; Kim et al., 2011). Hickey et al. (2003) observed that the increase in plasma cortisol in abruptly weaned calves was accompanied by a decrease in serum IFN- $\gamma$  concentration. Recently, the effect of abrupt weaning on lymphocyte distribution and neutrophil function has been examined in calves (Lynch et al., 2010a). This study showed that lymphocyte distribution was altered by weaning. Total white blood cell count was increased in abruptly weaned calves on day 2 post-weaning compared to non-weaned control calves. Additionally, CD4<sup>+</sup>, CD8<sup>+</sup> and WC1<sup>+</sup> lymphocytes and neutrophil function were decreased on day 2 post weaning in abruptly separated calves compared to non-weaned controls. Although no attempt was made to quantify cortisol concentrations in blood or feces, the authors concluded that the stress associated with weaning impairs immune competence in beef cattle. A similar study investigating the effects of cessation of milk feeding in dairy calves on inflammatory cytokines, cortisol and leukocyte subtypes was also conducted by Kim et al. (2011). This study found that plasma cortisol concentrations were increased in calves 5 days after the succession of milk feeding. This increase was accompanied by decreased IFN- $\gamma$  and increased tumor necrosis factor (TNF) alpha. The neutrophil to lymphocyte ratio was also increased on day 5 post cessation of milk feeding. The proportion of CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes were also numerically increased at 5 days, but this increase was not statistically significant. Based on these results the authors concluded that the succession of milk feeding is stressful to calves based on the observed elevations serum cortisol. They also concluded that this stressor is sufficient to result in alterations in inflammatory cytokines, which may result in immune attenuation.



### *Stress Hormones and Neutrophil Function*

Neutrophils play an important function in innate immunity because they are the first line of defense against invading pathogens (Burton et al., 2005). Neutrophils in the circulating pool have the ability to marginate and traverse blood vessels when signaled by various chemokines originating from inflamed tissue. At the site of tissue damage, neutrophils are able to phagocytize foreign molecules and cellular debris as well as undergo a respiratory burst aimed at killing invading pathogens. Following completion of these functions neutrophils undergo programmed cell death known as apoptosis. Research has shown that stress hormones, most notably glucocorticoids, alter these functions (Burton et al., 2005).

Neutrophils express high levels of glucocorticoid receptors in their cytoplasm while in the circulatory pool. Upon glucocorticoid binding of this receptor, it is translocated into the nucleus where gene transcription is ultimately altered. Burton et al. (2005) indicates that genes involved with migration (CD62L) and programmed cell death (Fas) are primarily altered by glucocorticoid stimulation. Additionally, neutrophil bactericidal activity is depressed, which is linked to increased disease susceptibility (Lynch et al., 2010a; Burton et al., 2005).

One of the best examples of this phenomenon in nature is parturition in the bovine. During this time period the concentration of circulating cortisol is elevated and increased disease susceptibility in postpartum cows is well documented (Burton et al., 1995). High levels of cortisol are associated with decreases in gene transcripts that code for apoptosis of neutrophils as well as an increase in neutrophil derived anti-apoptotic gene transcripts. Furthermore, genes important to vascular adhesion and tissue migration

of neutrophils are also down regulated at times when plasma cortisol concentration is elevated. Lastly, genes known to be important for production of reactive oxygen species and respiratory burst functions of neutrophils have been shown to be down regulated in the presence of high plasma cortisol (Burton et al. 2005). These findings have led researchers to hypothesize that changes in neutrophil function play an important role in increased disease susceptibility at times of stress (Burton et al. 1995; Weber et al. 2001; Weber et al. 2004; Burton et al. 2005).

## Conclusion

*Eimeria* spp. infections occur wherever cattle are kept, thus they impose a constant threat to animal health and production. Although millions of dollars are spent annually on the control of coccidial infections in the USA cattle industry (Fitzgerald, 1980) little information is available on the impact of these organisms in grazing cattle. Calves are naturally infected with these organisms shortly after birth and shed high levels of a diverse population of *Eimeria* spp. in their first year of life. A severe life threatening disease may result from heavy infections with the pathogenic species *E. bovis* and *E. zuernii*. Immunity to relapse of disease develops following the initial exposure which becomes more complete with age. Cell mediated immunity is believed to be most important in primary *Eimeria* spp. infection in several animal species including cattle. The immune response to challenge infection is less understood and it is unclear whether adaptive cell mediated immunity or innate immunity is most important in abrogating early infection. Although immunity develops following primary *Eimeria* spp. exposure, stressful events such as weaning, transport and harsh weather have been associated with outbreaks of clinical coccidiosis in cattle. Studies aimed at reproducing this disease have

largely been unsuccessful. Administration of glucocorticoids has, however, resulted in complete dissolution of immunity in poultry and exacerbation of clinical disease in cattle. Stress in the form of weaning and transport has been shown to result in decreased immune function and increased susceptibility to disease in cattle. It is still not clear whether stressful events shift the immune response away from a Th1 and toward a Th2 type response in cattle as they do mice. Decreases in circulating CD4<sup>+</sup> and CD8<sup>+</sup> lymphocyte subtypes as well as decreases in Th1-type cytokines following weaning and transport in cattle do, however, support this concept. Likewise, stressful events such as transport and parturition in cattle are also associated with diminished neutrophil function. These facts provide leverage to the argument that stress-induced depression of the immune system is responsible for stress associated outbreaks of coccidiosis in calves.

The source of *Eimeria* spp. oocysts that result in outbreaks of stress associated coccidiosis is still unclear. Previous reports describing outbreaks of coccidiosis in calves indicate that weather conditions were not suitable for oocyst sporulation nor could oocysts be recovered from the calves' environment. It is possible however, that exposure to high levels of infectious oocysts occurred prior to initiation of the studies. No information is available regarding the level of oocyst buildup on pasture in the eastern United States. Further research is needed to completely characterize the immune response to *Eimeria* spp. infection in cattle during times of stress. Additional research is necessary to elucidate the temporal relationship of stress and *Eimeria* spp. oocyst exposure required to precipitate disease. If stress-induced immune suppression in addition to challenges with high doses of *Eimeria* spp. oocysts are required to induce disease then measures

taken to separate stressful events from oocyst exposure should be recommended to prevent disease.

### **Chapter 3: Variation in *Eimeria* oocyst count and species composition in weanling beef heifers**

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#### **Abstract**

Rectal fecal samples were collected daily on 10 consecutive days in November 2004 from 11 weaned beef heifers to assess daily variation in fecal oocyst count and species composition. Subsequent samples were collected from the same animals on 15 April 2005 and 9 June 2005. Oocyst numbers were determined by the Modified McMaster's test and species were identified by examination of oocysts recovered with the Wisconsin sugar flotation technique. Soil samples were collected from the heifer pasture on 8 June 2005 and oocysts were quantified and identified to species. Mean fecal oocyst counts varied little at all sampling dates ranging from 134 – 377 oocysts per g. Ten *Eimeria* spp. were identified in fecal samples collected in November and April and 11 in June. *Eimeria bovis* was the most common species identified at all samplings. Mean species composition showed little variation during the 10-day sampling period in November, remained similar in April, and varied slightly in June. Twelve *Eimeria* spp.

were identified in soil samples in proportions similar to those seen in fecal samples. The results indicate that clinically normal weanling beef heifers are likely to be infected with a diverse, but relatively stable community of *Eimeria* spp.

### **Introduction**

Species of *Eimeria* are gastrointestinal coccidians that infect cattle worldwide. Reports indicate that calves are infected shortly after birth and shed relatively high numbers of oocysts during their first yr of life (Fitzgerald, 1962; Ernst et al., 1984, 1987; Diaz de Ramirez et al., 2001). Most studies reporting species prevalence have found that *Eimeria bovis* is the most prevalent species in calves less than 1 yr of age based on identification of fecal oocysts (Fitzgerald, 1962; Ernst et al., 1987; Hasbullah et al., 1990; Diaz de Diaz de Ramirez et al., 2001). In contrast, Parker et al. (1984), reported that *Eimeria zuernii* was the most prevalent oocysts in the feces of freshly weaned beef calves in Australia and Svennson et al. (1993) found *E. alabamensis* most prevalent in dairy calves (4-16 mo of age) just after turnout in Sweden. No reports exist, however, that describe daily variation in fecal oocyst counts and species composition in calves. The objective of the present study was to assess daily variation in fecal oocyst count and species composition in clinically normal weanling beef calves and to determine if changes occurred after a period of several mo.

### **Materials and Methods**

Eleven beef heifers (Angus, Charolais, and Hereford breeding), maintained at the Virginia Tech Beef Center, Blacksburg, Virginia, were surveyed for natural coccidia infection. In the previous yr no outbreaks of coccidiosis had been observed at this facility. Heifers ( $287 \pm 19$  days of age at the beginning of the study) were sampled for a period of

10 consecutive days (15 November 2004-24 November 2004). Subsequent samples were collected from 10 of the same heifers 5 mo later (15 April 2005) and from 9 of the heifers 7 mo later (9 June 2005). Heifers were weaned in September 2004 and maintained together on a permanent native grass lot used every yr for replacement animals with free access to water and mineral mix. The heifers were fed daily hay and corn silage. All heifers remained on the same lot from November to June at which time they were moved to summer pastures. They were clinically normal throughout the course of the survey. All fecal samples were collected per rectum from the cattle at approximately 0930 hr. Oocyst number in each fecal sample was calculated using the Modified McMaster's technique with a sensitivity of 25 oocysts per g (OPG) (Whitlock, 1948). A modified Wisconsin sugar flotation technique (Cox and Todd, 1962) was also performed on each fecal sample and 50 *Eimeria* spp. oocysts per sample were examined at 400X and identified to species based on oocyst morphology (Levine and Ivens, 1986). One individual (AL) carried out all oocyst counts and species identifications. Because oocysts of *E. cylindrica* and *E. ellipsoidalis* could not be distinguished reliably, all small cylindrical *Eimeria* spp oocysts measuring from 19-36 um by 8-18 um were designated *Eimeria cylindrica/ellipsoidalis*-like (Levine and Ivens, 1986).

Soil samples (approximately 100 g each) were also collected from different sites in the replacement heifer lot on 8 June 2005, shortly after the heifers were removed. All samples were collected from the top 2.5 cm of soil. A general field sample was obtained by collecting approximately 10 g of soil every 10 m in a diagonal across the field. Soil around the feed bunk and hay ring was also sampled by collecting approximately 10 g subsamples taken every 2 m from a distance of approximately 3 m away from and along

the length of the feed bunk and around the entire perimeter of the hay ring.

Approximately 10 g of soil were also collected from beneath 10 fecal pats that were several days old and selected on a first seen basis while walking a diagonal across the field. For each area (whole pasture, feed bunk area, hay ring area, and fecal pats), samples were mixed well and a 20 g sub-sample was analyzed using the modified Wisconsin sugar flotation technique (Coxx and Todd, 1962). Oocysts were identified to species based on bovine *Eimeria* spp. oocyst morphological characteristics (Levine and Ivens, 1986).

## Results

*Eimeria* spp. oocysts were seen in 94% of fecal samples collected during the 10-day period. Heifer fecal oocyst counts ranged from 0 -700 OPG throughout the sampling period with an overall mean oocyst count of 192 OPG. Arithmetic mean daily oocyst counts for the heifers remained similar over the course of the 10-day sampling period, ranging from 134–377 OPG. Similarly, oocyst counts from individual animals exhibited little variation over the course of the 10 days, with more variation in oocyst count seen between individuals within days. Those animals with relatively high oocyst counts at the start of the study maintained higher oocyst counts throughout, while those that began with lower oocyst counts maintained low levels over the sampling period. These individuals also maintained their relative high or low oocyst shedding tendency in samples collected in April and June. Ten *Eimeria* spp. were recovered from the heifers during this period (Table I). *Eimeria bovis* was the most common species found, present in 100% of all positive samples. *Eimeria zuernii* was the second most common species (96 %), followed by *E. alabamensis* and *E. auburnensis* (91%), *E.*



*cylindrica/ellipsoidallis*-like (80%), and *E. canadensis* (67%). All other species were present in fewer than 50% of positive samples.

Mean daily species compositions for the 10-day sampling period are presented in Figure 1. *Eimeria bovis* was the most numerous species identified on all 10 days, with a mean of 40% of the oocysts identified in each sample being *E. bovis*. The second most numerous species varied slightly from day-to-day among *E. zuernii*, *E. alabamensis* and *E. cylindrica/ellipsoidallis*-like. Overall, the mean species composition exhibited little daily variation.

Results from the 10 heifers sampled on 15 April 2005 were generally similar to the results obtained from the 10-day sampling period in November (Table I). The mean oocyst count (145 OPG) and species composition showed little change. Nine of the 10 *Eimeria* spp. previously seen were present (*E. bukidonensis* not seen). Oocysts of *E. brasiliensis*, which were not seen the previous November, were seen in 2 of the 10 animals. On 6 June 2005, the mean fecal oocyst count for the 9 heifers sampled was 173 OPG. *Eimeria bovis* made up the highest mean percentage of oocysts identified per sample, but the mean percentage of *E. canadensis* was only slightly lower (Table I). Oocysts of *E. canadensis* were first detected on 18 November 2004, and the proportion appeared to increase from November to the following June. In November, *E. canadensis* oocysts made up less than 10% of the oocysts counted in 8 of 11 animals. In June, however, only 1 of the 9 animals had less than 10% *E. canadensis*, and 6 heifers had greater than 20%. No animals had a lower percentage of *E. canadensis* in June compared to November.

All soil samples analyzed from the sites around the heifer's pasture contained coccidia oocysts. Eighty-four percent of the oocysts identified were sporulated. The sample collected from the feed bunk had the most coccidia oocysts, (1,532 oocysts in 20 g of soil). Samples analyzed from the hay ring, beneath fecal pats and from the field contained 284, 232, and 115 oocysts per 20 g of soil, respectively. The species composition was similar among all 4 sites. In total, 12 *Eimeria* spp. were seen in these samples. The mean percentages of oocysts identified in samples from all sites are presented in Table II. The percentages of oocysts identified in these samples were similar to the percentages reported for the heifer fecal samples, with *E. bovis* predominating. Although coccidian oocysts of wild mammals and birds may have been present in the soil and could not be differentiated from bovine *Eimeria* spp., the similarity in species distribution between manure and soil samples suggests that most of the recovered oocysts were of bovine origin.

### **Discussion**

There are no published reports describing short term daily variation in fecal oocyst count or *Eimeria* spp. composition in cattle. The results of this study, conducted on weanling beef heifers, found little daily variation in both fecal oocyst count and species composition over a 10-day period. Minimal variation was seen during this period, thus the next 2 samplings were carried out on a single day to assess longer-term changes in oocyst count and species composition. Little change was seen in mean fecal oocyst counts from the same heifers sampled approximately 5 and 7 mo later, although some differences in mean species composition was observed. *Eimeria brasiliensis* and *E. illinoisensis* were not seen in the first sampling, but were more common and numerous in

subsequent samples. Likewise, *E. canadensis* became almost as numerous as *E. bovis* by June (Table 1). This suggests that there may be host and/or environmental factors that lead to gradual changes in the dominant *Eimeria* spp. present in cattle as they age. Results of this study also indicate that weanling beef heifers are infected with a diverse community of *Eimeria* spp. This diversity is consistent with surveys of cattle from around the world (Ernst et al., 1984; Parker et al., 1984; Habushala et al., 1990; Svensson et al., 1993).

Soil samples collected from various locations on the heifer's lot also had a diverse community of *Eimeria* spp. oocysts. Oocysts of all 12 *Eimeria* spp., identified in fecal samples collected over the course of the whole study, were also seen in similar proportions in these soil samples. This result, together with the fact that the majority (84%) of oocysts recovered from the soil were sporulated, suggests that oocysts in the environment sporulate and remain viable in proportions similar to that passed in the feces, at least in the warmer mo of the yr.

Most published reports dealing with the epidemiology of coccidia infections in beef cattle focus on cow/calf pairs or freshly weaned calves (Fitzgerald, 1962; Parker et al., 1984; Ernst et al., 1987; Parker and Jones, 1987). Little information is available on levels of oocyst shedding in animals during their first yr after weaning. Oocysts counts were low in all samples, but may have been higher if samples were collected at times of stress such as weaning. The results of this study suggest that weanling heifers maintain a low level infection with a diverse community of *Eimeria* spp. Although the pathogenic species (*E. bovis* and *E. zuernii*) predominated, no clinical disease was observed throughout the study. Soil samples from the heifer's pasture indicate that there is a

diverse community of infective oocysts present in the animal's environment. The persistent low level infections and continued re-exposure in clinically normal animals suggest that by 9 mo of age, host and parasite have reached a relatively stable equilibrium.

Table 3.1: Prevalence of *Eimeria* spp. in fecal samples, and mean percentage of oocysts identified per sample for heifers sampled 15 November 2004 to 24 November 2004, 15 April 2005, and 9 June 2005.

<i>Eimeria</i> spp.	11/15/04-11/24/04		4/15/05		6/9/05	
	Prevalence (%)	Mean oocysts / sample (%)	Prevalence (%)	Mean oocysts / sample (%)	Prevalence (%)	Mean oocysts / sample (%)
<i>E. bovis</i>	100.0	39.7	100.0	41.5	100.0	27.3
<i>E. zuernii</i>	96.0	13.4	90.0	7.9	89.0	6.9
<i>E. alabamensis</i>	91.0	15.3	90.0	12.2	89.0	5.0
<i>E. auburnensis</i>	91.0	10.2	100.0	5.8	100.0	13.6
<i>E. cylindrica/ellipsoidalis</i> -like	80.0	9.2	100.0	17.5	78.0	3.4
<i>E. canadensis</i>	67.0	7.1	100.0	8.4	100.0	21.8
<i>E. subspherica</i>	46.0	2.0	40.0	1.6	0.0	0.0
<i>E. wyomingensis</i>	18.0	1.7	30.0	1.6	22.0	4.0
<i>E. pelita</i>	34.0	1.1	30.0	0.8	33.0	3.0
<i>E. bukidonensis</i>	13.0	0.4	0.0	0.0	33.0	4.8
<i>E. brasiliensis</i>	0.0	0.0	20.0	2.8	89.0	8.9
<i>E. illinoisensis</i>	0.0	0.0	0.0	0.0	33.0	3.0

Table 3.2: Mean percentage of oocysts identified in soil samples from representative sites on the heifer lot.

<i>Eimeria</i> spp.	Mean oocysts / sample (%)
<i>E. bovis</i>	40.6
<i>E. cylindrica/ellipsoidalis</i> -like	15.0
<i>E. alabamensis</i>	12.1
<i>E. zuernii</i>	12.0
<i>E. auburnensis</i>	4.7
<i>E. subspherica</i>	4.7
<i>E. canadensis</i>	2.5
<i>E. pelita</i>	2.4
<i>E. illinoisensis</i>	2.4
<i>E. brasiliensis</i>	1.6
<i>E. wyomingensis</i>	1.4
<i>E. bukidonensis</i>	0.5

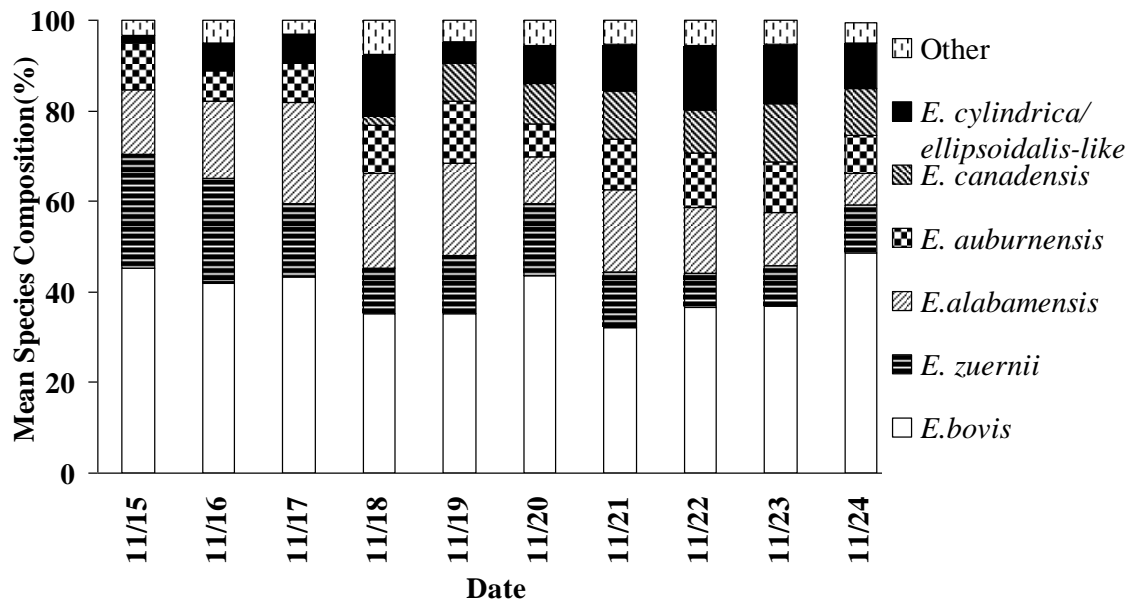


Figure 3.1: Mean daily species compositions for heifers sampled 15 November 2005 to 24 November 2005.

**Chapter 4: A study of the level and dynamics of *Eimeria* populations in naturally infected, grazing beef cattle at various stages of production in the central**

**Appalachian region**

**Abstract**

There is little information available on the dynamics of gastrointestinal parasites in grazing cattle in the central Appalachian region of the United States. The objective of this study was to describe the level of infection and species dynamics of *Eimeria* spp. in grazing beef cattle of various age groups over the course of a year in the central Appalachian region. Rectal fecal samples were collected from calves (n=72) monthly from May through October, cows (n=72) in May, July, and September and heifers (n=36) monthly from November through April. *Eimeria* spp. oocysts were detected in 399 of 414 (96%) fecal samples collected from the calves from May through October. Fecal oocyst counts in the calves were lower ( $P<0.05$ ) in May than all other months and no differences were detected from June through September. *Eimeria* spp. oocysts were detected in 198 of 213 (92%) fecal samples collected from the 36 replacement heifers monthly from November to April and mean monthly FOC did not differ during this time period. The prevalence of oocyst shedding increased to 100% in calves in September and remained near 100% in the replacement heifers during the sampling period. *Eimeria* spp. oocysts were also detected in 150 of 200 (75%) samples collected in May, July, and September from the cows and mean FOC did not differ significantly over the sampling period. *Eimeria* spp. composition was dominated by *E. bovis* in fecal samples collected from calves, replacement heifers and cows. Mixed *Eimeria* spp. infections were, however,



common in all groups with single species infections detected in only 2% of all samples. All 13 *Eimeria* spp. oocysts were identified throughout the sampling period.

### **Introduction**

The Appalachian region covers a large area in the eastern U.S.A. including portions of states from southern New York State to northern Mississippi, Georgia, and Alabama. In the West Virginia and Virginia Appalachian regions, interest in forage based beef production systems has increased in recent years. In a grass-based system with continuous use of pastures, internal parasitism may be a factor limiting production. However, there is only limited information on the epidemiology of internal parasites in the central Appalachian region, which restricts the ability make effective recommendations for producers on parasite control.

Coccidian parasites of the genus *Eimeria* parasitize the gastrointestinal tract of cattle worldwide and calves are exposed in early life in all conventional management systems (Boughton, 1945; Ernst et al., 1984; Ernst et al., 1987; Dauschies and Najdrowski, 2005). The highest prevalence of infection and incidence of disease is in calves less than one year of age (Fitzgerald, 1962; Ernst et al., 1984; Ernst et al., 1987; Diaz de Ramirez et al., 2001), although older cattle continue to shed oocysts (Cox and Todd 1962; Ernst et al., 1984; Cornelissen et al., 1995; Lucas et al., 2006). In most natural infections, regardless of animal age, mixed species infections are common (Ernst et al., 1984; Ernst et al., 1987; Cornelissen et al., 1995; Lucas et al., 2006), but only two species, *E. bovis* and *E. zuernii*, have been associated with clinical disease in the U.S.A. (Hammond, 1964; Ernst and Benz, 1986).

Numerous surveys in the U.S.A report the prevalence of *Eimeria* spp. oocysts in bovine feces (Lee and Armour, 1959; Cox and Todd, 1962; Fitzgerald, 1962; Marquardt, 1962; Nyberg et al., 1967; Jacobson and Worley, 1969; Jolly and Bergstrom, 1977; Grisi and Todd, 1978; Parker and Jones, 1987; Marshall et al., 1998). Most surveys focus on calves and report the number of samples positive at a given time. There are limited studies that describe repeated samplings of the same animals over time from this region (Ernst et al., 1987). The main objectives of this study were to characterize both the level of infection and species dynamics of *Eimeria* in naturally infected, grazing cattle at various stages of production in the central Appalachian region of the United States.

## **Materials and Methods**

### **Animal Management**

#### **Pre-weaning**

During the initial 6 month portion of the study, a group of 108 Angus-cross cow and spring born calf pairs pastured at the Shenandoah Valley Agricultural Research and Extension Center (SVAREC), Steele's Tavern, VA were surveyed for *Eimeria* spp. and strongylid infection. Pasture systems are described in detail by Scaglia et al. (2008), but briefly cows and calves grazed one of 6 different endophyte infected tall fescue (*Lolium arundinaceum* (Schreb.) S. J. Darbyshire) based pasture systems in groups of six cow-calf pairs at a stocking density of 0.67- 0.73 ha/animal unit until the calves were weaned (20 October 2005). There were three replicates of each pasture system, yielding a total of 18 separate pastures. Routine pre-weaning vaccinations were administered to all calves on 14 September 2005 and all calves were dewormed percutaneously (moxidectin, 0.5mg/kg) at weaning on 20 October 2005.

### Post-weaning

Once weaned, replacement heifer calves (n=36) were moved to wintering paddocks at SVAREC for the following 6 month portion of the study. These paddocks are used every year to develop weaned heifers and are fallow for the preceding 2-3 months. The heifer group consisted of 25 heifers that had been previously sampled and 11 heifers that were part of the farm's cow-calf group, but had not been sampled earlier, although they were managed similarly. Fescue pasture continued to serve as the major forage, but half of the heifers also received a supplement consisting of 75% wheat middling, 25% corn gluten fed at 1% of their body weight daily. Stocking density for both treatment groups was 0.32 ha/animal. Heifers were given routine pre-breeding vaccinations in March, 2006 and subsequently synchronized and artificially inseminated.

### Sampling

#### Pre-weaning

Seventy two calves (4/replicate, 26-76 days of age) were weighed and fecal samples were collected per rectum monthly beginning on 2 May 2005 and ending one week prior to weaning on 13 October 2005. Blood samples for serum pepsinogen analysis were also collected via jugular vein from the 72 calves at all sampling dates beginning in July as an indirect assessment of *Ostertagia ostertagi* infection. Rectal fecal samples were collected from the cows (n=72) (> 2 years of age) on 2 May 2005, 14 July 2005, and 14 September 2005.

#### Post-weaning

Calves were weaned on 20 October 2005 and weights, blood and fecal samples were collected from the 36 replacement heifers (246-281 days of age) monthly from November, 2005 through April, 2006. .

#### Fecal Sample Analysis

##### *Eimeria* spp.

Oocyst number in each calf and heifer sample was calculated using the Modified McMaster technique with a sensitivity of 25 oocysts per g (OPG) (Whitlock, 1948). A modified Wisconsin sugar flotation technique (Cox and Todd, 1962) was also performed on each *Eimeria* positive fecal sample from all calves pre-weaning and from heifers after weaning. The species of 50 *Eimeria* oocysts per sample were identified based on oocyst morphology (Levine and Ivens, 1986). An estimate of intensity of each *Eimeria* species in calf and heifer samples was then obtained by relating the proportion of species identified in the Wisconsin sugar flotation test to the total oocyst count obtained by the modified McMaster technique. Fecal samples (5 g) collected from the cows were examined by the Wisconsin sugar flotation technique. Oocysts were counted, and a maximum of 50 oocysts identified to species. One individual (AL) carried out all species identifications. Because oocysts of *E. cylindrica* and *E. ellipsoidalis* could not be distinguished reliably, all small cylindrical *Eimeria* spp. oocysts measuring from 19-36  $\mu\text{m}$  by 8-18  $\mu\text{m}$  were designated *Eimeria cylindrica/ellipsoidalis*-like (Levine and Ivens, 1986).

##### Strongylid nematodes

Data on parasitic strongylid nematodes was additionally compiled from samples collected during this study and the data are presented in appendices 2 and 3. Strongylid

egg numbers in all calf and cow fecal samples were calculated using the Modified McMaster technique and Modified Wisconsin techniques as described for the coccidia. In order to investigate strongylid species composition, coproculture was conducted. All pre-weaning calf samples starting with the July sample were individually cultured. Likewise, heifer samples collected from November through April were individually cultured. For all coprocultures, approximately 50 g of feces was mixed with approximately 5 g of vermiculite in a 12-oz plastic cup. The cups were covered with a bilayer of cheesecloth and left at 22°C for 14 days, at which time third stage larvae were harvested from the sample using a modified Baermann technique (Zajac and Conboy, 2006). Composite fecal samples from the cows were made by combining feces from all cows across replicates within a pasture system. Each composite fecal sample consisted of approximately 25 g of feces from 12 cows (4 cows from each of the 3 replicate pasture systems) for a total of approximately 300 g of feces in 6 composite samples. The 6 approximately 300 g fecal samples were mixed with approximately 15 g of vermiculite in a 500 ml plastic container. The containers were incubated as previously described, and third stage larvae harvested using the modified Baermann technique (Zajac and Conboy, 2006). For all samples, coprocultures were set up on the day of collection.

After collection, third stage larvae were preserved by adding an equal volume of 10% formalin solution and stored at 4 °C. Larval identifications were carried out by thoroughly mixing each formalin fixed sample and placing 3 drops of the larval suspension onto a glass slide and adding a cover glass. The first 100 larvae were identified to genus based on morphologic characteristics (Bowman, 2009; van Wyk et al. 2004).

## Serum pepsinogen

Serum pepsinogen concentration was also determined in samples collected monthly from calves and heifers in this study and results are presented in appendix 2. All blood samples were collected via jugular venipuncture into evacuated serum separator tubes. Samples were placed on ice until transport to the lab where they were allowed to warm to room temperature and then centrifuged at 3000 x g for 10 minutes for separation of serum. Serum was harvested and stored at -20 °C. Serum pepsinogen concentrations were determined according to the method described by Berghen et al. (1987). Briefly, 1 ml of serum was mixed with 5 ml of buffered albumin substrate (0.1 M glycine, 0.1 M NaCl, 0.27N HCl, 0.2% bovine serum albumin) and 3 ml of mixture was transferred to another tube. This mixture was incubated in a 37°C water bath for 24 hours. The remaining 3 ml of mixture was denatured with 5 ml of trichloroacetic acid (0.4%) and incubated for 24 hours at 4°C. Following incubation, the serum and buffered albumin substrate mixture was denatured by addition of 5 ml of trichloroacetic acid (0.4%). Each sample was centrifuged at 3000 x g and 2 ml of supernatant was transferred to new tubes. To each 2 ml sample 5 ml of 0.25 N NaOH and 0.5 ml of Folin Ciocalteu's reagent (diluted 1:3) was added. Samples were then mixed by gentle agitation and 1 ml of each sample was transferred to a plastic cuvette. After 30 minute incubation at room temperature, the absorbance of each sample was measured at 680 nm. To calculate the amount of tyrosine produced, standard tyrosine solutions (0.1 µmol/ml, 0.2 µmol/ml and 0.3 µmol/ml) were prepared and 5 ml of 0.25 N NaOH and 0.5 ml of Folin Ciocalteu's reagent (diluted 1:3) was added. Absorbance was measured at 680 nm after 30 minute

incubation and a standard curve was generated. Pepsinogen concentrations are expressed in milliunits (mU) of tyrosine.

#### Rainfall

Monthly rainfall totals were recorded from April 2005 through April 2006. The data was gathered from the USDA-NRCS maintained weather station number 2088 on site at the SVAREC. (<http://www.wcc.nrcs.usda.gov/nwcc/site?sitenum=2088&state=va>)

#### Statistics

The natural logs of all fecal oocyst counts (FOC), fecal egg counts (FEC), and serum pepsinogen levels (PEP) were calculated and analyzed by a repeated measures analysis of variance using the MIXED procedure of SAS (SAS Institute, Inc., Cary, NC) for all groups (calves, cows, heifers, and steers). The model statement contained the fixed effects of treatment (forage system for cows and calves, 1% supplementation vs no supplementation for heifers), time (sampling date) and the treatment x time interaction. Treatment effects were only detected for FOC in calves in May. Additionally no treatment effects were detected for calves, heifers and cows for FEC and PEP. Data were pooled across treatments by sampling day and subsequently analyzed by ANOVA for time effects. When significant time effects ( $P < 0.05$ ) were detected Tukey's pairwise comparisons were made to separate means.

### **Results**

*Eimeria* spp. oocysts were seen in 399 of 414 (96%) fecal samples collected from the calves from May through October. Oocyst counts in positive samples ranged from 25 (limit of detection) to 52,000 oocysts per gram (OPG). Clinical coccidiosis was not observed during the study, although all calves shed oocysts during the study period. Most

calves shed oocysts for the duration of the study and a few animals shed large numbers of oocysts. Mean fecal oocyst counts (FOC) for the calves are shown in Table 4.1. The mean FOC was lower ( $P<0.05$ ) in May than all other months. Monthly FOC from June through September did not differ, while the FOC for October was lower ( $P<0.05$ ) than the FOC for both July and September. After weaning, the heifers and steer calves were separated, but fecal sampling continued on the heifers and steer data is published in Lucas et al. (2007). The mean heifer FOC in November was similar to October's preweaning sample. All animals remained clinically normal and no post-weaning rise in FOC was observed. *Eimeria* spp. oocysts were detected in 198 of 213 (92%) of fecal samples collected from the 36 replacement heifers monthly from November to April. Oocyst counts in positive samples ranged from 25 to 10,925 OPG. Mean FOC for the heifers exhibited little fluctuation during the winter, with no differences noted (Table 4.1).

Monthly oocyst prevalence values (positive samples/total samples) for calves and heifers varied minimally throughout the duration of the study (Table 4.1). The lowest prevalence was observed in May (84%) when the calves were the youngest (26-76 days old), and then increased to 100% in September and remained near 100% in the heifers throughout the winter.

Fecal oocyst counts and *Eimeria* spp. composition (% oocysts identified/sample) expressed as a function of calf age are shown in Figure 4.1. The calves reached peak oocyst shedding at 24-27 weeks of age, but the mean FOC did not increase uniformly as the calves aged. A closer look at individual FOC revealed that 6 of the 26 calves in the 4-7 week age group had a FOC greater than 2000 OPG and 2 calves in this group had FOC



above 10,000 OPG. Comparatively fewer (3 of 37) calves in the 8-11 week age group had FOC above 2000 OPG and no calves had FOC greater than 3000 OPG. *E. cylindrica/ellipsoidalis*-like and *E. subspherica* made up a larger proportion of the oocysts shed in the feces of calves aged 4-7 weeks compared to all other age groups. Following this period, the *Eimeria* spp. infection was dominated by *E. bovis* in both calves and heifers.

*Eimeria* spp. oocysts were also detected in 150 of 200 (75%) samples collected in May, July, and September from the cows. Mean FOC and prevalence values, however, were lower in the cows, exhibiting a downward trend along with prevalence from May through September (Table 4.2). Similar to the calves and heifers the *Eimeria* spp infection in the cows was also dominated by *E. bovis*. At all three sampling dates, *E. canadensis* was the second most numerous species.

## **Discussion**

The large number of cattle that were available for investigation in the current study provided us with an opportunity to assess both the dynamics and level of infection of *Eimeria* populations in cattle as they aged and progressed through various stages of production over the seasons of the year in a region where there has not been extensive study. Many studies investigating natural *Eimeria* infections in cattle focused on young calves. In the present study, calves on pasture became infected with a wide variety of *Eimeria* spp. early in life. While prevalence remains high throughout life, calves less than one year of age shed the highest numbers of oocysts (Fitzgerald, 1962; Ernst et al., 1987; Cornelissen et al., 1995, Diaz de Ramirez et al., 2001). The results of this study are similar to those obtained by Ernst et al. (1987), who followed similarly aged calves

and cows for four grazing seasons. Mean FOC peaked in the calves in that study in either July or August when the calves averaged 5-6 months of age. Even though FOC declined in the calves in this study by 3-fold from September to October, the prevalence of oocyst shedding showed no decline and 100% of calves were still shedding oocysts at the final sampling just prior to weaning.

In the current study, *E. bovis* was found to be the most numerous and common *Eimeria* spp. detected in the fecal samples from calves, heifers and cows, a result common in other studies (Ernst et al., 1987; Hasbullah et al., 1990 Cornelissen et al., 1995). By having the exact birthdates of all calves involved in this study we were able to examine more closely the dynamics of *Eimeria* spp. composition in the calves as they aged. The average proportion of *E. bovis* in the samples stabilized by approximately 12 weeks of age and it remained dominant in the calves as they aged and progressed through the stages of production and seasons of the year. Calves aged 4-7 weeks shed a comparatively higher proportion of *E. subspherica*, *E. cylindrica/ellipsoidalis*-like, and *E. alabamensis* than did older calves. The explanation for this dynamic of *Eimeria* spp. shedding may be related to the prepatent period of the species observed. If all calves are assumed to be infected shortly after birth, *Eimeria* spp. with the shortest prepatent period would be observed in higher proportions at a younger age. The prepatent periods for *E. subspherica* (12 days), *E. cylindrica/ellipsoidalis*-like (10 days), and *E. alabamensis* (6-11 days) are shorter than that of *E. bovis* (20 days). These results are similar to those reported by Diaz de Ramirez and associates (2001), in which the feces from housed dairy calves were analyzed daily from age 12 to 90 days. The prevalence values for *Eimeria* spp. oocysts in cow samples in the present study, however, appeared to be higher than

those reported in other studies involving similarly aged cows (Ernst et al., 1984; Cornelissen et al., 1995). This discrepancy is most likely related to the use of a more sensitive technique in the current study. In those studies the *Eimeria* spp infection in adult cows was also dominated by *E. bovis*. The higher level of *E. canadensis* seen in the cows in this study is also similar to that reported by Ernst et al. (1984). This species is not considered pathogenic and these findings may suggest that it has a predilection for older cattle.

The objective of this study was to collect information on the prevalence and transmission of gastrointestinal parasites that might limit production in pastured beef cattle in the central Appalachian region. We observed that grazing beef calves, weanling heifers, steers, and adult cows in this region are naturally infected with a diverse community of *Eimeria* spp. The infection patterns of these parasites closely match that reported in cows and calves from northern Georgia (Ernst et al., 1984). Parasitic infection is a constant element that requires consideration in at nearly all phases of grazing cattle production in the central Appalachian region. With that said, clinical signs of disease associated with *Eimeria* spp. infection were not observed. The observed infections were omnipresent, but dynamic over time as the animals aged and progressed through the stages of production and seasons of the year. This work indicates that gastrointestinal parasitism is a factor that must be considered, but can be managed in pasture based beef production systems the central Appalachian region.

Table 4.1: Mean individual *Eimeria* spp. fecal oocyst counts and prevalence (%) in positive samples and mean total fecal *Eimeria* spp. oocyst counts, prevalence (%) and 95% confidence intervals for calves (n=72) sampled monthly May - October, 2005 and heifers (n=36) sampled monthly November and December 2005, and January - April, 2006. Total *Eimeria* fecal oocyst count (FOC) means within calves and heifers with no common superscripts are different (P<0.05).

<i>Eimeria</i> spp.	Calves						Heifers					
	Mean Oocyst/Sample (Prevalence)						Mean Oocyst/Sample (Prevalence)					
	May	June	July	Aug	Sept	Oct	Nov	Dec	Jan	Feb	Mar	Apr
<i>bovis</i>	32.6 (73)	125.4 (88)	395.4 (100)	291.7 (98)	346.2 (100)	188.1 (97)	66.9 (97)	156.5 (94)	42.2 (94)	68.2 (97)	88.6 (100)	87.3 (100)
<i>zuernii</i>	8.3 (60)	20.4 (82)	32.7 (85)	21 (81)	31.3 (83)	18.5 (91)	12.6 (88)	9.6 (81)	29.9 (97)	17.3 (97)	14.5 (97)	8.4 (90)
<i>cylindrica/ellipsoidalis</i> -1	11.0 (56)	11.1 (74)	15.3 (68)	24.1 (86)	21.2 (79)	8.1 (74)	12.2 (91)	13.0 (84)	12.5 (91)	10.1 (88)	11.7 (97)	4.0 (72)
<i>auburnensis</i>	6.7 (51)	23.8 (84)	20.4 (80)	10.6 (89)	11.0 (69)	6.5 (69)	2.0 (53)	4.8 (71)	4.3 (59)	4.2 (72)	2.7 (71)	1.4 (52)
<i>alabamensis</i>	6.0 (49)	4.7 (48)	7.3 (60)	10.6 (69)	44.5 (90)	14.6 (87)	5.2 (68)	3.5 (58)	26.0 (97)	9.1 (84)	3.3 (68)	1.9 (48)
<i>canadensis</i>	4.2 (45)	8.2 (64)	4.0 (48)	2.1 (34)	2.8 (37)	5.9 (59)	2.7 (50)	3.8 (55)	15.8 (81)	12.1 (75)	8.3 (81)	7.2 (83)
<i>illinoisensis</i>	3.6 (36)	2.7 (44)	5.3 (53)	9.8 (73)	3.7 (43)	8.3 (71)	0.9 (26)	0.7 (26)	1.4 (28)	0.2 (13)	1.0 (32)	0.60 (21)
<i>pellita</i>	2.4 (35)	5.0 (56)	14.5 (73)	10.1 (66)	3.6 (43)	2.0 (44)	4.4 (59)	0.07 (3)	0.21 (13)	0.1 (6)	0.24 (16)	0.63 (24)
<i>subspherica</i>	1.4 (24)	0.9 (24)	1.6 (28)	2.4 (36)	1.9 (32)	0.8 (23)	0.4 (15)	0.16 (10)	0.17 (6)	0.6 (25)	0.23(6)	0.0 (0)
<i>brasiliensis</i>	0.2 (7)	0.6 (16)	0.7 (20)	1.3 (27)	0.6 (14)	0.9 (23)	21 (41)	2.1 (39)	0.13 (6)	>0.05 (3)	0.2 (13)	0.3 (10)
<i>wyomingensis</i>	0.0 (0)	0.3 (10)	0.3 (12)	0.2 (8)	0.4 (11)	0.3 (9)	1.3 (29)	0.1 (3)	0.0 (0)	>0.05 (3)	0.2 (6)	>0.05 (7)
<i>bukidonensis</i>	<0.05 (2)	0.4 (8)	0.0 (0)	0.2 (6)	0.6 (11)	0.3 (10)	3.0 (44)	0.1 (3)	0.2 (13)	0.2 (6)	0.4 (10)	0.1 (7)
Total <i>Eimeria</i> FOC	139.6 <sup>a</sup> (84)	376.9 <sup>bc</sup> (96)	905.2 <sup>b</sup> (99)	621.4 <sup>bc</sup> (99)	847.9 <sup>b</sup> (100)	389.4 <sup>c</sup> (100)	170.2 <sup>a</sup> (97)	193.0 <sup>a</sup> (94)	166.6 <sup>a</sup> (94)	142.0 <sup>a</sup> (92)	139.0 <sup>a</sup> (94)	86.0 <sup>a</sup> (85)
95% Confidence Interval	72-269	230-671	625-1312	427-905	591-1216	295-514	105-275	101-369	92-302	77-262	76-255	40-182

Table 4.2: Mean individual *Eimeria* spp. fecal oocyst counts and prevalence (%) in positive samples and total *Eimeria* fecal oocyst counts (FOC) and prevalence (%) in cows sampled 2 May 2005, 15 July 2005, and 14 September 2005. Total *Eimeria* fecal oocyst count means with no common superscripts are different (P<0.05)

<i>Eimeria</i> spp.	Mean Oocyst/Sample (Prevalence)		
	May	July	September
<i>bovis</i>	0.80 (89)	0.42 (67)	0.28 (81)
<i>zuernii</i>	0.08 (26)	0.19 (60)	0.08 (33)
<i>cylindrica/ellipsoidalis</i>	0.05 (16)	0.02 (10)	0.02 (9)
<i>auburnensis</i>	0.31 (58)	0.08(31)	0.04 (23)
<i>alabamensis</i>	0.06 (21)	0.10 (42)	0.01 (5)
<i>canadensis</i>	0.40 (56)	0.21 (39)	0.14 (47)
<i>illinoisensis</i>	0.01 (7)	0.03(17)	0.01(7)
<i>pellita</i>	0.01 (7)	0.02 (6)	0.01(2)
<i>subspherica</i>	0.00 (0)	<0.005 (2)	0.00 (0)
<i>brasiliensis</i>	0.01 (4)	0.01 (6)	0.01 (6)
<i>wyomingensis</i>	<0.005 (2)	0.01 (6)	0.01 (7)
<i>bukidonensis</i>	0.00 (0)	0.01 (6)	0.01 (7)
Total <i>Eimeria</i> FOC	1.73 <sup>a</sup> (86)	0.96 <sup>ab</sup> (77)	0.60 <sup>b</sup> (63)
95% Confidence Interval	1.1-2.5	0.60-1.3	0.40-0.80

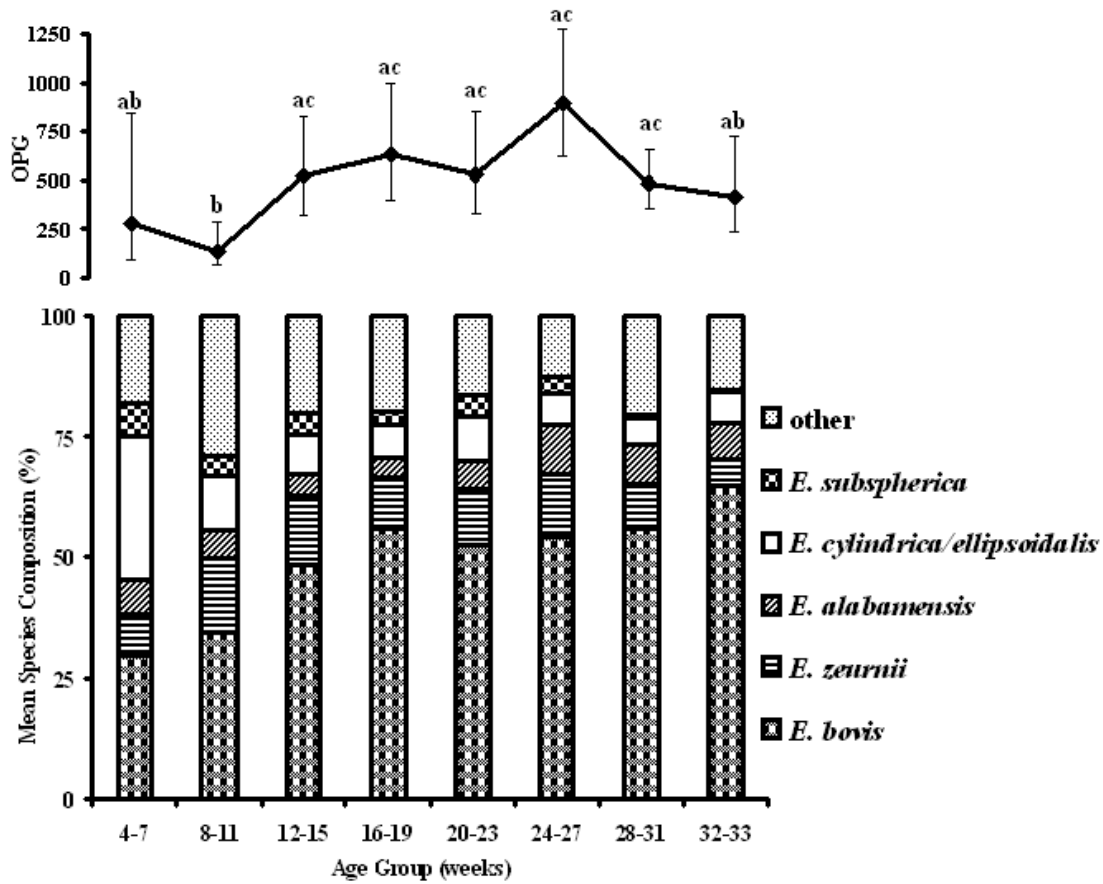


Figure 4.1: Mean *Eimeria* fecal oocyst numbers per gram of feces (OPG) and *Eimeria* species composition (%) for calves by age group at sampling date. Mean fecal oocyst counts with no common superscripts are different ( $P < 0.05$ ). Error bars represent 95% confidence intervals.

## Chapter 5: The effect of weaning method on coccidial infections in beef calves

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### Abstract

Weaning is often cited as a stressful event that can precipitate clinical coccidiosis in cattle. Recently, two-stage weaning methods have been investigated as a means to reduce stress in calves. The objective of this study was to investigate the effect of weaning method on fecal oocyst count (FOC) and *Eimeria* spp. composition. A group of 108 beef calves (190-240 days of age) were allocated to 3 treatments on day -7: a fenceline weaned group where calves could see and hear their dams, but were physically separated from them by a fence (FL), a noseclipped group where suckling was prevented (NC), and a group left with their dams to be traditionally weaned (TW) by abrupt separation on day 0. On day 0, noseclips were removed from the NC calves and all groups were completely separated from their dams. The steers (n=50) were transported 172 km to another farm, and heifers (n=58) were taken to distant fields on the same farm. Rectal fecal samples were collected from all calves on days -7, 0, and 14. Steers were also

sampled on days 7, 28, and 42. A group of steers (n=24) from each of the treatments previously described were fitted with pedometers and intensively observed on days 1-4. The Modified McMaster's test and Wisconsin sugar flotation test were used for quantification and identification of *Eimeria* spp. oocysts. Clinical coccidiosis was not observed during the study. Fecal oocyst counts did not differ between treatment groups on any sampling day. Differences between steer and heifer FOC were detected. Treatment had no effect on species composition, but time effects were detected in the steers. The mean percentage of *E. bovis* was lower ( $P<0.05$ ) on days 28 and 42 than all other sampling days. The decrease in the percentage of *E. bovis* was accompanied by an increase ( $P<0.05$ ) in the percentage of *E. canadensis* on days 28 and 42. Behavioral observation showed that TW steers exhibited higher levels of stress related behavior ( $P<0.05$ ) following complete separation, although this was not reflected in FOC.

Keywords: *Eimeria*, Cattle, Weaning, Stress

### **Introduction**

Coccidia of the genus *Eimeria* are protozoan parasites that infect cattle worldwide. All calves managed under conventional systems are exposed and become infected early in life (Boughton, 1945; Ernst et al., 1984; Ernst et al., 1987; Dauschies and Najdrowski, 2005). Thirteen *Eimeria* spp. are reported to infect cattle in the U.S. (Levine and Ivens, 1967; Ernst and Todd, 1977; Fayer, 1980) and mixed infections are very common under natural conditions (Marquardt, 1962; Nyberg et al. 1967; Grisi and Todd, 1978; Ward et al., 1979; Ernst et al., 1984; Ernst et al., 1987). Although mixed infections dominate in the U.S., only 2 species, *E. bovis* and *E. zuernii*, are found commonly in association with clinical disease, and are therefore considered pathogenic (Hammond, 1964; Ernst and Benz, 1986).



The highest prevalence of oocyst shedding and disease incidence occurs in calves less than a year of age (Fitzgerald, 1962; Ernst et al., 1984; Ernst et al., 1987; Diaz de Ramirez et al., 2001). Management practices that expose calves to high numbers of infective oocysts (such as crowding and commingling in areas where sanitation is deficient) can serve to increase both the level of infection and incidence of clinical disease (Boughton, 1945; Stromberg et al., 1986; Step et al., 2002). Interestingly, outbreaks of coccidiosis have also been reported in calves following periods of stress when increased exposure to infective oocysts is not evident (Jolley and Bergstrom, 1977; Marshall et al., 1998). Outbreaks of coccidiosis following harsh, winter conditions have been reported by several researchers (Fitzgerald, 1962; Marquardt, 1962; Niilo, 1970). Similarly, the stress associated with weaning is believed to precipitate outbreaks of disease. In the U.S. and Australia, post-weaning increases in oocyst shedding and onset of clinical coccidiosis have been reported in calves (Fitzgerald, 1962; Parker et al., 1984). The reasons that stress may precipitate outbreaks of coccidiosis are not fully understood. Researchers have hypothesized that stages of the parasite may become arrested in the host tissue, and stress-induced immune suppression could reactivate these stages leading to onset of clinical disease (Marquardt et al. 1976, Speer et al. 1985). This hypothesis was supported by the findings of Lindsay et al. (1990), who were able to detect first generation merozoites of *E. bovis* in the mesenteric lymph nodes of experimentally infected calves. Even in light of these findings further research on this topic has stalled and the exact mechanism for stress-induced outbreaks of coccidiosis in cattle remains unknown.

Recently, two-stage weaning methods have been explored as a means to reduce stress levels in calves at weaning. Two-stage weaning involves a period where nursing is prevented between cow-calf pairs before the complete separation of dam and calf. Price et al. (2003) found that

calves weaned using the fenceline method had higher average daily gains, spent more time eating, and less time walking ( $P < 0.05$ ) than traditionally weaned, abruptly separated control calves. Likewise, Haley et al. (2005) found that calves fitted with noseclips to prevent nursing for a period before separation spent less time walking, more time eating, and vocalized less ( $P < 0.05$ ) than abruptly separated, control calves. Furthermore, Hickey et al. (2003) found that abruptly weaned calves had elevated ( $P < 0.05$ ) concentrations of plasma cortisol, and noradrenaline, and attenuation of immune function when compared to unweaned control calves. Since weaning stress may be associated with outbreaks of coccidiosis and two-stage weaning methods may alter weaning stress levels, the objective of this study was to investigate the effect of weaning method on coccidia oocyst counts and species composition in beef calves.

## **Materials and Methods**

### **Animal management and sampling**

A group of 108 Angus-cross calves born at the Shenandoah Valley Agricultural Research and Extension Center (SVAREC), Steele's Tavern, VA were used in this study. Calves grazed one of 6 different endophyte infected tall fescue (*Festuca arundinacea* Schreb) based pasture systems with their dams in groups of six cow-calf pairs at a stocking density of 0.67- 0.73 ha/animal unit until weaning. There were three replicates of each pasture system, yielding a total of 18 separate pastures. On 13 October 2005 (day -7) pre-weaning treatments were allocated at the level of pasture such that all calves (190-240 days of age) within each pasture received the same treatment. The treatments were: 1) a fenceline weaned group where calves and dams were placed in adjacent paddocks separated by a fence to serve as a physical barrier to prevent suckling, but allow calves to see and hear their dams, (FL), 2) a group where calves were fitted with an anti-suckling noseclip device (Calf Weaner®, Nasco Farm and Ranch) to prevent suckling and placed

back in the same paddocks with their dams (NC), and a group allowed to remain with their dams until day 0 (TW). These treatments were applied for 7 days and then the noseclips were removed and all calves were totally separated from their dams (day 0). This final separation yielded 2 groups of calves weaned by a two-stage method (FL and NC), and one group traditionally weaned by abrupt separation (TW). On day 0 all steers (n=50) were transported a distance of 172 km from the SVAREC to Kentland Farm, Blacksburg, VA. After their arrival the steers were allocated into groups within weaning treatment and stocked on pure stand grass pastures consisting of endophyte infected tall fescue, endophyte-free tall fescue, novel-endophyte infected tall fescue, or Lakota (*Bromus catharticus*) prairie grass. The steers remained on these pastures for the remainder of the study. Heifers (n=58) stayed at the SVAREC, but were moved to distant fields where they grazed fescue based pastures for the remainder of the study. Fecal samples were collected per rectum from all calves on days -7, 0 and 14. Additional rectal fecal samples were collected from the steers on days 7, 28, and 42.

As part of a concurrent research project a group of 24 steers equally selected from each of the treatments previously described were fitted with pedometers and intensively observed on days 1-4 to assess levels of stress related behavior (Boland et al., 2006)

#### Fecal sample analysis

Oocyst number in each fecal sample was calculated using the Modified McMaster's technique with a sensitivity of 25 oocysts per g (OPG) (Whitlock, 1948). A modified Wisconsin sugar flotation technique (Cox and Todd, 1962) was also performed on each fecal sample and 50 *Eimeria* spp. oocysts per sample were examined at 400X and identified to species based on oocyst morphology (Levine and Ivens, 1986). One trained individual (AL) carried out all oocyst counts and species identifications. Because oocysts of *E. cylindrica* and *E. ellipsoidalis* could

not be distinguished reliably, all small cylindrical *Eimeria* spp. oocysts measuring from 19-36  $\mu\text{m}$  by 8-18  $\mu\text{m}$  were designated *Eimeria cylindrica/ellipsoidalis*-like (Levine and Ivens, 1986).

## Statistics

Oocyst count and *Eimeria* spp. composition data were analyzed by a repeated measures analysis of variance using the MIXED procedure of SAS (SAS Institute, Inc., Cary, NC). The model statement contained the fixed effects of treatment, time, and the treatment x time interaction. Data were analyzed using animal(treatment) as the error term. In this model animal was the experimental unit. When significant treatment interactions were observed ( $P < 0.05$ ), differences among treatments were examined using the slice option. For oocyst count data a natural logarithmic transformation was applied to obtain a symmetrical Gaussian distribution.

## Results

*Eimeria* spp. oocysts were seen in 463 of 467 (99%) fecal samples collected over the study. Oocyst counts in positive samples ranged from 25 (limit of detection) to 11,450 oocysts per gram (OPG). All calves were clinically normal at each sampling and clinical coccidiosis was not observed throughout the study. Mean fecal oocyst counts (FOC) for the 58 heifers sampled on days -7, 0, and 14 are reported in Table 5.1. A post-weaning rise in mean FOC was not observed in the heifers, regardless of weaning treatment. Likewise, a post-weaning rise in mean FOC of the 50 steers additionally sampled on days 7, 28, and 42 was not observed (Table 5.1).

Weaning treatment had no significant effect on mean FOC. Numerical differences between weaning treatments, although not significant, were observed. The mean FOC for the NC calves (heifers and steers) was numerically higher than the other two groups on day 0 (Figures 1 and 2). On day 28 the FL steer's FOC was higher than the FOC of the other two groups. The TW steer's

FOC remained low on day 42 and it was lower than the FOC of the NC and FL steers (Figure 5.2).

Neither weaning treatment nor gender had an effect on species composition. Twelve *Eimeria* spp. were recovered at each sampling date. *Eimeria bovis* was the most common species recovered, present in 100% of all positive samples at each sampling date. Other more common species recovered at each sampling include *E. zuernii* (ranging in prevalence from 67 to 94%), *E. canadensis* (50-74%), *E. cylindrica/ellipsoidalis*-like (71-89%), and *E. alabamensis* (43-84%) (Table 5.2). There was a time effect on species composition observed in the steers. The mean percentage of *E. bovis* oocysts identified in each sample was lower ( $P < 0.05$ ) on days 28 and 42 than on all other sampling days. This decrease in the mean percentage of *E. bovis* oocysts was accompanied by an increase ( $P < 0.05$ ) in the mean percentage of *E. canadensis* oocysts identified on days 28 and 42 (Table 5.2).

### **Discussion**

The results of this study indicate that weaning method has little effect on post-weaning FOC. Recent studies by Price et al. (2003) and Haley et al. (2005) found that calves weaned using two-stage methods similar to the methods employed by this study vocalized more frequently, spent more time walking, and less time eating. Intense observation of the steers in this study showed similar behavioral results (Boland et al., 2006), which suggests higher post-weaning stress levels within the TW steers. This did not, however, translate into a greater post-weaning FOC in this group. No significant differences in FOC were observed throughout the study. On certain days, however, there were noticeable numerical differences in FOC between groups. The FOC for both the NC and FL groups were (steers and heifers) elevated on day 0 above the TW group, but this increase was most distinct in the FL group (Figures 5.1 and 5.2). The elevated FOC of this

group may be attributed to dehydration. The noseclips that were used on this group prevented nursing, but may have also limited water intake, available to the calves only by accessing ball waterers. The feces collected from this group on day 0 were dry and had the appearance of large sheep pellets. Physiological compensation for decreased water intake by the NC calves may have involved the retention of feces in the large intestine for longer periods to increase water reabsorption, thereby decreasing the digestive passage rate and subsequent FOC. Other numerical differences in FOC were not observed until late in the study (days 28 and 42) when the FOC of the TW steers was actually lower than the other groups. This is similar to the findings of Fitzgerald (1962), who also found that measures taken to reduce stress at weaning have little effect on post-weaning oocyst shedding. In that study differences in FOC were also not observed until 8 weeks post-weaning, leading to the conclusion that observed differences were not a result of weaning method. Likewise, the numerical differences observed in the present study are likely not a result of weaning method, but more likely related to possible differences in environment and management, given that they occurred several weeks after weaning and no clear pattern among the different treatments had emerged before that point (Figure 5.2).

A post-weaning increase in mean FOC for the entire group of calves, regardless of weaning treatment, was not detected. Although brief increases in FOC occurring between sampling dates cannot be completely discounted, individual FOC were generally low at all sampling dates. Fecal oocyst counts for positive samples ranged from 25 to 11,450 OPG, with only 12 % of all FOC exceeded 2000 OPG and 23% of those were from the NC group on day 0. This result conflicts with many studies reporting an increase in oocyst shedding following periods of stress (Fitzgerald, 1962; Marquardt, 1962; Niilo, 1970; Jolley and Bergstrom, 1977; Parker et al., 1984;

Marshall et al., 1998). Management of this group of calves could be a possible explanation for the lack of an observed increase in FOC. Although differing levels of stress may have been observed between weaning treatments, the stress level of the whole group of calves was probably low compared to calves in previous studies (Fitzgerald et al. 1962; Parker et al. 1984) and in typical U.S. cow-calf operations. Under conventional management systems calves are often handled for the first time at weaning, commingled in auction barns, and then hauled longer distances, often in cold weather, in route to feedlot finishing operations. Calves in the present study were acclimated to handling (handled monthly from birth), only transported short distances in very mild weather, and placed on pastures where the stocking density did not exceed 0.75 h/animal unit. Therefore, it is possible that any or all of these deviations in conventional management spared this group of calves from increases in FOC and onset of clinical disease.

*Eimeria* spp. composition was not affected by weaning treatment. There were, however, changes detected in the mean *Eimeria* spp. composition from the steers over time (Table 5.2). Increased oocysts shedding of the pathogenic species, *E. bovis* (Fitzgerald, 1962) and *E. zuernii* (Parker et al., 1984), has been reported in calves shortly after weaning, but increased oocyst shedding of those species did not occur in the present study. Species composition remained similar in all calves sampled through day 14 and differences were not evident in the steers until days 28 and 42 post-weaning (Table 5.2). These changes may have been a product of the change of steer location on day 0. The new pastures could have been contaminated with a proportion of infective oocysts different from the previous pastures, thereby leading to a change in fecal species composition. If the exposure to differing communities of infective oocysts was to result in a change in fecal species composition it is logical that it would be detected by day 28, given that the prepatent period of the 13 *Eimeria* spp. ranges from 7-24 days (Levine and Ivens, 1986).

Weather and host response can also not be excluded as possible factors influencing the observed change in species composition. The interactions between host, parasite, and environment are complex, but these results do show that *Eimeria* spp. composition can remain stable in calves, with only gradual changes occurring in the weeks following weaning.

The mechanism for stress associated relapse of bovine coccidiosis is still not understood. The results of this study indicate that although two-stage weaning methods may decrease levels of stress in calves, they may have little effect on subsequent post-weaning FOC. In the present study overall mean FOC, regardless of weaning treatment, remained low for the heifer calves last sampled 14 days after weaning, and was also unchanged in the 50 steer calves additionally sampled up to 42 days after weaning. As previously mentioned, stress levels in these calves were likely not comparable to levels within many commercial cow-calf operations, due to differences in the management of this herd. The weaning methods used, however, were comparable to methods employed throughout the U.S. Since none of these weaning methods resulted in an increase in FOC or disease, we can conclude that stress resulting from the physical separation of calf from dam alone may not be sufficient to elevate fecal oocyst shedding or cause outbreaks of coccidiosis.



Table 5.1: Geometric mean fecal oocyst count and 95 % confidence intervals (CI) for heifer calves sampled -7, 0, and 14 post-weaning and steer calves sampled -7, 0, 14, 28, and 42 days post-weaning.

Day	Heifers (n=58)	95% CI	Steers (n=50)	95% CI
-7	354	27-4,634	351	23-5,473
0	415	33-8,056	671	57-10,396
7	-	-	227	13-4,009
14	466	47-4,624	382	35-4,190
28	-	-	371	40-3,479
42	-	-	385	15-9,715

\*Steer calves transported 172 km on day 0.

Table 5.2: Mean percentage of oocysts identified per sample, and prevalence of *Eimeria* spp. in fecal samples from steer calves sampled -7, 0, 7, 14, 28, and 42 days post-weaning

<i>Eimeria</i> spp.	Mean Oocyst / Sample (%)						Prevalence (%)					
	-7	0	7	14	28	42	-7	0	7	14	28	42
<i>bovis</i>	51.6	63.8	63.4	64.3	48.6	45.6	100	100	100	100	100	100
<i>canadensis</i>	10.6	5.1	10.4	5.8	13.1	22.1	65	60	65	50	66	74
<i>zuernii</i>	10.1	7.0	4.6	7.9	8.4	7.4	86	87	67	94	80	77
<i>alabamensis</i>	9.6	10.0	4.6	4.2	4.6	1.7	80	84	63	59	64	43
<i>cylindrica/ellipsoidalis</i> -like	6.2	4.0	5.3	8.5	9.7	7.0	71	76	71	83	88	89
<i>auburnensis</i>	4.0	3.0	3.9	2.4	5.4	7.2	76	53	42	48	56	79
<i>illinoisensis</i>	3.3	2.7	3.0	4.8	2.4	1.5	63	56	50	61	44	34
<i>pellita</i>	2.2	1.3	1.7	0.5	3.1	1.9	47	27	35	15	38	38
<i>subspherica</i>	1.0	0.6	0.9	0.3	0.2	0.8	24	13	23	15	8	9
<i>brasiliensis</i>	0.6	2.0	0.5	0.2	1.1	2.6	20	13	4	9	16	28
<i>wyomingensis</i>	0.4	0.4	0.9	0.2	0.4	0.3	16	9	13	7	6	11
<i>bukidonensis</i>	0.4	0.2	0.8	0.9	3.0	1.9	8	4	13	22	26	19

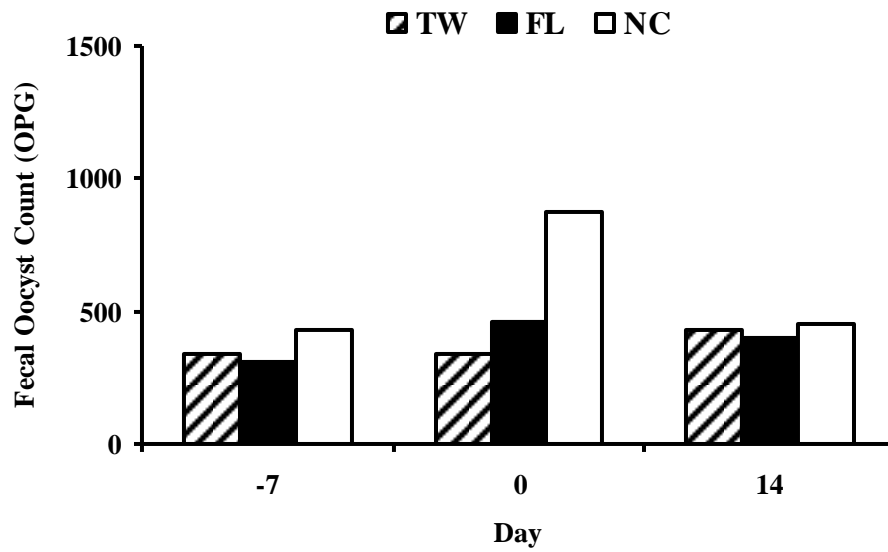


Figure 5.1: Mean fecal oocysts counts for 58 heifer calves weaned by traditional (TW), fenceline (FL), and noseclip (NC) weaning methods and sampled -7, 0, and 14 days post-weaning

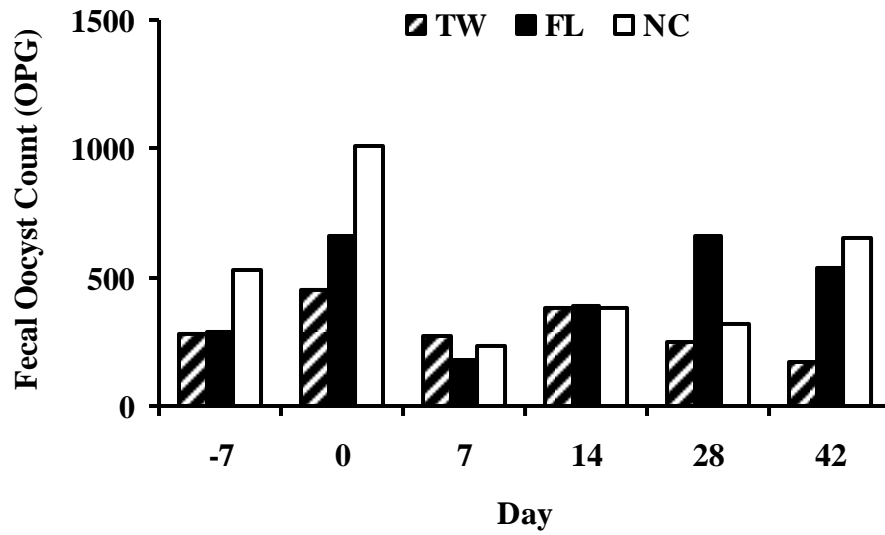


Figure 5.2: Mean fecal oocyst counts for 50 steer calves weaned by traditional (TW), fenceline (FL), and noseclip (NC) weaning methods and sampled -7, 0, 7, 14, 28, and 42 days post weaning.

## Chapter 6: A model of stress induced coccidiosis in cattle

### Abstract

Currently there is no reproducible model of stress-induced coccidiosis in cattle. Synthetic glucocorticoids and adrenocorticotrophic hormone (ACTH) alter immune function in cattle. The objectives of this study were first to identify a drug that would induce changes in immune parameters in beef calves similar to those seen following weaning and transport and, secondly, to use this agent in combination with varying doses of *E. bovis* to identify the number of oocysts required to increase fecal oocyst count. In the initial study 6 weaned beef calves were allocated to one of two treatments. 1) 3 calves given 25 IU cosyntropin via intramuscular injection and 0.0024 mg/kg epinephrine via subcutaneous injection once at time zero and 2) 3 calves given 0.35 mg/kg dexamethasone via intramuscular injection once at time zero. Blood was collected from all calves via jugular venipuncture at times 0, 1, 16 and 40 hours post treatment (PT). Increases in white blood cell (WBC) count and neutrophil:lymphocyte (N:L) ratio and decreases in circulating lymphocyte subtypes, lymphoproliferative responses and IFN- $\gamma$  production were detected at 16 and 40 hrs PT in the dexamethasone treated calves. No changes were noted in the ACTH/Epinephrine treated calves thus dexamethasone was selected for use in the second study. In this study 15 weaned beef calves were ranked by initial FOC and assigned to one of 3 treatments (n=5/treatment). The treatments were 1) calves given 500,000 sporulated *E. bovis* oocysts orally on day 0, 2) calves given 100,000 sporulated *E. bovis* oocysts orally on day 0, 3) calves given 20,000 sporulated *E. bovis* oocysts orally for 5 consecutive days starting on day 0. All calves were injected with 0.35 mg/kg of dexamethasone on day 7 post oocyst challenge (PC). Fecal oocyst count increased on days 23-24 in the 500,000 oocyst/calf group and this increase in FOC was accompanied by a trend toward a decrease in fecal dry matter. Additionally, increases

in white blood cell (WBC) count and neutrophil:lymphocyte (N:L) ratio and decreases in circulating lymphocyte subtypes, lymphoproliferative responses and IFN- $\gamma$  production were decreased in all treatment groups on day 8 PC as a result of dexamethasone administration. In conclusion, parenteral administration of dexamethasone along with a single *E. bovis* challenge with 500,000 oocysts may be used as a model for stress-induced coccidiosis in cattle.

### **Introduction**

Clinical coccidiosis in beef calves is often associated with stressful events such as weaning, shipping, and commingling (Fitzgerald, 1962; Marquardt, 1962; Niilo, 1970a; Jolley and Bergstrom, 1977; Marshall et al., 1998). Although thirteen species of *Eimeria* are known to infect cattle in North America only *E. bovis* and *E. zuernii* are regarded as pathogenic (Levine and Ivens, 1986). Calves develop immunity following primary infection that protects them from disease on subsequent challenge (Dauguschies et al. 1986; Fiege et al., 1992; Suhwold et al., 2010), thus outbreaks of coccidiosis have been associated with stress induced immunosuppression. Although antibody production reflects exposure to *Eimeria* (Lillehoj, 1987; Fiege et al., 1992), antibodies alone are not protective and in many host species cellular immunity is believed to confer protection. Immunity to *Eimeria* in mice can be adoptively transferred using mesenteric lymph node or spleen cells (Rose et al. 1988). Furthermore, athymic rats and mice develop a more severe primary *Eimeria* infection and have no immunity to subsequent challenge (Rose et al., 1992). Likewise, use of the specific T-cell inhibitor cyclosporin A in chickens prior to *Eimeria* infection resulted in a more severe infection, and prevented the development of protective immunity (Lillehoj, 1987). The use of synthetic glucocorticoids in poultry also enhanced primary *Eimeria* infection and abrogated protective immunity (Rose, 1970; Long and Rose, 1970).

Cell mediated immunity also appears to be important in the immune response to *Eimeria* infection in cattle. Experimental bovine *Eimeria* infection produces an elevation in peripheral CD4<sup>+</sup> and CD8<sup>+</sup> T-cells along with an increase in antigen specific IFN- $\gamma$  production (Taubert et al, 2008; Suhwold et al., 2010). Additionally, these changes were only observed on day 8 post infection. Lymphocytes carrying the CD4 and CD8 surface markers inversely correlated with the level and duration of oocyst excretion during primary infection and it is hypothesized that these T-cell populations are responsible for immunologic control of subsequent infections (Hermosilla et al., 1999). Because most beef calves shed *Eimeria* oocysts prior to weaning (Ernst et al., 1987; Lucas et al., 2006) protective immunity should be present in beef calves prior to the stress of weaning and shipping.

Stressful events modulate the immune system via the hypothalamic-pituitary-adrenal (HPA) axis in multiple species including cattle (Gwazdauskas et al., 1980; Paape et al., 1981; Roth et al., 1982; Roth et al., 1983; Minton 1994; Elenkov and Chrousos, 1999). The release of cortisol from the adrenal cortex is believed to alter the cytokine expression profile that shifts the immune response away from a T-helper type 1 (Th1) toward a T-helper type 2 (Th2) response (Elenkov and Chrousos, 1999; Viveros-Paredes et al., 2006). The stress of weaning and/or transport leads to elevations in circulating cortisol in cattle (Crookshank et al. 1979; Buckham-Sporer et al. 2008) with associated changes in differential white blood cell (WBC) count (Lynch et al., 2010a), decreased lymphoproliferative response (Chang et al., 1994), and decreased Th1 type cytokine production (Lynch et al., 2010b). These changes are consistent with a shift away from a Th1 type response. The Th1 branch of the immune system is responsible for protective immunity to *Eimeria* infection (Findly et al., 1993; Ovington et al., 1995; Rose et al., 1992, Shi et al., 2001; Smith and Hayday 2000) and it is plausible that a shift away from this type of

response during times of stress will increase cattle's susceptibility to *Eimeria* infection. This mechanism may explain why stress associated outbreaks of coccidiosis occur in the beef industry.

The source of the *Eimeria* that produces outbreaks of clinical disease after a stressful event is not known. It has been hypothesized that latent *Eimeria* stages within the calf's intestinal tract or lymphatics may be reactivated during times of stress (Speer et al. 1985; Lindsay et al., 1990). Conversely, continued exposure to the parasite in the animal's environment may overwhelm the calf's immunity during times of stress resulting in clinical disease.

To study the immune response to *Eimeria* infection during times of stress, a reproducible method of inducing stress at a level similar to that seen during weaning and transport is needed. Adrenocorticotrophic hormone (ACTH) alone (Wegner and Stott 1972; Gwazdauskas et al., 1980; Paape et al., 1977; Roth et al., 1982; Roth et al., 1983) or in combination with epinephrine (Sconberg et al., 1993; Nockels et al., 1996) induces weight loss and increased serum cortisol, creatine kinase and blood urea nitrogen . Furthermore, changes in WBC count, lymphoproliferative response and neutrophil function have been documented using this stress model. (Wegner and Scott 1972; Paape et al., 1977; Gwazdauskas et al., 1980; Roth et al., 1982; Roth et al., 1983). Administration of synthetic glucocorticoids has also been used to induce immune suppression similar to that seen during times of stress in cattle. Nonnecke and colleagues (1997) reported that parenteral administration of dexamethasone to Holstein bulls resulted in a 97% reduction in IFN- $\gamma$  secretion by peripheral lymphocytes stimulated with pokeweed mitogen from two to seven days after multiple dexamethasone injections. Flow cytometric analysis of peripheral leukocytes indicated prolonged decreases in the percentages of B-Cells, CD3<sup>+</sup> (T-Cells), WC1<sup>+</sup>, CD8<sup>+</sup>, and CD4<sup>+</sup> cells after dexamethasone injection. Likewise,



Menge and Nystrom (2008) reported a profound stress leukogram (leukocytosis, mature neutrophilia, and lymphocytopenia) and a decrease in circulating  $\gamma\delta$  T-cells in Jersey bulls after parenteral dexamethasone administration. They also observed a decrease in the percentage of proliferating lymphocytes in response to mitogen stimulation and a concurrent decrease in Th1 type cytokine (IFN- $\gamma$  and IL-2) mRNA in supernatants from these cell populations. Based on the results of these studies, the administration of either ACTH or dexamethasone may reliably induce immune suppression similar to that seen following weaning, transport and commingling in beef calves. As previously stated, immunity to *E. bovis* infection seems to be directed against developing first generation merozoites on day 8 post infection. By administering ACTH or dexamethasone on day 7 PC our goal was to produce immunosuppression that would affect immunity to *E. bovis* administered 8 days previously.

Multiple studies have been published in which calves were experimentally infected with varying numbers of *E. bovis* oocysts. (Friend and Stockdale; 1981; Lindsay 1990; Hermosilla et al, 1999; Taubert et al., 2008; Suhwold et al., 2010). In all of these studies, however, young, naïve dairy calves were used. Only one study is available in which an increase in fecal oocyst shedding was induced by challenging older calves with *Eimeria* oocysts (Niilo, 1970c). Interestingly, this researcher used low doses of dexamethasone in conjunction with *E. zuernii* challenge. There is no information available on the number of *E. bovis* oocysts or frequency of administration that will produce increased oocyst shedding and possibly induce clinical disease in naturally infected weaned beef calves. The objectives of our studies were first to identify a pharmacologic agent that would induce changes in immune parameters in beef calves similar to those seen after weaning and transport and, secondly, to use this agent in combination with

varying doses of *E. bovis* to identify the number of oocysts required to increase subsequent fecal oocyst count.

## **Materials and Methods**

### **Study 1**

#### **Animal management and sampling**

Six 5-6 mo old, Angus and Angus-cross, weaned beef calves were used in this study. The calves originated from the Shenandoah Agricultural Research and Extension Center, Steele's Tavern, VA and were transported together to Blacksburg, VA 4 days after weaning. Upon arrival, all calves were commingled in a single 1 hectare paddock for the duration of the study. They were provided ad libitum access to mixed grass hay and water. They were also supplemented daily with corn gluten feed at 0.5% of body weight for the duration of the study. Refer to appendix 4 (pg. 161) for nutrient analysis of hay and corn gluten feed. Two days after their arrival the calves were randomly allocated to one of two treatment groups (3 calves/group). The treatments were: 1) 25 IU cosyntropin (synthetic ACTH; Cortrosyn<sup>®</sup> – Amphastar Pharmaceuticals, Rancho Cocamonga, CA) via intramuscular injection and 0.0024 mg/kg epinephrine (Phoenix Pharmaceuticals, Burlingame, CA) via subcutaneous injection once at time zero and 2) 0.35 mg/kg dexamethasone (AgriLabs, St. Joseph, MO) via intramuscular injection once at time zero. Blood was collected from all calves via jugular venipuncture at time 0, 1, 16 and 40 hours post treatment (PT). All animal handling procedures were approved by the Virginia Tech Animal Care and Use Committee.

#### **Complete Blood Count and Leukocyte Differential**

Blood collected at 0, 16, and 40 hours into EDTA coated tubes was stored on ice until transport to the lab. Whole blood samples were submitted to the clinical pathology laboratory of

the Virginia/Maryland Regional College of Veterinary Medicine within 2 hrs of collection. Total leukocyte counts were carried out using the Advia 120 Automated Hematology Analyzer (Siemens Healthcare Diagnostics Inc, Deerfield, IL). The proportion of each leukocyte type present in the blood samples was determined by standard manual slide differential count.

### Lymphocyte Isolation

Peripheral blood mononuclear cells (PMBC) were isolated from blood collected at 0, 16, and 40 hours PT according to the method of Witonsky et al. (2003). Blood was collected via jugular venipuncture into tubes containing lithium heparin and immediately placed on ice for transport. Blood samples were allowed to warm to room temperature and diluted 1:2 with sterile phosphate-buffered saline (PBS). After mixing, 10 ml of diluted blood was layered over 5 ml of Lymphoprep® medium (Accurate Chemical and Scientific Corporation, Westbury, NY). Tubes were centrifuged for 30 minutes at 250 X g at room temperature with the centrifuge brake turned off. After centrifugation, the plasma was removed and discarded. The lymphocyte band was recovered and washed twice (250 X g for 10 minutes) with cold culture medium (RPMI 1640, 10% fetal bovine serum (FBS), 1 % Penicillin/Streptomycin/Amphotericin B). Residual erythrocytes were lysed by resuspending the pellet in 2 ml of culture medium and adding 4 ml of sterile, deionized water. After mixing for 18 seconds by pipette, 10 ml of culture medium was added. The lymphocyte suspension was then subjected to a final wash (250 X g for 10 minutes) and resuspended in 1 ml of cold culture medium. Viable cells were counted by diluting the lymphocyte suspension 1:3 in trypan blue (Invitrogen, Carlsbad, CA). Twenty  $\mu$ L of the lymphocyte/trypan blue suspension was transferred to a disposable counting chamber for automated cell counting and cell viability determination in a Cellometer® Auto (Nexcelcom

Bioscience LLC, Lawrence, MA) programmed to count round cells 6-12  $\mu\text{m}$  in diameter.

Isolated lymphocytes were then diluted to  $2 \times 10^6$  viable cells per ml in cold complete medium.

### Lymphocyte Proliferation

After dilution of isolated lymphocytes, lymphoproliferative responses were assessed as described by Mullarky et al. (2009). Briefly,  $2 \times 10^5$  cells/well were placed in 96 well round bottom plates (Fisher Scientific Company, Middleton VA) and measured in triplicate after stimulation with 1  $\mu\text{g}/\text{mL}$  of pokeweed mitogen (PWM). Background proliferation was determined with PBMC cultured in complete medium. After incubation for 66 h at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$ , 20  $\mu\text{l}$  cell proliferation reagent (CellTiter 96-Aqueous®, Promega, Madison, WI) was added to each well. The plates were then incubated ( $37^\circ\text{C}$  and 5%  $\text{CO}_2$ ) for an additional 4 hours and then read at 490 nm with a Bio-Tek microtiter well plate reader (SpectraMax M5 Multi-Mode Microplate Reader, Molecular Devices, Downingtown, PA).

### Flow Cytometry

Flow cytometric analysis was carried out on blood collected at 0, 16, and 40 hrs PT according to the technique of Gilbertie et al. (2010). Briefly, whole blood (3 ml) collected into EDTA Vacutainer tubes (Becton Dickson Inc., Franklin Lakes, NJ) was added to 12 ml ACK Lysis Buffer (8.3 g  $\text{NH}_4\text{Cl}$ , 1.0 g  $\text{KHCO}_3$ , 0.0327 g EDTA in 1L deionized water pH 7.2-7.4; Sigma-Aldrich, St. Louis, MO) and gently mixed by manual rotation for 3 minutes. Tubes were centrifuged at 300 X g at  $4^\circ\text{C}$  for 5 minutes. Supernatant was discarded and 1 ml of PBS+1% bovine serum albumin (BSA) was added to the remaining pellet. The pellet was washed three times with 1 ml of PBS with 1% BSA and finally resuspended with 1 ml of PBS+1% BSA. Viable cells (trypan blue exclusion test) were counted with a hemocytometer. Cells were diluted to  $2 \times 10^7$  viable cells/ml in PBS+1% BSA and 100  $\mu\text{l}$  of cell suspension was added to each well

of a 96 well V- bottom plate (Fisher Scientific Company, Middleton VA) in addition to 50  $\mu$ l of primary antibody at the after dilutions: CD4 (VMRD, Pullman, WA IL-A11, IgG<sub>2a</sub> isotype) 1:400, CD8 (VMRD BAQ 111A, IgM isotype) 1:400, WC1-N3  $\gamma\delta$  (VMRD CACTB32A, IgG<sub>1</sub> isotype) 1:400, and CD21 (VMRD BAQ15A, IgM isotype) 1:200. All the primary antibodies were added to separate wells except CD4 and CD8, which were added to the same well. Primary antibodies were incubated with the cells for 20 minutes in the dark on ice. After centrifugation at 300 X g for 5 minutes at 4°C, the primary antibody was decanted and the plates were washed (300 x g for 5 minutes at 4°C) two times with 200  $\mu$ l PBS+1%BSA. Secondary antibody (50  $\mu$ l) was added at the after dilutions: anti-IgG<sub>2a</sub> FITC (Invitrogen, Carlsbad, CA M32201) 1:400, anti-IgM APC (SouthernBiotech, Birmingham, AL 1140-11) 1:15, and anti-IgG<sub>1</sub> R-PE (SouthernBiotech, Birmingham, AL 1144-09) 1:20. 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 100  $\mu$ L cold PBS+1% BSA and 100  $\mu$ L 4% paraformaldehyde (Fisher Scientific Company, Middleton, VA) was added to each well. After incubation in the dark for 15 minutes on ice, plates were centrifuged at 300 x g for 5 minutes at 4°C. Supernatant was discarded and cells were resuspended in 100  $\mu$ L cold 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 events were collected and analysis was done by FlowJo<sup>®</sup> software (Tree Star, Inc., Ashland, OR). Upon analysis, brightly and dimly stained populations of CD8<sup>+</sup> and CD21<sup>+</sup> were observed. Additionally, a population of CD4 and CD8 double positive (CD4<sup>+</sup>/CD8<sup>+</sup>) cells was consistently observed in all samples (Appendix 6, pg 164). Gates were set to quantify each of these lymphocyte phenotypes. Percentages of each lymphocyte phenotype were reported. The total number of each lymphocyte

phenotype per  $\mu\text{l}$  of whole blood was calculated by multiplying each subtype percentage by the total number of lymphocytes determined by CBC and slide differential.

#### Whole Blood Stimulation

Whole blood mitogen stimulation was carried out on blood collected at 0, 16, and 40 hrs PT according to the method of Stabel and Whitlock (2001). Blood collected in lithium heparin Vacutainer tubes (Becton Dickson Inc., Franklin Lakes, NJ) was stored at ambient temperature until transport to the laboratory. One ml of whole blood was stimulated in triplicate with either complete medium (control), 5  $\mu\text{g}/\text{ml}$  Con A, or 5  $\mu\text{g}/\text{ml}$  PWM in individual wells of a 24 well flat-bottom culture plate (Fisher Scientific Company, Middleton VA). The plates were incubated at 37°C and 5 %  $\text{CO}_2$  for 24 hours. After incubation, blood was harvested from each well, transferred to microcentrifuge tubes, and centrifuged at 3000 x g for 20 minutes at 27°C. After centrifugation supernatants were pooled by stimulant and frozen at -20°C until cytokine analysis was performed.

#### Supernatant IFN- $\gamma$ ELISA

The procedure was modified from Souza et al. (2007) using the IFN- $\gamma$  Bovine Screening Set (Thermo Fisher Scientific Inc., Waltham, MA) and following manufacturer's instructions. In brief, 96 well, high efficiency binding plates (Fisher Scientific Company, Middleton VA) were coated with 100  $\mu\text{l}/\text{well}$  coating antibody diluted 1:100 in carbonate/bicarbonate buffer, sealed with a plate sealer and incubated overnight at room temperature. The coating antibody was then decanted and 300  $\mu\text{l}/\text{well}$  of blocking buffer (4% BSA, 5% sucrose in PBS; passed through a 0.2  $\mu\text{m}$  filter) was added and plates were incubated for 1 hour at room temperature. Blocking buffer was decanted and 100  $\mu\text{L}/\text{well}$  of standards or samples were added in duplicate and incubated for 1 hour at room temperature. In order for concentrations to fall on the standard curve provided,

the PWM-stimulated samples were diluted 1:10 (0 and 40 hrs PT) or 1:5 (16 hrs PT) with reagent diluent (4% BSA in D-PBS, passed through a 0.2 µm filter). The control and Con A stimulated samples were analyzed undiluted. After incubation, plates were washed three times with wash solution (0.05% Tween 20 in PBS). After the final wash, 100 µl/well of a biotin labeled detection antibody diluted 1:100 in reagent diluent was added and plates were incubated for 1 hour at room temperature. Plates were again washed three times with wash solution and 100 µl/well streptavidin-HRP diluted 1:400 in reagent diluent was added and the plates were incubated for 30 minutes at room temperature. After three additional washes, 100 µl/well of TMB substrate was added and plates were incubated in the dark for 20 minutes at room temperature. The reaction was stopped by adding 100 µl/well stop solution (2N H<sub>2</sub>SO<sub>4</sub>). The plate absorbance was read at 450 nm (SpectraMax M5 Multi-Mode Microplate Reader, Molecular Devices, Downington, PA). Sample concentrations were determined using the equation of a four parameter logarithmic standard curve.

#### Plasma Cortisol

Blood collected at 0, 1, and 16 hrs PT into EDTA coated Vacutainer tubes (Becton Dickson Inc., Franklin Lakes, NJ) was stored on ice until reaching the laboratory. Tubes were centrifuged at 3000 X g for 10 minutes. After centrifugation, plasma was harvested into microcentrifuge tubes, stored at -20<sup>0</sup>C and shipped overnight to the Michigan State University Diagnostic Center for Population and Animal Health (4125 Beaumont Road, Lansing, MI 48910-8104) within 24 hrs. Plasma cortisol concentration was determined by radioimmunoassay.

#### Study 2

##### *Eimeria bovis* Cultivation

The *E. bovis* oocysts used in this study were initially isolated from the feces of naturally infected calves in Virginia. Oocysts were isolated from the feces by sugar flotation according to the method of Jackson (1964). Sugar solution was removed from isolated oocysts with three water washes followed by resuspension of oocysts in 2% (w/v) potassium dichromate solution. The oocysts were incubated in 2% potassium dichromate for 3 days at room temperature. After the 3 day incubation, 100 µl of the oocyst suspension was placed on a glass slide, coverslipped, and microscopically examined at 400X. The first 100 oocysts encountered were identified to species based on oocyst morphology (Levine and Ivens, 1986) and characterized as sporulated or unsporulated based on presence of fully developed sporozoites. The inoculum was 85 % *E. bovis* and 96% of the oocysts were sporulated. Oocyst concentration was determined by averaging 3 counts obtained with a hemocytometer. The oocyst suspension was stored at 4°C in 2% potassium dichromate until further use.

Two, 10-day old, Jersey bull calves were used to amplify and further purify the oocyst inoculum. A modified Wisconsin sugar flotation technique (Cox and Todd, 1962) was performed on fecal samples collected from the calves prior to inoculation to ensure they were not shedding *Eimeria* spp. oocysts. Each calf was given 75,000 sporulated *E. bovis* oocysts orally and housed in an individual calf hutch for 21 days post infection. Daily fecal samples were collected and Wisconsin sugar flotation tests performed. Oocysts of *E. bovis* were first detected in the feces of both calves on day 19 post infection and each calf was euthanized by pentobarbital overdose on day 21 post infection. Immediately after euthanasia, the cecum from each calf was tied off at the ileocecal junction and removed from the animal for further processing. Each cecum was placed in a stainless steel pan, opened longitudinally, and the mucosa thoroughly washed with warm tap water. The cecal contents and rinse water were collected in a plastic container with an equal



volume of 4% potassium dichromate. The entirety of the cecal mucosa was additionally rinsed with 10% (v/v) bleach solution, scraped with a glass slide and rinsed with warm tap water. The mucosal scrapings and rinse water were collected in a plastic container with an equal volume of 4% potassium dichromate. Both cecal contents and scrapings from each calf were incubated at room temperature for 3 days. After incubation, oocysts were isolated from the cecal content and mucosal scrapings and processed as previously described for feces. The final oocyst suspension was determined to be 97% *E. bovis* (3% *E. auburnensis* and *E. cylindrical/ellipsoidalis*-like) and 98% of the oocysts were sporulated.

#### Animal Management and Experimental Infection

Fifteen 5-6 mo old, Angus and Angus-cross, weaned, beef calves were used in this study. The calves originated from the herd as calves in study 1 and were transported together to Blacksburg, VA 8 days after weaning. Upon arrival, all calves were managed as in study 1. A rectal fecal sample was collected from each calf prior to the initiation of the study and fecal oocyst count (FOC) was determined using the modified McMaster test. The calves were then blocked by FOC and randomly assigned within blocks to one of 3 treatment groups (n=5/group): 1) 500,000 sporulated *E. bovis* oocysts orally on day 0, 2) 100,000 sporulated *E. bovis* oocysts orally on day 0, 3) 20,000 sporulated *E. bovis* oocysts orally for 5 consecutive days starting on day zero. All calves were injected (IM) with 0.35 mg/kg of dexamethasone (Agrilabs) day 7 post oocyst challenge (PC). All calves were weighed on day -1 and 28. Blood was collected from all calves via jugular venipuncture at -1 and 8 days and rectal fecal samples were collected from all calves at -13 (weaning), -1, 8, and 19-28 days.

#### Fecal sample analysis

Oocyst number in each fecal sample was calculated using the Modified McMaster test with a sensitivity of 25 oocysts per g (OPG) (Whitlock, 1948). A modified Wisconsin sugar flotation technique (Cox and Todd, 1962) was also performed on samples collected on day -13, -1, 23-25, and 28 PC. The first 100 *Eimeria* spp oocysts encountered in each sample were examined at 400X and identified to species based on oocyst morphology (Levine and Ivens, 1986). One individual (AL) carried out all oocyst counts and species identifications. Because oocysts of *E. cylindrica* and *E. ellipsoidalis* could not be distinguished reliably, all small cylindrical *Eimeria* spp oocysts measuring 19-36 µm by 8-18 µm were designated *Eimeria cylindrica/ellipsoidalis*-like (Levine and Ivens, 1986).

Fecal dry matter analysis was also conducted on all fecal samples. Ten grams of thoroughly mixed feces was weighed into aluminum pans and placed into a 55° C drying oven. After 48 hours in the drying oven samples were weighed every 24 hours until the weight on consecutive days changed less than 1%. Final fecal dry matter was calculated as follows:

Dry matter (%) = (final weight/starting weight) \* 100.

#### Blood analysis

Complete Blood Count and leukocyte differential, lymphocyte isolation and proliferation procedures, whole blood stimulation and flow cytometry were carried out as described in Study 1 on Days -1 and 8. Additionally, PBMC were also stimulated with 5 µg/mL concanavalin A (Con A) in the lymphocyte proliferation procedure.

#### Supernatant IFN-γ ELISA

Supernatants were analyzed for IFN- γ concentration by ELISA as described in study 1. In order for values to fall on the standard curve provided, the PWM-stimulated samples were

diluted 1:10 (day -1 PC) or 1:5 (day 8 PC) with reagent diluent (4% BSA in D-PBS, 0.2  $\mu$ m filtered). The control and Con A stimulated samples were analyzed undiluted.

## Statistics

For both studies, white blood cell count (WBC), neutrophil:lymphocyte (N:L) ratio, lymphoproliferative response, lymphocyte subtypes, and IFN- $\gamma$  concentrations were analyzed by a mixed model analysis of variance using the GLIMMIX procedure of SAS version 9.2 (Cary, NC, USA). The model statement contained the fixed effects of treatment, time, and the time\*treatment interaction. Data were analyzed using calf(treatment) as the error term. In this model calf was the experimental unit. In study 2, fecal oocyst count (FOC) and fecal dry matter were also analyzed in the same manner. Fecal oocyst counts were normally distributed and no transformation was required. Average daily gain (ADG) was calculated for each calf in study 2 using weights from day -1 and 28. These data were analyzed by a mixed model analysis of variance using the MIXED procedure of SAS version 9.2 (Cary, NC, USA). The model statement contained treatment as a fixed effect. Where significant effects were detected in both studies they were further examined using the SLICE option of SAS version 9.2 (Cary, NC, USA).

## Results

### Study 1

In the dexamethasone group WBC count and N:L ratio were increased ( $P < 0.05$ ) at 16 and 40 hrs PT (Table 6.1). Time effects were detected in multiple cell types in the dexamethasone treated calves. The total number of each lymphocyte subtype was lower ( $P < 0.05$ ) at 16 and 40 hrs PT than it was prior to dexamethasone administration (0 hrs PI). The same time effects were observed for the dimly stained CD8<sup>+</sup> and CD21<sup>+</sup> lymphocyte subtypes and only data for the

brightly stained subtypes are presented (Table 6.1). The dexamethasone treated calves also had a lower ( $P<0.05$ ) percentage of WC1<sup>+</sup> lymphocytes at 16 and 40 hrs PT compared to time 0. The percentage of CD4<sup>+</sup>/CD8<sup>+</sup> lymphocytes ranged between 7-15% in all samples, but no treatment or time effects were detected. No change in WBC, N:L ratio or lymphocyte subtype were observed in the ACTH/epinephrine treated calves at 16 and 40 hrs PT, but these parameters did significantly differ from those of the dexamethasone treated animals in WBC count, N:L ratio, total numbers of CD4<sup>+</sup>, CD8<sup>+</sup>, WC1<sup>+</sup> and CD21<sup>+</sup> cells and % WC1<sup>+</sup> cells at 16 and 40 hours.

Lymphocyte proliferation in response to PWM stimulation was decreased ( $P<0.05$ ) in the dexamethasone treated group at 16 and 40 hrs PT (Figure 6.1) as compared to 0 hrs. No change in lymphoproliferative response was observed in the ACTH/Epinephrine treated group. Similarly, the concentration of IFN- $\gamma$  was decreased ( $P<0.05$ ) in whole blood supernatants from the dexamethasone treated group in response to both mitogen stimulants at 16 hrs PT and to PWM stimulation at 40 hrs PT (Table 6.2). The level of IFN- $\gamma$  production remained unchanged in the ACTH/epinephrine treated group.

Plasma cortisol concentration was increased ( $P<0.05$ ) in the ACTH/epinephrine treated group at 1 and 16 hrs PT (Figure 6.2). Time by treatment interactions were also detected for each treatment group. The plasma cortisol concentration in the ACTH/epinephrine group was higher ( $P<0.05$ ) at 16 hrs PT than at 0 or 40 hrs PI, while in the dexamethasone treated calves the plasma cortisol exhibited a declining trend ( $P=0.08$ ) at 16 and 40 hrs PI.

## Study 2

Mean FOC was higher ( $P<0.05$ ) on days 23-24 PC in the calves dosed with 500,000 sporulated *E. bovis* oocysts compared to the other two treatment groups (Figure 6.3). Likewise, FOC was higher ( $P<0.05$ ) for the 500,000 oocyst group on day 25 PC as compared to the 20,000

oocyst group but not different from the 100,000 oocyst group. The average percentage of *E. bovis* identified on day -13 for each treatment was 86%, 89%, and 92% for the 500,000, 100,000 and 20,000 groups respectively. *Eimeria bovis* oocysts continued to make up between 80-98% of the total oocysts identified in the 500,000 oocyst group on days 23-25, while the percentage of *E. bovis* in the other two groups ranged from 42-62% of the total oocysts identified during that time period. The 500,000 oocyst group also trended ( $P=0.08$ ) toward a decrease in fecal dry matter compared to the other two treatment groups on days 23-24 as well as the 20,000 oocyst group on day 25 ( $P=0.07$ ) (Figure 6.4).

All calves exhibited similar changes in WBC, N:L ratio, and lymphocyte subtype regardless of coccidia exposure (Table 6.3). In all groups there was increased WBC count and N:L ratio and decreased total  $CD4^+$ ,  $CD8^+$ , and  $WC1^+$  lymphocytes on day 8 PC as compared to day -1. The percentage of  $CD4^+$ ,  $CD8^+$ , and  $WC1^+$  positive lymphocytes was also decreased ( $P<0.05$ ) on day 8 PC compared to day -1. The same time effect was observed for dimly stained  $CD8^+$  and  $CD21^+$  lymphocyte subtypes as was observed for the brightly stained subtypes and only data for the brightly stained populations is presented (Table 6.3). The percentage of  $CD4^+/CD8^+$  ranged between 8-19% in all samples but no treatment or time effects were detected.

Lymphocyte proliferation in response to mitogen stimulation did not differ among groups (Figure 6.5). Lymphocytes isolated from all treatment groups proliferated less ( $P<0.05$ ) in response to mitogen stimulation on day 8 PC compared to day -1 PC. Similarly, concentration of IFN- $\gamma$  in whole blood supernatants did not differ among treatments, but IFN- $\gamma$  concentration was lower ( $P<0.05$ ) in supernatants from PWM stimulated whole blood in all three treatment groups on day 8 PC compared to day -1 PC (Table 6.4).

Average daily gains from day -1 through 28 PC did not differ. Average daily gain was, however, inversely related to daily oocyst dose. The calves given 500,000 oocysts gained 0.05 kg/day, the calves given 100,000 oocysts gained 0.15 kg/day, and the calves given 20,000 oocysts daily for 5 days gained 0.48 kg/day.

### **Discussion**

The initial objective of these studies was to identify a pharmacologic agent that induces changes in immune parameters in beef calves similar to those seen after stressors like weaning and transport. Previous studies have documented increased WBC and N:L (Colditz and Hennessy, 2001; Buckham-Sporer et al., 2008; Lynch et al., 2010a), decreased numbers of CD4<sup>+</sup>, CD8<sup>+</sup> and WC1<sup>+</sup> lymphocytes (Lynch et al., 2010a), decreased lymphocyte proliferation (Chang et al., 1994), and decreased IFN- $\gamma$  production (Gilbertie et al., 2010; Lynch et al., 2010b) have been observed after stressful events in cattle such as weaning and transport. In the current study, immune parameters changed in a similar manner to those described above in the dexamethasone treated calves while immune parameters in the calves given ACTH/Epinephrine did not. The development of a consistent stress leukogram (leukocytosis, mature neutrophilia, and lymphocytopenia) was evident in all dexamethasone treated calves 16 hrs PT and was still evident at 40 hrs PT. While similar changes in circulating lymphocyte subtypes and lymphoproliferative responses have been observed in other studies where dexamethasone was administered to cattle of various age groups (Nonnecke et al., 1997; Menge and Dean-Nystrom, 2008), this study confirms these changes in weaned beef calves. Nonnecke and colleagues (1997) also reported that dexamethasone administration decreased the production of IFN- $\gamma$  in supernatants harvested from enriched lymphocyte populations after mitogen stimulation. In the current study, IFN- $\gamma$  quantification was carried out on supernatants harvested from whole blood

stimulated with PWM and Con A. Although a significant decrease in the amount of IFN- $\gamma$  was detected in these supernatants at 16 and 40 hrs after dexamethasone administration, it is impossible to determine the exact cause. In our assay whole blood was used as the medium for stimulation with mitogen and therefore the total number of lymphocytes present in each sample was not consistent. The total number of lymphocytes and CD4<sup>+</sup>, CD8<sup>+</sup>, and WC1<sup>+</sup> subtypes were decreased in whole blood from the dexamethasone treated calves. Therefore, we cannot be certain that the decreased IFN- $\gamma$  production was due solely to reduced production of IFN- $\gamma$  after mitogen stimulation, since results may also reflect reduced lymphocyte numbers.

In both studies, a population of circulating CD4<sup>+</sup>/CD8<sup>+</sup> positive lymphocytes was observed in all samples. To the author's knowledge no other studies have reported this lymphocyte phenotype in cattle, but this cell population is reported to make up as much as 60% of circulating T-cells in swine (Pescovitz et al., 1994). The function of these lymphocytes is not known, but these researchers concluded that this cell type contains the majority of memory T-cells. Although this population was not altered by the treatments in either study, further research is warranted to evaluate the functionality of this population.

Plasma cortisol concentration decreased in the dexamethasone treated calves, which was expected because dexamethasone exerts a negative feedback on the hypothalamic pituitary adrenal axis, thus decreasing circulating amounts of endogenous cortisol (Mormede et al., 2007)

Changes in immune parameters were not detected in the calves given ACTH in combination with epinephrine in the current study. Although multiple (1-6) high (50-200 IU) doses of purified ACTH produce immunologic changes in cattle (Wegner and Stott 1972; Paape et al., 1977; Gwazdauskas et al., 1980; Roth et al., 1982; Roth et al., 1983), the effect of a single low dose of synthetic ACTH on immunological parameters in calves has not been investigated to

the author's knowledge. The use of a synthetic preparation of ACTH (Cortrosyn – Amphastar Pharmaceuticals) was chosen in our study because it is available commercially as an FDA approved animal health pharmaceutical. The dose of Cortrosyn<sup>®</sup> (25 IU) was selected based on work by Yoshida and Nakao (2006), who observed a 10-fold rise in plasma cortisol in cattle after this dose. Plasma cortisol concentration 1 hour after ACTH/epinephrine injection in this study was similar to that seen by Yoshida and Nakao (2006) and consistent with plasma cortisol concentrations reported in calves after transport (Crookshank et al. 1979; Buckham-Sporer et al. 2008). In those studies, however, the plasma cortisol peak was maintained for up to 24 hours, while in the present study the plasma cortisol level had returned to baseline by 16 hours PI. This rapid decline in plasma cortisol likely accounts for the absence of observed immunological changes in this group. In conclusion, it was determined that a single intramuscular injection of dexamethasone at 0.35 mg/kg was sufficient to produce changes in immune parameters similar to those observed after weaning and transport in beef calves.

In the second study we identified the number of sporulated *E. bovis* oocysts required to increase subsequent FOC in dexamethasone stressed calves. The changes in immune parameters 16 hrs after dexamethasone injection (day 8 PC) in study 2 were almost identical to those observed after the dexamethasone injection in study 1. The same magnitude of WBC count and N:L ratio increase were observed in all treatment groups regardless of *E. bovis* dose. Likewise, all groups exhibited similar changes in circulating lymphocyte subtypes, magnitude of lymphocyte proliferation and IFN- $\gamma$  production after dexamethasone administration. The author concludes that dexamethasone administration produces consistent immune suppression in weaned beef calves.



Although the immunologic parameters were altered to the similar degree among the three treatment groups, differences in subsequent FOC were observed. An increase in FOC (days 23-24 PC) was observed only in the calves given 500,000 sporulated *E. bovis* oocysts. Although the fecal dry matter only trended toward a decrease in this group, the fact that it was lowest at the time of peak oocyst shedding may indicate that it was a result of the *E. bovis* infection. The proportion of *E. bovis* oocysts identified also increased during the same time period. The changes in oocyst numbers and species composition were not observed in the groups receiving a single dose of 100,000 oocysts or 5 daily doses of 20,000 oocysts.

Our study is the first in which calves known to be previously infected with *E. bovis* were immune suppressed and simultaneously challenged with *E. bovis* oocysts. In other studies of *E. bovis* challenge infection, calves were not simultaneously immunosuppressed. Conversely, in a study conducted by Lindsay et al. (1990) calves were given synthetic glucocorticoids after recovering from primary *E. bovis* infection, but were not simultaneously challenged. In all studies neither an increase in FOC nor the onset of clinical disease were induced. In poultry, administration of either cyclosporin A (Lillehoj, 1987) or synthetic glucocorticoids (Rose, 1970; Long and Rose, 1970) in conjunction with *Eimeria* challenge led to subsequent oocyst shedding, cecal lesion scores, and reduced weight gains equal to those observed after primary infection. Likewise, Niilo (1970c) reported that dexamethasone administered to calves during patent *E. zuernii* infection increased oocyst output and worsened clinical signs. The effect of this apparent abrogation of immunity is interesting because in many species, including cattle, immunity to *Eimeria* infection is believed to be of cell mediated (Rose et al., 1987; Lillehoj, 1987; Rose et al. 1988; Suhwold et al., 2010). In fact, elevated circulating CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes in addition to *E. bovis* merozoite specific increase in IFN- $\gamma$  production have been observed 8 days

after *E. bovis* infection (Taubert et al, 2008; Suhwold et al., 2010). Because these changes were observed only on day 8 post infection, these researchers hypothesize that protective cellular immunity to *E. bovis* infection is directed to developing first generation merozoites in the small intestine. We administered dexamethasone at 7 days PC in this study to suppress cellular immunity while merozoites were replicating in the small intestine. The resultant decreases in circulating T-lymphocytes, lymphocyte proliferation, and IFN-  $\gamma$  production on day 8 PC are consistent with immunosuppression. Immune suppression alone was not sufficient to produce an increase in FOC because only the calves given 500,000 *E. bovis* oocysts had increased FOC. Although immunosuppression was induced by dexamethasone administration in all calves, a sufficient immune response was still present to eliminate a single challenge with 100,000 or 20,000 *E. bovis* oocysts for 5 consecutive days. The challenge with 500,000 oocysts apparently exceeded the calves' ability to mount a sufficient immune response allowing *E. bovis* replication within the intestine and increased FOC. In conclusion, these studies provide a model for further study of the immune response to *E. bovis* challenge in calves during times of stress. Continued work with this model is needed to clearly identify the mechanism responsible for stress associated outbreaks of coccidiosis in beef calves.

Table 6.1: Leukocyte counts and lymphocyte phenotypes of calves at 0, 16 and 40 hours after treatment with Dexamethasone or ACTH/Epinephrine (n=3/group). Values are least squares means and standard error (SE).

Cell Type	Dexamethasone			ACTH/Epi			SE
	0 hrs	16 hrs	40 hrs	0 hrs	16 hrs	40 hrs	
WBC	9500 <sup>a</sup>	21,300 <sup>b</sup>	21,000 <sup>b</sup>	8,200	9,800	7,900	2,547
N:L Ratio	0.4 <sup>a</sup>	6.8 <sup>b</sup>	5.4 <sup>b</sup>	0.2	0.5	0.2	1.2
CD4 <sup>+</sup> %	13.7	14.9	16.8	15.1	15.5	21.3	1.1
CD4 <sup>+</sup> Total	819 <sup>a</sup>	396 <sup>b</sup>	580 <sup>b</sup>	985	1,035	1,267	130
CD8 <sup>+</sup> %	16.5	13.5	22.9	18.7	13.9	22.7	1.7
CD8 <sup>+</sup> Total	1,007 <sup>a</sup>	388 <sup>b</sup>	792 <sup>b</sup>	1,252	945	1,389	124
WC1 <sup>+</sup> %	5.3 <sup>a</sup>	1.4 <sup>b</sup>	2.0 <sup>b</sup>	4.3	3.9	4.0	0.6
WC1 <sup>+</sup> Total	335 <sup>a</sup>	40 <sup>b</sup>	64 <sup>b</sup>	278	247	244	49
CD21 <sup>+</sup> %	39.0	34.6	34.6	36.6	27.9	48.3	3.3
CD21 <sup>+</sup> Total	2,446 <sup>a</sup>	1,042 <sup>b</sup>	1,689 <sup>b</sup>	2,384	1,794	2,892	270

Time effects (P<0.05) are indicated by different numerical superscripts within time points.

Table 6.2: IFN- $\gamma$  concentration (pg/ml) in whole blood supernatants collected from calves at 0, 16 and 40 hours after treatment with Dexamethasone or ACTH/Epinephrine and stimulated with culture medium (Control), concanavalin A (Con A) or pokeweed mitogen (PWM). Values are least squares means and standard error (SE).

Stimulation	Dexamethasone			ACTH/Epi			SE
	0 hrs	16 hrs	40 hrs	0 hrs	16 hrs	40 hrs	
Control	24	25	9	35	71	29	8
Con A	88 <sup>a</sup>	29 <sup>b</sup>	172 <sup>a</sup>	133	112	107	21
PWM	888 <sup>a</sup>	238 <sup>b</sup>	325 <sup>b</sup>	1,552	4,698	4,210	806

Time effects ( $P < 0.05$ ) are indicated by different numerical superscripts within time points.

Table 6.3: Leukocyte counts and lymphocyte phenotypes of calves on -1 and 8 days after challenge with a single dose of 500,000 sporulated *E. bovis* oocysts (1X500,000), a single dose of 100,000 sporulated *E. bovis* oocysts (1X100,000), or a daily dose of 20,000 sporulated *E. bovis* oocysts for 5 consecutive days (5X20,000) (n=5/group). All calves received dexamethasone on day 7 post challenge. Values are least squares means and standard error (SE).

Cell Type	1X500,000		1X100,000		5X20,000		SE
	Day -1	Day 8	Day -1	Day 8	Day -1	Day 8	
WBC	7,652 <sup>a</sup>	21,466 <sup>b</sup>	8,141 <sup>a</sup>	22,548 <sup>b</sup>	7,032 <sup>a</sup>	24,000 <sup>b</sup>	1,380
N:L Ratio	0.1 <sup>a</sup>	6.35 <sup>b</sup>	0.3 <sup>a</sup>	6.04 <sup>b</sup>	0.2 <sup>a</sup>	6.82 <sup>b</sup>	1.4
CD4 <sup>+</sup> %	19.6 <sup>a</sup>	14.4 <sup>b</sup>	18.4 <sup>a</sup>	15.91 <sup>b</sup>	15.0 <sup>a</sup>	13.8 <sup>b</sup>	1.9
CD4 <sup>+</sup> Total	1,131 <sup>a</sup>	585 <sup>b</sup>	1,030 <sup>a</sup>	451 <sup>b</sup>	749 <sup>a</sup>	455 <sup>b</sup>	133
CD8 <sup>+</sup> %	24.0 <sup>a</sup>	16.6 <sup>b</sup>	24.1 <sup>a</sup>	16.9 <sup>b</sup>	23.3 <sup>a</sup>	16.6 <sup>b</sup>	1.2
CD8 <sup>+</sup> Total	1,456 <sup>a</sup>	524 <sup>b</sup>	1,132 <sup>a</sup>	643 <sup>b</sup>	1,167 <sup>a</sup>	547 <sup>b</sup>	209
WC1 <sup>+</sup> %	6.3 <sup>a</sup>	0.8 <sup>b</sup>	7.0 <sup>a</sup>	0.9 <sup>b</sup>	7.2 <sup>a</sup>	1.2 <sup>b</sup>	1.2
WC1 <sup>+</sup> Total	370 <sup>a</sup>	24 <sup>b</sup>	356 <sup>a</sup>	32 <sup>b</sup>	336 <sup>a</sup>	41 <sup>b</sup>	69.4
CD21 <sup>+</sup> %	27.3	34.3	28.6	29.0	29.3	27.9	2.8
CD21 <sup>+</sup> Total	1,573	899	1,658	1,374	1,473	944	274

Time effects (P<0.05) are indicated by different numerical superscripts within time points.

Table 6.4: IFN- $\gamma$  concentration (pg/ml) in whole blood supernatants collected from calves on days -1 and 8 after challenge with a single dose of 500,000 sporulated *E. bovis* oocysts (1X500,000), a single dose of 100,000 sporulated *E. bovis* oocysts (1X100,000), or a daily dose of 20,000 sporulated *E. bovis* oocysts for 5 consecutive days (5X20,000) (n=5/group). All calves received dexamethasone on day 7 post challenge. Values are least squares means and standard error (SE).

Stimulant	1X500,000		1X100,000		5X20,000		SE
	Day -1	Day 8	Day -1	Day 8	Day -1	Day 8	
Control	44	34	57	125	58	28	22
Con A	424	356	292	396	865	278	271
PWM	1,422 <sup>a</sup>	236 <sup>b</sup>	1,701 <sup>a</sup>	105 <sup>b</sup>	2,447 <sup>a</sup>	249 <sup>b</sup>	747

Time effects (P<0.05) are indicated by different numerical superscripts within time points.

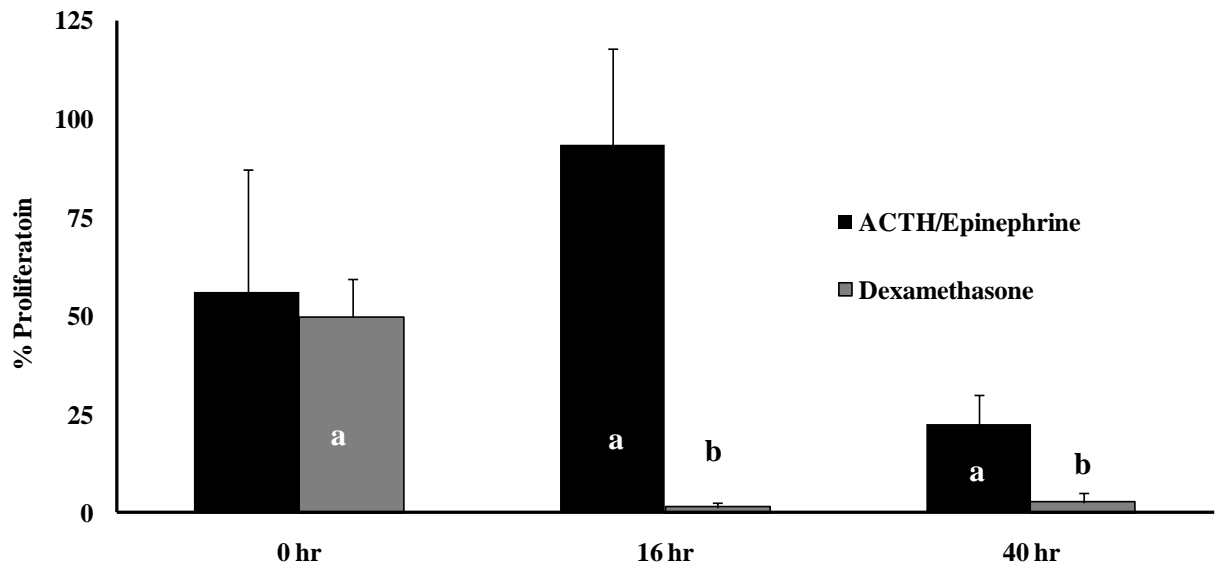


Figure 6.1: Lymphocyte proliferation after stimulation with pokeweed mitogen for calves at 0, 16 and 40 hours after treatment with Dexamethasone or ACTH/Epinephrine (n=3/group). Different letters within each time point represent treatment effects ( $P < 0.05$ ). Different letters within time points represent treatment effects ( $P < 0.05$ ). Different letters within each treatment at different time points represent time effects ( $P < 0.05$ ). Values are least squares means and error bars represent standard error.

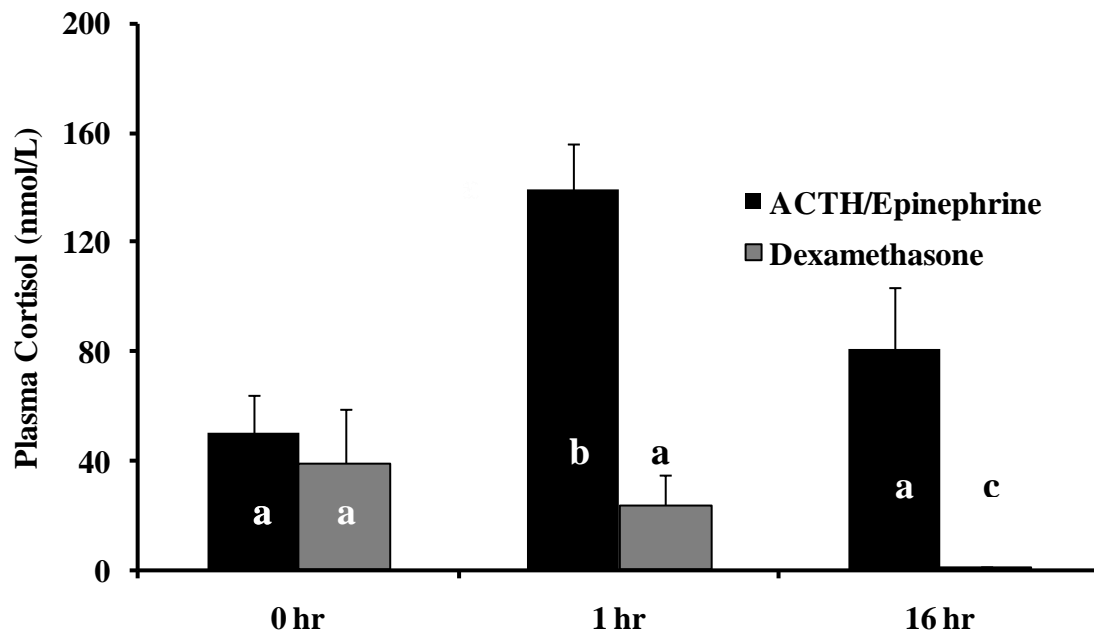


Figure 6.2: Plasma cortisol concentration for calves at 0, 1 and 16 hours after treatment with Dexamethasone or ACTH/Epinephrine (n=3/group). Different letters within each time point represent treatment effects ( $P < 0.05$ ). Different letters within each treatment at different time points represent time effects ( $P < 0.05$ ). Values are least squares means and error bars represent standard error.



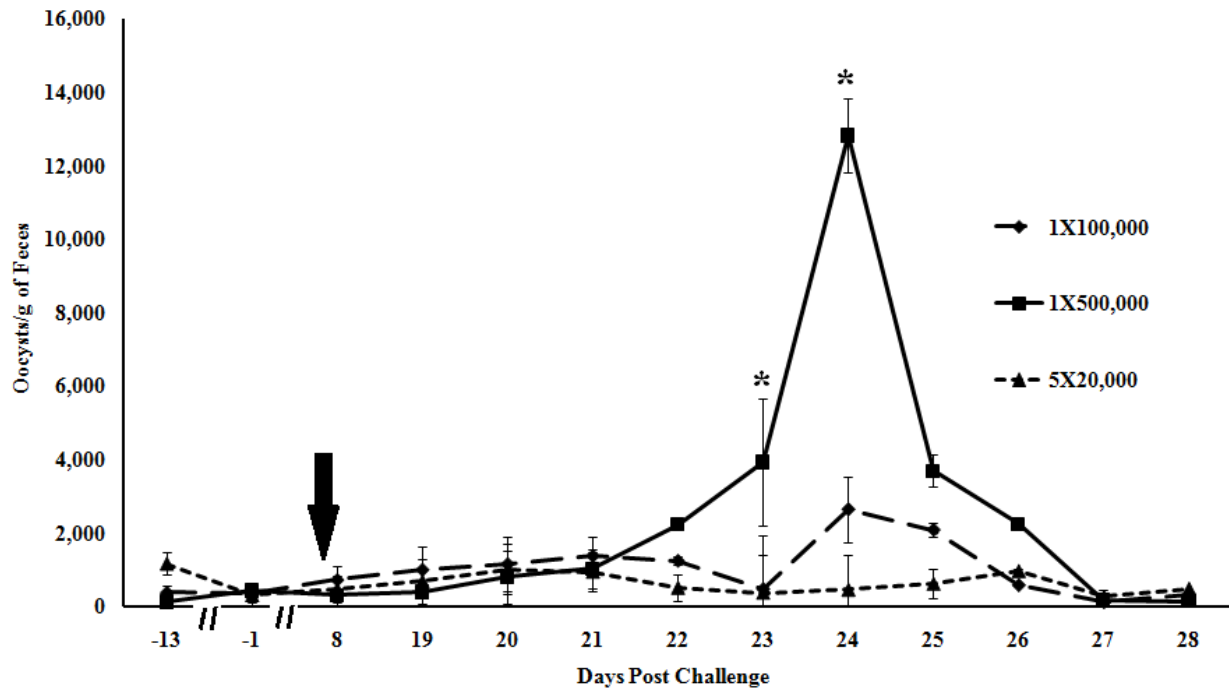


Figure 6.3: Fecal oocyst count/g of feces for calves challenged with a single dose of 500,000 sporulated *E. bovis* oocysts (1X500,000), a single dose of 100,000 sporulated *E. bovis* oocysts (1X100,000), or a daily dose of 20,000 sporulated *E. bovis* oocysts for 5 consecutive days (5X20,000) (n=5/group). All calves received dexamethasone on day 7 post challenge (arrow). Symbol (\*) indicates time points where treatment effects ( $P < 0.05$ ) were detected. Values are least squares means and error bars represent standard error.

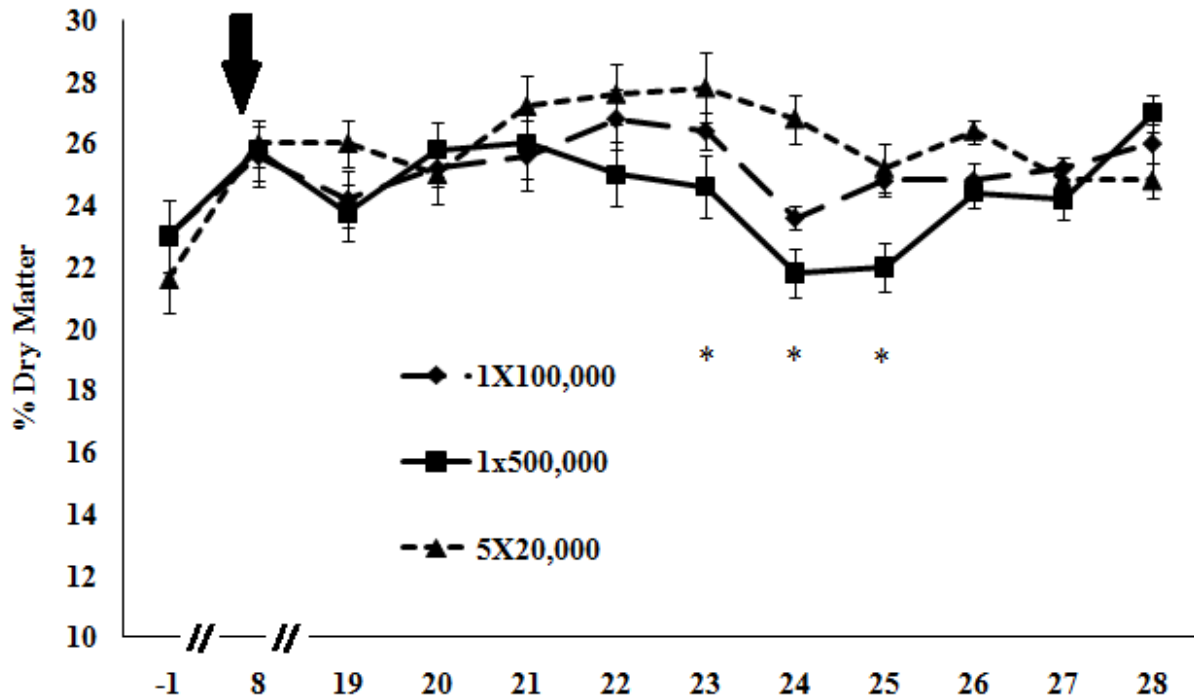


Figure 6.4: Percentage of fecal dry matter for calves challenged with a single dose of 500,000 sporulated *E. bovis* oocysts (1X500,000), a single dose of 100,000 sporulated *E. bovis* oocysts (1X100,000), or a daily dose of 20,000 sporulated *E. bovis* oocysts for 5 consecutive days (5X20,000) (n=5/group). All calves received dexamethasone on day 7 post challenge (arrow). Symbol (\*) indicates time points where treatment effects (P<0.05) were detected. Values are least squares means and error bars represent standard error.

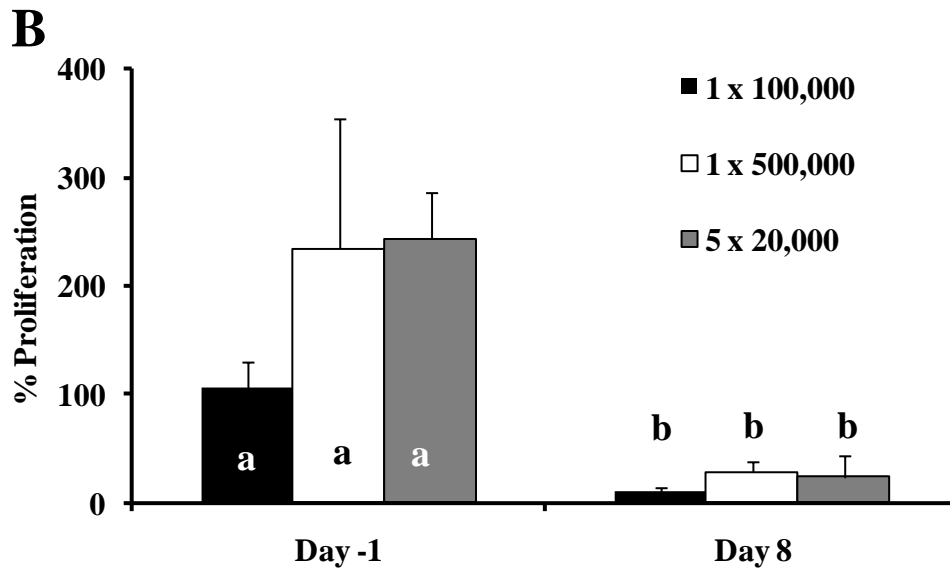
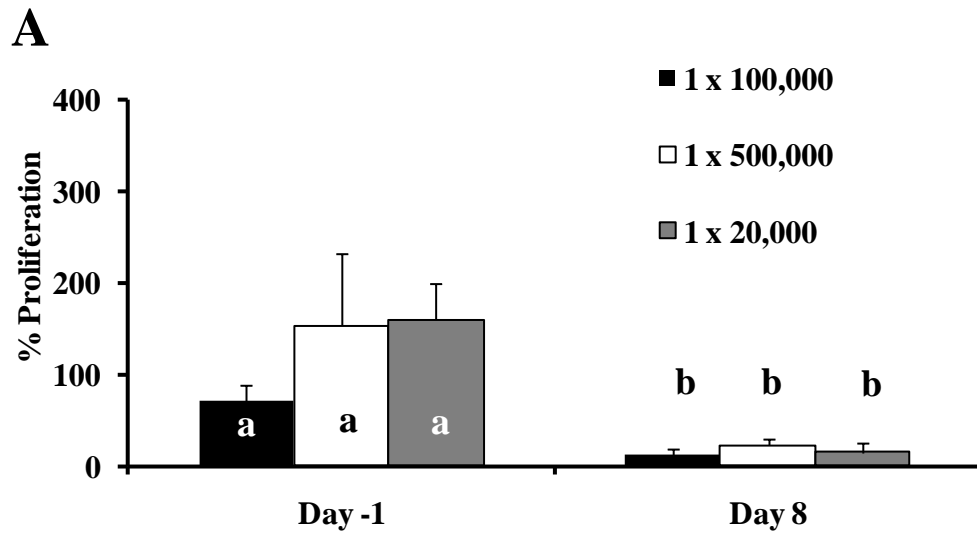


Figure 6.5: Lymphocyte proliferation after stimulation with pokeweed mitogen (A) or concanavalin A (B) for calves on days -1 and 8 after challenge with a single dose of 500,000 sporulated *E. bovis* oocysts (1X500,000), a single dose of 100,000 sporulated *E. bovis* oocysts (1X100,000), or a daily dose of 20,000 sporulated *E. bovis* oocysts for 5 consecutive days

(5X20,000) (n=5/group). All calves received dexamethasone on day 7 post challenge. Values are least squares means and error bars represent standard error.

## **Chapter 7: Immune response to *E. bovis* challenge in steroid stressed weanling beef calves**

### **Abstract**

Outbreaks of coccidiosis in beef calves are often associated with stressful events. The objective of this study was to investigate the immune response *E. bovis* infection during induced stress in beef calves. Thirty-two Angus-cross calves (6-7 mo.) were blocked by fecal oocyst count (FOC), sex and dam age and randomly assigned within blocks to one of four treatment groups (n=8/group): non-stressed non-challenged (NSNC), non-stressed challenged (NSC), stressed non-challenged (SNC), and stressed challenged (SC). Challenged calves were orally challenged with 1.5 million oocysts on day 0 and stressed calves were given dexamethasone (0.35 mg/kg) on day 7 post challenge (PC). Feces were collected from all calves on day -60, -1, 8 and daily from day 15-28 PC. Blood was collected from 4 calves per treatment on day -1, 8 and 21 PC for measurement of complete blood counts and lymphocyte phenotypes. In addition, lymphocytes were stimulated with *E. bovis* antigen, Con A and PWM to determine lymphoproliferative response and IFN- $\gamma$  and IL-4 production. Modified McMaster tests and fecal dry matter analysis were conducted on all fecal samples. The same four calves used for blood collection from the NSNC and SC group were euthanized on day 24 PC. The ceca were grossly scored for the presence of *Eimeria* spp. lesions. The mass of the ileocecal lymph node was recorded and lymphocytes were isolated from the ileocecal and colic lymph nodes for cell lymphocyte phenotyping. Additionally, tissue sections from the ileum and cecum were preserved for histological and immunohistochemical staining. Pathological changes seen in hematoxylin and eosin (H&E) stained slides were used to assign histopathologic lesion scores. Immunohistochemical staining was employed to quantify the number of lymphocyte subtypes in

intestinal sections. Dexamethasone treated calves had increased WBC count, neutrophil:lymphocyte ratios and IL-4 production on day 8 PC while lymphocyte proliferation in response to mitogen, total number of CD4+, CD8+, WC1+, and CD21+ lymphocytes, and IFN- $\gamma$  production were decreased. The fecal oocyst count increased in the SC group (day 21-26), but remained unchanged in other groups. Gross and histopathological lesion scores were higher for the ceca of SC compared to NSNC. The proportion of CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes were higher in regional lymph nodes of SC calves compared to the NSNC calves, but no differences in lymphocyte subtype infiltration of intestinal sections was observed. In conclusion, dexamethasone altered the cellular immune response, but no change in subsequent *E. bovis* oocyst shedding was detected unless dexamethasone was combined with *E. bovis* challenge. The author concludes that natural outbreaks of coccidiosis in stressed calves may require both immunosuppression and continued parasite exposure.

### **Introduction**

Routine management practices such as weaning, shipping, and commingling lead to increases in circulating stress hormones in beef calves (Crookshank et al. 1979; Buckham Sporer et al., 2008; Lynch et al., 2010b). High concentrations of these hormones, most notably cortisol, have been associated with alterations in immune function in many domestic species including cattle (Hickey et al., 2003; Arthington et al., 2005; Arthington et al., 2008, Lynch et al., 2010a, Lynch et al., 2010b). These alterations are characterized by cytokine expression profiles that shift the immune response away from a T-helper type 1 (Th1) toward a T-helper type 2 (Th2) response (Elenkov and Chrousos, 1999; Yang and Glaser, 2002; Viveros-Paredes et al., 2006). This shift may increase the stressed animal's susceptibility to pathogens that are controlled by a Th1 type immune response. The relationship between stress, immunity and disease susceptibility

is recognized in recently weaned calves, which are known to have an increased incidence of infectious diseases (Callan and Garry, 2002; Snowden, 2009).

Nearly all beef calves are infected with pathogenic *Eimeria* spp. prior to weaning (Ernst et al., 1987; Lucas et al., 2007). Although research investigating the immune response to *Eimeria* challenge in cattle is limited, clinical coccidiosis has not been experimentally reproduced in calves that have recovered from a primary *Eimeria* infection (Dauguschies et al. 1996; Fiege et al., 1992; Suhwold et al., 2010). Thus it seems likely that clinical coccidiosis should not occur in beef calves that have been previously exposed to *Eimeria*. Outbreaks of clinical coccidiosis in weaned beef calves have been reported, leading many to hypothesize that these outbreaks are associated with the stress of weaning (Fitzgerald, 1962; Marquardt, 1962; Niilo, 1970a; Niilo 1970b; Parker et al., 1986; Marshall et al., 1998). Several authors have suggested that a Th1 type immune response is required to prevent the development of disease in the face of continued parasite challenge (Dauguschies et al. 1996; Taubert et al., 2008; Suhwold et al., 2010). With this evidence in mind, we hypothesize that the outbreaks of clinical coccidiosis observed in recently weaned and transported beef calves are a result of stress-induced suppression of cell mediated immunity in combination with continued parasite exposure.

In order to study the immune response to *E. bovis* challenge in beef calves during times of stress a model simulating stress-induced immunologic change and coccidial challenge has been developed (Chapter 6). This model builds on the results obtained by Niilo (1970c) and employs the parenteral administration of a synthetic glucocorticoid (dexamethasone) in conjunction with a high oral dose of sporulated *E. bovis* oocysts to increase subsequent oocyst shedding in post-weaned calves. This stress model was employed in this study to investigate the immune response to *E. bovis* challenge in post-weaned beef calves during times of stress.

## Materials and Methods

### *Eimeria bovis* Cultivation

The *E. bovis* oocysts used in this study were initially isolated from the feces of naturally infected calves in Virginia. Oocysts were isolated from the feces by sugar flotation according to the method of Jackson (1964). Sugar solution was removed from isolated oocysts with three water washes followed by resuspension of oocysts in 2% (w/v) potassium dichromate solution. The oocysts were incubated in 2% potassium dichromate for 3 days at room temperature. After the 3 day incubation, 100 µl of the oocyst suspension was placed on a glass slide, coverslipped, and microscopically examined at 400X. The first 100 oocysts encountered were identified to species based on oocyst morphology (Levine and Ivens, 1986) and characterized as sporulated or unsporulated based on presence of fully developed sporozoites. The inoculum was 85 % *E. bovis* and 96% of the oocysts were sporulated. Oocyst concentration was determined by averaging 3 counts obtained with a hemocytometer. The oocyst suspension was stored at 4°C in 2% potassium dichromate until further use.

Two, 10-day old, Jersey bull calves were used to amplify and further purify the oocyst inoculum. A modified Wisconsin sugar flotation technique (Cox and Todd, 1962) was performed on fecal samples collected from the calves prior to inoculation to ensure they were not shedding *Eimeria* spp. oocysts. Each calf was given 75,000 sporulated *E. bovis* oocysts orally and housed in an individual calf hutch for 21 days post infection. Daily fecal samples were collected and Wisconsin sugar flotation tests performed. Oocysts of *E. bovis* were first detected in the feces of both calves on day 19 post infection and each calf was euthanized by pentobarbital overdose on day 21 post infection. Immediately after euthanasia, the cecum from each calf was tied off at the ileocecal junction and removed from the animal for further processing. Each cecum was placed



in a stainless steel pan, opened longitudinally, and the mucosa thoroughly washed with warm tap water. The cecal contents and rinse water were collected in a plastic container with an equal volume of 4% potassium dichromate. The entirety of the cecal mucosa was additionally rinsed with 10% (v/v) bleach solution, scraped with a glass slide and rinsed with warm tap water. The mucosal scrapings and rinse water were collected in a plastic container with an equal volume of 4% potassium dichromate. Both cecal contents and scrapings from each calf were incubated at room temperature for 3 days. After incubation, oocysts were isolated from the cecal content and mucosal scrapings and processed as previously described for feces. The final oocyst suspension was determined to be 97% *E. bovis* (3% *E. auburnensis* and *E. cylindrical/ellipsoidal*-like) and 98% of the oocysts were sporulated.

#### Animal Management and Experimental Infection

Thirty two 7-8 mo old, Angus and Angus-cross, weaned, beef calves were used in this study. The calves originated from the Shenandoah Agricultural Research and Extension Center, Steele's Tavern, VA and were transported together to Blacksburg, VA 34 days after weaning. Upon arrival, all calves were commingled in a single 10 hectare paddock for the duration of the study. They were provided ad libitum access to mixed grass hay and water and supplemented daily with corn gluten feed at 0.5% of body weight. Refer to appendix 4 (pg. 161) for nutrient analysis of hay and corn gluten feed. A rectal fecal sample was collected from each calf prior to the initiation of the study and fecal oocyst count (FOC) was determined using the modified McMaster test. The calves were blocked by gender (16 heifers and 16 steers), dam type (16 from mature cows and 16 from first calf heifers), and FOC and randomly assigned within blocks to one of 4 treatment groups (8 calves/treatment): 1) Non-stressed and non-challenged (NSNC) calves did not receive *E. bovis* oocysts or dexamethasone injection and served as untreated

controls 2) Non-stressed and challenged (NSC) calves were orally challenged with  $1.5 \times 10^6$  sporulated *E. bovis* oocysts on day 0, 3) Stressed and non-challenged (SNC) were given 0.35 mg/kg of dexamethasone (AgriLabs, St. Joseph, MO) via IM injection on Day 7 post challenge (PC), 4) Stressed and Challenged (SC) calves were orally challenged with  $1.5 \times 10^6$  sporulated *E. bovis* oocysts on day 0 and were given 0.35 mg/kg of dexamethasone via IM injection on Day 7 PC. All calves were weighed on day -1 and 23 PC. Blood was collected via jugular venipuncture from a randomly chosen subset of 4 calves from each treatment at -1, 8, and 21 days PC. Rectal fecal samples were collected from all calves at -60 (Weaning), -1, 8, and 19-24 days PC. On day 24 PC, the same 4 calves that were randomly selected for blood collection from each of the NSNC and SC groups were transported (approx. 1 km) to the Virginia-Maryland Regional College of Veterinary Medicine and euthanized by pentobarbital overdose. All animal handling procedures were approved by the Virginia Tech Animal Care and Use Committee.

#### Fecal Sample Analysis

Oocyst number in each fecal sample was calculated using the Modified McMaster test with a sensitivity of 25 oocysts per g (OPG, Whitlock, 1948). A modified Wisconsin sugar flotation test (Cox and Todd, 1962) was also performed on samples collected on day -60, -1, 8, 23-25, and 28 PC. The first 100 *Eimeria* oocysts encountered in each sample were identified to species based on oocyst morphology (Levine and Ivens, 1986). One individual (AL) carried out all oocyst counts and species identifications. Because oocysts of *E. cylindrica* and *E. ellipsoidalis* could not be distinguished reliably, all small cylindrical *Eimeria* spp. oocysts measuring 19-36  $\mu\text{m}$  by 8-18  $\mu\text{m}$  were designated *Eimeria cylindrica/ellipsoidalis*-like (Levine and Ivens, 1986).

Fecal dry matter analysis was also conducted on all fecal samples. Ten grams of thoroughly mixed wet feces was weighed into aluminum pans and placed into a 55°C drying oven. After 48

hours in the drying oven samples were weighed every 24 hours until the weight on consecutive days changed less than 1%. Final fecal dry matter was calculated using the formula:

Dry matter (%)=(final weight/starting weight) \* 100.

#### *E. bovis* Whole Oocyst Antigen Preparation

*Eimeria bovis* whole oocyst antigen (EbAg) was prepared as described by Hughes et al. (1989). Briefly,  $1 \times 10^6$  sporulated *E. bovis* oocysts were mixed with 1 ml of phosphate buffered saline (PBS) and 100 mg of 0.5 mm diameter zirconium beads in a 1.5 ml microcentrifuge tube. The oocysts were destroyed by placing the mixture in a bead beater (MiniBeadbeater<sup>®</sup>, Biospec Products, Bartlesville, OK) for 2 minutes. After initial beating, the microcentrifuge tube was placed on ice for 2 minutes and then placed back in bead beater for an additional 2 minute cycle. The homogenate was centrifuged at 11,000 x g for 10 minutes. The resultant supernatant was passed through a 0.2  $\mu$ m filter and dialyzed against RPMI-1640 medium. Protein concentration was determined using the Micro BCA Protein Assay (Thermo Fisher Scientific Inc., Waltham, MA) following manufacturer's instructions.

#### Complete Blood Count and Leukocyte Differential

Blood collected on day -1, 8, and 21 PC into EDTA coated tubes was stored on ice until transport to the lab. Whole blood samples were submitted to the clinical pathology laboratory of the Virginia-Maryland Regional College of Veterinary Medicine within 2 hrs of collection. Total leukocyte counts were carried out using the Advia 120 Automated Hematology Analyzer (Siemens Healthcare Diagnostics Inc). The proportion of each leukocyte type present in the blood samples was determined by standard manual slide differential count.

#### Lymphocyte Isolation

Peripheral blood mononuclear cells (PMBC) were isolated from blood collected on day -1, 8, and 21 PC according to the method of Witonsky et al. (2003). Blood was collected via jugular venipuncture into tubes containing lithium heparin and immediately placed on ice for transport. Blood samples were allowed to warm to room temperature and diluted 1:2 with sterile PBS. After mixing, 10 ml of diluted blood was layered over 5 ml of Lymphoprep® medium (Accurate Chemical and Scientific Corporation, Westbury, NY). Tubes were centrifuged for 30 minutes at 250 X g at room temperature with the centrifuge brake turned off. After centrifugation, the plasma was removed and discarded. The lymphocyte band was recovered and washed twice (250 X g for 10 minutes) with cold culture medium (RPMI 1640, 10% FBS, 1 % Penicillin/Streptomycin/Amphotericin B). Residual erythrocytes were lysed by resuspending the pellet in 2 ml of culture medium and adding 4 ml of sterile, deionized water. The mixture was mixed for 18 seconds by pipette and 10 ml of culture medium was added. The lymphocyte suspension was then subjected to a final wash (250 X g for 10 minutes) and resuspended in 1 ml of cold culture medium. Viable cells were counted by diluting the lymphocyte suspension 1:3 in trypan blue (Invitrogen, Carlsbad, CA). Twenty  $\mu\text{L}$  of the lymphocyte/trypan blue suspension was transferred to a disposable counting chamber for automated cell counting and cell viability determination in a Cellometer® Auto (Nexcelcom Bioscience LLC, Lawrence, MA) programmed to count round cells 6-12  $\mu\text{m}$  in diameter.

#### Flow Cytometry

Flow cytometric analysis was carried out on lymphocytes isolated from whole blood collected on day -1, 8, and 21 PC according to the technique of Gilbertie et al. (2010). Lymphocytes were diluted to  $2 \times 10^7$  viable cells/ml in PBS+1% bovine serum albumin (BSA) and 100  $\mu\text{l}$  of cell suspension was added to each well of a 96 well V- bottom plate (Fisher

Scientific Company, Middleton VA) in addition to 50  $\mu$ l of primary antibody at the following dilutions: CD4 (VMRD, Pullman, WA IL-A11, IgG<sub>2a</sub> isotype) 1:400, CD8 (VMRD BAQ 111A, IgM isotype) 1:400, WC1-N3  $\gamma\delta$  (VMRD CACTB32A, IgG<sub>1</sub> isotype) 1:400, and CD21 (VMRD BAQ15A, IgM isotype) 1:200. All the primary antibodies were added to separate wells except CD4 and CD8, which were added to the same well. The cells and primary antibodies were incubated for 20 minutes in the dark on ice. After centrifugation at 300 X g for 5 minutes at 4°C, the primary antibody was decanted and the plates were washed (300 x g for 5 minutes at 4°C) two times with 200  $\mu$ l PBS+1%BSA. Secondary antibody (50  $\mu$ l) was added at the following dilutions: anti-IgG<sub>2a</sub> FITC (Invitrogen, Carlsbad, CA M32201) 1:400, anti-IgM APC (SouthernBiotech, Birmingham, AL 1140-11) 1:15, and anti-IgG<sub>1</sub> R-PE (SouthernBiotech, Birmingham, AL 1144-09) 1:20. 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 100  $\mu$ L cold PBS+1%BSA and 100  $\mu$ L 4% paraformaldehyde (Fisher Scientific Company, Middleton, VA) was added to each well. After incubation in the dark for 15 minutes on ice, plates were centrifuged at 300 x g for 5 minutes at 4°C. Supernatant was discarded and cells were resuspended in cold 100 $\mu$ L 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 events were collected and analysis was done by FlowJo<sup>®</sup> software (Tree Star, Inc., Ashland, OR). Upon analysis, brightly and dimly stained populations of CD8<sup>+</sup> and CD21<sup>+</sup> were observed. Additionally, a population of CD4 and CD8 double positive cells (CD4<sup>+</sup>/CD8<sup>+</sup>) was consistently observed in all samples (Appendix 6, pg 164). Gates were set to quantify each of these lymphocyte phenotypes. Percentages of each lymphocyte phenotype were reported. The total number of each lymphocyte

phenotype per  $\mu\text{l}$  of whole blood was calculated by multiplying each subtype percentage by the total number of lymphocytes determined by CBC and slide differential.

### Lymphocyte Proliferation

Lymphoproliferative responses were assessed on lymphocytes isolated from whole blood collected on day -1, 8, and 21 PC as described by Mullarky et al. (2009). Briefly,  $2 \times 10^5$  cells/well were placed in a 96 well round bottom plate (Fisher Scientific Company, Middleton VA) and measured in triplicate after stimulation with 1  $\mu\text{g}/\text{ml}$  of pokeweed mitogen (PWM), 5  $\mu\text{g}/\text{ml}$  of concanavalin A (Con A), or 10  $\mu\text{g}/\text{ml}$  of *Eimeria bovis* antigen (EbAg). Background proliferation was determined with PBMC cultured in complete medium. After incubating for 90 h at 37°C and 5% CO<sub>2</sub>, 100  $\mu\text{l}$  of cell supernatant was collected from each well, pooled within stimulation type, and stored at -20°C for subsequent cytokine analysis. After supernatant removal, 20  $\mu\text{l}$  cell proliferation reagent (CellTiter 96-Aqueous®, Promega, Madison, WI) was added to each well and the plates were incubated (37°C and 5% CO<sub>2</sub>) for an additional 4 hours and finally read at 490 nm with a Bio-Tek microtiter well plate reader (SpectraMax M5 Multi-Mode Microplate Reader, Molecular Devices, Downington, PA).

### Supernatant IFN- $\gamma$ and IL-4 ELISA

The procedure was modified from Souza et al. (2007) using the IFN- $\gamma$  and IL-4 Bovine Screening Set (Thermo Fisher Scientific Inc., Waltham, MA) and following manufacturer's instructions. In brief, 96 well, high efficiency binding plates (Fisher Scientific Company, Middleton VA) were coated with 100  $\mu\text{l}/\text{well}$  coating antibody diluted 1:100 in carbonate/bicarbonate buffer, sealed with a plate sealer and incubated overnight at room temperature. The coating antibody was then decanted and 300  $\mu\text{l}/\text{well}$  of blocking buffer (4% BSA, 5% sucrose in PBS; passed through a 0.2  $\mu\text{m}$  filter) was added and plates were incubated

for 1 hr at room temperature. Blocking buffer was decanted and 100  $\mu$ L/well of standards or samples were added in duplicate and incubated for 1 hr (IFN-  $\gamma$ ) or 1.5 hrs (IL-4) at room temperature. In order for values to fall on the standard curve provided for each cytokine, the PWM and Con A stimulated samples were diluted 1:25 for IFN- $\gamma$  analysis and 1:3 for IL-4 analysis with reagent diluent (4% BSA in D-PBS, passed through a 0.2  $\mu$ m filter). The control and EbAg stimulated samples were analyzed undiluted at all time points. After incubation, plates were washed three times with wash solution (0.05% tween 20 in D-PBS). After the final wash, 100  $\mu$ l/well of a biotin labeled detection antibody diluted 1:100 in reagent diluent was added and plates were incubated for 1 hour at room temperature. Plates were again washed three times with wash solution and 100  $\mu$ l/well streptavidin-HRP diluted 1:400 in reagent diluent was added and the plates were incubated for 30 minutes at room temperature. After 3 additional washes, 100  $\mu$ l/well of TMB substrate was added and plates were incubated in the dark for 20 minutes at room temperature. The reaction was stopped by adding 100  $\mu$ l/well stop solution (2N H<sub>2</sub>SO<sub>4</sub>). The plate absorbance was read at 490 nm (SpectraMax M5 Multi-Mode Microplate Reader, Molecular Devices, Downington, PA). Sample concentrations were determined using the equation of a four parameter logarithmic standard curve.

#### Tissue collection and analysis

##### Ileocecal and Colic Lymph Nodes

Within 30 minutes of euthanasia, each calf's ileocecal junction was identified and manipulated to allow for visualization of the ileocecal fold. The ileocecal lymph node was removed from each calf, freed of mesenteric fat, weighed and then placed in cold culture medium and stored on ice until further analysis. Similarly, the most proximal colic lymph node

was removed from each calf, freed of mesenteric fat, and stored on ice in culture medium until further analysis.

Lymphocyte isolation was carried out on the collected lymph nodes using sterile technique according to the method of Suhwold et al. (2010). Each lymph node was sectioned into 1 cm cubes using a scalpel blade and gently teased through 180  $\mu\text{m}^2$  nylon mesh (Spectrum Labs, Rancho Dominguez, CA) into ice cold culture medium. The resultant cell suspension was transferred to a 15 ml centrifuge tube and pelleted by centrifugation at 300 x g for 5 minutes at 4°C. The cell pellets were washed three times with PBS plus 1% BSA and finally resuspended in 1 ml PBS plus 1% BSA. Viable cells (trypan blue exclusion test) were counted with a hemocytometer. Cells were diluted to  $2 \times 10^7$  viable cells/ml in PBS+1% BSA and cell surface staining and flow cytometric analysis for CD4<sup>+</sup>, CD8<sup>+</sup>, WC1<sup>+</sup>, and CD21<sup>+</sup> lymphocytes was carried out as described for lymphocytes isolated from whole blood. For each sample, 10,000 events were collected and analysis was done by FlowJo<sup>®</sup> software (Tree Star, Inc., Ashland, OR). Percentages of each lymphocyte subtype were reported.

#### Ileum and Cecum

After removal of lymph nodes, the ileum was transected 1 cm proximal to the ileocecal junction and lifted from the abdominal cavity by freeing the mesenteric attachments. A 1 cm long portion of ileum was removed from each calf, freed of visible mesenteric fat and opened longitudinally along the mesenteric border. Half of each ileal section was cryopreserved by being placed into a 1 cm<sup>3</sup> tissue mold containing enough optimal cutting temperature (OCT) medium (Tissue-Tek<sup>®</sup>) to completely cover the tissue section. The molds were placed at -80°C for 24 hours to allow for solidification of the OCT medium. Upon solidification, the OCT embedded tissue blocks were removed from the molds and stored at -80°C until further



immunohistochemical analysis. The other half of each ileal section was stored in 10% neutral buffered formalin until dehydrated and embedded in paraffin according to standard procedures.

The entire length of each cecum was freed from its mesenteric attachment, removed from the abdominal cavity and placed into a steel pan. The cecum was opened longitudinally along the mesenteric border and cecal content was removed from the mucosal surface by gentle rinsing with warm tap water. The mucosal surface from each calf was inspected by a single individual and assigned a gross lesion score (0-4) based on the scoring system described by Johnson and Reid (1970). After the gross lesion score was assigned, a 2 cm<sup>2</sup> section of cecum was removed from the cecal apex. Half of each cecal section was cryopreserved and the other half stored in 10% neutral buffered formalin

Immunohistochemical staining for the detection of CD4<sup>+</sup>, CD8<sup>+</sup>, WC1<sup>+</sup>, and CD21<sup>+</sup> lymphocytes was carried out on the cryopreserved sections of ileum and cecum as described by Suhwold et al. (2010). A 4 µm thick section of each tissue was placed onto a glass slide and endogenous peroxidase inactivated by incubation with 1 ml of 0.5% H<sub>2</sub>O<sub>2</sub> for 30 minutes at room temperature. The H<sub>2</sub>O<sub>2</sub> was discarded and the samples washed 5 times for 5 minutes each in tris-buffered saline (TBS) at room temperature. The samples were then probed with 500 µl of monoclonal antibody (anti-bovine CD4, anti-bovine CD8, anti-bovine WC1 and anti-bovine CD21, AbD Serotec, Raleigh, NC) diluted 1:100 in PBS for 1 hour at 37°C. After incubation, the sections were washed three times in PBS and incubated in 500 µl of secondary antibody (rabbit anti-mouse IgG:horseradish peroxidase, AbD Serotec, Raleigh NC) diluted 1:50 in PBS for 1 hour at 37°C. The sections were then washed three additional times with tris-buffered saline (TBS). After the last wash, the sections were covered with 500 µl of substrate (0.048 g DAB dissolved in 800 µl 3% H<sub>2</sub>O<sub>2</sub> and added to 80 ml of imidazole buffer) for 5 minutes. The

sections were again washed 3 times with TBS. The samples were then counterstained for 15 seconds with hematoxylin. The counter stain was removed by washing with tap water for 5 minutes. The sections were then mounted in Clear Mount<sup>®</sup> mounting medium (Fisher Scientific Company, Middleton VA) and stored until microscopic examination.

Immunostained intestinal sections were examined microscopically at 200X magnification. To quantify immunostained lymphocyte subtypes digital photomicrographs of 5 microscopic fields per section were obtained using NIS-Elements: Basic Research software (version 3.10, Nikon Instruments Inc., Melville, N.Y.). To limit bias, photomicrographs of image fields were obtained only when intestinal glands filled the entire microscopic field. Each captured image encompassed a 614 X 491  $\mu\text{m}$  area. Once all images were captured, the software was used to count the total number of immunostained lymphocytes per image. The counts for all 5 images were averaged and results reported as mean number of immunostained lymphocyte subtype per vision field.

Standard H&E stained sections of intestine were prepared by the Virginia-Maryland Regional College of Veterinary Medicine histopathology lab and examined by an anatomic pathologist. Each section was given a numerical histopathologic lesion score (0-4), based on the presence and extent of inflammation, necrotic debris within the epithelial glands, epithelial glandular fibrosis, and coccidial stages.

#### Statistics

Fecal oocyst counts (FOC) were normally distributed and no transformation was required. Fecal oocyst count, fecal dry matter percentage, white blood cell count (WBC), neutrophil:lymphocyte (N:L) ratio, lymphocyte subtype isolated from blood, IFN- $\gamma$  and IL-4 concentrations were analyzed by a mixed model analysis of variance using the GLIMMIX

procedure of SAS version 9.2 (Cary, NC, USA). The model statement contained the fixed effects of treatment, time, and the time\*treatment interaction. Data were analyzed using calf(treatment) as the error term. In this model animal was the experimental unit. Average daily gain (ADG), lymphocyte subtype isolated from lymph nodes, immunohistochemistry data and histopathologic lesion score were analyzed by a mixed model analysis of variance using the MIXED procedure of SAS version 9.2 (Cary, NC, USA). The model statement contained treatment as a fixed effect. Where significant effects were detected they were further examined using the SLICE option of SAS version 9.2 (Cary, NC, USA). Lymphocyte proliferation data and cecal lesion scores were not normally distributed and normality could not be restored by data transformation. These data were analyzed using a Friedman Chi-square test. Where significant treatment effects or time effects ( $P < 0.05$ ) were detected direct comparisons were made using the Friedman Chi-square test.

## Results

Mean FOC was higher ( $P < 0.05$ ) on days 21-26 PC in the SC group compared to the other treatment groups (Figure 7.1). The average percentage of *E. bovis* identified on day -60 for each treatment was 85%, 82%, 91%, and 81% for the NSNC, NSC, SNC and SC respectively.

*Eimeria bovis* oocysts continued range between 65-85% of the total oocysts identified in the SC group on days 23-25, while the percentage of *E. bovis* in the other three groups ranged from 5-40% of the total oocysts identified during that time period. *Eimeria auburnensis* constituted the majority of oocysts identified in these samples. The SC group also had lower ( $P < 0.05$ ) fecal dry matter as compared to the other two treatment groups on days 23-26 (Figure 7.2).

Dexamethasone administration, regardless of *E. bovis* challenge, increased WBC and N:L ratio and decreased total number of all lymphocyte subtypes on day 8 PC (Table 7.1). The

percentage of CD8<sup>+</sup> and WC1<sup>+</sup> positive lymphocytes was also decreased (P<0.05) on day 8 PC in the SNC and SC groups. The percentage of CD4<sup>+</sup> lymphocytes was increased on day 8 PC in only the SC group. Additionally, the percentage and total number of CD21<sup>+</sup> lymphocytes were decreased on day 21 in the dexamethasone injected calves. Treatment and time effects for the dimly stained CD8<sup>+</sup> and CD21<sup>+</sup> subtypes were identical to the brightly stained populations, thus only data for the brightly stained populations are presented (Table 7.1). The percentage and total number of CD4<sup>+</sup>/CD8<sup>+</sup> was decreased in all treatment groups on day 8 PC as compared to day -1 and 21 PC. Lymphocyte proliferation in response to mitogen stimulation is shown in Figure 7.3. Lymphocytes isolated from the NSC group exhibited greater (P<0.05) lymphoproliferative response to EbAg, PWM and Con A on day 8 PC as compared to the other treatment groups. Lymphocytes isolated from the SNC and SC exhibited decreased (P<0.05) lymphoproliferative response to stimulation with Con A on day 8 PC. The concentration of IFN- $\gamma$  in supernatants from non-stimulated (Control) lymphocytes was highest (P<0.05) on day -1 PC for the SC calves (Table 7.2a). On day 8 PC the concentration of IFN- $\gamma$  in supernatants from PWM stimulated lymphocytes was highest (P<0.05) in NSC calves and lowest (P<0.05) in SC calves. An increase (P<0.05) in IL-4 production was detected in response to Con A stimulation on day 8 PC in the SNC and SC groups (Table 7.2b).

Gross cecal lesion scores were higher (P<0.05) in the SC calves euthanized on day 24 PC compared to NSNC calves (Table 7.3). Similarly, histologic cecal lesion score was higher (P<0.05) in the SC calves compared to the NSNC calves. Ileocecal lymph node mass did not differ between treatments (P=0.58). Percentages of CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes isolated from the ileocecal lymph node were higher (P<0.05) in SC calves as compared to NSNC calves while the percentage of WC1<sup>+</sup> lymphocytes were lower (P<0.05) in the SC calves (Table 7.4a). The

percentage of CD4<sup>+</sup> lymphocytes in the colic lymph nodes from SC calves was higher as compared to the NSNC calves and the percentage of WC1<sup>+</sup> lymphocytes were lower in colic lymph nodes from the SC calves as compared to the NSNC calves. No differences were observed in the number of lymphocyte subtypes detected by immunohistochemical staining in ileal or cecal sections (Table 7.4b)

Average daily gains from day -1 through 23 PC are shown in figure 7.4. The NSNC calves gained the most weight (P<0.05), NSC and SNC calves were intermediate and SC calves gained the least (P<0.05).

### **Discussion**

The source of infectious oocysts in previous reports of clinical coccidiosis in recently stressed calves has been questioned (Fitzgerald, 1962; Niilo, 1970a; Niilo, 1970b; Parker et al., 1984; Parker et al., 1986). In these studies, environmental temperatures at the time of the outbreaks were unsuitable for *Eimeria* spp. oocyst sporulation (Marquardt et al. 1960) and an attempt to obtain oocysts from the calves' environment was unsuccessful (Fitzgerald, 1962). The authors speculated that latent *Eimeria* stages within the host tissues may have reactivated to produce clinical signs after periods of stress associated immune suppression (Lindsay et al., 1990). In the present study both challenge with large numbers of *E. bovis* oocysts and immune suppression were required to increase subsequent FOC in weanling beef calves. Increases in FOC were not observed in the NSC and SNC calves, which may indicate that *E. bovis* challenge or stress in the form of immunosuppressive doses of dexamethasone alone are not sufficient to increase *E. bovis* oocyst shedding.

Physiologic changes were also documented as a result of *E. bovis* challenge and dexamethasone administration in this study. In the SC calves a decrease in fecal dry matter corresponded to the period of peak *E. bovis* oocyst shedding. This finding is likely due to the

negative impacts on intestinal physiology known to be imparted by *E. bovis* replication within the large intestine (Dauguschies et al., 1986). A similar finding was reported by Niilo (1970c) who observed an exacerbation of clinical signs of *E. zuernii* infection when dexamethasone was administered during patent *E. zuernii* infections. The presence of gross and histopathologic lesions consistent with *E. bovis* infection in the SC calves and absence of lesions in the NSNC calves also indicate that intestinal integrity was altered in these calves. Damage to the colon in the SC calves likely contributed to their inability to gain weight at the same rate as calves in the other treatments. Although ADG was significantly lower in the SC calves than all other treatments, *E. bovis* challenge and dexamethasone injection alone also had an effect on ADG. Hammon et al. (2003) reported that dexamethasone administration decreased ADG in veal calves. These authors speculated that this effect was a result of the catabolic state of the physiologic response induced by this synthetic glucocorticoid. Depression of weight gain has been reported after primary *E. bovis* infection (Hammond, 1964) in calves, but no studies have investigated the impact of *E. bovis* challenge infection on weight gain in weaned beef calves. We conclude that *E. bovis* challenge may have an impact on ADG, even in the absence of increased oocyst shedding.

In many species, including cattle, immunity to *Eimeria* infection is believed to be cell mediated (Rose et al., 1987; Lillehoj, 1987; Rose et al. 1988; Suhwold et al., 2010). Studies by Taubert et al (2008) and Suhwold et al. (2010) reported increased lymphoproliferative responses and IFN- $\gamma$  production by lymphocytes 8 days after primary *E. bovis* infection. The authors hypothesized that the cell mediated immune response to primary *E. bovis* infection is directed against first generation merozoites replicating in the small intestine 8 days after infection. The immune response to *E. bovis* challenge has not been similarly investigated. Dexamethasone was

administered in this study on day 7 PC to induce immune suppression during replication of first generation merozoites in the small intestine of challenged calves. The administration of dexamethasone induced a stress leukogram characterized by leukocytosis with a mature neutrophilia and lymphocytopenia as described in other studies (Nonnecke et al., 1997; Menge and Dean-Nystrom, 2008). The total number of circulating CD4<sup>+</sup>, CD8<sup>+</sup>, WC1<sup>+</sup> and CD21<sup>+</sup> lymphocytes was decreased in dexamethasone injected calves regardless of *E. bovis* challenge on day 8 PC. Decreases in these subtypes were not observed in calves that were infected but not treated with dexamethasone on day 8 PC. This indicates that fluctuations in lymphocyte subtypes were related to dexamethasone injection and not *E. bovis* challenge, which is consistent with previous findings (Menge and Nystrom, 2008; Taubert et al., 2008; Suhwold et al., 2010). The continued changes in CD21<sup>+</sup> lymphocyte populations in only the dexamethasone treated calves on day 21 may indicate that the effects of glucocorticoid injection persists for longer in B-cells as compared to T-cells. Circulating CD4<sup>+</sup>/CD8<sup>+</sup> were detected in all samples throughout this study. Treatment had no effect on this lymphocyte population, but this cell type was decreased in all treatments on day 8 PC. Given that the magnitude of decrease was similar in the NSNC calves it is likely that this effect is spurious and not due to *E. bovis* challenge or dexamethasone injection. As stated in chapter 6, the function of this cell population in cattle requires further investigation.

The increase in the percentage and lymphoproliferative responses to mitogen stimulation were decreased dexamethasone injected calves on day 8 PC. In contrast, the lymphoproliferative responses to stimulation with EbAg, PWM and Con A were increased in NSC on day 8 PC. Similarly, IFN- $\gamma$  production was increased in NSC and decreased in SC calves in lymphocyte supernatants stimulated with PWM on day 8 PC. Furthermore, dexamethasone injection

increased IL-4 production in response to stimulation with Con A regardless of *E. bovis* challenge on day 8 PC. Thus it appears that the cell mediated immune response caused by *E. bovis* challenge was suppressed in all dexamethasone treated calves on day 8. The diminished cell mediated immunity in response to *E. bovis* challenge in the SC groups likely explains the increase in FOC observed in this group and may similarly explain stress related outbreaks of coccidiosis observed in the field.

Comparison of lymphocyte subtypes in regional lymph nodes and intestinal sections were made between NSNC and SC calves euthanized at day 24 PC. The proportion of CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes were increased and proportion of WC1<sup>+</sup> lymphocytes were decreased in ileocecal lymph nodes from SC calves. Additionally, the proportion of CD4<sup>+</sup> lymphocytes were increased and proportion of WC1<sup>+</sup> lymphocytes decreased in colic lymph nodes from SC calves. No treatment difference was detected in the number of lymphocyte subtypes infiltrating the ileal or cecal mucosa at day 24 PC. An EbAg specific proliferative response in lymphocytes isolated from ileocecal lymph nodes in primary *E. bovis* infected calve has been reported (Taubert et al., 2008; Suhwold et al., 2010). Additionally, CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes were increased in the cecal mucosa 40 days after primary *E. bovis* infection. These changes were not, however, observed after challenge infection (Suhwold et al., 2010). Unfortunately, due to resource limitations comparisons between NSC and SC calves could not be made in this study so it is impossible to say for sure whether the changes in lymphocyte subtypes were due to dexamethasone injection or *E. bovis* challenge.

The unchanged numbers of lymphocyte subtypes in the colonic mucosa of calves in response to *E. bovis* challenge has led some authors to speculate that the immune response to *E. bovis* challenge does not depend on cell mediated immunity (Taubert et al., 2008). These authors



speculate that *E. bovis* infection is abrogated early in its course by the innate immune response. Neutrophils, a component of innate immunity have been shown to actively phagocytize and trap *E. bovis* sporozoites in neutrophil extracellular trapping structures (Behrendt et al., 2008 and Behrendt et al., 2010). Although no investigation of neutrophil function in response to *E. bovis* challenge and dexamethasone injection was carried out in this study, neutrophil function is altered after dexamethasone injection (Burton et al., 2005). The involvement of stress-induced suppression of neutrophil function in stress associated outbreaks of coccidiosis requires further investigation.

Overall, we conclude that *E. bovis* challenge and immunosuppression is likely required to increase *E. bovis* oocyst shedding. This relationship of *E. bovis* exposure and immune suppression likely accounts for the stress associated outbreaks of coccidiosis observed in beef cattle in the United States. Many recently weaned calves are marketed at local livestock markets in the eastern United States. These calves are then loaded onto transport trailers for travel to distant feedlots. Both of these practices have the potential to expose calves to high numbers of *E. bovis* oocysts, although no direct investigation of the presence of *E. bovis* oocysts at these locations has been made. Transport and commingling of calves after weaning is known to be stressful (Crookshank et al., 1979). Thus, the temporal relationship of *E. bovis* exposure and subsequent stress-induced suppression of the immune system occurs frequently in the U.S. cattle industry. Based on these finding, management recommendations that involve decreasing the stress of weaning and transport in calves and separating these events from exposure to high levels of *E. bovis* oocysts in the environment should be recommended.

Table 7.1: Leukocyte counts and lymphocyte phenotypes of calves (n=4/group) on days -1, 8 and 21. Not stressed challenged (NSC) and stressed challenged (SC) calves were given a single dose of  $1.5 \times 10^6$  sporulated *E. bovis* oocysts on day 0. Stressed not challenged (SNC) and SC calves were given Dexamethasone on day 7. Not stressed not challenged (NSNC) calves served as untreated controls. Values are least squares means and standard error (SE).

Cell Type	Day -1				Day 8				Day 21				SE
	NSNC	NSC	SNC	SC	NSNC	NSC	SNC	SC	NSNC	NSC	SNC	SC	
WBC	11,730	10,675	10,130 <sup>a</sup>	10,423 <sup>a</sup>	10,235	9,548	17,448 <sup>b</sup>	17,815 <sup>b</sup>	9,900	10,175	9,625 <sup>a</sup>	10,143 <sup>a</sup>	1,125
N:L Ratio	0.3	0.3	0.3 <sup>a</sup>	0.3 <sup>a</sup>	0.4 <sup>1</sup>	0.3 <sup>1</sup>	5.5 <sup>2,b</sup>	5.0 <sup>2,b</sup>	0.2	0.2	0.3 <sup>a</sup>	0.3 <sup>a</sup>	2.4
CD4 <sup>+</sup> %	10.3	11.6	10.4	11.6 <sup>a</sup>	10.0	11.4	12.0	15.5 <sup>b</sup>	12.6	12.4	12.6	13.0 <sup>b</sup>	1.1
CD4 <sup>+</sup> Total	831	816	808 <sup>a</sup>	864 <sup>a</sup>	710	756	332 <sup>b</sup>	461 <sup>b</sup>	953	836	799 <sup>a</sup>	831 <sup>a</sup>	99
CD8 <sup>+</sup> %	12.2	11.9	12.6 <sup>a</sup>	11.0 <sup>a</sup>	12.3	10.6	9.6 <sup>b</sup>	8.9 <sup>b</sup>	14.3	12.1	17.7 <sup>c</sup>	15.7 <sup>c</sup>	1.1
CD8 <sup>+</sup> Total	992	855	984 <sup>a</sup>	817 <sup>a</sup>	872	733	248 <sup>b</sup>	267 <sup>b</sup>	1,001	841	1,172 <sup>a</sup>	986 <sup>a</sup>	129
WC1 <sup>+</sup> %	6.6	6.9	7.4 <sup>a</sup>	6.0 <sup>a</sup>	5.3	5.0	1.8 <sup>b</sup>	2.0 <sup>b</sup>	6.8	5.7	5.2 <sup>a</sup>	4.6 <sup>a</sup>	1.2
WC1 <sup>+</sup> Total	529	487	590 <sup>a</sup>	435 <sup>a</sup>	381	336	47 <sup>b</sup>	58 <sup>b</sup>	492	392	320 <sup>a</sup>	282 <sup>a</sup>	102
CD21 <sup>+</sup> %	32.0	25.9	25.5	23.6	30.3	23.1	26.1	31.1	35.7 <sup>1</sup>	33.5 <sup>1</sup>	23.8 <sup>2</sup>	26.6 <sup>2</sup>	2.4
CD21 <sup>+</sup> % Total	2,533	1,892	1,978 <sup>a</sup>	1,754 <sup>a</sup>	2,154	1,581	683 <sup>b</sup>	963 <sup>b</sup>	2,682 <sup>1</sup>	2,299 <sup>1</sup>	1586 <sup>b</sup>	1676 <sup>b</sup>	242
CD4 <sup>+</sup> /CD8 <sup>+</sup> %	12.6 <sup>a</sup>	14.0 <sup>a</sup>	14.6 <sup>a</sup>	12.9 <sup>a</sup>	9.1 <sup>b</sup>	8.4 <sup>b</sup>	9.2 <sup>b</sup>	7.4 <sup>b</sup>	13.1 <sup>a</sup>	11.8 <sup>a</sup>	14.8 <sup>a</sup>	16.5 <sup>a</sup>	1.0
CD4 <sup>+</sup> /CD8 <sup>+</sup> Total	1,018 <sup>a</sup>	988 <sup>a</sup>	1,138 <sup>a</sup>	932 <sup>a</sup>	641 <sup>b</sup>	557 <sup>b</sup>	191 <sup>b</sup>	278 <sup>b</sup>	989 <sup>a</sup>	793 <sup>a</sup>	966 <sup>a</sup>	1,084 <sup>a</sup>	103

Treatment effects (P<0.05) are indicated by different numerical superscripts within time points.

Time effects (P<0.05) are indicated by different alphabetical superscripts for each treatment within row.

Table 7.2: IFN- $\gamma$  (A) and IL-4 (B) concentration (pg/ml) in lymphocyte supernatants collected from calves (n=4/group) on days -1, 8 and 21. Not stressed challenged (NSC) and stressed challenged (SC) calves were given a single dose of  $1.5 \times 10^6$  sporulated *E. bovis* oocysts on day 0. Stressed not challenged (SNC) and SC calves were given Dexamethasone on day 7. Not stressed not challenged (NSNC) calves served as untreated controls. Values are least squares means and standard error (SE).

### IFN- $\gamma$

Stimulant	Day -1				Day 8				Day 21				SE
	NSNC	NSC	SNC	SC	NSNC	NSC	SNC	SC	NSNC	NSC	SNC	SC	
Control	62	42	31	133	87	63	39	55	32	22	57	29	23
EbAg	77	87	54	142	94	83	26	90	84	50	44	117	25
Con A	6,075	10,276	6,881	9,712	4,543	5,538	6,389	3,335	5,179	7,195	8,200	4,977	2,133
PWM	9,899	19,383	16,712	13,267 <sup>a</sup>	14,024 <sup>1,2</sup>	17,708 <sup>1</sup>	10,931 <sup>1,2</sup>	6,886 <sup>2,b</sup>	12,908	14,686	14,033	14,387 <sup>a</sup>	2,599

### IL-4

Stimulant	Day -1				Day 8				Day 21				SE
	NSNC	NSC	SNC	SC	NSNC	NSC	SNC	SC	NSNC	NSC	SNC	SC	
Control	11	7	6	9	7	10	6	6	6	7	7	9	3
EbAg	10	7	9	13	8	9	9	7	13	7	6	17	4
Con A	1,356	1,867	1,331 <sup>a</sup>	1435 <sup>a</sup>	974	1,599	2,052 <sup>b</sup>	2,422 <sup>b</sup>	1,188	1,230	581 <sup>a</sup>	1,150 <sup>a</sup>	509
PWM	383	332	372	347	349	333	255	175	297	291	202	243	87

Treatment effects ( $P < 0.05$ ) are indicated by different numerical superscripts within time points.

Time effects ( $P < 0.05$ ) are indicated by different alphabetical superscripts for each treatment within row.

Table 7.3: Histologic (ileum and cecum) and gross (cecum) lesion scores from calves (n=4/group) euthanized on sampling day 24. Stressed challenged (SC) calves were given a single dose of  $1.5 \times 10^6$  sporulated *E. bovis* oocysts on day 0 and were given Dexamethasone on day 7. Not stressed not challenged (NSNC) calves served as untreated controls. Values are least squares means and standard error (SE).

Treatment	Histologic		Gross
	Ileum	Cecum	Cecum
NSNC	0.50	0.25 <sup>a</sup>	0.0 <sup>a</sup>
SC	0.75	2.75 <sup>b</sup>	1.0 <sup>b</sup>
SE	0.4	0.5	0.1

Treatment effects ( $P < 0.05$ ) are indicated by different numerical superscripts within columns.

Table 7.4: Percentage of lymphocyte phenotypes identified by flow cytometry (A) in ileocecal and colic lymph nodes and number of lymphocyte phenotypes per 614 x 461  $\mu\text{m}$  area identified by immunohistochemical staining and microscopic evaluation (B) in ileal and cecal sections for calves euthanized on sampling day 24. Stressed challenged (SC) calves were given a single dose of  $1.5 \times 10^6$  sporulated *E. bovis* oocysts on day 0 and were given Dexamethasone on day 7. Not stressed not challenged (NSNC) calves served as untreated controls. Values are least squares means and standard error (SE).

### A

Treatment	Ileocecal				Colic			
	CD4 <sup>+</sup>	CD8 <sup>+</sup>	WC1 <sup>+</sup>	CD21 <sup>+</sup>	CD4 <sup>+</sup>	CD8 <sup>+</sup>	WC1 <sup>+</sup>	CD21 <sup>+</sup>
NSNC	15.0 <sup>a</sup>	16.0 <sup>a</sup>	0.9 <sup>a</sup>	21.0	12.0 <sup>a</sup>	16.0	0.8 <sup>a</sup>	25.0
SC	20.0 <sup>b</sup>	21.0 <sup>b</sup>	0.3 <sup>b</sup>	19.0	15.0 <sup>b</sup>	20.0	0.4 <sup>b</sup>	25.0
SE	1.3	1.7	0.0	2.4	1.2	2.0	0.0	3.4

### B

Treatment	Ileum				Cecum			
	CD4 <sup>+</sup>	CD8 <sup>+</sup>	WC1 <sup>+</sup>	CD21 <sup>+</sup>	CD4 <sup>+</sup>	CD8 <sup>+</sup>	WC1 <sup>+</sup>	CD21 <sup>+</sup>
NSNC	166.5	133.0	155.2	155.2	130.1	126.2	99.8	119.6
SC	144.8	149.8	162.1	148.0	113.7	61.9	102.7	70.7
SE	28.6	10.7	8.2	9.0	19.2	29.3	4.5	13.9

Treatment effects ( $P < 0.05$ ) are indicated by different alphabetical superscripts within columns.

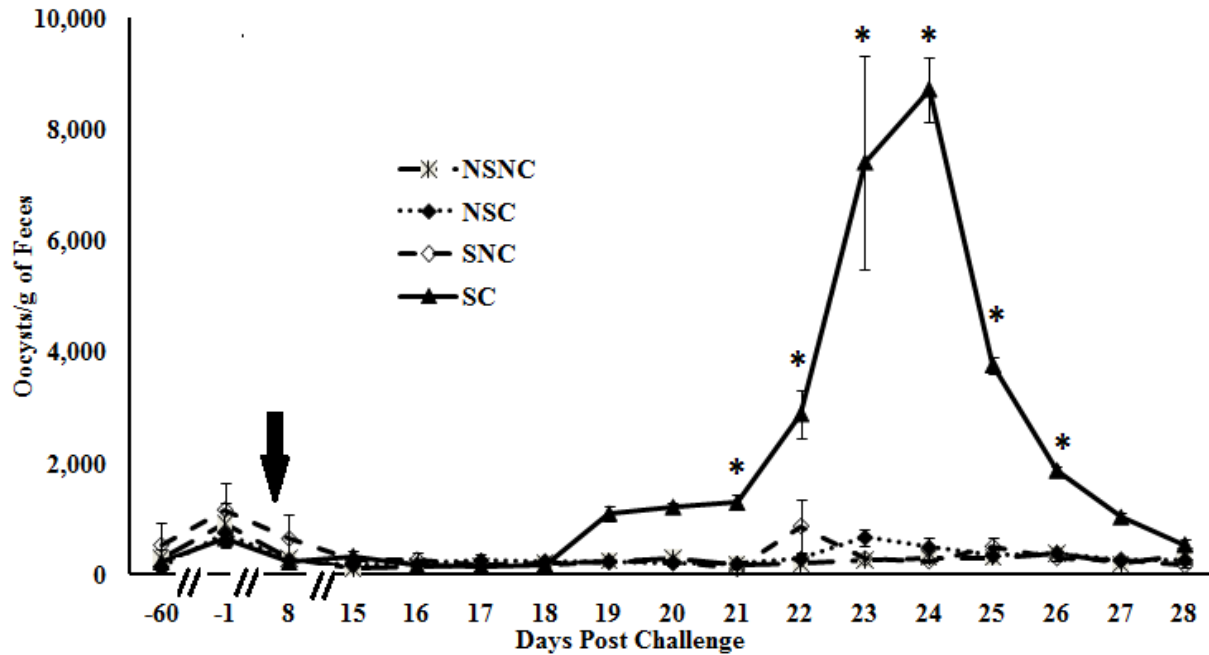


Figure 7.1: Fecal oocyst count/g of feces for calves (n=8/group) on day -60, -1, 8, and 15-28. Not stressed challenged (NSC) and stressed challenged (SC) calves were given a single dose of  $1.5 \times 10^6$  sporulated *E. bovis* oocysts on day 0. Stressed not challenged (SNC) and SC calves were given Dexamethasone on day 7 (arrow). Not stressed not challenged (NSNC) calves served as untreated controls. Symbol (\*) indicates time points where treatment effects ( $P < 0.05$ ) were detected. Values are least squares means and error bars represent standard errors.

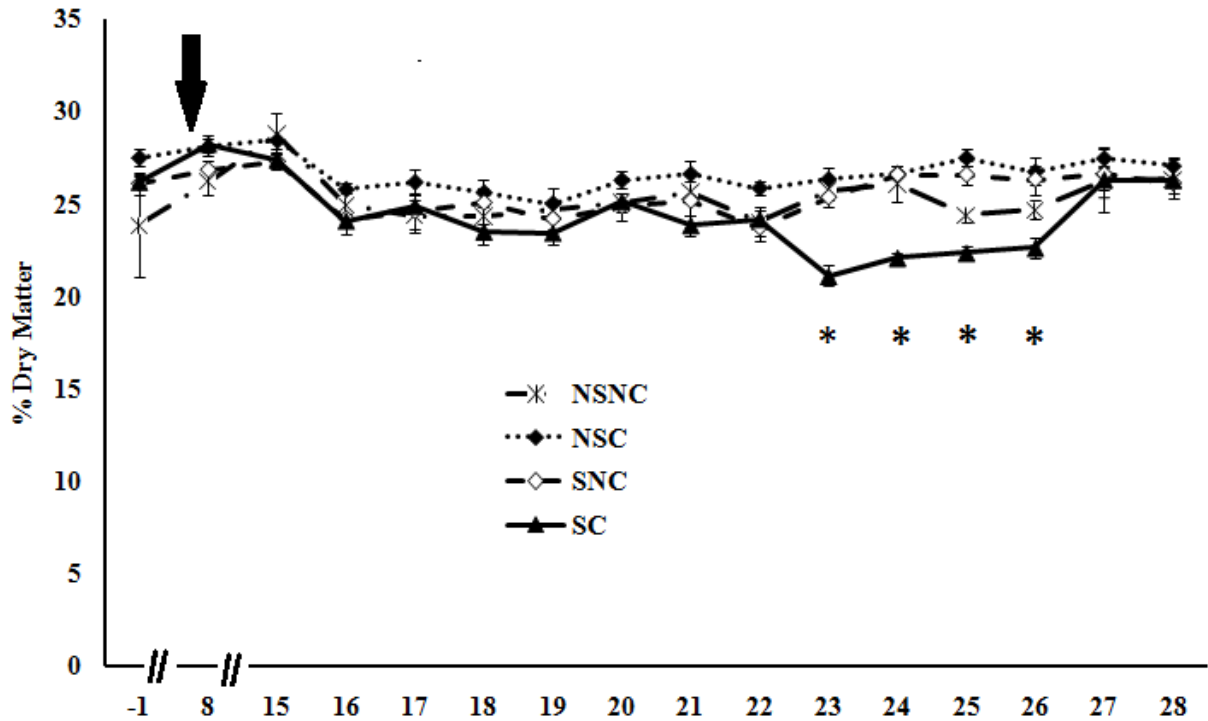


Figure 7.2: Percentage of fecal dry matter for calves (n=8/group) on day -1, 8, and 15-28. Not stressed challenged (NSC) and stressed challenged (SC) calves were given a single dose of  $1.5 \times 10^6$  sporulated *E. bovis* oocysts on day 0. Stressed not challenged (SNC) and SC calves were given Dexamethasone on day 7 (arrow). Not stressed not challenged (NSNC) calves served as untreated controls. Symbol (\*) indicates time points where treatment effects ( $P < 0.05$ ) were detected. Values are least squares means and error bars represent standard errors.



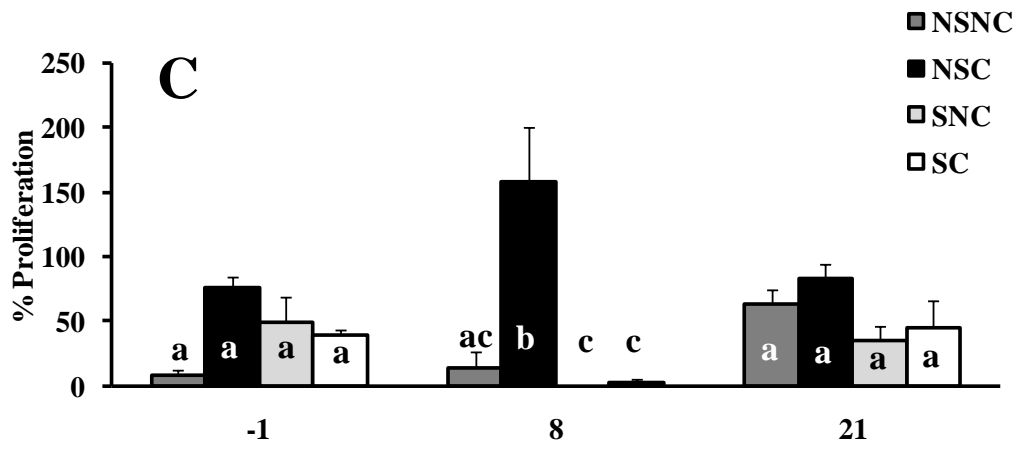
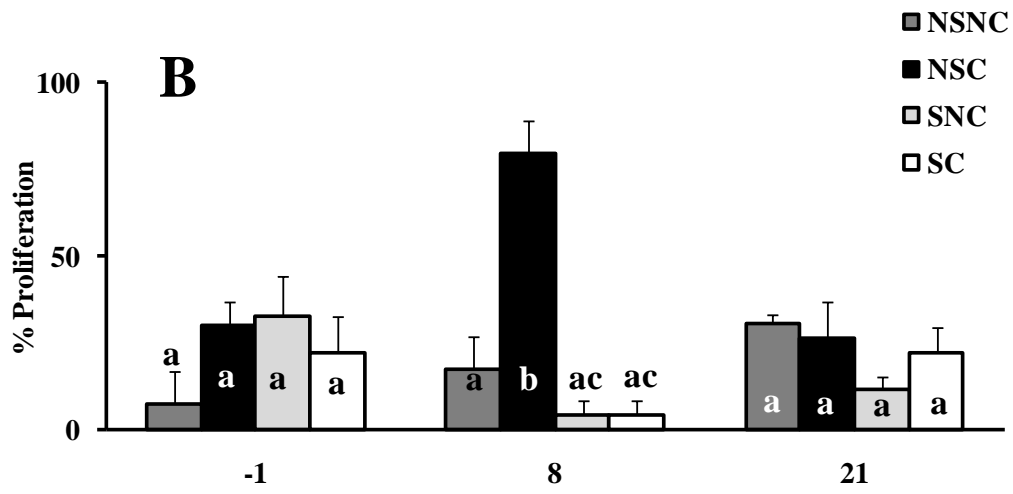
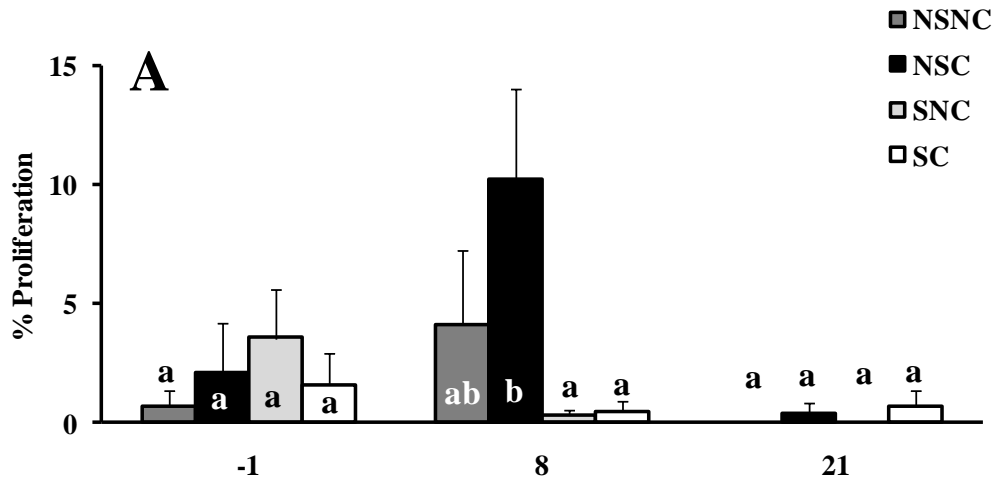


Figure 7.3: Lymphocyte proliferation after stimulation with *Eimeria bovis* antigen (A) pokeweed mitogen (B) or concanavalin A (C) for calves (n=4/group) on day -1, 8, and 21. Not stressed challenged (NSC) and stressed challenged (SC) calves were given a single dose of  $1.5 \times 10^6$  sporulated *E. bovis* oocysts on day 0. Stressed not challenged (SNC) and SC calves were given Dexamethasone on day 7 (arrow). Not stressed not challenged (NSNC) calves served as untreated controls. Different letters within each time point represent treatment effects ( $P < 0.05$ ). Different letters within each treatment at different time points represent time effects ( $P < 0.05$ ). Values are least squares means and error bars represent standard error.

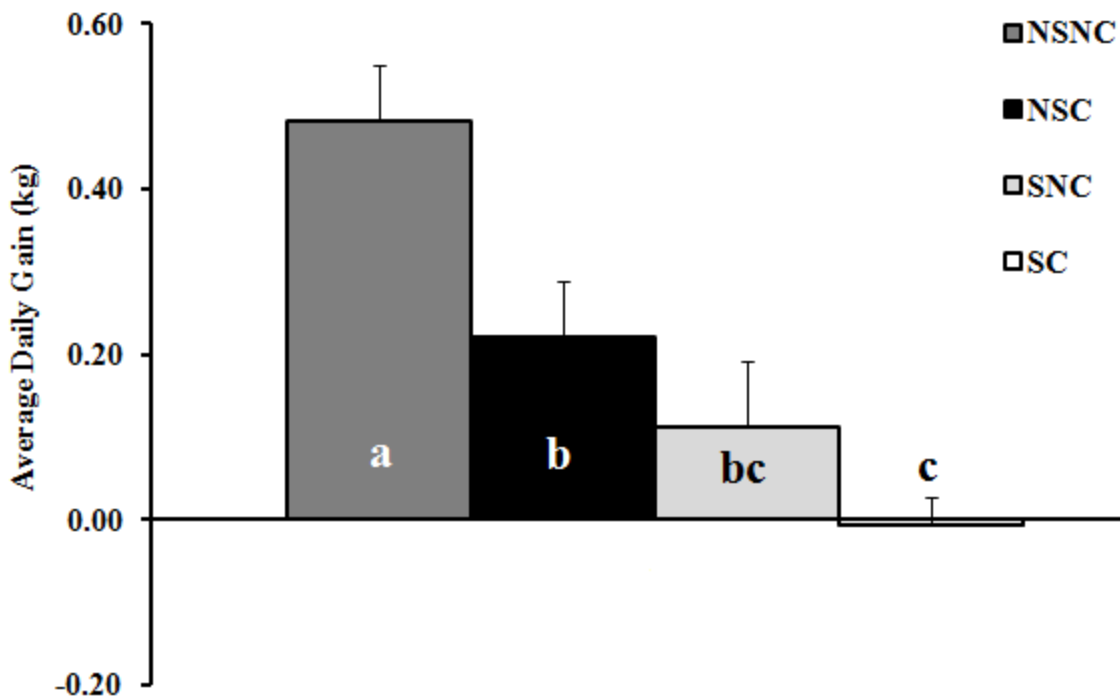


Figure 7.4: Average daily gain for calves (n=8/group) weighed on day -1 and 23. Not stressed challenged (NSC) and stressed challenged (SC) calves were given a single dose of  $1.5 \times 10^6$  sporulated *E. bovis* oocysts on day 0. Stressed not challenged (SNC) and SC calves were given Dexamethasone on day 7 (arrow). Not stressed not challenged (NSNC) calves served as untreated controls. Differing letters indicate treatment effects ( $P < 0.05$ ). Values are least squares means and error bars represent standard error.

## Chapter 8

### Summary and Conclusion

Pasture based cattle production systems are common in the central Appalachian region of the United States. In these systems the nutritive needs of the cow and calf are met through utilization of the abundant forages in the area. Often, weaned calves are backgrounded on pasture before being transported to feedlots west of the Mississippi river. Additionally, replacement heifers are commonly developed on pasture in these systems. Information on the infection dynamics of gastrointestinal parasites in grazing cattle in this region is not available. This dissertation provides the first year round description of the infection dynamics of eimerian parasites in grazing cattle in the central Appalachian region.

Extensive information is provided on *Eimeria* spp. infection dynamics in grazing cattle of various ages and stages of production. This dissertation provides the first year round description of *Eimeria* spp. infection dynamics in grazing cattle. Although coccidiosis associated with heavy *E. alabamensis* infection in pastured cattle is reported from Sweden, no cases of coccidiosis were observed in the cattle in these studies. With the understanding that our observations encompass a limited number of cattle, the author proposes that *Eimeria* spp. infection is unlikely to produce clinical disease in pastured cattle. Anecdotal reports of outbreaks of coccidiosis in recently weaned pastured cattle in the central Appalachian region are relatively common. Our observations, however, do not support these reports and suggest that when outbreaks of diarrhea are observed in recently weaned pastured calves other etiologies should be considered. Thorough diagnostic workup should be undertaken to rule out nutritional, viral, bacterial, and other parasitic etiologies as well as to assess the level of *Eimeria* spp. infection before a definitive diagnosis is made in such cases. Furthermore, we suggest that the indiscriminate use of

coccidiostatic agents aimed at reducing outbreaks of coccidiosis in pastured cattle should be reconsidered. It must be remembered, however that our studies make no attempt to correlate subclinical *Eimeria* spp. infection with weight gain.

Outbreaks of coccidiosis are still reported in recently weaned and transported calves shortly after their arrival to feedlots. Neither the source of the pathogenic *Eimeria* spp. oocysts nor the temporal relationship of exposure to these oocysts and stress is completely clear. By coupling administration of the synthetic glucocorticoid, dexamethasone, with *E. bovis* challenge a model of stress-induced coccidiosis was developed. This model was repeated using a larger number of calves with the same effect. In addition to increased oocyst counts, clinical affects including decreased weight gain, decreased fecal dry matter and increased gross and histopathologic lesions were induced by dexamethasone injection and *E. bovis* challenge. The utility of this model is not limited to investigation of the immune response to *E. bovis* challenge in stressed calves. The testing of chemotherapeutic agents as well as investigation of the effects of other intracellular pathogens on weaned calves could also be undertaken by employing this model.

The author concludes that stress and continued *E. bovis* challenge may be required in order to increase subsequent oocyst shedding in weaned beef calves. Stress or *E. bovis* challenge alone was not sufficient to produce the same rise in fecal oocyst count in the weaned beef calf in our studies. These findings are important because when outbreaks of coccidiosis occur in the stressed calf the source of *Eimeria* spp. oocysts should be investigated. Additionally, when clinical cases of coccidiosis are confirmed in pastured calves not only should the source of *Eimeria* spp. oocysts be sought, but recommendations should be made to reduce stress in these

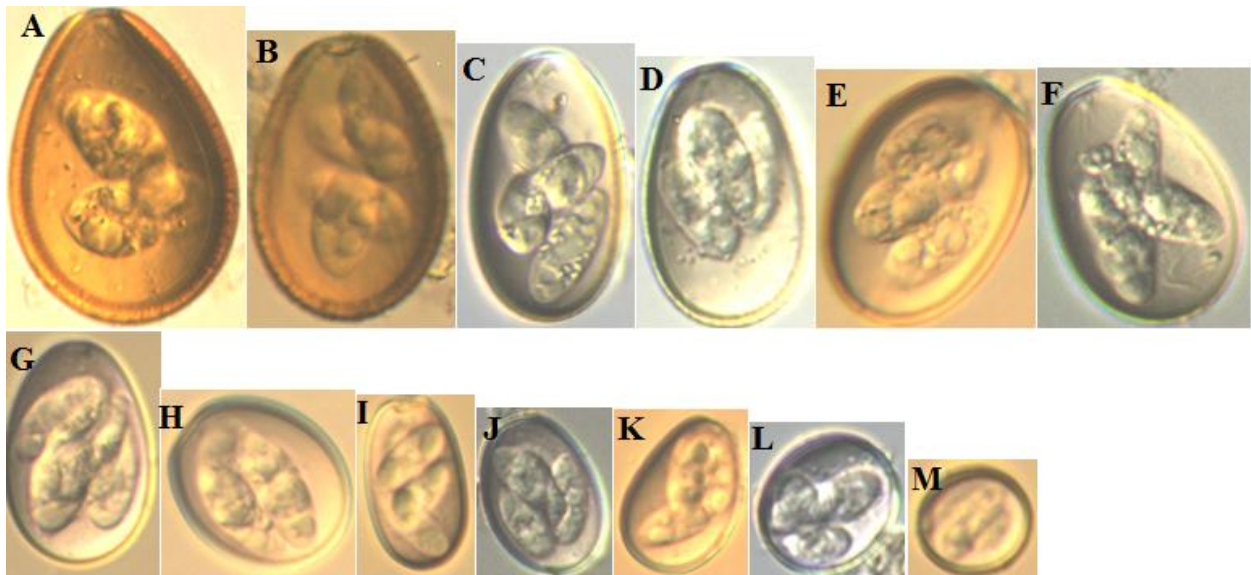
calves. If exposure to high numbers pathogenic *Eimeria* spp. oocysts can be limited, even in stressed calves it appears that outbreaks of clinical disease won't occur.

Although cell mediated immunity appears to be most important in primary and challenge infection in rodents and poultry and to primary infection in cattle, no research is available on the immune response to challenge infection in weaned beef cattle. Our studies are the first to investigate the immune response to *E. bovis* challenge in weaned beef calves. Using previously exposed, weaned beef calves is important because they are the subset of calves where outbreaks of coccidiosis are most prevalent. The increase in the Th1 cytokine IFN- $\gamma$  and increase in lymphocyte proliferative response to EbAg in only the *E. bovis* challenged calves suggests that immunity to challenge is cell mediated. Suppression of cell mediated immunity after dexamethasone injection was confirmed in weaned beef calves in our studies. Furthermore, the changes induced by dexamethasone administration approximate those seen after weaning in beef calves. Depression of cell mediated immunity during times of stress may explain the increase in susceptibility to coccidiosis observed in weaned beef calves after transport to feedlots in the United States.

In conclusion, gastrointestinal parasitism is common in cattle maintained on pasture based systems in the central Appalachian region. Although the author reports extensive information on the dynamics of strongylid and *Eimeria* spp. shedding in pastured cattle, data was only collected across a limited number of years. Climatic conditions have large impacts on the environmental stages of these organisms and further work is warranted to investigate the consistency of the present findings in years with different climatic conditions. Parasitism of represents a complicated dynamic of host, parasite and environmental interaction. Although our model of stress-induced coccidiosis appears to be repeatable, further research is required to

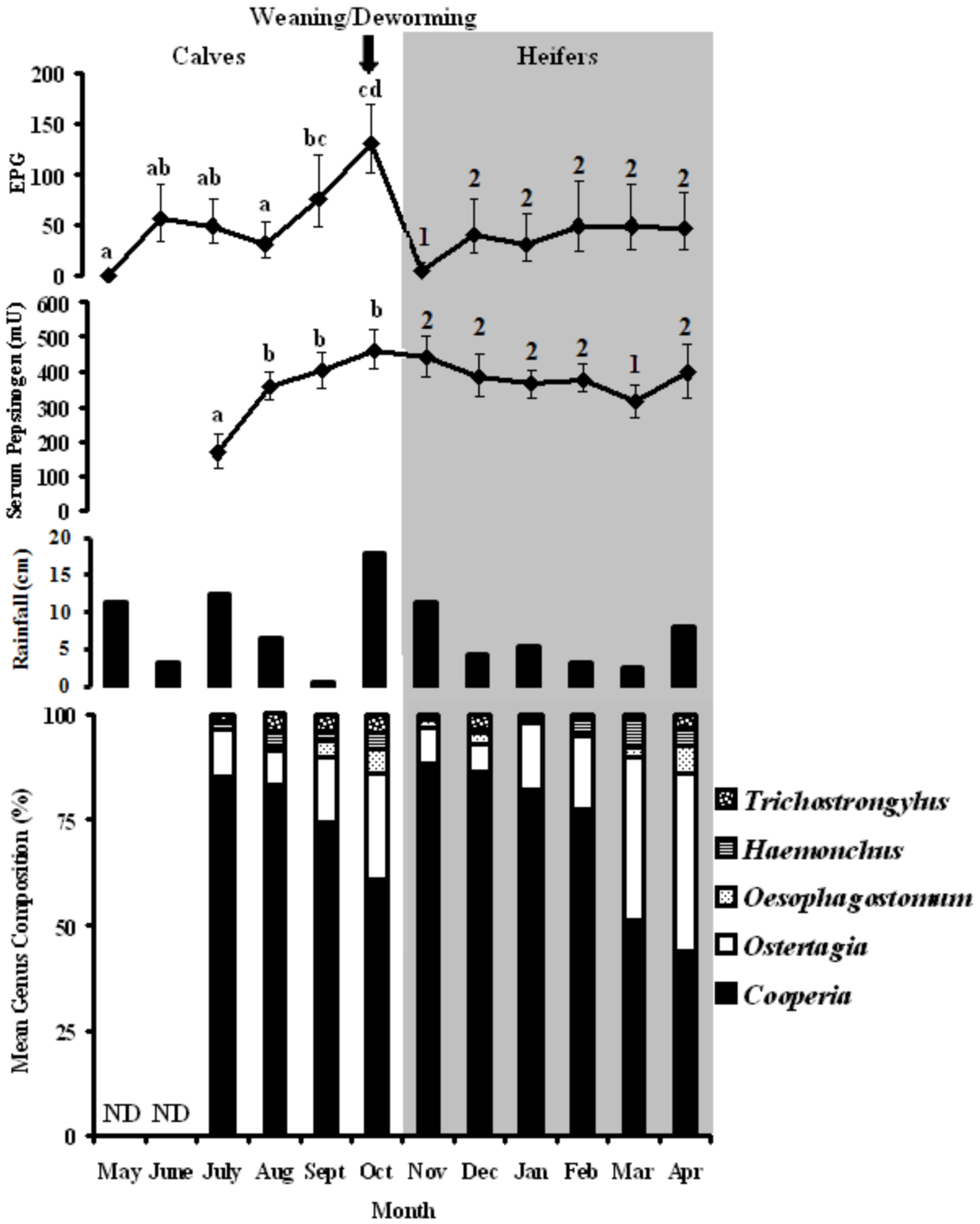
investigate its repeatability of this model using *E. bovis* oocysts isolated from different geographical regions and challenging different types of cattle. Nonetheless, this model provides a means to study the immune response to *E. bovis* challenge in weaned beef calves and subsequent studies are required to more clearly elucidate the nature of this response.

Appendix 1: *Eimeria* species oocysts isolated from feces of grazing calves in Virginia. Oocysts were sporulated in potassium dichromate for 48 hours and identified to species based on morphological characteristics described in Levine and Ivens (1986). *E. bukidonensis* (A), *E. wyomingensis* (B), *E. auburnensis* (C), *E. pelita* (D), *E. brasiliensis* (E), *E. canadensis* (F), *E. bovis* (G), *E. illinoisensis* (H), *E. cylindrica* (I), *E. ellipsoidalis* (J), *E. alabamensis* (K), *E. zuernii* (L) and *E. subspherica* (M).





Appendix 2: Mean fecal egg count (EPG), serum pepsinogen level (mU), and 3<sup>rd</sup> stage larvae species composition in coprocultures (%) for calves (n=72) sampled monthly May - October, 2005 and heifers (n=36) sampled monthly November and December 2005, and January - April, 2006. No data (ND) are available for fecal culture/larval identification and serum pepsinogen levels in May and June. Monthly rainfall totals are also shown. Mean fecal egg counts and serum pepsinogen levels with no common superscripts within calves (letters) and heifers (numbers) are different (P<0.05). Error bars represent 95% confidence intervals.



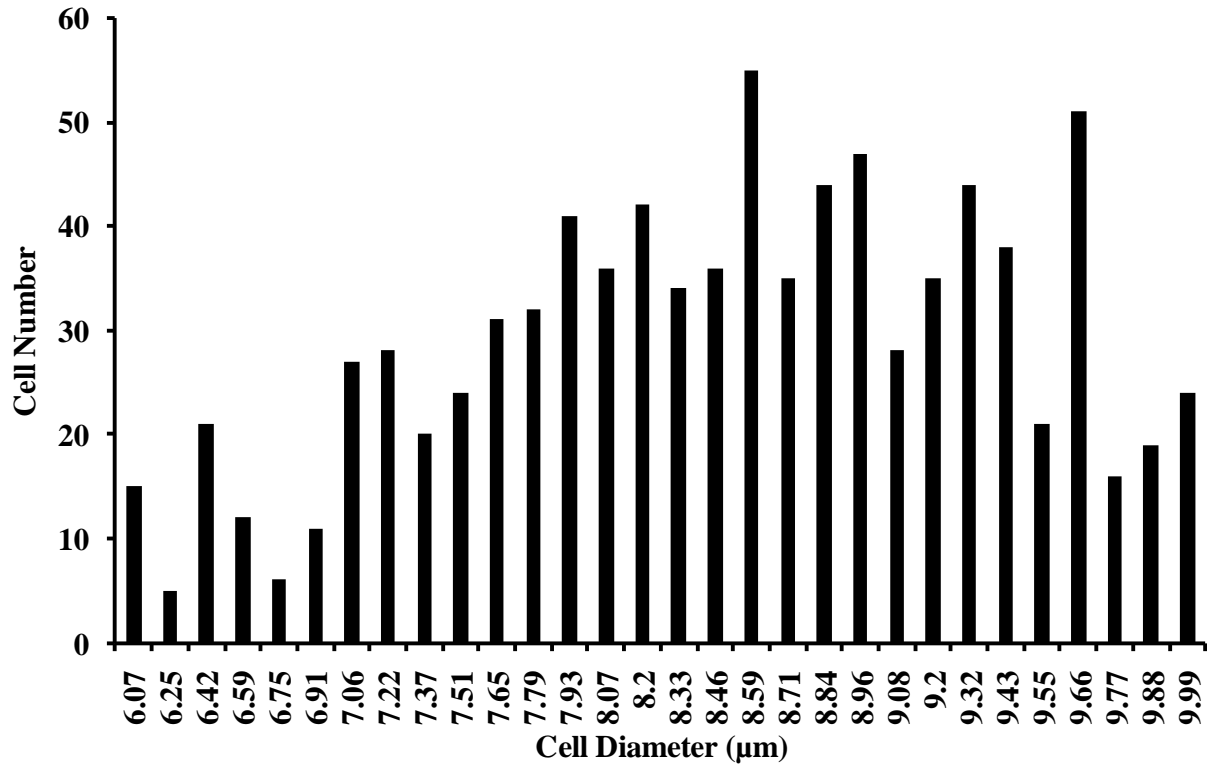
Appendix 3: Mean percentage of strongylid larvae from composite coprocultures and mean total strongylid fecal egg count (FEC) for cows sampled 2 May 2005, 15 July 2005, and 14 September 2005. Total strongylid fecal egg count (FEC) means with different superscripts differ ( $P < 0.05$ ).

Strongyle Genus	Mean Larvae/Sample (%)		
	May	July	September
<i>Ostertagia</i>	28.7	27.2	10.3
<i>Haemonchus</i>	0.3	11.6	26.6
<i>Oesophagostomum</i>	35.8	8.8	24.2
<i>Trichostrongylus</i>	35.2	48.3	31.8
<i>Cooperia</i>	0.0	4.1	7.1
Total Strongylid FEC	1.8 <sup>a</sup>	1.6 <sup>a</sup>	1.0 <sup>b</sup>
95% Confidence Interval	1.3-2.3	1.3-1.9	0.7-1.3

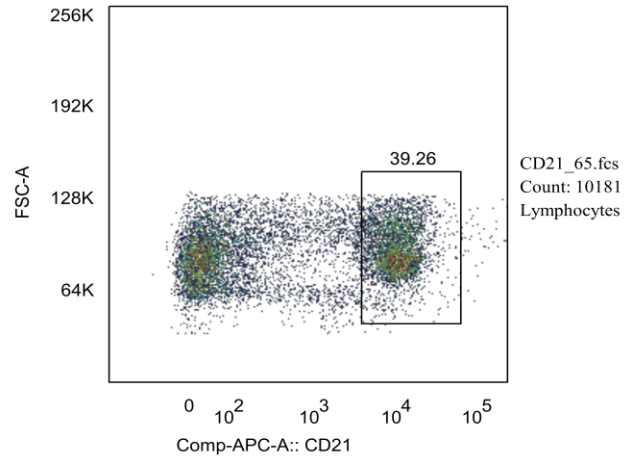
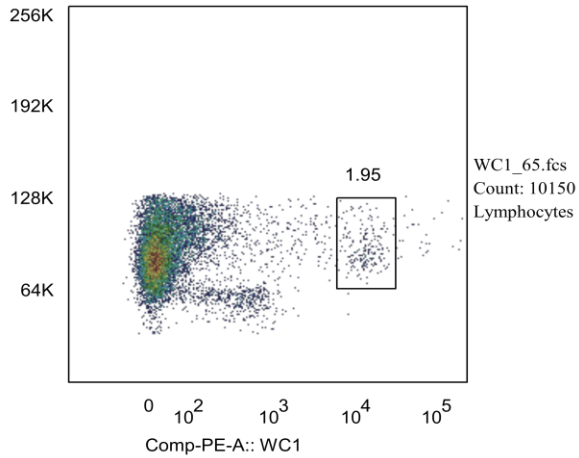
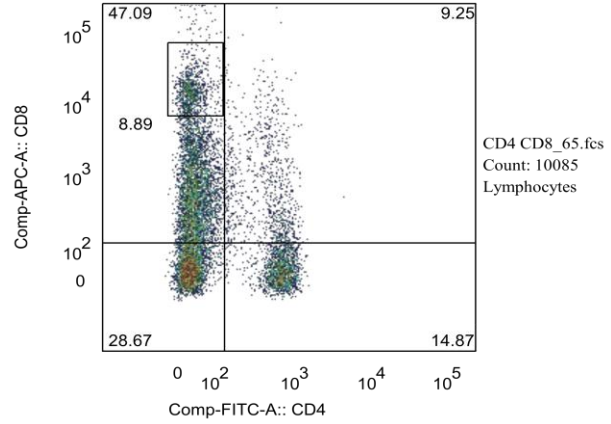
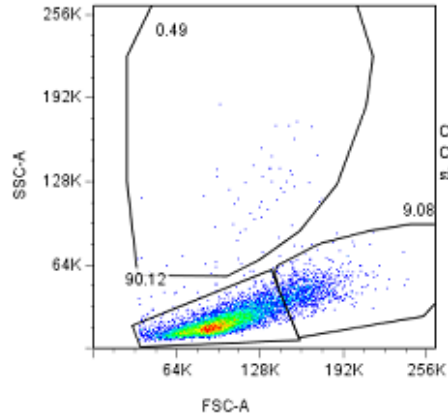
Appendix 4: Nutrient analysis from mixed grass hay and corn gluten feed. Mixed grass hay was provided ad libitum to calves in chapters 6 and 7 and corn gluten feed was offered to these calves at 0.5% of body weight daily. Nutrient analysis was carried out by Cumberland Valley Analytical Services Inc., Maugansville, MD.

<u>Mixed Grass Hay</u>	
Moisture	11.3%
Crude Protein	8.9%
Acid Detergent Fiber	42.2%
Neutral Detergent Fiber	69.2%
<u>Total Digestible Nutrients</u>	<u>55.3%</u>
<u>Corn Gluten Feed</u>	
Moisture	14.5%
Crude Protein	23.1%
Acid Detergent Fiber	13.1%
Neutral Detergent Fiber	34.7%
<u>Total Digestible Nutrients</u>	<u>88.2%</u>

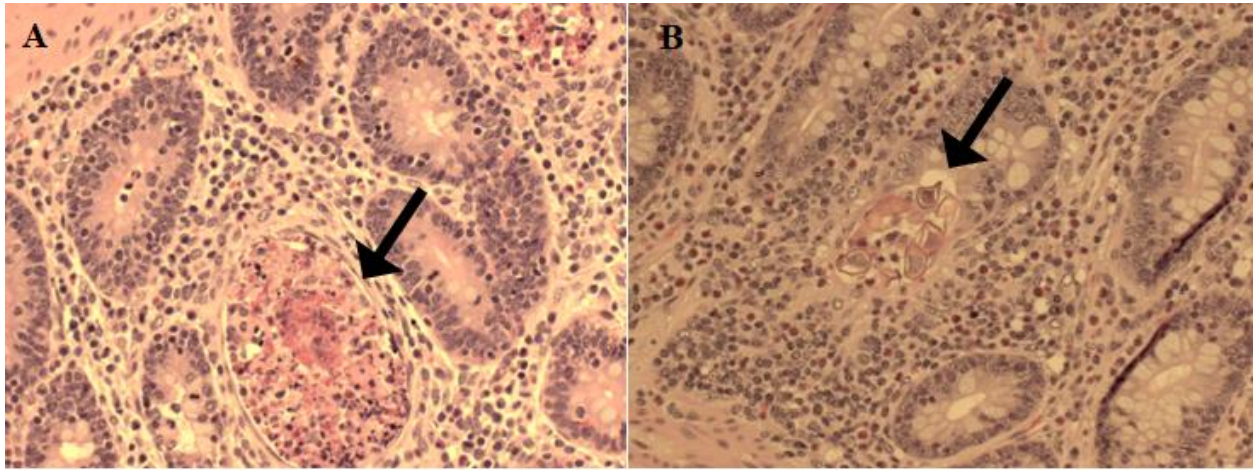
Appendix 5: Representative histogram of cell sizes isolated from calves using Lymphoprep<sup>®</sup> and counted by the Cellometer<sup>®</sup> Auto (Nexcelcom Bioscience LLC, Lawrence, MA).



Appendix 6: Representative flow cytometry plots for lymphocyte subtypes isolated from calves 16 hours after dexamethasone administration. Leukocytes were from whole blood using lymphoprep<sup>®</sup>. Plots were generated with FlowJo Software and BD FACSAria.

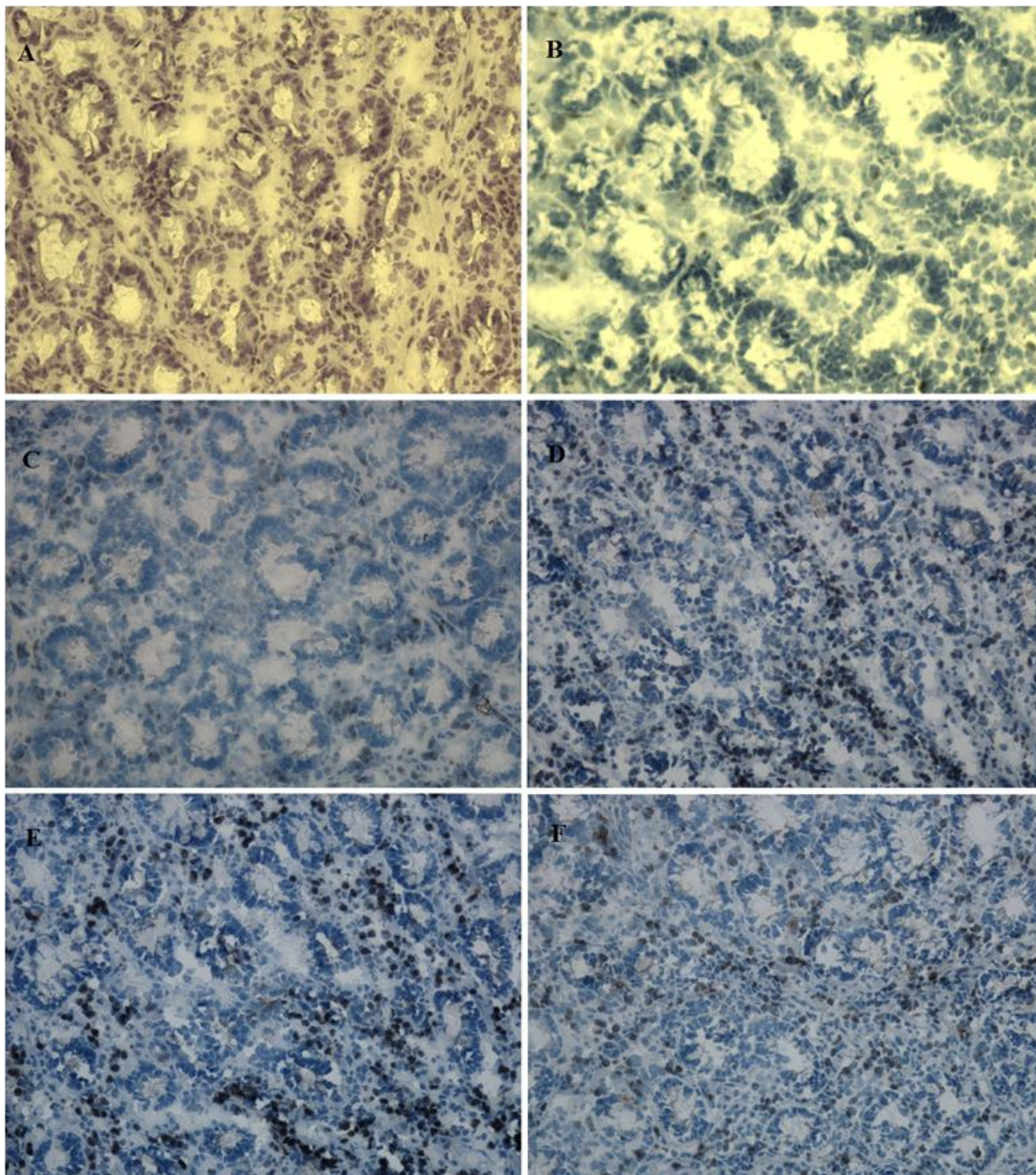


Appendix 7: Representative H&E stained cecal sections from calves that were challenged with  $1 \times 10^6$  *E. bovis* oocysts, given dexamethasone on day 7 post challenge and euthanized on day 24 post challenge. Crypt necrosis (A) and *Eimeria* spp. sexual stages (B) are indicated by the arrows.



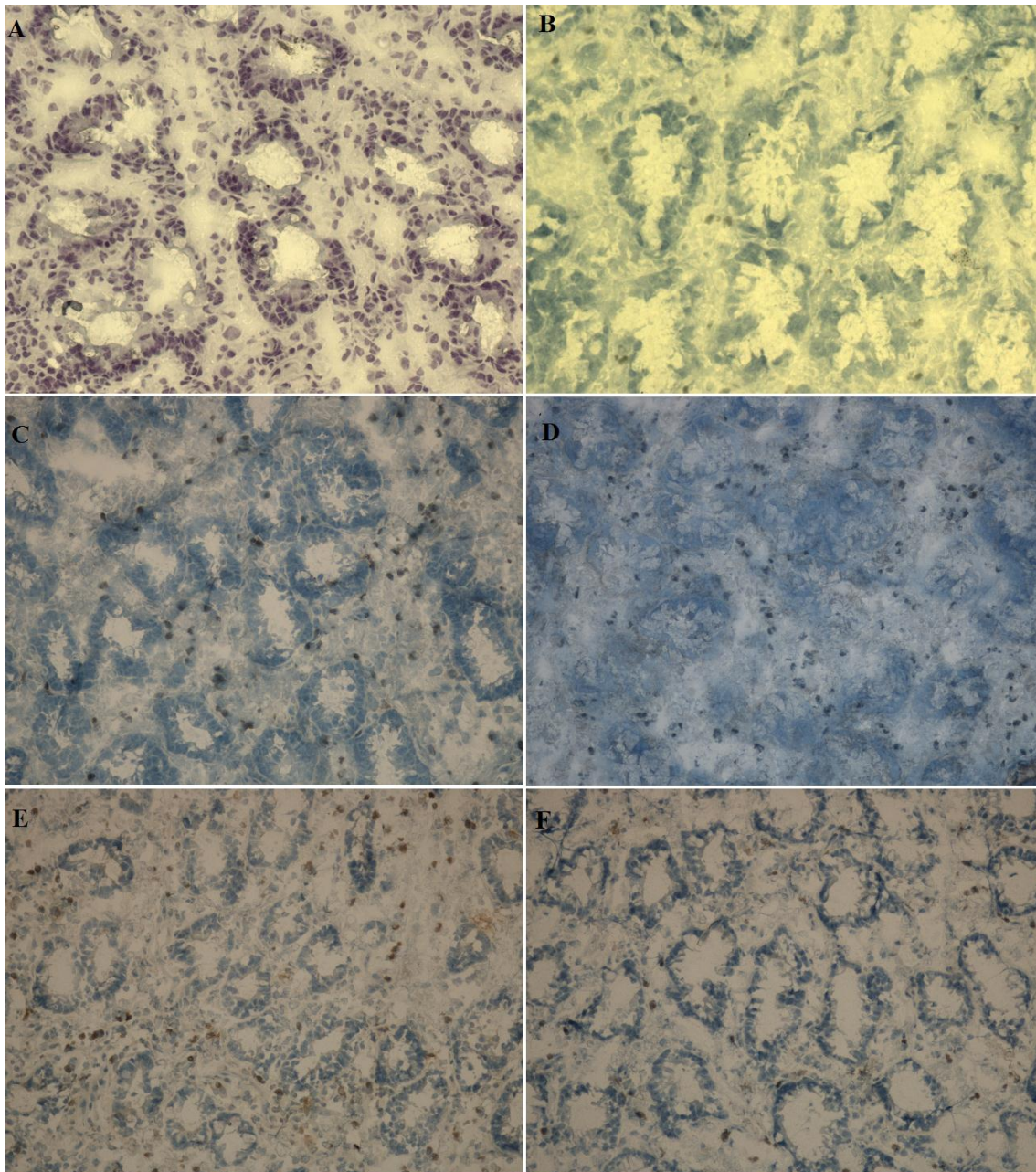


Appendix 8: Examples of immunohistochemistry staining for CD4<sup>+</sup> (C) CD8<sup>+</sup> (D), WC1<sup>+</sup>(E), and CD21<sup>+</sup> (F) lymphocytes in ileal sections from calves euthanized on day 24 of the study. Stressed and challenged (SC) (n=4) calves were challenged with  $1.5 \times 10^6$  sporulated *E. bovis* oocysts orally on day 0 and received dexamethasone on day 7. Not stressed and not challenged (NSNC) (n=4) calves received no *E. bovis* challenge or dexamethasone. Negative controls consisting of DAB substrate only (A) and secondary antibody only (B) were carried out.





Appendix 9: Examples of immunohistochemistry staining for CD4<sup>+</sup> (C) CD8<sup>+</sup>(D), WC1<sup>+</sup>(E), and CD21<sup>+</sup> (F) lymphocytes in cecal sections from calves euthanized on day 24 of the study. Stressed and challenged (SC) (n=4) calves were challenged with  $1.5 \times 10^6$  sporulated *E. bovis* oocysts orally on day 0 and received dexamethasone on day 7. Not stressed and not challenged (NSNC) (n=4) calves received no *E. bovis* challenge or dexamethasone. Negative controls consisting of DAB substrate only (A) and secondary antibody only (B) were carried out.



### Literature Cited

- Abrahamsen, M.S., 1998. Bovine T cell responses to *Cryptosporidium parvum* infection. *International Journal of Parasitology*. 28, 1083-1088.
- Arthington, J.D. Qiu, X., Crooke, R.F., Vendramini, J.M.B., Araujo, D.B., Chase, C.C., Jr. Coleman, S.W., 2008. Effects of preshipping management on measures of stress and performance of beef steers during feedlot receiving. *Journal of Animal Science*. 86, 2016-2023.
- Arthington, J.D., Spears, J.W., Miller, D.C., 2005. The effect of early weaning on feedlot performance and measures of stress in beef calves. *Journal of Animal Science*. 83, 933-939.
- Behrendt, J.H., Clauss, W., Zahner, H., Hermosilla C., 2004. Alternative mechanism of *Eimeria bovis* sporozoites to invade cells in vitro by breaching the plasma membrane. *Journal of Parasitology*. 90, 1163-1165.
- Behrendt, J.H., Hermosilla, C., Hardt, M., Failing, K., Zahner, H., Taubert, A., 2008. PMN-mediated immune reactions against *Eimeria bovis*. *Veterinary Parasitology*. 151, 97-109.
- Behrendt, J.H., Ruiz, A. Zahner, H., Taubert, A., Hermosilla, C., 2010. Neutrophil extracellular trap formation as innate immune reactions against the apicomplexan parasite *Eimeria bovis*. *Veterinary Immunology and Immunopathology*. 133, 1-8.
- Berghen, P., Dorny, P., Vercruyssen, J., 1987. Evaluation of a simplified blood pepsinogen assay. *American Journal of Veterinary Research*. 48, 664-669.

- Blackwell, G.J., Cornuccio, M., DiRosa, R.J., 1980. Macro cortin: A polypeptide causing the antiphospholipase effect of glucocorticoids. *Nature*. 187, 147-149.
- Blotta, M.H., Dekruyff, R.H., Umetsu, D.T., 1997. Corticosteroids inhibit IL-12 production in human monocytes and enhance their capacity to induce IL-4 synthesis in CD4+ lymphocytes. *Journal of Immunology*. 158, 5589-5595.
- Boland, H.T., Scaglia, G., Swecker, W.S., Jr., Burke, N.C., 2008. Effects of alternate weaning methods on behavior, blood metabolites and performance of beef calves. *Professional Animal Scientist*. 24, 539-551.
- Boughton, D.C., 1942. An overlooked macroscopic intestinal lesion of value in diagnosing bovine coccidiosis. *The North American Veterinarian*. 23, 123-175.
- Boughton, D.C., 1945. Bovine coccidiosis: From carrier to clinical case. *The North American Veterinarian*. 26, 147-153.
- Bowman, D., 2009. *Georgi's Parasitology for Veterinarians*. Elsevier. Independence Square West, Philadelphia, Pennsylvania.
- Breazile, J.E., 1988. The physiology of stress and its relationship to mechanisms of disease and therapeutics. *The Veterinary Clinics of North America, Food Animal Practice*. 4, 441-480.
- Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D.S., Weinrach, Y., Zychlinsky, A., 2004. Neutrophil extracellular traps kill bacteria. *Science*. 303. 1532-1535.

- Broom., D.M., Johnson, K.G., 1993. Stress and animal welfare. *Animal Behavior Series*. 28, 8-11.
- Brown, W.C., Davis, W.C., Dobbelaere, D.A.E., Rice-Ficht, A.C., 1994. CD4+ T cell clones obtained from cattle chronically infected with *Fasciola hepatica* and specific for adult worm antigen express both unrestricted and Th2 cytokine profiles. *Infection and Immunology*. 62, 818-827.
- Brown, W.C., Estes, D.M., 1997. Type I and type II responses in cattle and their regulations. *Cytokines in Veterinary Medicine*. CAB international, Wallingford, UK, pp. 15-33.
- Brown, W.C., Rice-Ficht, A.C., Estes, D.,M., 1998. Bovine type 1 and type 2 responses. *Veterinary Immunology and Immunopathology*. 63, 45-55.
- Buckham-Sporer, K.R., Weber, P.S.D., Burton, J.L., Early, B., Crowe, M.A., 2008. Transportation of young beef bulls alters circulating physiological parameters that may be effective biomarkers of stress. *Journal of Animal Science*. 86, 1325-1334.
- Burger, H.J., 1983. *Eimeria* infektionen beim Rind. *Berl. Munch. Tierarztl. Wochenschr.* 96, 350-357.
- Burton, J.L., Erskine, R.J., 2003. Mastitis and immunity: some new ideas for an old disease. *Veterinary Clinics of North America, Food Animal Practice*. 19, 1-45.
- Burton, J.L., Kehrli, M.E., 1995. Regulation of neutrophil adhesion molecules and shedding of *Staphylococcus aureus* in milk of cortisol and dexamethasone-treated cows. *American Journal of Veterinary Research*. 56, 997-1006.

- Burton, J.L., Madsen, S.A., Chang, L.C., Weber, P.S.D., Buckham, K.R., Dorp, R., Hickey, M.C., Early, B., 2005. Gene expression signatures in neutrophils exposed to glucocorticoids: a new paradigm to help explain “neutrophil dysfunction” in parturient dairy cows. *Veterinary Immunology and Immunopathology*. 105, 197-219.
- Busato, A., Lentze, T., Hofer, D., Burnens A., Hentrich B., Gaillard, C., 1998. A coase control study of potential enteric pathogens for calves raised in cow-calf herds. *Journal of Veterinary Medicine B*. 45, 519-528.
- Callan, R.J., Garry, F.B., 2002. Biosecurity and bovine respiratory disease. *Veterinary Clinics of North America, Food Animal Practice*. 18, 57-77.
- Chang, X., Mallard, B.A., Mowat, D.N., 1994. Proliferation of peripheral blood lymphocytes of feeder calves in response to chromium. *Nutrition Research*. 14, 821:864.
- Chitko-McKown, C.G., Ruef, B.J., Rice-Ficht, A.C., Brown, W.C., 1995. Interleukin-10 down regulates proliferation and expression of interleukin-2 receptor P $\alpha$  chain and interferon- $\gamma$  but not interleukin-2 or interleukin-4 by parasite-specific helper T cell clones obtained from cattle chronically infected with *Babesia bovis* or *Fasciola hepatica*. *Journal of Interferon Cytokine Research*. 15, 915-922.
- Christensen, J.F., 1941. The oocysts of coccidia from domestic cattle in Alabama (U.S.A) with descriptions of two new species. *Journal of Parasitology*. 27, 203-220.
- Coico, R., Sunshine, G., 2009. *Immunology: a short course*. Wiley-Blackwell, Hoboken, New Jersey. pp. 8-100.

- Colditz, I.G., Hennessey, D.W., 2001. Associations between immune system, growth and carcass variables in cattle. *Australian Journal of Experimental Agriculture*. 41, 1051-1056.
- Conlogue, G., Foreyt, W.J., Wescott, R.B., 1984. Bovine coccidiosis: protective effects of low-level infection and coccidiosis treatments in calves. *American Journal of Veterinary Research*. 45, 863-866.
- Cooper, T.R., Trunkfield, H.R., Zanella, A.J., Booth, W.D., 1989. An enzyme-linked immunosorbent assay for cortisol in the saliva of man and domestic farm animals. *Journal of Endocrinology*. 123, 13-16.
- Cornelissen, A.W.C.A., Verstegen, R., van den Brand, H., Perie, N.M., Eysker, M., Lam, T.J.G.M., Pijpers A., 1995. An observational study of *Eimeria* species in housed cattle on Dutch dairy farms. *Veterinary Parasitology*, 56, 7-16.
- Cox, D.D., Todd, A.C., 1962. Survey of gastrointestinal parasitism in Wisconsin dairy cattle. *Journal of the American Veterinary Medical Association*. 141, 706-709.
- Crookshank., H.R., Elissalde, M.H., White, R.G., Clanton, R.G., Smalley, H.E., 1979. Effect of transportation and handling of calves upon blood serum composition. *Journal of Animal Science*. 48, 430-435.
- Daugshies, A., Akimaru, M., Burger, H.J., 1986. Experimentelle *Eimeria bovis* infection beim kalb. 1. Parasitologische und klinische Befunde *Dtsch. Tieraztl. Wschr.* 93, 393-397.
- Daugshies, A., Burger, H.J., Akimaru, M., 1998. Apparent digestibility of nutrients and nitrogen balance during experimental infection of calves with *Eimeria bovis*. *Veterinary Parasitology*. 77, 93-102.

- Dauguschies, A., Najdrowski, M., 2005. Eimeriosis in cattle: Current understanding. *Journal of Veterinary Medicine B*. 52, 417-427.
- DeKruyff, R.H., Fang, Y., Umetsu, D.T., 1998. Corticosteroids enhance capacity of macrophages to induce Th2 cytokine synthesis in CD4+ lymphocytes by inhibiting IL-12 production. *Journal of Immunology*. 160, 2231-2237.
- Diaz de Ramirez, A., Hernandez, A., Garcia, A., Ramirez-Iglesia, L.N., 2001. Excretion of oocysts of *Eimeria* spp. during the first three months of life in calves from dairy farms in western Venezuela. *Revista Científica, Facultad de Ciencia Veterinarias, Universidad del Zulia* 11, 207-212
- Elenkov, I.J., Chrousos, G.P., 1999. Stress hormones, Th1/Th2 patterns, pro/anti-inflammatory cytokines and susceptibility to disease. *Trends in Endocrinology and Metabolism*. 10:359-368
- Elenkov, I.J., Papanicolaou, D.A., Wilder, R.L., Chrousos, G.P., 1996. Modulatory effects of glucocorticoids and catecholamines on human interleukin-12 and interleukin-10 production: clinical implications. *Proceedings of the Association of American Physicians*. 108, 374-381.
- Ernst, J.V., Benz, G.W., 1986. Intestinal coccidiosis in cattle. *Veterinary Clinic of North America: Food Animal Practice*. 2, 283-291.
- Ernst, J.V., Ciordia, H., Stuedemann, J. A., 1984. *Coccidia* in cows and calves on pasture in north Georgia (U.S.A.). *Veterinary Parasitology* 15: 213-221

- Ernst, J.V., Stewart, T.B., Whitlock, D.R., 1987. Quantitative determination of coccidian oocysts in beef calves from the coastal plain area of Georgia (U.S.A.). *Veterinary Parasitology*. 23, 1-10.
- Ernst, J.V., Todd, K.S., Jr., 1977. New geographic record and redescription of the sporulated oocyst of *Eimeria pellita* Supperer, 1952 from Alabama cattle. *Proceedings of the Helminthological Society of Washington*. 44, 221-223.
- Faber, J.E., Kollmann, D., Heise, A., Bauer, C., Failing, K., Burger, H.J., Zahner, H., 2002. *Eimeria* infections in cows in the periparturient phase and their calves: oocysts excretion and levels of specific serum and colostrum antibodies. *Veterinary Parasitology*. 106, 1-17.
- Fayer, R., 1980. Epidemiology of protozoan infections: The coccidia. *Veterinary Parasitology*. 6, 75-103.
- Fiege, N.D., Klatte, D., Kollmann, D., Zahner, H., Burger, H.J., 1992. *Eimeria bovis* in cattle: colostrum transfer of antibodies and immune response to experimental infections. *Parasitology Research*. 78, 32-38.
- Findly, R.C., Roberts, S.J., Hayday, A.C., 1993. Dynamic response of murine gut intraepithelial T cells after infection by the coccidian parasite *Eimeria*. *European Journal of Immunology*. 23:2557-2564.
- Fiorentino, D.F., Bond, M.W, Mosmann, T.R., 1989. Two types of mouse T Helper cell: IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *Journal of Experimental Medicine*. 490, 2081-2095.



- Fitzgerald, P.R., 1962. Coccidia in Hereford calves on summer and winter ranges and in feedlots in Utah. *Journal of Parasitology*. 48, 347-351.
- Fitzgerald, P.R., 1967. Results of continuous low-level inoculations with *Eimeria bovis* in calves. *American Journal of Veterinary Research*. 28, 659-665.
- Fitzgerald, P.R., 1980. The economic impact of coccidiosis in domestic animals. *Advances in Veterinary Science and Comparative Medicine*. 24, 121-143.
- Fitzgerald, P.R., Mansfield, M.E., 1972. Effects of bovine coccidiosis on certain blood components, feed consumption, and body weight changes of calves. *American Journal of Veterinary Research*. 33, 1391-1397.
- Fitzgerald, P.R., Mansfield, M.E., 1979. Efficacy of lasalocid against coccidia in cattle. *Journal of Parasitology*. 65, 824-825.
- Fitzgerald, P.R., Mansfield, M.E., 1984. Control of bovine coccidiosis with monensin in nonresistant newborn calves. *American Journal of Veterinary Research*. 45, 1984-1988.
- Fitzgerald, P.R., Mansfield, M.E., 1989a. Effects of intermittent and continuous administration of decoquinate on bovine coccidiosis in male calves. *American Journal of Veterinary Research*. 50, 961-964.
- Fitzgerald, P.R., Mansfield, M.E., 1989b. Effects of inoculations with *Eimeria zuernii* on young calves treated with decoquinate or narasin with or without dexamethasone. *American Journal of Veterinary Research*. 50, 1056-1059.

- Fluharty, F.L., Loerch, S.C., Turner, T.B., Moeller, S.J., Lowe, G.D., 2000. Effects of weaning age and diet on growth and carcass characteristics in steers. *Journal of Animal Science*. 78, 1759-1767.
- Fox, J.E., 1985. Coccidiosis in cattle. *Modern Veterinary Practice*. 66, 113-116.
- Friend, S.C.E., Stockdale, P.H.G., 1981. Experimental *Eimeria bovis* infection in calves: a histopathological study. *Canadian Journal of Comparative Medicine*. 44, 129-140.
- Gilbertie, J.M., LeRoith, T., Wahlberg, M., Witonsky S., Swecker, W.S., 2010. Increases in cortisol due to weaning stress and subsequent alterations in immune function in beef calves. MS thesis. Virginia Tech, Blacksburg, VA.
- Grandin, T., 1997. Assessment of stress during handling and transport. *Journal of Animal Science*. 75, 249-257.
- Grisi, L, Todd, A.C., 1978. Prevalence of gastrointestinal parasitisms among milking cows in Wisconsin, Pennsylvania, and North Carolina. *American Journal of Veterinary Research*. 39, 51-54.
- Gwazdauskas, F.C., Paape, M.J., Peery, D.A., McGilliard, M.L., 1980. Plasma glucocorticoid and circulating blood leukocyte responses in cattle after sequential intramuscular injections of ACTH. *American Journal of Veterinary Research*. 41, 1052-1056.
- Haley, D.B., Bailey, D.W., Stookey, J.M., 2005. The effects of weaning beef calves in two stages on their behavior and growth rate. *Journal of Animal Science*. 83, 2205-2214.

- Hammon, H.M., Sauter, S.N., Reist, M., Zbinden, Y., Philipona, C., Morel, C., Blum, J.W.,  
2003. Dexamethasone and colostrum feeding affect hepatic gluconeogenic enzymes  
differently in neonatal calves. *Journal of Animal Science*. 81, 3095-3106.
- Hammond, D.M., 1964. Coccidiosis of cattle: Some unsolved problems. Thirtieth Faculty Honor  
Lecture, Utah State University, Logan Utah.
- Hammond, D.M., 1973. The coccidia. *Eimeria*, *Isospora*, *Toxoplasma* and related genera.  
University Park Press. Baltimore Maryland.
- Hammond, D.M., Clark, G.W., Miner, M.L., Trost, W.A., Johnson, A.E., 1956. Treatment of  
experimental bovine coccidiosis with multiple small doses and single large doses of  
sulfamethazine and sulfabromo-methazine. *American Journal of Veterinary Research*. 20,  
708-713.
- Hammond, D.M., Fayer, R., Miner, M.L., 1966. Amprolium for control of experimental  
coccidiosis in cattle. *American Journal of Veterinary Research*. 27, 199-206.
- Hasbullah, Akiba, Y., Takano, H., Ogimoto, K., 1990. Seasonal distribution of bovine coccidia  
in beef cattle herd in the university farm. *Japanese Journal of Veterinary Science*.  
52,1175-1179.
- Heath, H.L., Blagburn, B.L., Elsasser, T.H., Pugh, D.C., Sanders L.G., Sartin, E.A., Steele, B.,  
Sartin, J.L., 1997. Hormonal modulation of the physiologic response of calves infected  
with *Eimeria bovis*. *American Journal of Veterinary Research*. 58, 891-896.
- Heise, A., Peters W., Zahner, H., 1999b. Microneme antigens of *Eimeria bovis* recognized by  
two monoclonal antibodies. *Parasitology Research*. 85, 457-467

- Heise, A., Peters, W., Zahner, H., 1999a. Phosphocholine epitopes in *Eimeria bovis*.  
*Experimental Parasitology*. 92, 279-282
- Hermosilla, C., Barbisch, B., Heise, A., Kowalik, S., Zahner, H., 2002. Development of *Eimeria bovis* in vitro: suitability of several bovine, human and porcine endothelial cell lines, bovine fetal gastrointestinal, Madin-Darby bovine kidney (MDBK) and African green monkey kidney (VERO) cells. *Parasitology Research*. 88, 301-307.
- Hermosilla, C., Burger, H.J., Zahner, H., 1999. T cell responses in calves to a primary *E. bovis* infection: phenotypical and functional changes. *Veterinary Parasitology*. 84, 49-64.
- Hickey, M.C., Drennan, M., Earley, B., 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. *Journal of Animal Science*. 814, 2847-2855.
- Hughes, P.A., Thomas, K.R., Speer, C.A., 1988. Antigen-specific lymphocyte transformation induced by oocyst antigens of *Eimeria bovis*. *Infection and Immunity*. 56, 1518-1525.
- Hughes, P.A., Whitmire, W.M., Speer, C.A., 1989. Immunity patterns during acute infection by *Eimeria bovis*. *Journal of Parasitology*. 75, 86-91.
- Hultgren, J., 1998. Risk factors of displaced abomasums in Wisconsin dairy herds. 32, 56-57
- Jackson, A.R., 1964. The isolation of viable coccidial sporozoites. *Parasitology*. 54, 87-93.
- Jacobson, R.H., Worley, D.E., 1969. Incidence and distribution of helminth parasites and coccidia in Montana cattle. *American Journal of Veterinary Research*. 30,113-116.

- Jolley, W.R., Bardsley, K.D., 2006. Ruminant Coccidiosis. *Veterinary Clinics of North America, Food Animal Practice*. 22, 613-621.
- Jolley, W.R., Bergstrom, R.C., 1977. Summer coccidiosis in Wyoming calves. *Veterinary Medicine Small Animal Clinics*. 71, 218-219.
- Jubb, K.V.F., Kennedy, P.C., Palmer, N., 2007. Jubb, Kennedy, and Palmer's pathology of domestic animals. Elsevier Saunders. New York, New York.
- Kelso, A., 1995. Th1 and Th2 subsets: paradigms lost? *Immunology Today*. 16, 374-379.
- Kim, M.H., Yang, J.Y., Upadhyaya, S.D., Lee, H.J., Yun, C.H., Ha, J.K., 2011. The stress of weaning influences serum levels of acute-phase proteins, iron-binding proteins, inflammatory cytokines, cortisol, and leukocyte subsets in Holstein calves. *Journal of Veterinary Science*. 12, 151-157.
- Lee, R.P., Armour, J., 1959. The coccidia oocysts of Nigerian cattle. *The British Veterinary Journal*. 115, 6-17.
- Lefcourt, A.M., Elsasser, T.H., 1995. Adrenal responses in Angus X Hereford cattle to the stress of weaning. *Journal of Animal Science*. 73, 2669-2676.
- Levine, N.D., Ivens, V., 1967. The sporulated oocysts of *Eimeria illinoisensis* n. sp. and of other species of *Eimeria* of the ox. *Journal of Protozoology*. 14, 351-360.
- Levine, N.D., Ivens, V., 1986. The coccidian parasites (Protozoa, Apicomplexa) of Artiodactyla. *Illinois Biological Monographs No. 55*, University of Illinois Press, Urbana, Illinois, pp. 66-79.

- Lillehoj, H.S., 1987. Effects of immunosuppression on avian coccidiosis: cyclosporin A but not hormonal bursectomy abrogates host protective immunity. *Infection and Immunology*. 55, 1616-1621.
- Lillehoj, H.S., Bacon, L.D., 1991. Increase of intestinal intraepithelial lymphocytes expressing CD8 antigen following challenge with *Eimeria acervulina*. *Avian Diseases*. 35, 294-301.
- Lillehoj, H.S., Trout, J.M., 1996. Avian gut-associated lymphoid tissues and intestinal immune responses to *Eimeria* parasites. *Clinical Microbiology Reviews*. 9, 349-360.
- Lindsay, D.S., Blagburn, B.L., 2001. Antiprotozoan Drugs. *Veterinary Pharmacology and Therapeutics*. 8th Edition. Iowa State University Press. Ames, Iowa, pp. 1002-1004.
- Lindsay, D.S., Dubey, J.P., Fayer, R., 1990. Extraintestinal stages of *Eimeria bovis* in calves and attempts to induce relapse of clinical disease. *Veterinary Parasitology*. 36, 1-9.
- Long, P.L., 1982. *The Biology of the Coccidia*. University Park Press. Baltimore Maryland.
- Long, P.L., Rose, M.E., 1970. Extended schizogony of *Eimeria mivati* in betamethasone-treated chickens. *Parasitology*. 60, 147-155.
- Lucas, A.S., Swecker, G., Lindsay, D.S., W.S., Scaglia, Elvinger, F., Zajac, A.M., 2007. The effect of weaning method on coccidial infections in beef calves. *Veterinary Parasitology*. 145, 228-233.
- Lucas, A.S., Swecker, W.S., Scaglia, G., Lindsay, D.S., Zajac, A.M., 2006. Variation in *Eimeria* oocyst count and species composition in weanling beef heifers. *Journal of Parasitology*. 92, 1115-1117.

- Lynch, E.M., Earley, B., McGee, M., Doyle, S., 2010a. Characterization of physiological and immunological responses in beef cows to abrupt weaning and subsequent housing. *BMC Veterinary Research*. 6, 37
- Lynch, E.M., Early, B., McGee, M., Doyle, S., 2010b. Effect of abrupt weaning at housing on leukocyte distribution, functional activity of neutrophils, and acute phase protein response of beef calves. *BMC Veterinary Research*. 6, 39.
- Marquardt, W.C., 1962. Subclinical infection with coccidia in cattle and their transmission to susceptible calves. *Journal of Parasitology*. 48, 270-275.
- Marquardt, W.C., 1976. Some problems of host and parasite interaction in the coccidia. *Journal of Protozoology*. 23, 287-290.
- Marquardt, W.C., Senger, C.M., Seghetti, L., 1960. The effect of physical and chemical agents on the oocysts of *Eimeria zurnii*. *Journal of Protozoology*. 7, 186-189.
- Marsh, H., 1938. Healthy cattle as carriers of coccidia. *Journal of the American Veterinary Medical Association*. 92, 184-194.
- Marshall, R.N., Catchpole, J., Green, J.A., Webster, K.A., 1998. Bovine coccidiosis in calves following turnout. *Veterinary Record*. 143, 366-367.
- Matjila, P.T., Penzhorn, B.L., 2002. Occurrence and diversity of bovine coccidia at three localities in South Africa. *Veterinary Parasitology*. 104, 93-102.
- Menge, C. Dean-Nystrom, E.A., 2008. Dexamethasone depletes  $\gamma\delta$ T Cells and alters the activation state and responsiveness of bovine peripheral blood lymphocyte subpopulations. *Journal of Dairy Science*. 91, 2284-2298.

- Miner, M.L., Jensen, J.B., 1976. Decoquate in the control of experimentally induced coccidiosis of calves. *American Journal of Veterinary Research*. 37, 1043-1045.
- Minton, J.E., 1994. Function of the hypothalamic-pituitary-adrenal axis and the sympathetic nervous system in models of acute stress in domestic farm animals. *Journal of Animal Science*. 72:1891-1898.
- Moberg, G.P., 1987. A model for assessing the impact of behavioral stress on domestic animals. *Journal of Animal Science*. 65, 1228-1235.
- Moberg, G.P., Wood, V.A., 1982. Effect of differential rearing on the behavioral and adrenocortical response of lambs to a novel environment. 8, 269-279.
- Mormede, P., Andanson S., Auperin, B., Beerda, B., Guemene, D., Malmkvist, J., Mantec, X., Manteuffel, G., Prunet, P., van Reenen, C.G., Sabine, R., Veissier, I., 2007. Exploration of the hypothalamic-pituitary-adrenal function as a tool to evaluate animal welfare. *Physiology and Behavior*. 92, 317-339.
- Mosmann, T.R., Coffman, R.L., 1989. Th1 and Th2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annual Review Immunology*. 7, 145-173.
- Mosmann, T.R., Sad, S., 1996. The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunology Today*. 12, 128-146.
- Mostl, E., Maggs, J.L., Schrotter, G., Besenfelder, U., Palme, R., 2002. Measurement of cortisol metabolites in faeces of ruminants. *Veterinary Research Communications*. 26, 127-139.



- Mundt, H.C., Bangoura, B., Rinke, M., Rosenburch, M., Dauschies, A., 2005. Pathology and treatment of *Eimeria zuernii* coccidiosis in calves: investigations of an infection model. *Parasitology International*. 54, 223-230.
- Mundt, H.C., Rodder F., Mengel, H., Bangoura, B., Ocak, M., Dauschies A., 2007. Control of coccidiosis due to *Eimeria bovis* and *Eimeria zuernii* in calves with toltrazuril under field conditions in comparison with diclazuril and untreated controls. *Parasitology Research*. 101, supplement 1, S93-S104.
- Niilo L., 1970a. Bovine coccidiosis in Canada. *Canadian Veterinary Journal*. 11, 91-98.
- Niilo L., 1970b. Experimental winter coccidiosis in sheltered and unsheltered calves. *Canadian Journal of Comparative Medicine*. 34, 20-25.
- Niilo L., 1970c. The effect of dexamethasone on bovine coccidiosis. *Canadian Journal of Comparative Medicine*. 34, 325-328.
- Nockels, C.F., Odde, K.G., Craig, A.M., 1996. Vitamin E supplementation and stress affect tissue alpha-tocopherol content of beef heifers. *Journal of Animal Science*. 74:672-677.
- Nonnecke, B.J., Burton, J.L., Kehrli, M.E., 1997. Associations between function and composition of blood mononuclear leukocyte populations from Holstein bulls treated with dexamethasone. *Journal of Dairy Science*. 80, 2403-2410.
- Norcross, M.A., Siegmund, O.H., Fraser C.M., 1974. Amprolium for coccidiosis in cattle: a review of efficacy and safety. *Veterinary Medicine and Small Animal Clinician*. 69, 459-465.

- Nyberg, P.A., Helfer, D.H., Knapp, S.E., 1967. Incidence of bovine coccidia in western Oregon. Proceedings of the Helminthological Society of Washington. 34, 13-14.
- Ovington, K.S, Alleva, L.M. Kerr E.A., 1995. Cytokines and immunological control of Eimeria spp. International Journal of Parasitology. 25:1331-1351.
- Paape, M.J., Gwazdauskas, F.C., Guidry A.J., Weinland, B.T., 1977. Concentrations of corticosteroids, leukocytes, and immunoglobulins in blood and milk after administration of ACTH to lactating dairy cattle: Effects on phagocytosis of Staphylococcus aureus, by polymorphonuclear leukocytes. American Journal of Veterinary Research. 42: 2081-2087.
- Parker, R.J., Boothby, K., Polkinghorne, I., Holroyd, R.G., 1984. Coccidiosis associated with post-weaning diarrhoea in beef calves in a dry tropical region. Australian Veterinary Journal. 61, 181-183.
- Parker, R.J., Jones, G.W., 1987. The development of eimerian infections during the first eight months of life in unweaned beef calves in a dry tropical region of Australia. Veterinary Parasitology. 25, 1-7.
- Parker, R.J., Jones, G.W., Ellis, K.J., Heater, K.M., Schroter, K.L., Tyler, R., Holroyd, R.G., 1986. Post-weaning coccidiosis in beef calves in the dry tropics: experimental control with continuous monensin supplementation via intra-ruminal devices and concurrent epidemiological observations. Tropical Animal Health and Production. 18, 198-208.

- Peardon, D.L., Bilkovich, F.R., Todd, A.C., Hoyt, H.H., 1965. Trials of candidate bovine coccidiostats: efficacy of amprolium, lincomycin, sulfamethazine, chlorquine sulfate and di-phenate-70. *American Journal of Veterinary Research*. 26, 683-687.
- Pellerdy, L.P., 1974. *Coccidia and coccidiosis*. Berlin; Hamburg. Parey, 1974.
- Pescovitz, M.D., Sakopoulos, A.G., Gaddy, J.A., Husmann, R.J., Zuckermann, F.A., 1994. Porcine peripheral blood CD4<sup>+</sup>/CD8<sup>+</sup> dual expressing T-cells. *Veterinary Immunology and Immunopathology*. 43, 53-62.
- Price, E.O., Harris, J.E., Borgwardt, R.E., Sween, M.L., Connor, J.M., 2003. Fenceline contact of beef calves with their dams at weaning reduces the negative effects of separation on behavior and growth rate. *Journal of Animal Science*. 81, 116-121.
- Radostits, O.M., Gay, C.C., Hinchliff, K.W., Constable, P.D., 2007. *Veterinary Medicine: a textbook of the diseases of cattle, horses, sheep, pigs, and goats*. Elsevier Saunders, New York, New York.
- Rodriguez, S.D., Palmer, G.H., McElwain, T.F., McGuire, T.C., Ruef, B.J., Chitko-McKown, C.G., Brown, W.C., 1996. CD4<sup>+</sup> T helper lymphocyte response against *Babesia bigemina* rhoptry-associated protein-1. *Infection and Immunology*. 64, 2079-2087.
- Romagnani, S., 1994. Lymphokine production by human T cells in disease states. *Annual Review Immunology*. 12, 227-257.
- Rose, M.E., 1970. Immunity to coccidiosis: effect of betamethasone treatment of fowls on *Eimeria mivati* infection. *Parasitology*. 60, 137-146.

- Rose, M.E., Hesketh P., 1987. *Eimeria tenella*: effects of immunity on sporozoites within the lumen of the small intestine. *Experimental Parasitology*. 63, 337-344.
- Rose, M.E., Hesketh, P. Wakelin, D., 1992. Immune control of murine coccidiosis CD4+, and CD8+ T lymphocytes contribute differentially in resistance to primary and secondary infections. *Parasitology*. 105:349-354.
- Rose, M.E., Hesketh, P., 1982. Immunity to coccidia in chickens and adoptive transfer with peripheral blood lymphocytes and spleen cells. *Parasite Immunology*. 4, 171-185.
- Rose, M.E., Wakelin, D., Joysey, H.S., Hesketh, P., 1988. Immunity to coccidiosis: adoptive transfer in NIH mice challenged with *Eimeria vermiformis*. *Parasite Immunology*. 10, 59-69.
- Roth, J.A., Kaeberle, M.L., 1983. Suppression of neutrophil and lymphocyte function induced by a vaccinal strain of bovine viral diarrhea virus with and without administration of ACTH. *American Journal of Veterinary Research*. 44:2366-2372.
- Roth, J.A., Kaeberle, M.L., 1982. Effect of glucocorticoids on the bovine immune system. *Journal of the American Veterinary Medical Association*. 180, 894-901.
- Roth, J.A., Kaeberle, M.L., Hsu, W.H., 1982. Effects of ACTH administration on bovine polymorphonuclear leukocyte function and lymphocyte blastogenesis. *American Journal of Veterinary Research*. 43:412-416.
- Sanchez, R.O., Romero, J.R., Founroge, R.D., 2008. Dynamics of *Eimeria* oocysts excretion in dairy calves in the province of Buenos Aires (Argentina), during their first 2 months of age. *Veterinary Parasitology*. 151, 133-138.

- Sartin, J.L., Shores, M.A., Schartz, D.D., Kemppainen, R.J., Baker, J., 2000. Reduced growth of calves and its reversal by use of anabolic agents. *Domestic Animal Endocrinology*. 19, 85-92.
- Scaglia, G., Swecker, W.S., Fontenot, J.P., Fiske, D., Fike, J.H., Abaye O.A., Clapham, W., Hall J.B., 2008. Forage systems for cow-calf production in the Appalachian region. *Journal of Animal Science*. 86, 2032–2042.
- Schneider, D., Ayeni, A.O., Durr, U., 1972. Physical resistance of coccidial oocysts: a review. *Deutsche Tierärztliche Wochenschrift*. 79, 561-569.
- Sconberg, S., Nockels C.F., Bennett, B.W., Bryninckx W., Blancquaert, A.M., Craig A. M., 1993. Effects of shipping, handling, adrenocorticotrophic hormone, and epinephrine on alpha-tocopherol content of bovine blood. *American Journal of Veterinary Research*. 54:1287-1293.
- Senger, C.M., Hammond, D.M., Thorne, J.L., Johnson A.E., Wells, G.M., 1959. Resistance of calves to reinfection with *Eimeria bovis*. *Journal of Protozoology*. 6, 51-58.
- Shi, M. Huther, S., Burkardt, E., Zahner, H., 2001. Lymphocyte subpopulations in the caecum mucosa of rats after infections with *Eimeria* separate: early responses in naïve and immune animals to primary and challenge infections. *International Journal of Parasitology*. 31:49-55.
- Smith, A.L., Hayday, A.C., 2000. An alpha beta T-Cell independent immunoprotective response towards gut coccidia is supported by gammadelta cells. *Immunology* 101:325-332.

- Snowder, G., 2009. Genetics, environment and bovine respiratory disease. *Animal Health Research Reviews*. 10, 117-119.
- Souza, M., Cheetham, S.M., Azevedo, M.S.P., Costantini, V., Saif, L.J., 2007. Cytokine and antibody responses in gnotobiotic pigs after infection with human norovirus genogroup II.4 (HS66 strain). *Journal of Virology*. 81, 9183-9192.
- Speer, C.A., Reduker, D.W., Burgess, D.E., Whitmire, W.M., Splitter, G.A., 1985. Lymphokine-induced inhibition of growth of *Eimeria bovis* and *Eimeria papillata* (Apicomplexa) in cultured bovine monocytes. *Infection and Immunity*. 50, 566-571.
- Step, D.L., Streeter, R.N., Kirkpatrick, J.G., 2002. Bovine coccidiosis-A review. *The Bovine Practitioner*. 36, 126-135.
- Stockdale, P.H.G., 1981. Effects of monensin on coccidiosis in ruminants. *Veterinary Medicine and Small Animal Clinician*. 76, 1575-1578.
- Stockdale, P.H.G., 1987. The pathogenesis of the lesions produced by *Eimeria zuernii* in calves. *Canadian Journal of Comparative Medicine*. 41, 338-344.
- Stookey, J. M., Schwartzkopf-Fenswein, K. S., Waltz, C. S., Watts J. M., 1997. Effects of remote and contact weaning on behaviour and weight gain of beef calves. *Journal of Animal Science*. 75(Suppl. 1)(157 (Abstr)).
- Stromberg, B.E., Schlotthauer, J.C., Hamann, K.J., Saatara Oz, H., Bemrick, W.J., 1986. Experimental bovine coccidiosis: Control with Monensin. *Veterinary Parasitology*. 22, 135-140.

- Suhwold, A., Hermosilla, C., Seeger, T., Zahner, H., Taubert, A., 2010. T cell reactions of *Eimeria bovis* primary and challenge-infected calves. *Parasitology Research*. 106: 595-605.
- Svennson C., 1997. The survival and transmission of oocysts of *E. alabamensis* in hay. *Veterinary Parasitology*. 69, 211-218.
- Svennson C., 2000. Excretion of *E. alabamensis* oocysts in grazing calves and young stock. *Journal of Veterinary Medicine B*. 47, 105-110
- Svennson, C.H., Olofsson, H., Ugglå, A., 1996. Immunisation of calves against *Eimeria alabamensis* coccidiosis. *Applied Parasitology*. 37, 209-216.
- Svensson, C., 1994. Bovine coccidiosis with special reference to *Eimeria alabamensis* infection in grazing calves. Dissertation Swedish University of Agricultural Sciences. Uppsala, Sweden.
- Svensson, C., Hooshmand-Rad, P., Pehrson, B., Tornquist, M., Ugglå A., 1993. Excretion of *Eimeria* oocysts in calves during their first three weeks after turn-out to pasture. *Acta vet. Scand*. 34: 175-182.
- Szabo, S.J., Kim, S.T., Costa, G.L., Zhang, X., Fathman C.G., Glimcher L.H., 2000. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell*. 100, 655-669.
- Szanto, J. Mohan, R.N., Levine N.D., 1964. Prevalence of coccidia and gastrointestinal nematodes in beef cattle in Illinois and their relation to shipping fever. *Journal of the American Veterinary Medical Association*. 144, 741-746.

- Taubert, A., Hermosilla, C., Suhwold, A., Zahner, H., 2008. Antigen-induced cytokine production in lymphocytes of primary and challenge infected calves. *Veterinary Immunology and Immunopathology*. 126, 309-320.
- Terlouw, E.M.C., Lawrence, A.B., Koolhaas, J.M., Cockram, M., 1993. Relationship between feeding, stereotypes, and plasma glucose concentrations in food-restricted and restrained sows. *Physiology and Behavior*. 54, 189-193.
- Tilbrook, A.J., Clarke, I.J., 2006. Neuroendocrine mechanisms of innate states of attenuated responsiveness of the hypothalam-pituitary adrenal axis to stress. *Frontiers in Neuroendocrinology*. 27, 285-307.
- Trout, J.M., Lillehoj, H.S., 1995. *Eimeria acervulina*: evidence for the involvement of CD8+ T lymphocytes in sporozoite transport and host protection. *Poultry Science*. 74, 1117-1125.
- Uetake, K., Ishiwata, T., Abe, N., Eguchi, Y., Tanaka, T., 2006. Hormonal and metabolic relation to restraint and human handling in growing-fattening steers. *Animal Science Journal*. 22, 370-374.
- United States Department of Agriculture, Natural Resource Conservation Service,  
<http://www.wcc.nrcs.usda.gov/nwcc/site?sitenum=2088&state=va>
- van Wyk, J.A., van Caberet, J., Michael, L.M., 2004. Morphological identification of nematode larvae of small ruminants and cattle simplified. *Veterinary Parasitology*. 119, 277-306.
- Viveros-Paredes, J.M., Puebla-Perez, A.M., Gutierrez-Coronado, O., Sandoval-Ramirez, L., Villasenor-Garcia., M.M., 2006. Dysregulation of the Th1/Th2 cytokine profile is



- associated with immunosuppression induced by the hypothalamic-pituitary-adrenal axis activation in mice. *International Immunopharmacology*. 6, 774-781.
- Ward J.K., Ferguson, D.L., Parkhurst, A.M., 1979. Gastrointestinal parasites in beef cows. *Journal of Animal Science*. 49, 306-309.
- Weber, P.S.D., Madsen, S.A., Smith, G.W., Ireland, J.J., Burton, J.L., 2001. Pre-translation regulation of neutrophil L-selectin in glucocorticoid-challenged cattle. *Veterinary Immunology and Immunopathology*. 83, 213-240.
- Weber, P.S.D., Toelboell, T., Chang, L.C., Tirrell, J.D., Saama, P.M., Smith, G.W., Burton, J.L. 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. *Journal of Leukocyte Biology*, 75, 815-827.
- Wegner, T.N., Stott, G.H., 1972. Serum minerals, leukocyte profiles, and plasma corticoids in dairy heifers after an injection of corticotropin. *Journal of Dairy Science*. 55: 1464-1469.
- Whitlock, H. V., 1948. Some modifications of the McMaster helminth egg-counting technique apparatus. *Journal of the Council for Scientific and Industrial Research*. 21, 177-180.
- Yang, E.V., Glasser, R., 2002. Stress-induced immunomodulation and implications for health. *International Immunopharmacology*. 2, 315-324.
- Yoshida, C., Nakao, T., 2006. Plasma cortisol and progesterone responses to low doses of adrenocorticotrophic hormone in ovariectomized lactating cows. *Journal of Reproduction and Development*. 6, 797-803.

Zajac, A.M., Conboy G.A., 2006. Veterinary Clinical Parasitology. Blackwell Publishing Professional, 2121 State Avenue, Ames, Iowa 50014. pp. 12-13.