BOVINE RESPIRATORY DISEASE: EFFECTS ON PERFORMANCE, IMMUNE RESPONSE, CYTOKINES AND GENE EXPRESSION

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INTRODUCTION

Bovine respiratory disease (**BRD**) is the most common and detrimental disease of beef cattle production during the post-weaning phase, causing approximately 75% of morbidity and over 50% of mortality in feedlots (Smith, 1998). Despite extensive research conducted over several years, BRD continues to result in the greatest economic loss to the beef industry. Although mortality due to BRD is a concern, morbidity of cattle most likely costs the industry more considering the expenses associated with medications, labor involved with treatment, premature culling due to chronic conditions, and the expense of reduced performance during and after the illness (Smith, 1998; Gagea et al., 2006).

Bovine respiratory disease is a multi-factorial disease. Environmental conditions, stressors, and active infection with a number of respiratory viruses can predispose cattle to pneumonia caused by several bacterial pathogens (Czuprynski et al., 2004). Predominant pathogens isolated from feedlot cattle suggest a viral/bacterial synergism between bovine viral diarrhea virus (**BVDV**), which predisposes cattle to a secondary bacterial infection with *Mannheimia haemolytica*, *Pasteurella multocida, Histophilus somni,* and *Mycobacterium bovis* . It is believed that the high prevalence of beef cattle herds infected with BVDV around the world may be a consequence of the ability of noncytopathic BVDV (**ncpBVDV**) to establish lifelong infections after *in utero* infection during early pregnancy, and thus generate a reservoir of persistently infected (**PI**) animals (Charleston et al., 2001).

Due to the detrimental effect of BVDV leading to secondary bacterial infections, the overall objective of the work presented herein was to determine the effects of timing of BVDV

exposure relative to a *M. haemolytica* challenge on short-term animal performance, immune response, and cytokine expression in economically important tissues of beef cattle.

CHAPTER II

LITTERATURE REVIEW

Bovine Respiratory Disease

Bovine respiratory disease is the most costly beef cattle disease in the United States. Costs associated with BRD prevention, treatment, morbidity, and mortality have been estimated from $13.90 (Snowder et al., 2006) to $15.57 (Faber et al., 1999) per animal. Babcock et al. 2009 indicated that BRD costs the beef industry more than $690 million annually. Decreased performance and poor carcass merit were associated with a decline of $23.23, $30.15, and $54.01 in carcass value when comparing cattle never treated to cattle treated once, twice, or three or more times, respectively, for clinical signs of BRD (Schneider et al., 2009).

Bovine respiratory disease results from a complex, multi-factorial interaction of stressors, animal susceptibility, and respiratory pathogens. The infectious agents (i.e., pathogens) related to BRD are found everywhere among cattle populations. Usually, one or a combination of stressors are necessary to initiate BRD (Cusack et al., 2007). Frequently, bacterial pneumonia is preceded by a viral respiratory infection. Bovine viral diarrhea virus, bovine herpesvirus-1 (**BHV-1**), also known as infectious bovine rhinotracheitis virus (**IBRV**), parainfluenza type 3 virus (**PI3V**), bovine respiratory syncytical virus (**BRSV**), *Mycoplasma bovis*, *M. haemolytica*, *Pasteurella multocida*, and *Haemophilus somnus* are the viruses and bacteria frequently associated with BRD. However, among the infectious agents, BVDV and *M. haemolytica* are the predominant pathogens isolated from BRD in feedlot cattle at necropsy (Fulton et al., 2005; 2006b; Katsuda et al., 2008).

Incidence of BRD has been reported in many studies with variability from 5% to 66% in feedlot cattle (Snowder et al., 2006). A report by Schneider et al. 2009 suggested, that the incidence of BRD increases sharply after 5 days of arrival to feedlots and can remain high until approximately 80 days on feed. A recent study analyzed records from 5,976 cattle and showed that 105 cattle died (1.43%) in the feedlot; 49% of deaths were associated with BRD, 40% of deaths were due to reasons not related to BRD, and 11% of deaths were connected to undetermined causes. An earlier study revealed that only 35% of steers received treatment for BRD from birth to harvest, whereas 72% had pulmonary lesions evident at slaughter (Wittum et al., 1996). A survey conducted in Australia showed that 68, 13, 57 and 27% of cattle entering the feedlot were serologically positive for BVDV, BHV1, PI3V and BRSV, respectively. All animals that were serologically negative at arrival were retested at harvest and 94, 76, 78 and 71% sero-converted to BVDV, BHV1, PI3V and BRSV, respectively. Only 10.3% of the cattle that serologically converted to one or more respiratory viruses had clinical signs detected (Cusack et al., 2003). These data indicate that there is continued need for research studying management practices with potential for preventing risk of BRD, and providing methods for early and objective diagnosis of this disease.

Impact of BRD on Animal Performance. The effects of BRD on subsequent performance in feedlot cattle have been investigated with varying results. It seems logical that animals that remain healthy during the entire feeding period would perform better than calves that suffered from BRD. Incidence of BRD in the feedlot has been reported to decrease average daily gain (**ADG**) during both the acclimation period (0.37 ± 0.03 kg/d) and the overall testing period (0.07 ± 0.01 kg/d; Schneider et al., 2009). Reports for a 28-day receiving period indicated that ADG was 0.23 kg/d (Smith, 1998) and 0.14 kg/d (Bateman et al., 1990) lower for calves that

became sick compared with calves that remained healthy. Citing health records, Bateman et al. (1990) and Wittum et al. (1996) reported that calves treated for BRD had 0.06 kg/d lower ADG than those not treated. A Canadian report (Morck et al., 1993) showed that calves experiencing a single episode of BRD had 0.18 kg/d lower ADG than those remaining healthy, and those sick two or more times had 0.33 kg/d lower ADG. The association between ADG and pulmonary lesions at harvest in feedlot cattle has been reported (Wittum et al., 1996). A decrease in ADG of 0.07 kg/d during the feeding period was associated with pulmonary lesions found at slaughter (Wittum et al., 1996). In addition, steers with presence of lung lesions had lower performance and carcass traits, including ADG, hot carcass weight (**HCW**), internal fat, and marbling scores compared with steers without lesions.

Interestingly, lung lesions were observed in almost an equal number of steers that received treatment for BRD (33%) and steers not treated (29%; Gardner et al., 1999). However, the greatest loss of production has been observed in cattle that had active bronchial lymph nodes at harvest (Gardner et al., 1999; Schneider et al., 2009). There is often a discrepancy between records of cattle treated for BRD and lung lesions at slaughter. Not all treated cattle will have lung lesions at slaughter and not all healthy cattle will be free of lung lesions. In steers treated for BRD, 78% had pulmonary lesions, whereas 68% of steers not treated also had pulmonary lesions (Wittum et al., 1996). Data from 1,665 cattle records revealed that 64.4% of cattle harvested had lung lesions (Schneider et al., 2009). The authors did not find influence of presence of lung lesions on performance and carcass traits considered in this study. However, incidence of BRD decreased HCW and marbling score by 8.16 ± 1.38 kg and 0.13 ± 0.04 marbling units, respectively, in treated cattle (Schneider et al., 2009).

Stress Factors. The effects of mixing calves at the auction barn, transportation, castration, and sorting at the feedyard may increase the incidence of BRD as a result of either commingling with unfamiliar cattle or by increasing the chance of contacting pathogens (Snowder et al., 2006; Rice et al., 2008). A study conducted at Oklahoma State University showed that calves from a single ranch origin had greater ADG than calves commingled at the feedlot or calves coming from an auction barn (Step et al., 2008). Weaning management program (weaned on the ranch or weaned and vaccinated on the ranch) did not have an effect on ADG. During the first 45 days after arrival at the feedlot, dry matter intake (**DMI**) was not affected by cattle origin. In addition, weaning and vaccination management pre- and post-arrival to the feedyard did not affect gain efficiency. Calves from a single source ranch were less likely to be treated for BRD than calves coming from an auction market. Calves from a single source and retained on the ranch for 45 days were healthier and consequently had lower health costs than calves coming from an auction market or weaned and immediately shipped to the feedlot (Step et al., 2008). These data suggest that preconditioning programs can decrease the incidence of BRD.

Bovine Viral Diarrhea Virus

Bovine viral diarrhea virus is a single stranded RNA virus member of the genus *Pestivirus* and the family *Flaviviridae* (Baker, 1995; Baule et al., 2001). Bovine viral diarrhea virus has been classified into biotypes and genotypes. Biotypes are based on their ability to induce a cytopathic effect in cell culture. There are two biotypes for BVDV: cytopathic and noncytopathic (Fulton et al., 2006). The noncytopathic biotype is the most common virus isolated from acute infections, while the cytopathic biotype is commonly found, together with

the noncytopathic biotype, in animals suffering from mucosal disease (**MD**). Mucosal disease occurs in PI animals with a noncytopathic biotype that become super-infected with a cytopathic biotype strain (Baule et al., 2001). In North America there are two genotypes based on antigenic characteristics: BVDV1 and BVDV2, and recently divided subgenotypes BVDV1a, BVDV1b, BVDV2a, and BVDV2b (Fulton et al., 2006). Noncytopathic BVDV and BVDV1 genotypes were the most commonly isolated from cattle, which died from fibrinous pneumonia causes. In addition, BVDV1 is the most common strain involved in acute respiratory disease of cattle in association with *M. haemolytica* and *P. multocida* (Robert et al., 2002).

Role of BVDV in Feedlot Cattle Health. Studies have suggested that persistently infected (**PI**) cattle are the main source of BVDV for surrounding susceptible cattle. Persistently infected cattle are calves that were infected *in utero* between 42 to 125 d of their gestation period. Persistently infected calves shed virus in all secretions throughout their lives. Fulton et al. (2005) reported that the primary BVDV strain in PI calves was BVDV1b, and 64.4% of cattle seroconvert to BVDV1b after being exposed (Fulton et al., 2005). Although seroconversion has been reported, Booker et al. (2008) reported that the presence of PI calves in feedlot pens does not always have a negative effect on animal health or performance. Supporting this finding, Elam et al. (2008) reported that exposure to a PI calf either for a short period (60 h) or for the entire feeding period (125 d) did not affect overall performance (BW, DMI, ADG and gain efficiency) in calves previously vaccinated, freshly weaned, and transport-stressed compared with non-exposed calves. A recent study by Hessman et al. (2009) analyzed the data from 15,348 cattle in 167 lots occupying 172 pens. The authors reported that the presence of BVDV PI cattle within pens resulted in an increase in cost of production caused by negative effects of the BVDV disease, an increased number of deaths, and diminished animal performance. However, when

the performance data was analyzed individually, they observed that some of the BVDV PI cattle penmates had acceptable performance and health outcomes.

The contradictory results among previous experiments could be due to the vaccination and prophylactic management programs used. For example, Fulton et al. (2005) commingled vaccinated with non-vaccinated calves, and calves were vaccinated 3 days prior to exposure to PI calves with either a modified live or killed viral vaccine. In the experiments of Booker et al. (2008) and Elam et al. (2008), all cattle were vaccinated with a modified live viral vaccine and received metaphylaxis. Therefore, one could speculate that less pathogenic effects were observed in the latter two experiments due to the vaccination program used and differences in health protocols followed.

Impact of BVDV on Secondary Infections. The importance of bacterial–viral synergism or interactions in BRD has been recognized for several years (Baker, 1995). It seems clear that viral-induced immune suppression is a significant factor that permits both viruses and bacteria to establish in the lungs, and there is increasing understanding of the details of the underlying mechanisms that are involved. In addition, psychological stressors on respiratory viral infections in mice lend credence to the possibility that similar complex interactions occur among the psychological, physical, and nutritional stressors associated with weaning and transport in calves. A disease model of BHV-1 respiratory infection followed by aerosol challenge with *M. haemolytica* produced clinical signs of the disease and a mortality rate of 30 to 70% (Hodgson et al., 2005). A combination of weaning and shipping immediately prior to BHV-1 infection and *M. haemolytica* challenge resulted in twofold greater mortality due to BRD (80%) in calves experiencing the combination of social reestablishment and transport compared with transportation alone (Hodgson et al., 2005). Analysis of gene expression in peripheral blood

mononuclear cells (**PBMC**) from calves following BHV-1 infection showed increased production of interferon (**IFN**), toll-like receptor 2 (**TLR2**) and TLR4. In addition, changes related with lipid metabolism, ion transport, and cell growth were observed (Hodgson et al., 2005). It was suggested that increased levels of serum corticosteroids enhanced immunosuppression as a consequence of the viral-bacterial synergy that resulted in fatal BRD following infection by *M. haemolytica* (Hodgson et al., 2005).

BVDV Immunosuppression. As mentioned co-infections of viruses and bacteria are important in the pathogenesis of BRD (Baker, 1995). Bovine viral diarrhea virus is a virus that can cause respiratory disease on its own (Fulton et al., 2005), and can also make animals more susceptible to bacterial infections (Baker, 1995). Acute BVDV in cattle shows transient immunosuppression as a result of the virus' predilection for cells that play critical roles in the host immune system. During the initial infection the virus has major effects on thymic and follicular T-lymphocytes, as well as follicular B-lymphocytes, often resulting in a severe decline in circulating numbers of lymphocytes and suppression of functional activities of these cells. Similarly, granulocytes and monocytes are highly susceptible to BVDV infections; BVDV infection results in a decrease in numbers and suppression of function (Brewoo et al., 2007). In addition, BVDV infection may have an important impact on cell-mediated immunity by inducing changes in the proportions of lymphocyte subpopulations in blood. The proportions of cytotoxic T-lymphocytes (**CD8+**) and helper T-lymphocytes (**CD4+**) decrease, while the workshop cluster 1 (**WC1+**) cells are considered to be unaffected by BVDV infection (Brodersen and Kelling, 1999). Cytotoxic T-lymphocytes secrete molecules that destroy the cell to which they have bound by cytotoxic effects on somatic infected cells. Helper T-lymphocytes are essential for both the cell-mediated and antibody-mediated branches of the immune system. These CD4+ cells

bind to antigen presented by antigen-presenting cells (APCs) like phagocytic macrophages and dendritic cells and can help B cells make antibodies against pathogens (Janeway et al., 2005). Fikri et al. (2000) described WC1+ cells as a major subset of bovine γδT-cells that express a bovine species-specific cell surface molecule and play an important role in the early stages of infection.

The impairment effects produced by acute BVDV infection occurred within 10 days of infection, with initial hyperplasia of the germinal centers of all lymphoid organs followed by lymphoid depletion (Baule et al., 2001). In addition, BVDV diminishes humoral antibody production, depresses monocyte chemotaxis, and compromises the myeloperoxidase antibacterial system in polymorphonuclear leukocytes (Cusack et al., 2003). An experiment in calves showed that total leukocytes, neutrophils, and lymphocytes decreased in calves inoculated with BVDV (Gånheim et al., 2005). In contrast, calves challenged with *M. haemolytica* had an increased number of leukocytes and neutrophils, while lymphocyte count decreased. However, calves that received the BVDV administration followed by *M. haemolytica* 5 days later had significantly lower total leukocyte and lymphocyte (remained lower throughout the experiment) counts. In addition, the numbers of CD8+, CD4+, and WC1+ lymphocytes decreased significantly after compared with before inoculation mainly in the BVDV and BVDV/*M. haemolytica* groups. The lower values were most pronounced in the BVDV/*M. haemolytica* group (Gånheim et al., 2005). It appears that BVDV enhances secondary colonization of the lungs by other BRD pathogens and exacerbates the pulmonary pathology they generate. The direct cytopathic effects of BVDV in the airways result in acute cattarrhal inflammation in the nasal cavity and trachea, and focal intralobular interstitial pneumonia (Baule et al., 2001).

Timing of Bacterial Challenge Following BVDV Inoculation. Gånheim et al. (2003)

observed significant differences in clinical signs and acute phase response, as measured by the acute phase proteins haptoglobin (**Hp**), serum amyloid A (**SAA**), and fibrinogen in calves infected with BVDV and *M. haemolytica* compared with calves infected with the infectious agents independently, emphasizing the importance of co-infections. Clinical signs were also recorded and were most severe in the BVDV/*M. haemolytica* group. The signs were mild to moderate in the BVDV group, while no or very mild signs were observed in the *M. haemolytica* group. The authors concluded that BVDV had a marked suppressive effect on several important lymphocyte subpopulations, which resulted in a poor immune response to *M. haemolytica* infection with negative effects on the clinical outcome.

In addition, timing of bacterial challenge relative to PI BVDV exposure may play a role BRD pathogenesis. Burciaga-Robles et al. (2009) reported low antibody production to *M. haemolytica leukotoxin* in cattle previously infected with BVDV 12 hours prior to *M. haemolytica* challenge. In addition, neutrophil concentrations were decreased by BVDV in steers exposed to PI BVDV cattle. Zhang et al. (1997) observed that lymphocyte subset differentiation showed a significant reduction in the $CD4^+$ T-cell concentration in relation with CD8 in calves infected with bovine immunodeficiency virus. This coincided with a rapid replication of bovine immunodeficiency virus, suggesting immunosuppression during the three to seven weeks post bovine immunodeficiency virus inoculation. In addition, antibody production in response to vaccination with BVDV and herpes virus-1 was dcreased from days 11 to 65 and from days 9 to 35, respectively (Zhang et al., 1997). Based in these studies we could speculate that virus infection produces immunosuppression compromising the immune response to a secondary infection.

Mannheimia Haemolytica

Mannheimia haemolytica is a Gram-negative, non-motile, non-spore forming, fermentative, oxidative-positive, facultative anaerobic coccobacillus and member of the family *Pasteurellaceae*, genus *Mannheimia* (Rice et al., 2008). *Mannheimia haemolytica* lives in the nasopharynx and tonsils of healthy cattle as a commensal organism, where it preserves a symbiotic relationship with its host (Deshpande et al., 2002; Narayanan et al., 2002; Rice et al., 2008). However, when animals experience stress due to shipping or other causes, *M. haemolytica* multiplies rapidly, reaches the lungs during inhalation, and sets up an active infection of the alveolar epithelium (Narayanan et al., 2002). *Mannheimia haemolytica* is the principal bacterium isolated from respiratory disease in feedlot cattle (Rice et al., 2008). Serotype 1 and lately serotype 6 have been the most frequently isolated serotypes from bovine pneumonic lungs (Davies et al., 2001; Deshpande et al., 2002; Ewers et al., 2004). An earlier study suggested that *M. haemolytica* serotype 2 (less pathogenic) switched to serotype 1 (more pathogenic) in 80 to 100% of the pnemonia cases (Corbiel and Gogolewski, 1985). *Mannheimia haemolytica* causes extensive economic losses to the global cattle industry, and particularly in North America (Deshpande et al., 2002; Rice et al., 2008), Germany (Ewers et al., 2004), and Japan (Katsuda et al., 2008). Characteristic lesions for this disease result from the strong influx of neutrophils accompanied by accumulation of fibrin, finally causing necrosis of the alveolar spaces (Ewers et al., 2004).

Many studies have attempted to understand the mechanisms associated with the *M. haemolytica* switch from commensal to pathogen. Cold stress and transportation were demonstrated to cause an incremental increase in cortisol levels with inhibition of lymphocyte blastogenesis (Rice et al., 2008). The complement system is a major effecter system in innate immunity, and its activation by the classical, alternative or lectin pathways generates opsonins,

inflammatory mediators and cytolytic protein complexes. Complement immune response is thought to be compromised in calves coming from auction barns, and the compromised immune system could alter the *M. haemolytica* equilibrium with the host (Rice et al., 2008). If viral and bacterial agents break down the antimicrobial barrier of β-defensins within the host and serous and mucous secretions within the respiratory tract, *M. haemolytica* will pass from a commensal microorganism to pathogenic bacteria (Rice et al., 2008).

Physical Barriers. The respiratory epithelial surface constitutes the first line of defense against the pathogens of BRD. It provides mechanical, chemical and microbiological barriers to infection (Janeway et al., 2005). The pathogens attempt to establish an infection by adhering to and colonizing surfaces. The respiratory epithelium secretes mucus, which coats the pathogens preventing their adherence. More importantly, the pathogens are expelled in the flow of mucus driven by the constant upward movement of the epithelial cilia. The surfactant proteins A and D that bathe the lung epithelial surfaces coat the surfaces of pathogens and make them more susceptible to phagocytosis by macrophages and neutrophils (Srikumaran et al., 2007). *Mannheimia haemolytica* may overcome the clearance mechanism through the ability to break down mucus via neurominidase or extracellular proteases, making the mucus less viscous. Compromised adherence to mucus could increase colonization (Corbiel and Gogolewski, 1985). Therefore, damage to the epithelium would be clearly advantageous for the pathogens (Srikumaran et al., 2007).

After serotype 1 strains have colonized the bovine upper respiratory tract they replace other serotypes by mechanisms unknown to date (Ewers et al., 2004). Ultimately, bacteria reach alveolar spaces leading to the beginning of an inflammatory cascade by *M. haemolytica*

leukotoxins (**LKT**) and lipopolysaccharide (**LPS**), causing activation of the complement system and release of cytokines (Ewers et al., 2004).

Phagocytosis. When the microorganisms cross the natural barriers and begin to replicate within the lung tissue of the host they are immediately recognized by the alveolar macrophages (**AM**) also called resident macrophages (Janaway et al., 2005). Macrophages are located in the submucosal tissues, and are the first cells that encounter the pathogens (Liggitt, 1985). Macrophages continuously mature from blood monocytes that leave the circulation and migrate into various tissues (Srikumaran et al., 2007). Neutrophils, also known as polymorphonuclear neutrophils (**PMN**), are abundant in blood and not present in healthy tissue. Both AM and PMN play a central role in innate immunity to recognize, ingest and destroy many pathogens. Macrophages recognize pathogens by means of their cell-surface (Janaway et al., 2005).

Toxins of Mannheimia haemolytica. *Mannheimia haemolytica* serotype 1 produces a variety of virulence factors that play an important role during the pathogenesis of bovine pneumonic pasteurellosis. Among these, LKT and LPS have recently been demonstrated to be the primary virulence factors of the pneumonia pasteurellosis pathogenesis (Rice et al., 2008). The *M. haemolytica* leukotoxin is a member of the family of exotoxins produced by gram-negative bacteria called repeats in toxins (**RTX**). The LKT production increments are a result of the bacterial growth being higher during the logarithmic phase when the pulmonary disease is taking effect. The toxin is a protein that can occur singly or as a polymer, and it has specific toxic effects on ruminant leukocytes, mainly PMN, with wide pathological effects resulting in acute fibrinous pleuropneumonia (Deshpande et al., 2002; Narayanan et al., 2002).

Recent evidence suggests that *M. haemolytica* LKT binding to bovine leukocytes is mediated by the β2-integrin CD11a/CD18, which subsequently induces activation and death of

these cells (Leite et al., 2003). The β2-integrins have a common β-subunit, CD18, which associates with three distinct β chains, CD11a, CD11b, and CD11c, to generate three different β2-integrins, CD11a/CD18 (**LFA-1**), CD11b/CD18 (**Mac-1**), and CD11c/CD18 (**CR4**), respectively. *Mannheimia haemolytica* LKT binds to all three β2-integrins confirming that bovine CD18 is necessary to mediate LKT-induced cytolysis of ruminant leukocytes (Jeyaseelan et al., 2000; Deshpande et al., 2002; Dassanayake et al., 2007).

Leukotoxins of Mannheimia haemolytica. Binding LKT to CD18 stimulates signaling events leading to elevation of intracellular [Ca^{2+}], tyrosine phosphorylation of the cytosolytic domain of CD18, and cytolysis of bovine leukocytes (Jeyaseelan et al., 2000; Dassanayake et al., 2007; Lawrece et al., 2008). Once LKT of *M. haemolytica* utilizes the cell adhesion molecule LFA-1 to cause activation and cytolysis of neutrophils and macrophages in the alveolar spaces, it triggers the production and accumulation of proinflammatory mediators with a continuous intra-alveolar replication of *M. haemolytica*. These events result in an uncontrollable inflammatory response leading to lung injury that is characteristic of bovine pneumonic pasteurellosis (Jeyaseelan et al., 2000). Lymphocyte function-associated antigen 1 is a binding receptor for *M. haemolytica* that allows pore-forming on the lymphocyte's membrane surface (Lally et al., 1997), leading to the initiation of cytolysis with increasing intracellular [Ca2+] (Lawrence et al., 2007). Increased LFA-1 expression by PMN exposed to LKT and LPS was associated with increased LKT binding and cell death (Leite et al., 2003). Leukotoxin activity against target cells is dose-dependent. As the LKT concentration increases the cell damage goes from respiratory burst to degranulation and apoptosis. Apoptosis is due to membrane pore formation damage (Narayanan et al, 2002).

Lipopolyssaccharide (LPS). Lipopolysaccharide is a component of gram-negative bacteria outer membrane and is considered an endotoxin. Lipopolysaccharide as well as LKT increases intracellular [Ca2+] via phospholipase C and protein tyrosine kinase signal transduction pathways. Activation of both signal transduction pathways leads to a calcium-dependent activation of transcription factor NFκB. Activation of NFκB leads to increased expression and secretion of interleukin (**IL**)-1β and tumor necrosis factor α (**TNFα**) suggesting that these proinflammatory cytokines play an important role in lung injury in pneumonic cattle (Yoo et al., 1995). Studies from 1985 through 2009 support that outer membrane protein A and lipoprotein 1 contribute to adherence of *M. haemolytica* to bovine respiratory epithelial cells (Kisiela and Czuprynski, 2009). Lipoprotein 1 is a portion of the center of the LPS molecule which results in lung tissue damage and toxicity. Endotoxins activate the complement of coagulation cascades leading to vessel dilation. Vascular permeability and coagulation leads to accumulation of inflammatory cells, edema, and both intravascular and extravascular fibrin in the lung. Endotoxin also activates granulocytes and macrophages which leads to protection against bacteria that contain endotoxins and to increased tissue damage (Corbiel and Gogolewski, 1985).

Lipopolysaccharide and Cytokine Production. Both TNFα and IL-1β mRNA expression in bovine alveolar macrophages are stimulated by the production of LPS coming from *M. haemolytica* (Yoo et al., 1995). Calves challenged intratracheally with *M. haemolytica* had increased concentrations of TNFα that reached a peak 2 h after inoculation but returned to undetectable levels after an additional 4 h (Horadagoda et al., 1994; Yoo et al., 1995). Similar results were observed in macrophage cell cultures where expression of TNFα and IL-1β reached a peak 1 h after LPS stimulation and returned to undetectable levels after 24 h. This experiment also showed that gene expression of cytokines is LPS dose dependent (Yoo et al., 1995). These

results support a role for LPS from *M. haemolytica* in the induction of inflammatory cytokines in bovine pneumonic pasteurellosis. Other experiments have indicated that LKT main effects were in bovine monocytes, with minor effects on alveolar macrophages releasing cytokines IL-1 and TNFα (Stevens and Czuprynski, 1995). These authors suggested the formation of a complex synergism between LKT and LPS. Exposure of bovine PMN to LKT or LPS induces expression of inflammatory cytokines, which in turn can increase LFA-1 expression and conformational activation (Leite et al., 2003).

Cytokines

Interferons are antiviral proteins produced by cells in response to viral infection (Janeway et al., 2005). Interferon is also considered the most important innate immune defense antiviral cytokine (Chase et al., 2004). The production of α/β-IFN is the first line of defense from the host against viral infections during the double-replication (dsRNA) phase (Iqbal et al., 2004). In vitro studies have shown that neither cpBVDV nor ncpBVDV infection induces IFN response in cell cultures, and induction of IFN-stimulated genes has been blocked by paramyxovirus (Chen et al., 2007). In contrast to in vitro studies, trials developed in calves infected with ncpBVDV showed strong α/β and γ IFN response in gnotobiotic animals. The response was related to low concentrations of transforming growth factor-β (**TGF-β**) in serum. Therefore, it appears that immunosuppression by ncpBVDV is not caused by low IFN response or high levels of TGF-β in vivo (Carleston et al., 2001). The α/β IFN have three major functions. First, they induce resistance to viral replication in uninfected cells by activating genes that cause the destruction of mRNA and inhibit the translation of viral and some host proteins. Second, they enhance major histocompatability I (**MHC I**) expression in somatic cells, consequently improving their resistance to natural killer (**NK**) cells. They may also stimulate synthesis of MHC I molecules in

newly virus-infected cells, thus making them more susceptible to killing by CD8, cytotoxic T cells. Third, they activate NK cells, which then kill virus-infected cells selectively (Peterhands et al., 2003; Janaway et al., 2005).

The action of toll-like receptors (**TLR**) is to activate phagocytes and tissue dendritic cells in response to insults by secreting chemokines and cytokines, and to express the co-stimulatory substances that in turn will activate adaptive immunity (Janaway et al., 2005). Toll-like receptor 4 on macrophages signals the presence of LPS when LPS associates with CD14, the macrophage receptor for LPS (Janaway et al., 2005). The binding of bacterial LPS activates TLR4 on macrophages. Toll-like receptors are built of transmembrane receptors that contain extracellular leucine-rich repeat (**LRR**) motifs and an internal toll/interleukin-1 receptor (**TIR**) domain, which is essential for the activation of NFκB. Myeloid differentiated gene 88 (**MyD88**) interacts with IL-1 receptor associated kinase (**IRAK**) and TNF receptor-associated factor 6 (**TRAF6**) which in turn activate NFκB and MAPKs. Then COX-2 and pro-inflammatory cytokines are activated (Lee et al., 2003; Janaway et al., 2005). Ultimately, the activation of TLR4 by LPS stimulates macrophages to produce important cytokines such as TNFα, IL-8, IL-1β, IL-6 and IL-12. Tumor necrosis factor-α is an inducer of local inflammatory response that helps to contain infections; it also has systemic effects, many of which are harmful. Interleukin-8 is also involved in the local inflammatory response and is a chemotactic for monocytes, neutrophils and T lymphocytes to the site of infection (Zhu et al., 2003). Interleukin-1β, IL-6 and TNFα have a crucial role in inducing the acute-phase response in the liver and induce fever, which favors effective host defense. The cytokine IL-12 is essential in many immune activities including activation and production of IFNγ and NK cells, and favors the differentiation of CD4 T cells

into the Th1 subset during an adaptive immune response (Lafleur et al., 2001; Janaway et al., 2005).

Cytokines Released by Phagocytes. Cytokines produced by macrophages contribute to host defense by elevation of body temperature, which is caused mainly by TNFα, IL-1β, and IL-6, termed endogenous pyrogens. Fever is generally beneficial to host defense; most pathogens grow better at lower temperatures and adaptive immune responses are more intense at elevated temperatures. These cytokines act on the hypothalamus, altering the body's temperature regulation, and on muscle and fat cells, altering energy mobilization to increase body temperature (Janaway et al., 2005). Cytokines TNFα, IL-1β, and IL-6 activate hepatocytes to synthesize acute phase proteins (**APP**), and bone marrow endothelium to release neutrophils. The APP act as opsonins, whereas the disposal of opsonized pathogens is augmented by the enhanced recruitment of the neutrophils from the bone marrow.

Acute Phase Proteins

The acute phase response is a set of reactions which occur as an early protection mechanism of the host against infection. Pathogens are encountered by macrophages, and then macrophages release cytokines (IL-1, IL-6 and TNFα) which travel via the bloodstream to the liver, where they stimulate liver cells to initiate APP synthesis (Janaway et al., 2005). Both Hp and SAA are APP that have been studied in cattle (Nikunen et al., 2007). Haptoglobin concentrations in healthy cattle are often undetectable but during an acute phase response bovine haptoglobin can increase 50 ± 100 fold, making it the most prominent APP in cattle. Serum amyloid-A is a moderate APP in cattle increasing around 2 ± 5 fold during an acute phase response (Heegaard et al., 2000). Serum amyloid-A is considered a rapid bovine APP in response to *M. haemolytica* infections (Horadagoda et al., 1994). The acute phase response is

thought to be beneficial to the injured organism with the aim of restoring the disturbed physiological homeostasis (Heinrich et al., 1990). However, Nikunen et al. (2007) mentioned that functions of APP in host defense during inflammation are not fully understood (Nikunen et al., 2007). Some imitate the actions of antibodies, but unlike antibodies, these proteins have broad specificity for pathogen-associated molecular patterns (Janaway et al., 2005).

Heinrich et al. (1990) described that the acute phase response is characterized by localized changes such as the aggregation of platelets and clot formation, dilatation and leakage of blood vessels, accumulation of leukocytes, and activation of stromal cells to release biological response modifiers. The release of mediators by resident and infiltrating cells then results in the initiation of systemic responses, including fever, leukocytosis, activation of complement and clotting systems, alterations in the plasma concentration of trace minerals, and changes in liver metabolism, including the production of APP (Heinrich et al., 1990). Alternatively, measurement of the acute phase response may reflect the severity of the disease process and serve as a prognostic indicator. Assessment of the acute phase response during BRD could be a valuable aid in the diagnosis and prognosis of this condition (Godson et al., 1996).

Conclusions

Several immune evasion/immunosuppressive strategies have been developed by the pathogens of BRD. These mechanisms developed by one pathogen not only help that pathogen, but also the others, resulting in exacerbation of the disease. For example, when innate immune function is compromised with BVDV infection, it leads to dysfunction of leukocytes that in turn causes immunosuppression and allows *M. haemolytica* to migrate to the lungs, proliferate, elaborate the virulence determinants and cause disease. Infection of the cells of the immune

system by BVDV, inhibition of proliferation of the cells by BVDV, and induction of apoptosis compromise the orchestration of the immune response against the pathogens. Production of IFNs is the first line of defense from the host against viral infections, and it appears that immunosuppression by ncpBVDV does not result from lack of IFN response. Cytolysis of all the subsets of leukocytes by *M. haemolytica* leukotoxin eliminates the cells that are critical for mounting the innate and adaptive immune responses. In addition, it results in the release of the toxic chemicals that cause acute damage to the pulmonary epithelium.

Bovine respiratory disease is indeed a complex disease syndrome. The severity of the disease and the rate of mortality in a herd very likely depend on the host immune system, microorganism pathogenesis and abundance, and the number of pathogens infecting the animals concurrently. The complex etiology and the multitude of immune evasion strategies developed by these pathogens probably explain the failure of vaccines to provide complete protection against this disease complex. Given the complex etiology of BRD attributed to BVDV and *M. haemolytica*, it is unlikely that any single strategy will be completely effective in preventing the disease. A combination of more definitive diagnostic methods, more efficacious vaccines, improved therapeutic agents and better management practices will be needed, and the search for solutions is likely to involve researchers for some time to come.

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Effect of timing of *Mannheimia haemolytica* challenge following short-term (72 hour) exposure to bovine viral diarrhea virus type 1b on animal performance and immune reponse in beef steers

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ABSTRACT:

Objectives - To determine the effects of timing of an intratracheal *Mannheimia haemolytica* challenge relative to 72-hour exposure to bovine viral diarrhea type 1b (BVDV1b) persistently infected calves (PI) on serum antibody production, total and differential white blood cell count (WBC), clinical signs, and performance of feedlot steers.

Animals, Procedures and Experimental Design - Twenty-four steers (initial BW = 276 ± 31 kg) were randomly allocated to one of three treatments (eight animals/treatment) in a randomized complete block design. Treatments were: 1) steers not challenged with BVDV or *M. haemolytica* (**CON**); 2) steers intratracheally challenged with *M. haemolytica* 84 hours after being exposed to calves PI with BVDV1b for 72 hours (**LateCh**); and 3) steers intratracheally challenged with *M. haemolytica* 12 hours after being exposed to calves PI with BVDV1b for 72 hours (**EarlyCh**).

Results - Delaying the *M. haemolytica* challenge for 84 hours after exposure to calves PI with BVDV increased clinical attitude of BRD and the acute phase response compared with delaying *M. haemolytica* challenge for 12 hours after BVDV exposure. The increased clinical and acute phase response were associate with decreased did not affect short-term performance.

Conclusions - Timing of BVDV exposure relative to a *M. haemolytica* challenge influences immune response in growing beef cattle.

Clinical Relevance - Understanding the physiological changes in morbid animals will lead to improved strategies for decreasing severity and economic losses associated with BRD.

Key Words - Herd health-beef, Bovine respiratory tract disease, Bovine viral diarrhea virus, Immunity to bacteria

INTRODUCTION

Bovine respiratory disease (**BRD**) is the most common and economically detrimental disease of cattle during the post-weaning period, causing approximately 75% of morbidity and over 50% of mortality in feedlots (Smith, 1998). Regardless of extensive research conducted over several years, BRD remains the most detrimental disease in the beef cattle industry. Although mortality due to BRD is a concern, morbidity of cattle most likely costs the industry more considering the expenses associated with medications, labor involved with treatment, premature culling due to chronic conditions and the expense of decreased performance and carcass quality during and after the illness (Smith, 1998; Gardner et al., 1999; Gagea et al., 2006). Bovine respiratory disease is a multi-factorial disease that involves environmental conditions and other stressors, and active infection with a number of respiratory viruses could predispose cattle to pneumonia from several bacterial pathogens (Czuprynski et al., 2004). A predominant viral/bacterial synergism that exists between pathogens isolated from feedlot cattle includes bovine viral diarrhea virus (**BVDV**)**,** which can predispose cattle to a secondary bacterial infection including *Mannheimia haemolytica*. It is believed that the high prevalence of beef cattle herds infected with BVDV around the world may be a consequence of the ability of noncytopathic BVDV (**ncpBVDV**) to establish lifelong infections after *in utero* infection during early pregnancy, and thus generate a reservoir of persistently infected (**PI**) animals (Charleston et al., 2001).

A recent experiment evaluated the effects of an intratracheal challenge with *M. haemolytica* with or without previous exposure (72 hours) to steers PI with BVDV1b (Burciaga-Robles et al., 2009a). We observed changes in serum antibody production, total and differential white blood cell count (WBC), cytokine concentrations, and blood gases consistent with an

immune challenge in beef cattle. Exposure to calves PI with BVDV type 1b and intratracheal challenge with *M. haemolytica* resulted in decreased antibody production to *M. haemolytica* leukotoxin and increased serum concentrations of IFNγ, IL-1β and TNFα, suggesting that exposure of naïve calves to PI calves increases potential for secondary infections and detriment to animal health and performance. Decreased rectal temperature and lymphocytes were observed from 36 to 72 and 18 to 96 hours, respectively, after the *M. haemolytica* challenge in steers that were exposed to steers PI with BVDV1b. Therefore, we hypothesized that steers exposed to BVDV 1b for 72 hours, and challenge with *M. haemolytica* 84 hours latter would have a lower immune responses than steers receiving the *M. haemolytica* challenge immediately (12 hours) after exposer to BVDV 1b. Our objective was to determine the effects of timing of BVDV exposure relative to a *M. haemolytica* challenge on animal performance and immune response in growing beef cattle.

MATERIALS AND METHODS

Animals

All procedures were approved by the Oklahoma State University Institutional Animal Care and Use Committee (Protocol# AG0616). A total of 24 Angus crossbred steers (initial BW = 276 ± 31 kg) were housed at the Nutrition Physiology Research Center, Oklahoma State University, Stillwater to determine the effects of timing of bacterial challenge relative to viral exposure on performance and immune response in receiving cattle. All animals were considered clinically healthy and were seronegative to all pathogens involved in the study as determined with paired serum samples collected 14 days apart prior to the start of the experiment.

Treatments

The 24 steers were randomly allocated to one of three treatments (8 steers/treatment) arranged as a randomized complete block design. Treatments were: 1) steers not challenged with BVDV or *M. haemolytica* (**CON**); 2) steers intratracheally challenged with *M. haemolytica* 84 hours after being exposed to calves persistently infected (**PI**) with BVDV1b for 72 hours (**LateCh**); and 3) steers intratracheally challenged with *M. haemolytica* 12 hours after being exposed to calves PI with BVDV1b for 72 hours (**EarlyCh**). To facilitate sample collection, steers were blocked by body weight (**BW**) into 2 groups of twelve and the challenge procedures and sample collections were staggered by a 2 week interval between periods. Steers exposed to the calves PI with BVDV were transported approximately 3.2 km to the Willard Sparks Beef Research Center, Stillwater, OK where they were commingled in a 6 × 10.8 m pen with 2 steers previously confirmed as being PI with BVDV1b via immuohistochemistry and genotyping (Fulton et al., 2006). The PI subtype was determined by sequencing the 5'-untranslated region. Steers not exposed to calves PI with BVDV1b were not transported the short distance to prevent risk of exposure to the virus. For both the EarlyCh and LateCh groups, the length of exposure to the PI calves was 72 hours. After the time of BVDV exposure, calves were returned to the Nutrition Physiology Research Center where they remained for the remainder of the experiment. Steers challenged with *M. haemolytica* received 10 mL of a solution containing 6×10^9 CFU of *M. haemolytica* serotype 1 that was reconstituted and grown prior to the challenge as described by Mosier et al. (1998). Steers not challenged with *M. haemolytica* (CON) were intratracheally dosed with 10 mL of a phosphate–buffered saline (**PBS**) solution (pH 7.4; Sigma Aldrich, St. Louis, MO). Inoculation with the *M. haemolytica* culture or PBS solution was performed as described by Dowling et al. (2002) with modifications as described by Burciaga-Robles et al.

(2009a). Challenge with *M. haemolytica* occurred on the same day for all appropriate treatment groups beginning at 0800.

The experiment consisted of 24 days during which the animals were kept in individual pens (3.7 × 3.7 m) with the exception of days 0 to 4. During those days, animals were placed in metabolic stanchions to allow for the challenge procedures and collection of blood samples (Burciaga-Robles et al., 2009a). During the experiment, steers were offered feed at 3% of the BW; feed was delivered twice daily. The diet (Table 1) was formulated to meet or exceed nutrient requirements (NRC, 1996). Animals were weighed on day -7, 0, 4, and 17. Average daily gain was calculated using body weight and days on feed and gain-to-feed was calculated using dry matter intake for the corresponding periods.

Sample Collection

Rectal Temperature, Respiration Rate, and Subjective Clinical Attitude Score. Rectal temperatures were recorded using a digital veterinary thermometer (GLA M-500; GLA Agricultural Electronics, San Luis Obispo, CA) and respiration rates were measured by counting flank movements for 1 min with a stopwatch as described by Legates et al. (1991). In addition, all steers were monitored by trained personnel throughout the length of the experiment for clinical signs of morbidity. The clinical evaluation used has been described by Step et al. (2008). Briefly, the subjective criteria included depression (e.g., hanging head, sunken eyes, arched back and difficulty getting up from lying down), abnormal appetite, and respiratory signs (e.g., labored breathing). Based on the severity of signs, the evaluator assigned a numeric score ranging from 1 to 4, where 1 was assigned for mild, 2 for moderate, 3 for severe, and 4 for moribund (steer would not rise from recumbency or assistance was needed). Rectal temperature,

respiration rate, and subjective clinical attitude score were recorded prior to BVDV exposure and at -96, -2, 2, 4, 6, 8, 12, 18, 24, 36, 48, 72 and 96 hours following the *M. haemolytica* challenge.

Serum Haptoglobin. Blood samples (Clott activator, Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ) were collected via jugular venipuncture with an 18 × 1 gauge needle (-96 -2, 2, 6, 12, 18, 24, 36, 48, 72, 96 and 168 hours following the 0 hour *M. haemolytica* challenge). Samples collected were allowed to clot for 24 hours at 4^{o}C. After the clotting time, chilled blood samples were centrifuged at 3,000 × g at 4^{o}C for 20 min. Serum was harvested in 2 mL centrifuge tubes and stored at -20^{o}C until further analyses were performed. Once all the serum samples were collected, a Bovine Haptoglobin ELISA test (Immunology Consultants Lab, Portland OR) was used to determine the haptoglobin concentration of each serum sample. Prior to the analysis, serum samples were diluted 1:10,000 in Tris-buffered saline with Tween 20 (pH 4.0; Sigma-Aldrich, St Louis, MO). The intra- and inter-assay coefficients of variation were below 5%.

Hemogram. Blood samples (EDTA, Becton Dickinson Vacutainer Systems) were collected via jugular venipuncture with an 18 guage needle at -168, -96, -12, -2, 6, 18, 72, 96, 168 and 336 hours following the *M. haemolytica* challenge. Samples were immediately submitted to a commercial lab (Antech Diagnostics, Stillwater, OK) for total and differential white blood cell count determination, total red blood cells, total platelets, hemoglobin, hematocrit, mean corpuscular volume (**MCV**), and mean corpuscular hemoglobin (**MCH**) concentration.

Mannheimia haemolytica Antibodies. Serum samples from day -7, 0, 4, 7, and 14 were used to determine antibodies to whole bacterial cell (**WC**) and leukotoxin (**LKT**) for a formalin killed *M. haemolytica* serotype 1 by an ELISA test as described by Confer et al. (1995; 1996).

Antibody responses were expressed as nanograms of immunoglobulin binding based on a set of IgG standards on each plate. The intra- and inter-assay coefficients of variation were below 5%.

Bovine Viral Diarrhea Virus Antibodies. Serum samples from days -7, 0, 4, 7, 14, 28 and 42 were submitted to the Oklahoma Animal Disease and Diagnostic Laboratory where a virus neutralization test in Madin–Darby bovine kidney cell monolayers in 96-well microtiter plates was used to quantify virus-neutralizing antibodies to BVDV. The viruses used were CP BVDV1a (singer strain), CP BVDV1b (TGAC 8HB), and CP BVDV2a (125-C). A 1:4 dilution was the lowest tested, and titers of less than 1:4 were considered negative.

Statistical Analyses

The experiment was designed as a randomized complete block; animal served as the experimental unit. Data for BVDV antibody titers, *M. haemolytica* whole cell and leukotoxin antibody titers, rectal temperature, clinical attitude score, and haptoglobin concentrations were analyzed using repeated measures analysis of the MIXED procedure of SAS with a non-structured covariance structure and slice output option (SAS Inst., Inc., Cary, NC). The model for all variables included the main effects of treatment (CON, EarlyCh and LateCh) and their interactions with time. When a EarlyCh and or LateCh × time interaction was significant ($P \leq 0.05$), the slice output option was used to determine the time points at which the time effect was different across treatments. Least squares means were separated using the pdiff statement of SAS.

RESULTS

Body weight was not affected ($P \geq 0.67$) by timing of *M. haemolytica* challenge in relation to BVDV exposure (Table 2). From day 0 to 4, EarlyCh and LateCh steers had lower (*P*

= 0.0004) ADG than CON steers. From day 5 to 17, LateCh steers compensated and had greater (P = 0.01) ADG than CON and EarlyCh steers. Across the entire period, EarlyCh steers had lower (P = 0.03) ADG than CON and LateCh steers. From day 0 to 4, dry matter intake (kg/d) was greatest (P = 0.0005) for CON steers, intermediate for LateCh steers, and lowest for EarlyCh steers. In addition, exposure to steers PI with BVDV for 72 hours decreased (P = 0.02) dry matter intake as a percent of BW from day 0 to 4, regardless of timing of *M. haemolytica* challenge. From day 0 to 4, gain:feed was lower (P = 0.001) for steers exposed to steers PI with BVDV compared with CON steers. Across the entire experiment, gain:feed was greatest (P = 0.03) for CON steers, intermediate for LateCh steers, and lowest for EarlyCh steers.

There was a treatment × time interaction (P < 0.0001) for BVDV antibody titers (Figure 1). Antibody titers increased across time for steers exposed to steers PI with BVDV, and on d 28 and 42, BVDV antibody titers were greater (P < 0.05) for steers exposed to BVDV compared with CON steers. In addition, BVDV antibody titers were greater (P < 0.05) for LateCh compared with EarlyCh steers. There was a treatment × time interaction (P < 0.0001) for *M. haemolytica* whole cell (Figure 2a) and leukotoxin (Figure 2b) antibodies. Antibody titers for *M. haemolytica* whole cells and leukotoxin increased across time for steers challenged with *M. haemolytica*, and were greater on d 7 and 14 following *M. haemolytica* challenge compared with CON steers.

Rectal temperatures were greater for challenged steers from 4 to 72 hours following the challenge compared with CON steers (treatment × time interaction, P < 0.0001; Figure 3). In addition, rectal temperature was greater for LateCh compared with EarlyCh steers from 12 to 18 hours after the *M. haemolytica* challenge. Subjective clinical attitude score responded with a treatment × time interaction (P < 0.0001; Figure 4). Challenged steers had a greater subjective

clinical attitude score than CON steers beginning at 4 (LateCh) or 12 (EarlyCh) hours post *M. haemolytica* challenge, and continuing through 96 h. In addition, subjective clinical attitude score was greater for LateCh compared with EarlyCh steers from 8 to 96 hours after the challenge. Similar to rectal temperature and clinical attitude score, haptoglobin concentrations responded with a treatment × time interaction ($P < 0.0001$; Figure 5). Haptoglobin concentrations were greater for steers exposed to steers PI with BVDV and challenged with *M. haemolytica* from 18 to 168 h following the challenge, and were greater ($P < 0.05$) for LateCh compared with EarlyCh steers from 48 to 96 hours after the *M. haemolytica* challenge.

There was a treatment × time interaction ($P = 0.007$) for total white blood cells (Figure 6a). Steers in the LateCh group had lower ($P < 0.05$) white blood cells at -24 and -2 hours of *M. haemolytica* challenge, whereas steers in the EarlyCh group had lower ($P < 0.05$) white blood cells at 72 and 96 hours after *M. haemolytica* challenge compared with steers on the remaining treatments. Both EarlyCh and LateCh steers had greater ($P < 0.05$) total white blood cells than CON steers 18 hours after *M. haemolytica* challenge. There was a BVD × MH interaction ($P = 0.02$) for neutrophils (Figure 6b). Neutrophils were lower ($P < 0.05$) in CON and LateCh steers compared with EarlyCh steers at -12 hours, and remained lower for LateCh steers at time -2 hours. Similar to the total white blood cells, both EarlyCh and LateCh steers had greater ($P < 0.05$) total white blood cells than CON steers 18 hours after *M. haemolytica* challenge. At 96 hours after *M. haemolytica* challenge, EarlyCh steers had lower ($P < 0.05$) neutrophils than CON and LateCh steers. Lymphoctes were lower ($P < 0.05$) for LateCh steers beginning at -12 hours, and for EarlyCh steers beginning at -2 hours compared with CON steers (Figure 6c). Lymphocytes remained lower ($P < 0.05$) through 72 hours for LateCh and 96 hours for EarlyCh steers compared with CON.

Monocytes, eosinophils, and basophils were not affected ($P \geq 0.20$) by exposure to BVDV and challenge with *M. haemolytica* (Table 3). Similarly, hematocrits, hemoglobin, mean corpuscular volume, and mean corpuscular hemoglobin were not affected ($P \geq 0.22$) by treatments. Concentration of platelets were greatest ($P = 0.001$) for CON, intermediate for EarlyCh, and lowest for LateCh. Red cells tended ($P = 0.08$) to be affected by treatments.

DISCUSSION

Experiments have evaluated the effects of PI BVDV calves in feedyards as a main source of BVDV infection among sero-negative cattle within pens. Fulton et al. (2005) used vaccinated and non-vaccinated calves exposed to calves PI with BVDV1b to determine infection with BVDV in the feedyard. When a PI animal was included in a pen, seroconversion occurred in 70% to 100% of non-vaccinated penmates (Fulton et al., 2005). Our results confirm those of prior studies demonstrating that PI calves can serve as a natural method of challenge to sero-negative BVDV steers for immunological studies. In addition, we confirmed that PI BVDV1b calves can serve as an effective source of infection to healthy animals as an experimental model (Burciaga-Robles et al., 2009a). We detected seroconversion to BVDV1b for all steers exposed to PI calves. Similar results have been observed using intranasal inoculation of BVDV type 2 in young calves (Archambault et al., 2000) and with calves infected with BVDV type 1 (Kelling et al., 2007).

Multiple products and components of *M. haemolytica* serotype 1 have been proposed as virulence factors. However, **LKT** and lipopolysaccaride (**LPS**) are considered the most important factors in the pathogenesis of these bacteria (Rice et al., 2008). In the present experiment serum concentrations of *M. haemolytica* whole cell antibodies and *M. haemolytica*

LKT antibodies were increased across time for both EarlyCh and LateCh groups. However, there was no effect of timing *M. haemolytica* challenge in relation to BVDV exposure between EarlyCh and LateCh treatments. Using a similar challenge model, Burciaga-Robles et al. (2009a) reported that *M. haemolytica* LKT antibodies increased over time, and on d 7, 14, and 28 were greatest for steers challenged with *M. haemolytica*, lowest for steers not challenged with *M. haemolytica*, and intermediate for steers exposed to BVDV1b followed by an intratracheal challenge with *M. haemolytica*.

Morbidity resulting from BRD decreased feedlot performance, potentially due to the febrile response that is known to accelerate protein and energy metabolism (Jim et al., 1993), and also the decrease in dry matter intake could decrease growth rate associated with BRD (Thompson et al., 2006). Schneider et al. (2009) reported that BRD decreased ADG during both the acclimation period (4 to 6 weeks; 0.37 ± 0.03 kg) and for the overall test period (0.07 ± 0.01 kg). Smith (1998) reported that for the first 28 days of a receiving period ADG was 0.23 kg lower for calves that became sick compared with healthy calves. In addition, calves treated for BRD had 0.06 kg lower ADG than those not treated (Bateman et al., 1990). In the present experiment, ADG was decreased for EarlyCh whereas LateCh steers appeared to compensate from days 5 through 17. Reasons for this difference are unclear, but may suggest that a decrease in ADG occurs when a secondary bacterial infection occurs within 24 hours of BVDV exposure. Dry matter intake expressed as kg/d and as a percentage of body weight were decreased from day 0 to 4 for both EarlyCh and LateCh groups following *M. haemolytica* challenge; however, the greatest depression in dry matter intake (kg/d) occurred in the EarlyCh group. As rectal temperature, clinical attitude score, and haptoglobin concentration were greater for the LateCh compared with the EarlyCh group, it appears that the depression in dry matter intake is most

likely driving the performance results observed in the present experiment, and not differences associated with clinical signs or immune response. In support, gain efficiency was decreased from days 0 to 17 both EarlyCh and LateCh steers compared with CON, but was lowest for the EarlyCh steers.

Increased body temperature (or fever) and depression are part of the clinical signs of acute BRD infection. Burciaga-Robles et al. (2009a) reported no effect of exposure to steers PI with BVDV on rectal temperature during the first 24 hours following an intratracheal *M. haemolytica* challenge. However, from 36 to 72 hours animals exposed to steers PI with BVDV had a higher rectal temperature compared with animals that received only *M. haemolytica* and controls. In the present experiment, rectal temperatures were elevated from 4 to 72 hours after *M. haemolytica* challenge. A similar study observed that all calves infected with BVDV had a mildly affected general appearance and an elevated body temperature (>39.5ºC) 7 days post infection (Gånheim et al., 2005). In their experiment, only two of the calves in the *M. haemolytica* group were mildly depressed and three had a rise in body temperature (>39.5ºC) the day after inoculation. However, the most severe clinical signs were observed in calves challenged with both BVDV and *M. haemolytica*. All calves had fever and depression which started 1 to 3 days post *M. haemolytica* or 6 to 8 days after BVDV inoculation, and lasted for 3 to 11 days (Gånheim et al., 2005). These results indicate a viral-bacterial synergism on alteration of body temperature and clinical attitude. We did not detect significant alterations in rectal temperature or clinical attitude in steers exposed to PI BVDV calves until following the *M. haemolytica* challenge. Rectal temperatures went up to >40ºC at 4 hours after *M. haemolytica* challenge and peaked at 6 and 8 h; peak temperatures were >40.7ºC and >41.3ºC for EarlyCh and LateCh steers, respectively. Clinical attitude score was also increased soon (4 hours) after *M.*

haemolytica challenge, closely related to rectal temperatures. The greater and more extended increase in rectal temperature and clinical attitude score for LateCh compared with EarlyCh steers suggests that timing of immunosuppression due to BVDV in relation to bacterial challenge may alter the severity of response to the disease.

Haptoglobin is one of several proteins produced by the liver of cattle during the acute-phase response, and is not detectable in the serum of healthy animals (Wittum et al., 1996). We observed significant changes in serum haptoglobin concentrations after challenge with *M. haemolytica*, with a more pronounced effect for the LateCh group from 48 to 96 hours of the experiment. Similarly, Gånheim et al. (2003) observed significant differences in clinical signs and haptoglobin in calves infected with both BVDV or *M. haemolytica* compared with calves infected with the individual infectious agents, emphasizing the importance of co-infections. A study using 1 to 2 week old calves intranasally infected with bovine respiratory syncytial virus (BRSV) showed elevated serum haptoglobin concentrations peaking at 7 to 8 days after inoculation. The elevation of haptoglobin concentrations corresponded to the severity of clinical signs and the presence of lung lesions (Heegaard et al., 2000). It is important to mention that in the experiment of Heegaard et al. (2000) *P. multocida* was isolated from lungs at necropsy. However, the secondary bacterial infection was not correlated with elevation of haptoglobin. Our study shows that timing of the bacterial challenge relative to BVDV exposure may be important in production of Hp. The controversy between our results and the observations of Heegaard et al. (2000) may be due to the pathogenesis of bacteria involved, pathogen doses, and timing of *M. haemolytica* (12 or 84 hours) following exposure to PI BVDV calves.

Archambault et al. (2000) reported that BVDV inoculation induced leukopenia, mainly due to a decrease in lymphocytes, but also in neutrophils and monocytes, although the decrease

in monocyte numbers was not significant. In the present experiment, total white blood cells were decreased at -12 and -2 hours relative to *M. haemolytica* challenge for the LateCh steers, and a not significant tendency at 96 hours after *M. haemolytica* challenge for the EarlyCh steers. Similar to Archambault et al. (2000), in our study the decrease in white blood cells was mainly due to the reduction of lymphocyes as a result of exposure to BVDV. It appears from the present experiment that the decrease in total white blood cells and lymphocytes was related to the timing of BVDV exposure, although timing of BVDV exposure did not influence the subsequent response to challenge with *M. haemolytica*. Similarly, Burciaga-Robles et al. (2009a) reported that neutrophils increased in response to *M. haemolytica* challenge and lymphocytes decreased in response to exposure to BVDV, but there was not a BVDV exposure × *M. haemolytica* interaction. In contrast, Gånheim et al. (2005) reported that lymphopenia was more severe in calves with dually inoculated BVDV and *M. haemolytica*, which was associated with more severe clinical signs. Differences in challenge procedures and age and weight of the calves may explain differences among experiments.

In conclusion, total white blood cells and lymphocytes were decreased by BVDV exposure and neutrophils were increased by the bacterial challenge. Delaying the *M. haemolytica* challenge for 84 hours after exposure to calves PI with BVDV increased rectal temperature, clinical severity score, and serum haptoglobin concentrations compared with delaying *M. haemolytica* challenge for only 12 hours after BVDV exposure. However, the increased clinical and acute phase response did not affect short-term performance, which appeared to be driven by the greater decrease in dry matter intake for EarlyCh steers. However, it should be noted that long-term effects of BVDV on animal performance have been reported

(Burciaga-Robles et al., 2009b), and differences observed early in the feeding period may not reflect the potential for long-term consequences of BVDV.

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Table 1. Composition of diets (DM basis)

Ingredient, % of diet	Diet
Corn dent	46.6
Dried corn distillers grain	9.0
Alfalfa hay	40.0
Synergy 19-14[1]	1.00
Wheat midds	1.12
Limestone, 38%	1.27
Dicalcium phosphate	0.25
Salt	0.33
Manganous oxide	0.003
Zinc sulfate	0.020
Potassium chloride	0.260
Magnesium oxide	0.110
Vitamin A-3,000	0.003
Vitamin E-50%	0.002
Rumensin 80[2]	0.018
Tylan 40[2]	0.010
Nutrient, % DM basis[3]	
DM, %	89.3
NE_m, Mcal/kg	1.79
NE_g, Mcal/kg	1.12
NDF, %	26.5
Crude fat, %	3.89
Crude protein, %	14.8
Ca, %	1.05
P, %	0.38
K, %	1.10

[1]Westway Feed Products, New Orleans, LA.

[2]Elanco Animal Health, Indianapolis, IN.

[3]All values are from laboratory analyses and are presented on a 100% DM basis (except DM).

Table 2. Effects of PI BVDV exposure and *M. haemolytica* intratracheal challenge on performance in beef steers.

	Treatments[1]				
Item	CON	EarlyCh	LateCh	SEM	*P* - value
BW, kg					
Initial	270	275	283	17.67	0.67
d 0	282	294	290	16.53	0.77
d 4	291	287	280	16.64	0.77
d 17	305	299	309	12.24	0.84
ADG, kg					
d 0 to 4	2.21[a]	-1.51[b]	-2.62[b]	0.74	0.0004
d 5 to 17	1.14[a]	0.88[b]	2.26[c]	0.31	0.01
d 0 to 17	1.39[a]	0.32[b]	1.12[a]	0.27	0.03
DMI, kg/d					
d 0 to 4	6.58[a]	5.13[b]	5.80[c]	0.24	0.0005
d 5 to 17	6.38	6.06	6.72	0.31	0.33
d 0 to 17	6.42	5.84	6.36	0.22	0.15
DMI, %BW					
d 0 to 4	2.31[a]	1.81[b]	1.85[b]	0.13	0.02
d 5 to 17	2.51	2.08	2.26	0.17	0.49
d 0 to 17	2.19	2.02	2.16	0.14	0.39
Gain:Feed, kg/kg					
d 0 to 4	0.32[a]	-0.31[b]	-0.50[b]	0.14	0.001
d 5 to 17	0.17[a]	0.12[b]	0.33[c]	0.05	0.01
d 0 to 17	0.22[a]	0.04[b]	0.17[c]	0.05	0.03

[1]CON steers not challenged with BVDV or *M. haemolytica*; LateCh steers intratracheally challenged with *M. haemolytica* 84 hours after being exposed to calves PI with BVDV1b for 72 hours and EarlyCh steers intratracheally challenged with *M. haemolytica* 12 hours after being exposed to calves PI with BVDV1b for 72 hours.

[a,b,c]Within a row means with different superscripts are different ($P < 0.05$).

Table 3. Effects of PI BVDV exposure and *M. haemolytica* intratracheal challenge on hemogram of beef steers

Item	Treatments[1]			SEM	*P*-value	
	CON	EarlyCh	LateCh		Treatment	Treatment × hour
Monocytes/µL	344.2	378.6	355.6	46.5	0.77	0.87
Eosionphils/µL	238.5	304.9	207.2	48.8	0.30	0.73
Basophils/µL	124.1	102.4	100.1	10.3	0.20	0.02
Hematocrit, %	33.66	33.11	33.45	1.01	0.73	0.99
Hemoglobin, g/100 mL	12.26	11.89	12.07	0.29	0.45	0.79
Mean corpuscular volume, fL	37.71	37.28	38.6	0.79	0.22	0.84
Mean corpuscular hemoglobin, %	13.83	13.52	13.93	0.20	0.35	0.99
Platelets, 10^3/µL	649[a]	566[b]	478[c]	31.7	0.001	0.62
Red cells, 10^6/µL	8.80	8.83	8.43	0.22	0.08	0.01

[1]CON steers not challenged with BVDV or *M. haemolytica*; LateCh steers intratracheally challenged with *M. haemolytica* 84 hours after being exposed to calves PI with BVDV1b for 72 hours and EarlyCh steers intratracheally challenged with *M. haemolytica* 12 hours after being exposed to calves PI with BVDV1b for 72 hours.

[a,b,c]Within a row means with different superscripts are different ($P < 0.05$).

Figure 1. Serum concentrations of bovine viral diarrhea virus (BVDV) neutralization antibody titers in calves exposed to steers PI with BVDV for 72 hours followed by an intratracheal challenge on d 0 with 6×10^9 CFU of *M. haemolytica* serotype 1 (MH) 12 (EarlyCh) or 84 (LateCh) hours after BVDV exposure compared with non-exposed or challenged steers (CON). There was a treatment × time interaction ($P < 0.0001$; SEM= 8.60). Values plotted represent Least squares means ± standard error of the mean, calculated for 8 animals per experimental group. [a,b]Within day, Least squares means with different letters are different ($P < 0.05$).

Figure 2. Serum concentration of *M. haemolytica* whole cell antibodies (Figure 2a) and *M. haemolytica* leukotoxin antibodies (Figure 2b) in calves exposed to steers PI with BVDV for 72 hours followed by an intratracheal challenge on d 0 with 6×10^9 CFU of *M. haemolytica* serotype 1 (MH) 12 (EarlyCh) or 84 (LateCh) hours after BVDV exposure compared with non-exposed or challenged steers (CON). There was a treatment × time interaction ($P < 0.009$; SEM = 0.293; Figure 2a) for *M. haemolytica* whole cell antibodies, and a treatment × time interaction ($P < 0.02$; SEM = 0.135; Figure 2b) for *M. haemolytica* leukotoxin antibodies. Values plotted represent Least squares means ± standard error of the mean, calculated for 8 animals per experimental group for both *M. haemolytica* whole cell antibodies and for *M. haemolytica* leukotoxin antibodies. [a,b]Within day, Least squares means with different letters are different ($P < 0.05$).

Figure 3. Rectal temperature of calves exposed to steers PI with BVDV for 72 hours followed by an intratracheal challenge on d 0 with 6×10^9 CFU of *M. haemolytica* serotype 1 (MH) 12 (EarlyCh) or 84 (LateCh) hours after BVDV exposure compared with non-exposed or challenged steers (CON). There was a treatment × time interaction ($P < 0.0001$; SEM = 0.06). Values plotted represent Least squares means ± standard error of the mean, calculated for 8

animals per experimental group. [a,b,c]Within a day, Least squares means with different letters are different ($P < 0.05$).

Figure 4. Clinical attitude score of calves exposed to steers PI with BVDV for 72 hours followed by an intratracheal challenge on d 0 with 6×10^9 CFU of *M. haemolytica* serotype 1 (MH) 12 (EarlyCh) or 84 (LateCh) hours after BVDV exposure compared with non-exposed or challenged steers (CON). There was a treatment × time interaction ($P < 0.0001$; SEM = 0.08). Values plotted represent Least squares means ± standard error of the mean, calculated for 8 animals per experimental group. [a,b]Within a day, Least squares means with different letters are different ($P < 0.05$).

Figure 5. Serum haptoglobin concentrations in calves exposed to steers PI with BVDV for 72 hours followed by an intratracheal challenge on d 0 with 6×10^9 CFU of *M. haemolytica* serotype 1 (MH) 12 (EarlyCh) or 84 (LateCh) hours after BVDV exposure compared with non-exposed or challenged steers (CON). There was a treatment × time interaction ($P < 0.0001$; SEM = 95.76). Values plotted represent Least squares means ± standard error of the mean, calculated for 8 animals per experimental group. [a,b,c]Within a day, Least squares means with different letters are different ($P < 0.05$).

Figure 6. Total white blood cell (Figure 6a), neutrophil (Figure 6b) and lymphocyte (Figure 6c) counts in calves exposed to steers PI with BVDV for 72 hours followed by an intratracheal challenge on d 0 with 6×10^9 CFU of *M. haemolytica* serotype 1 (MH) 12 (EarlyCh) or 84 (LateCh) hours after BVDV exposure compared with non-exposed or challenged steers (CON). There were treatment × time interactions ($P < 0.007$, SEM = 0.66, Figure 6a; $P < 0.0001$, SEM = 312.24, Figures 6b; and $P < 0.03$; SEM = 350.54; Figure 6c). Values plotted represent Least squares means ± standard error of the mean, calculated for 8 animals per

experimental group. [a,b]Within a day, Least squares means with different letters are different ($P < 0.05$).

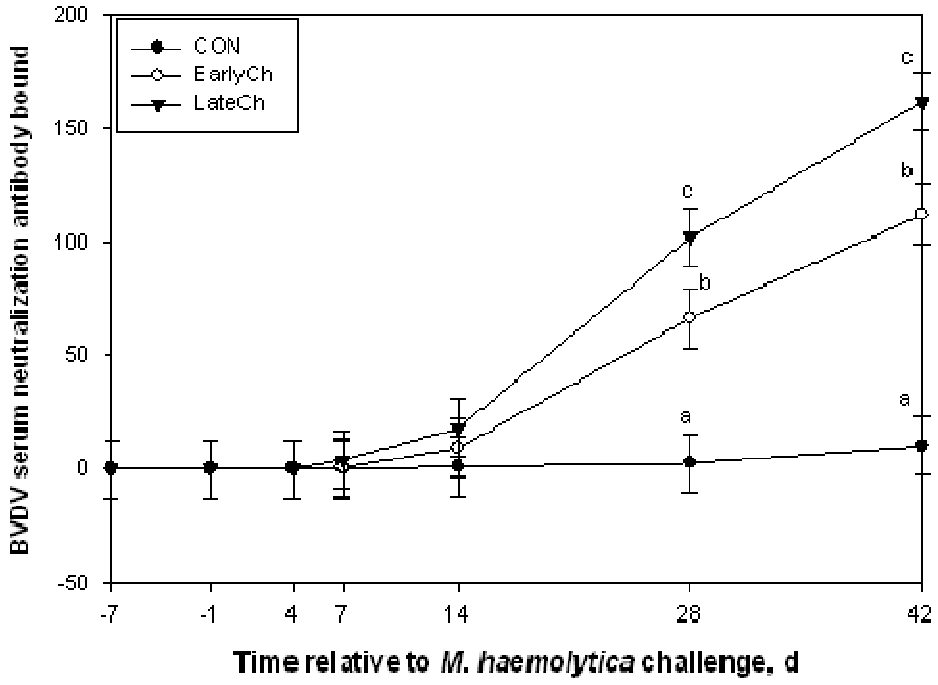
CON
EarlyCh
LateCh
BVDV serum neutralization antibody bound
200
150
100
50
0
-50
-7
-1
4
7
14
28
42
a
b
c
a
b
c
Time relative to M. haemolytica challenge, d

Figure 1.

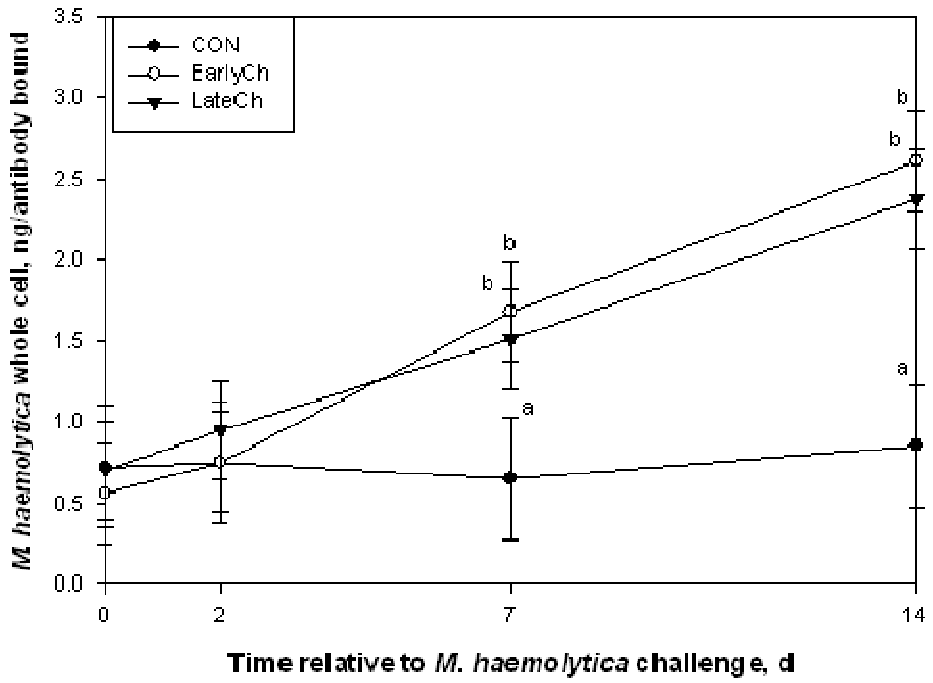
CON
EarlyCh
LateCh
M haemolytica whole cell, ng/antibody bound
Time relative to M. haemolytica challenge, d
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
0
2
7
14
a
b
b
b
a
b

Figure 2a.

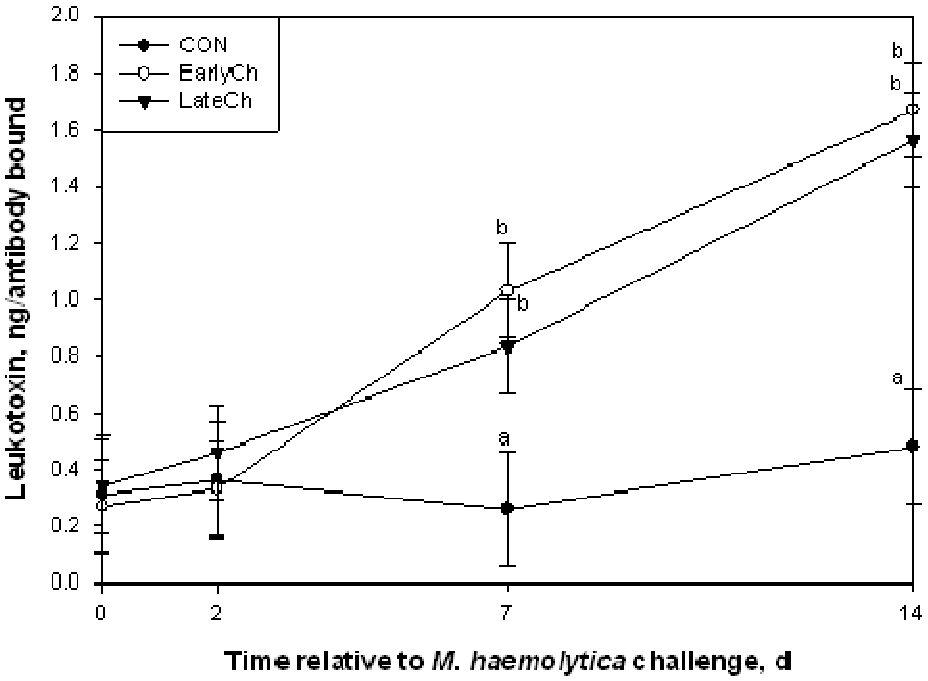
CON
EarlyCh
LateCh
Leukotoxin, ng/antibody bound
Time relative to M. haemolytica challenge, d
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
2
7
14
a
b
b
b
b
a

Figure 2b.

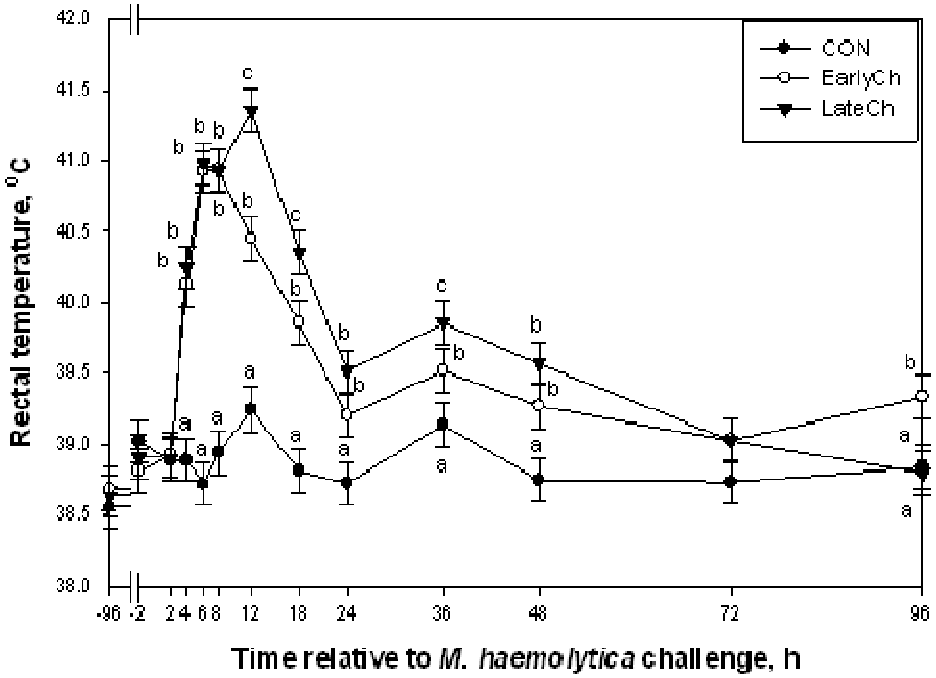
CON
EarlyCh
LateCh
Rectal temperature, °C
Time relative to M. haemolytica challenge, h

Figure 3.

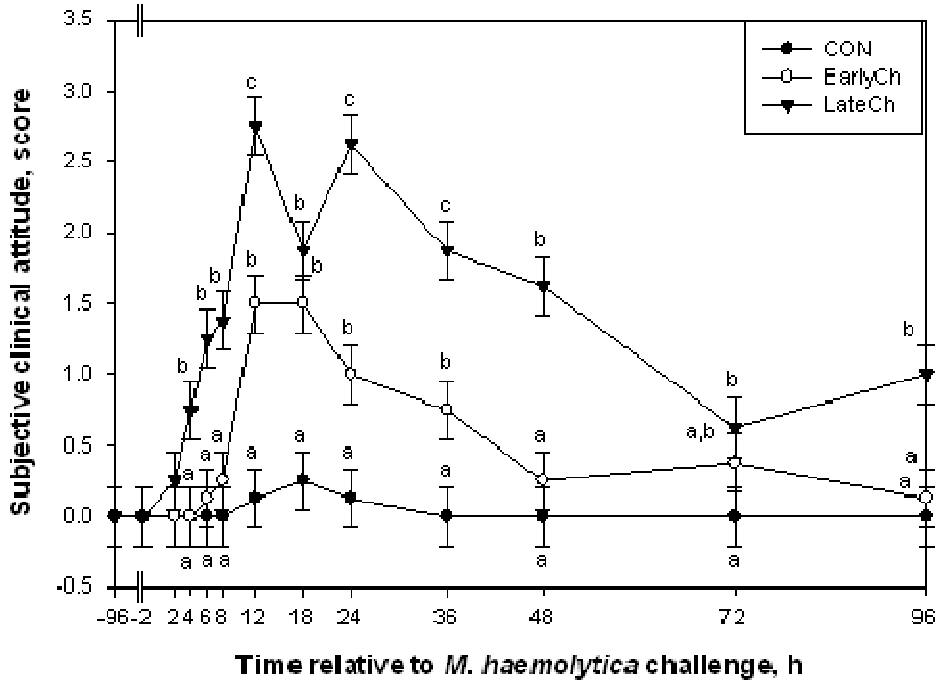
CON
EarlyCh
LateCh
Subjective clinical attitude, score
Time relative to M. haemolytica challenge, h

Figure 4.

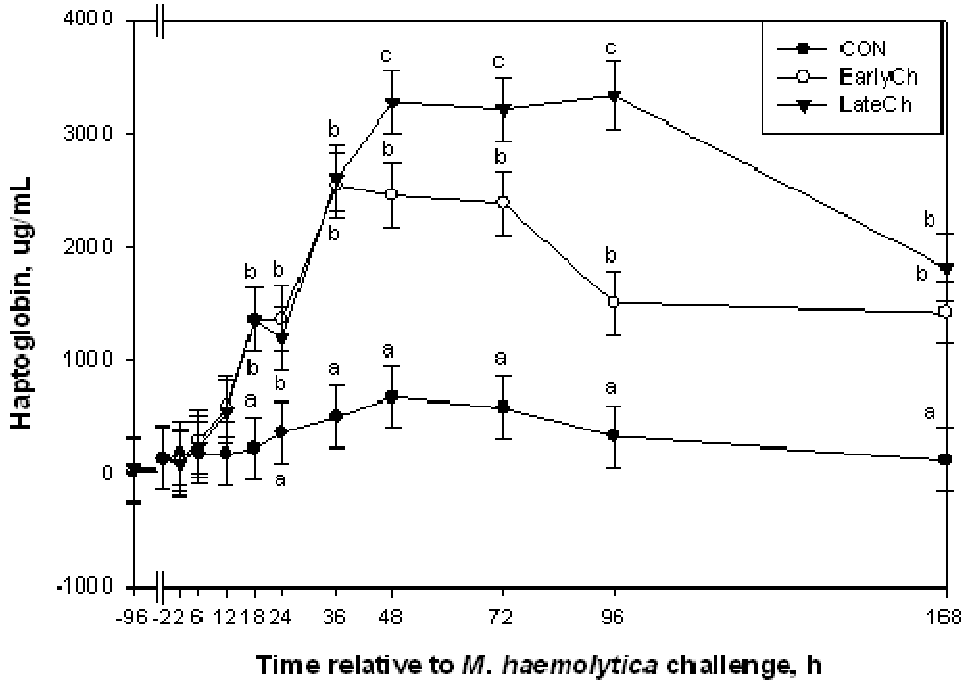
4000
3000
2000
1000
0
-1000
Haptoglobin, ug/mL
CON
EarlyCh
LateCh
-96
-22
6
12
18
24
36
48
72
96
168
Time relative to M. haemolytica challenge, h

Figure 5.

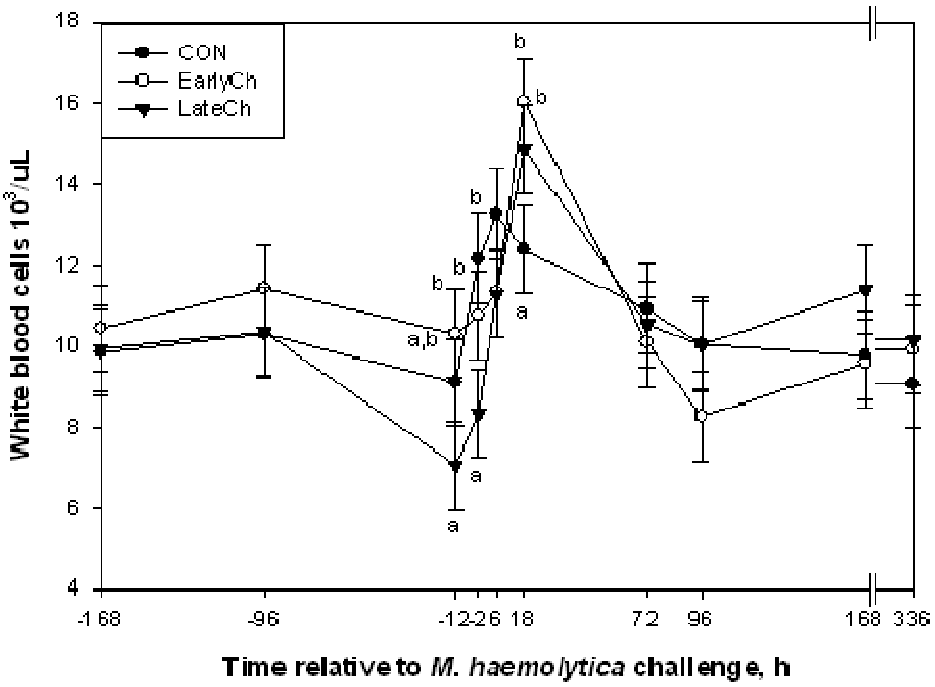
CON
EarlyCh
LateCh
White blood cells 10³/uL
Time relative to M. haemolytica challenge, h
18
16
14
12
10
8
6
4
-168
-96
-12
-2
6
18
72
96
168
336
a
a,b
b

Figure 6a.

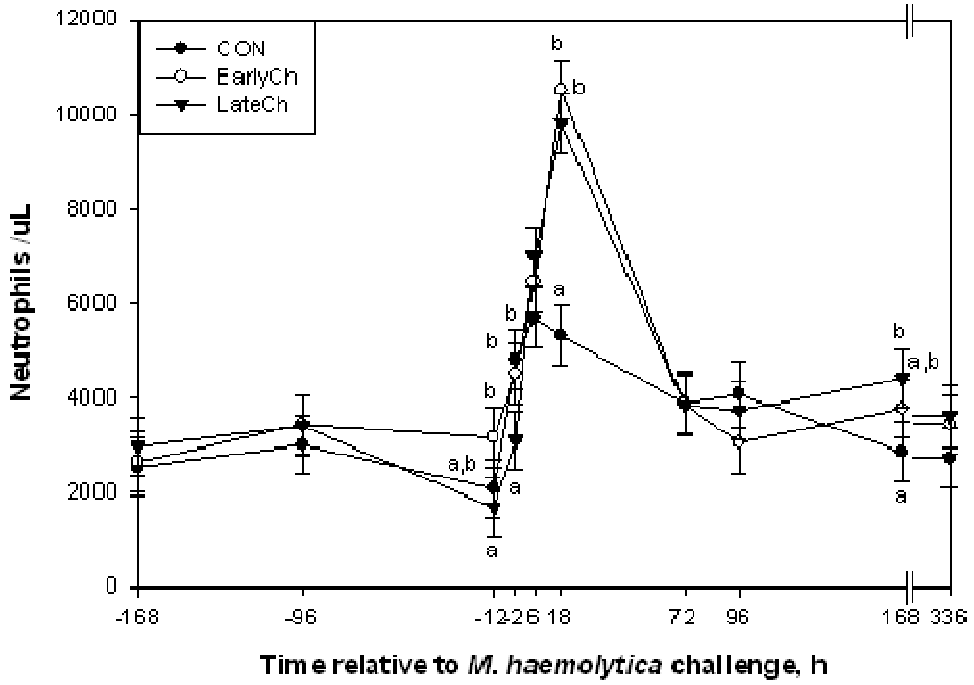
CON
EarlyCh
LateCh
Neutrophils /uL
12000
10000
8000
6000
4000
2000
0
-168
-96
-12
-2
6
18
72
96
168
336
Time relative to M. haemolytica challenge, h

Figure 6b.

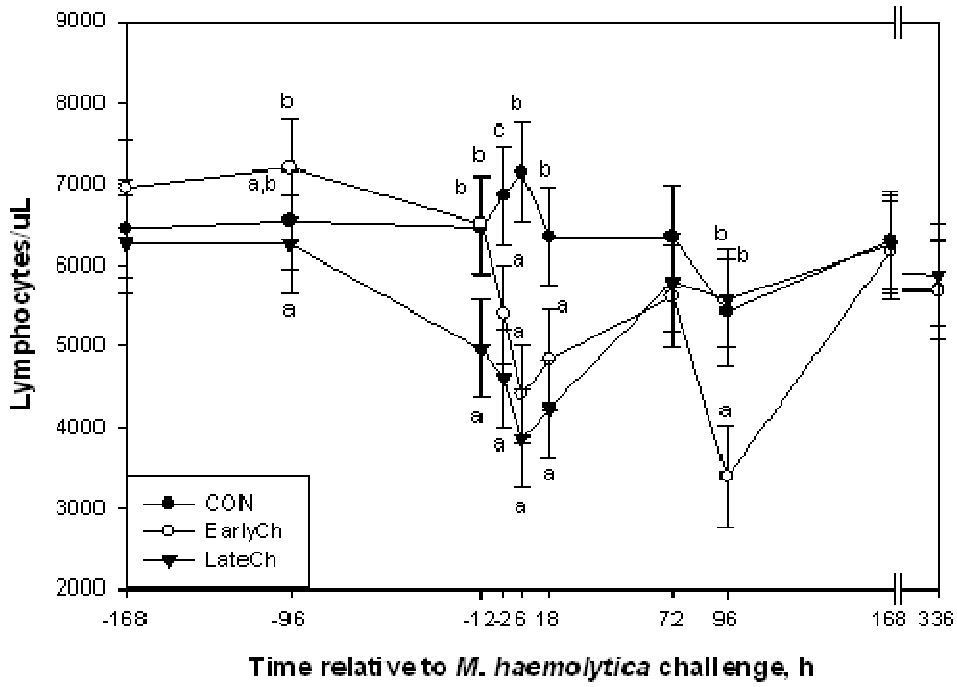
Lymphocytes/uL
9000
8000
7000
6000
5000
4000
3000
2000
CON
EarlyCh
LateCh
-168
-96
-12
-2
6
18
72
96
168
336
Time relative to M. haemolytica challenge, h

Figure 6c.

Effect of timing of *Mannheimia haemolytica* challenge following short-term exposure to bovine viral diarrhea virus type 1b on serum cytokine concentrations and muscle and fat gene expression changes in growing beef steers

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ABSTRACT: Few studies have evaluated the effects of *Mannheimia haemolytica* challenge following exposure to calves persistently infected (**PI**) with bovine viral diarrhea virus (**BVDV**). We hypothesized that immunosuppression following exposure to BVDV could alter the response to a secondary bacterial infection in growing beef steers. Our objective was to determine the effects of two different timings of an intratracheal *Mannheimia haemolytica* (**MH**) serotype 1 challenge following short-term exposure (72 hours) to BVDV type 1b on serum concentrations of cytokines and gene expression of cytokines in subcutaneous fat (**SCF**) and *longissimus dorsi* muscle (**LM**) in growing beef steers. Eighteen steers (initial BW = 269.46 ± 31.32 kg) were randomly allocated to one of three treatments (six animals/treatment) in a randomized complete block design. Treatments were: 1) steers not challenged with BVDV or *M. haemolytica* (**CON**); 2) steers intratracheally challenged with *M. haemolytica* 84 hours after being exposed to calves PI with BVDV1b for 72 hours (**LateCH**); and 3) steers intratracheally challenged with *M. haemolytica* 12 hours after being exposed to calves PI with BVDV 1b for 72 hours (**EarlyCH**). Blood samples were collected during the first 336 hours for serum cytokine analysis and LM and SCF biopsies were collected from -168, 12, 24, 48 and 72 hours relative to MH challenge. Serum concentrations of IFNγ ($P<0.007$), TNFα ($P<0.0001$), and IL-6 ($P=0.014$) were increased in LateCh steers compared to CON and EaryCh steers. Expression of TLR4, NFκB, TNFα and IL-6 in LM were upregulated ($P<0.02$) for EarlyCh steers compared with LateCh and CON steers. Similarly, TLR4 ($P<0.03$) and IL-6 ($P<0.02$) were upregulated in SCF for EarlyCh and LateCh steers compared with CON steers. No significant differences were found for gene expression of TNFα and NFκB in SCF. We conclude that the differences in gene expression profiles in

LM and SCF and an increment in serum cytokine concentrations during induction of the acute phase protein response to pathogens commonly associated with BRD could ultimately decrease animal growth and carcass quality in growing beef steers.

Keywords: beef cattle, bovine respiratory disease, *Mannheimia haemolytica*, cytokines, gene expression.

INTRODUCTION

Bovine viral diarrhea virus (**BVDV**) and *Mannheimia haemolytica* are pathogens involved in bovine respiratory disease (**BRD**). BVDV type 1b is the most common virus isolated from calves arriving at feedlots (Fulton et al., 2005), and *M. haemolytica* serotype 1 is the most common bacteria isolated from lungs of cattle suffering from BRD (Katsuda et al., 2008). Lipopolysaccharide (**LPS**) is a component of the outer membrane of *M. haemolytica*, which is recognized by Toll-like receptor 4 (**TLR4**) inside the host. TLR4 triggers an immune response with production of proinflammatory cytokines interleukin (**IL**)-6 and tumor necrosis factor α (**TNFα**). An increase in protein catabolism appears to be mediated in part by pro-inflammatory cytokines. Nuclear factor *kappa* B (**NFκB**) transcription factor, a major determinant of inflammatory gene expression, is activated by LPS in adipocytes and this induction is coupled to the production of IL-6 and TNFα (Jacobi et al., 2004). Bacterial infection stimulates the production of a large amount of the proinflammatory cytokines interleukin-1 (**IL-1**), IL-6, and TNFα, by macrophages/monocytes and neutrophils (Matsumura et al., 2000). Subsequently, a wide variety of pathologic and host defense reactions are induced, such as fever, pain and synthesis of acute-phase proteins (Matsumura et al., 2000). Innate

immunity generally includes macrophages and neutrophils. However, recent findings have implicated adipocytes and myofibers as participants in innate immunity (Gabler and Spurlock, 2007). We developed a model to simulate a natural infection of BRD by exposing seronegative calves to steers persistently infected (**PI**) with BVDV type 1b followed by an intratracheal *M. haemolytica* challenge. Few studies have been conducted in vivo to evaluate the effects of BRD immune response on gene expression in myofibers and adipose tissue. The objective of our experiment was to evaluate the effects of timing of *M. haemolytica* following BVDV exposure on pro-inflammatory cytokine serum concentrations and gene expression in *longissimus dorsi* muscle and subcutaneous fat in growing steers.

MATERIALS AND METHODS

Animals

All procedures for the present experiment were approved by the Oklahoma State University Institutional Animal Care and Use Committee (Protocol# AG0616). A total of 18 Angus crossbred steers (initial BW = 269.46 ± 31.32 kg) were housed at the Nutrition and Physiology Research Center, Oklahoma State University, Stillwater to determine the effects of viral exposure and bacterial challenge with pathogens that have been associated with BRD on pro-inflammatory cytokine serum concentrations and gene expression in *longissimus dorsi* muscle and subcutaneous fat in growing steers. All animals were considered clinically healthy and were seronegative to all pathogens involved in the study as determined with paired serum samples collected 14 d apart prior at the start of the experiment.

Treatments

The 18 steers were allocated to one of three treatments (6 steers/treatment) arranged as a randomized complete block design. Treatments were: 1) steers not challenged with BVDV or *M. haemolytica* (**CON**); 2) steers intratracheally challenged with *M. haemolytica* 84 hours after being exposed to calves PI with BVDV1b for 72 hours (**LateCh**); and 3) steers intratracheally challenged with *M. haemolytica* 12 hours after being exposed to calves PI with BVDV1b for 72 hours (**EarlyCh**). To facilitate sample collection, steers were blocked by body weight into 2 groups of twelve and the challenge procedures and sample collections were staggered by a 2 week interval between periods. Steers exposed to the calves PI with BVDV were transported approximately 3.2 km to the Willard Sparks Beef Research Center, Stillwater, OK where they were commingled in a 6 × 10.8 m pen with 2 steers previously confirmed as being PI with BVDV1b via immuohistochemistry and genotyping (Fulton et al., 2006). The PI subtype was determined by sequencing the 5'-untranslated region. Steers not exposed to steers PI with BVDV1b were not transported the short distance to prevent risk of exposure to the virus or other potential respiratory pathogens. For both the EarlyCh and LateCh groups, the length of exposure to the PI calves was 72 hours. After the time of BVDV exposure, calves were returned to the Nutrition Physiology Research Center where they were housed for the remainder of the experiment. Steers challenged with *M. haemolytica* received 10 mL of a solution containing 6×10^9 CFU of *M. haemolytica* serotype 1 that was reconstituted and grown prior to the challenge as described by Mosier et al. (1998). Steers not challenged with *M. haemolytica* (CON) were intratracheally dosed with 10 mL of a phosphate–buffered saline (**PBS**) solution (pH 7.4; Sigma Aldrich,

St. Louis, MO). Inoculation with the *M. haemolytica* culture or PBS solution was performed as described by Dowling et al. (2002) with modifications as described by Burciaga-Robles et al. (2009). Challenge with *M. haemolytica* occurred on the same day for all appropriate treatment groups beginning at 0800.

The experiment consisted of 24 days during which the animals were kept in individual pens (3.7 × 3.7 m) with the exception of days 0 to 4. During those days, animals were placed in metabolic stanchions to allow for the challenge procedures and collection of blood and biopsy samples (Burciaga-Robles et al., 2009). During the experiment, steers were offered feed at 3% of the body weight delivered twice daily. The diet was formulated to meet or exceed nutrient requirements (NRC, 1996).

Sample Collection

Blood samples (Clot activator, Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ) were collected via jugular venipuncture with an 18 gauge × 1 inch needle at -168 -12, -2, 2, 6, 12, 18, 24, 36, 48, 72, 96 and 168 hours following the 0 hour *M. haemolytica* challenge. Samples collected were allowed to clot for 24 hours at 4^{o}C. After the clotting time, chilled blood samples were centrifuged at 3,000 × g at 4^{o}C for 20 min. Serum was harvested in 2 mL centrifuge tubes and stored at -20^{o}C until further analyses were performed.

Tissue biopsies were collected from the *longissimus dorsi* muscle (**LM**) and subcutaneous fat (**SCF**) of steers at -168, 12, 24, 48 and 72 hours relative to *M. haemolytica* challenge. Steers were restrained in metabolism stanchions, hair was removed from each biopsy site, and 8 mL of a commercial solution containing 20 mg/mL of lidocaine HCl were injected at each biopsy site with local anesthetic at least 10 min

before the surgical procedure. The biopsy site was surgically scrubbed with a commercially available iodine solution (Betadine Surgical Scrub, Purdue Products, Stamford, CT) followed by a rinse with a 70% isopropyl solution. A 1-cm incision was made along the dorsal area of the thoracic and lumbar region with a sterile scalpel. Tissue was collected (1.0 g) from the LM utilizing a sterile Bergstrom biopsy needle (approximately 10 cm long and 4 mm internal diameter). Subcutaneous fat biopsies were taken by dissecting tissue (approximately 100 mg) through a 2.5 cm skin incision around the sacrococcygeal region. After collection, tissues were quickly placed in an RNase-free polyethylene tube and snap-frozen in liquid N and later stored at -80ºC until processed for analysis. All biopsies were taken from separate incisions made at least 10 cm apart to minimize the potential effect of the previous biopsy on gene expression. In addition, for SCF the biopsy site was alternated between the left and right side of the animal with each subsequent biopsy taken. For LM, all biopsies were collected from the right side of the animal. The incisions were closed with a sterile non-absorbable Braunamide suture (Braun, Bethlehem, PA). All steers were monitored for swelling for 24 to 48 hours after the biopsy and sutures were removed from each incision site 10 days after the surgical procedure.

Laboratory Analyses

Serum Cytokines. Before cytokine analysis, all serum samples were diluted 1:1 in Tris-buffered saline with Tween 20 (pH 8.0; Sigma-Aldrich). After samples were diluted, cytokine [interferon γ (**IFNγ**), IL-6, and TNFα] concentrations in serum were measured in duplicate with commercially available ELISA kits (IFNγ and IL-6, Pierce Protein Research Products, Thermo Scientific, Rockford, IL; TNFα, R&D, Minneapolis,

MN) with reagents provided by the manufacturer unless otherwise specified. Briefly, 96 well plates (Microtiter 96-well plates, Thermo Scientific, Waltham, MA) were coated overnight at room temperature with the appropriate bovine specific coating antibody in Tris-buffered saline with Tween 20 (pH 8.0; Sigma-Aldrich). After the incubation, the coating solution was aspirated followed by the addition of 300 μL of ELISA ultra block solution (AbD Serotec, Raleigh, NC) to each well, and incubated at room temperature for 1 hour. This step was followed by aspiration of the blocking solution, addition of 100 μL of samples or standards to wells, and incubation at room temperature for 1hour. Following sample incubation, standards and samples were aspirated and a three-step wash was performed using a manual plate washer (Nunc Immuno Plate 12; Thermo Fisher Scientific, Rochester, NY). Three hundred microliters of ELISA wash buffer (AbD Serotec) were added during each wash followed by aspiration. Following the initial three-step wash, the appropriate detection antibody [biotin-labeled antibody in Tris-buffered saline with Tween 20 (pH 8.0; Sigma-Aldrich)] was added to each well and incubated for 1 hour at room temperature, followed by the three-step wash as described before. Following the washing step, 100 μL of horse radish peroxidase-labeled streptavid in Tris-buffered saline with Tween 20 (pH 8.0; Sigma-Aldrich) were added to each well and incubated at room temperature for 30 min, followed by a final three-step wash.

For the colorimetric determination of cytokines, 100 μL of 3,3',5,5'-tetramethylbenzidine substrate solution were added to each well and incubated for 20 min at room temperature followed by the addition of 100 μL of stop solution (0.16 *M* sulfuric acid) and optical density was measured at 450 and 550 nm using a plate reader (Multiskan Spectrum; Thermo Scientific, Waltham, MA). Detection limits for the

cytokine procedures were: 2,000 to 31 pg/mL for IFNγ; 5,000 to 78 pg/mL for IL-6; and 8,500 to 133 pg/mL for TNFα based on maximum and minimum concentrations of the recombinant bovine cytokine standards, respectively. Concentrations of cytokines in serum samples were calculated by subtracting the 450 nm absorbance from the 550 nm absorbance to account for any optical imperfections in the plates. Then, readings were subtracted from the blank and a 4-point parameter logistic method standard curve was developed for each plate to calculate the concentrations of the cytokine in each sample. All intra-assay coefficients of variation were lower than 5% and inter-assay coefficients of variation below 7.5%.

RNA Extraction and Reverse-Transcription Polymerase Chain Reaction. Total RNA was recovered from LM and SCF tissues using Trizol reagent (Invitrogen, Carlsbad, CA, USA) with modifications. Approximately 25 to 30 mg of LM and SCF tissue were hand ground with a mortar and pestle in 1 mL of Trizol. All contents in the mortar were transferred to a 2 mL centrifuge tube and centrifuged at 12,000 × *g* for 10 min. The supernatant was extracted (avoiding the fat layer) and placed into a new centrifuge tube (1.5 mL). The extracted supernatant was incubated for 5 min at room temperature (15 to 25°C) to ensure the complete dissociation of nucleoprotein complexes. Chloroform (0.2 mL/mL of Trizol) was added and tubes were shaken for 15 s and incubated for 10 min at room temperature (15 to 25°C). Tubes were centrifuged at 12,000 × *g* for 15 min at 2 to 8°C, and the aqueous phase was transferred to a new tube and precipitated with isopropanol (0.5 mL/mL of Trizol). Contents were mixed by inversion and incubated for 5 to 10 min at room temperature, centrifuged at 12,000 × *g* for 10 min at 2 to 8°C, and supernatant discharged. Pellets were rinsed with 75% ethanol

(1 mL ethanol/mL of Trizol) and stored at -80°C for at least 30 min or overnight. Tubes were spun at 12,000 × *g* for 30 min, the pellet wased with 70% ethanol and resuspended in diethyl pyrocarbonate (**DEPC**) water.

A 1% agarose gel with ethidium bromide was used to determine the quality of the RNA. The quantity was determined using a Nanodrop™ ND-100 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Thereafter, 1 μg of RNA was reverse-transcribed using QuantiTect® Reverse Transcription (QIAGEN, Valencia, CA) for cDNA synthesis with integrated removal of genomic DNA contamination. The cDNA was then used as the template for real-time qRT-PCR.

Quantitative Two-Step Real-Time RT-PCR. Differential expression of genes (TLR4, TNFa, NFκB and 18s rRNA as a housekeeping gene) in LM was quantified following the two-step qRT-PCR reaction for FastStart Universal SYBR Green Master ROX (Roche Applied Science, Mannheim, Germany) using a iCycler iQ Real Time PCR System (BioRad, Milpitas, CA). Thermal cycling conditions were 95ºC for 10 min followed by 38 repetitive cycles of 94ºC for 15 s, 60ºC for 15 s annealing temperature, and 72ºC for 20 s. Immediately following RT-PCR, a melt curve analysis was conducted by bringing the reaction to 95ºC for 1 min, 55ºC for 1 min, then increasing the temperature by 0.5ºC from 55ºC to 94.5ºC. Primers (Table 1) were designed using the Primer Quest interface of Integrated DNA Technologies, Inc (Coralville, IA). Interleukin-6 was quantified in LM using TaqMan probes, as described below.

Expression analysis of genes in SCF and IL-6 in muscle were performed by two-step qRT-PCR. A ribosomal 18S rRNA control kit (Applied Biosystems, Inc., Foster City, CA) was used as a housekeeping gene to normalize samples for any variation in

RNA loading (Voge et al., 2004; Spicer and Aad, 2007). Based on preliminary optimization results, 120 ng total cDNA in duplicate wells were amplified in a total reaction volume of 20 μL consisting of 300 nM forward primer, 300 nM reverse primer, and 200 nM fluorescent (FAM/TAMRA) probe for TLR4 and TNFα target genes; 50 nM of 18S rRNA primers and 100 nM of the 18S rRNA VIC-labeled probe. PerfeCta™ Multiplex qPCR Supermix (QUANTA BIOSCIENCE™, Gaithersburg, MD) master mix at 20 μL final volume reaction was used. Primers and probes (Table 1) for NFκB and IL-6 were supplied in the TaqMan Regents kit from Applied Biosystems with TAMRA quencher. Thermal cycler conditions for PCR were 50°C for 2 min, 95°C for 3 min, followed by 45 cycles of 95°C for 15 sec, and 65°C for 60 sec for annealing/extend data collection. Primers and fluorescent probes for TLR4 and TNFα were designed using Primer Express software (Applied Biosystems Inc.) and the Primer Quest interface of Integrated DNA Technologies, Inc. using the criteria as described by Grado-Auir (2008).

Quantification of Target Gene Expression. Relative quantification of target gene expression was evaluated using the comparative threshold cycle method (Aplied Biosystems, 2001). As previously described (Voge et al., 2004; Aad et al., 2006), the abundance of mRNA was estimated setting an arbitrary threshold (CT) on the FAM or VIC curves in the geometric portion of the RT-PCR amplification plot after examining the log view. Then, the ΔCT was determined by subtracting the 18S rRNA CT from the target gene CT value. The ΔΔCt was normalized to the average of the ΔCt at time point zero of the control group. The fold change was calculated using the $2^{-\Delta\Delta CT}$ method (Applied Biosystems, 2001).

Statistical Analysis

Data for serum cytokines and the ΔCt from the comparative threshold cycle method were analyzed using a repeated measures analysis of the MIXED procedure of SAS (2003), with a non-structured covariance structure and slice output option (SAS Inst., Inc., Cary, NC). For all cytokine and ΔCt data, values from serum and biopsy samples collected before (-168 hours) steers were exposed to steers PI with BVDV were used as a covariate. The slice output option for both serum cytokines and fold change was used to determine the time points at which there were differences among treatments.

RESULTS

There were treatment × time interaction ($P < 0.0001$) for serum IFNγ concentration (Figure 1a). Serum IFNγ concentrations were greater at -12, -2 and 2 hours ($P < 0.01$) for LateCh steers than CON and at -12 hours compared with EarlyCh steers. There was a treatment × time interaction ($P < 0.0001$) for serum TNFα concentration (Figure 1b). Serum TNFα concentrations were greater ($P < 0.05$) at -12, 36, 72 and 96 hours compared with the EarlyCh steers and at 36, 72 and 96 hours compared with the CON steers. There was a treatment × time interaction ($P < 0.0001$) for serum IL-6 concentration (Figure 1c). Serum IL-6 concentrations ($P < 0.05$) were greater in LateCh steers at 36, 48, and 72 hours compared with EarlyCh and at 36 and 72 hours compared with CON steers. Overall there were no significant differences ($P > 0.10$) in serum cytokine concentrations for EarlyCh steers compared with CON steers.

Gene expression of TLR4 in LM was up-regulated ($P < 0.0001$) for EarlyCh steers compared with CON and LateCh steers (Figure 2a). Greater ($P < 0.05$) expression

of TLR4 was observed at 12, 24, 48, and 72 hours following *M. haemolytica* challenge. Similar results were observed for NFκB ($P < 0.003$; Figure 2b), TNFα ($P < 0.0001$; Figure 2c) and IL-6 ($P < 0.009$; Figure 2d) in LM for EarlyCh compared with CON and LateCh steers.

Gene expression of TLR4 in SCF was up-regulated ($P < 0.01$) for EarlyCh and LateCh steers compared with CON steers at 48 and 72 hours after *M. haemolytica* challenge (Figure 3a). In addition, expression was greater ($P < 0.05$) for EarlyCh compared with LateCh steers at 24, 48 and 72 hours. Gene expression of NFκB and TNFα in adipose tissue did not differ ($P > 0.10$) among treatments (data not shown). Expression of IL-6 in adipose tissue was up-regulated ($P = 0.02$) for EarlyCh and LateCh steers compared with CON steers at 24, 48, and 72 hours and at 12 hours following *M. haemolytica* challenge for LateCh compared with EarlyCh and CON steers.

DISCUSSION

Serum Cytokine Response

Interferon-γ. Interferons are cytokines produced by mammalian cells in response to viral infection or other stressors (Loo and Gale, 2007). Interferon-γ is induced by a unique set of stimuli and is produced only by T lymphocytes and natural killer (**NK**) cells. Stimulation of T cells results in the induction of IFNγ mRNA. Similarly, Franchini et al. (2006) observed that once ncp BVDV infection takes place in host T lymphocytes, dendritic cells are stimulated to produce IFNγ. To investigate whether the cellular antiviral response was impaired by ncp BVDV infected cells, Baigent et al. (2002) used cell cultures to determine the effect of blocked transcriptional responses to α/β IFN.

They showed that pre-existing infection of cells with ncp BVDV was not able to produce α/β IFN when stimulated with a secondary virus. This also demonstrated that NFκB induction by a second viral infection was not compromised by the previous infection of cells with ncp BVDV. In our study, IFNγ (Fig 1a) serum concentrations were elevated soon after PI BVDV exposure for LateCh steers at -12, -2 and 2 h and decreased soon after *M. haemolytica* challenge. Our results are in agreement with reports in the literature, which have shown increases in IFNγ concentrations after viral infection (Loo and Gale, 2007). In contrast, there was no increase in IFNγ for EarlyCh steers after exposure to steers PI with BVDV and throughout the remainder of the experiment. Reasons for this lack of response are unclear, but may suggest an interaction between timing of exposure to BVDV and *M. haemolytica* challenge, which cannot be determined from the present experiment.

Tumor necrosis factor-α. Bacterial infection stimulates the production of pro-inflammatory cytokines such as IL-1, IL-6, and TNFα by macrophages/monocytes and neutrophils. Subsequently, a wide variety of pathologic and host defense reactions are induced, such as fever, pain, synthesis of acute phase proteins, decrease in serum levels of iron, and granulocytosis (Matsumura et al., 2000). Our results are in agreement with previous results in that bacterial infection (*M. haemolytica*) resulted in a gradual increase in serum TNFα concentrations in LateCh steers. Because we used *M. haemolytica*, which is a gram-negative bacteria that produces leukotoxins (**LKT**) and contains lipolpolysacharide (**LPS**) in its outer membrane, we presume that TNFα elevation could be affected by LKT, LPS or both. Our results agree with those reported by Vels et al.

(2009) in healthy dairy cows that responded with an increased TNFα concentration in plasma after LPS administration.

In contrast, no significant changes in serum TNFα concentrations were observed for EarlyCh steers in the present experiment. An *in vitro* study with bovine bone marrow-derived macrophages infected with ncp BVDV and cp BVDV showed a decreased production of TNFα upon stimulation with LPS (Alder et al., 1996). Alder et al. (1996) concluded that decreased production of TNFα in infected macrophages might contribute to the well-documented immunosuppression in animals infected with BVDV. Perhaps steers in the EarlyCh group were more immunosuppressed than LateCh steers at the time of *M. haemolytica* challenge in the present experiment, resulting in no increase in cytokine concentrations.

Interleukin-6. A recent experiment implicated myocytes as a source of pro-inflammatory cytokines in the presence of bacterial LPS (Borge et al., 2009). Both TNFα and IL-6 in muscle interstitial fluid and plasma were elevated by LPS. Concentration of TNFα in plasma was 6 times higher than muscle interstitial fluid at 30 and 90 min after LPS administration. As was expected there were no significant elevations in serum IL-6 concentrations before *M. haemolytica* challenge in the present experiment. However, after challenge serum IL-6 concentrations for LateCh steers were increased. Our results agree with those reported by Burciaga-Robles et al. (2009) where serum concentrations of IL-6 were higher for steers challenged with *M. haemolytica* than controls. Another study conducted by Yong-Woon et al. (2007) demonstrated that plasma TNFα and IL-6 levels increased by LPS injection in rats at 0.5 and 2 h, and at 2

and 4 h, respectively. Similar to TNFα, there were no significant changes in serum concentrations of IL-6 for EarlyCh steers throughout the experiment compared with CON and LateCh steers. Lack of response might be attributed to the immunosuppression effects of ncp BVDV on cytokine production (Alder et al., 1996) related to the timing of MH challenge.

In general, our results are in agreement with reports cited in the literature. However, time and duration of cytokine response differ from results where LPS administration has been used. We speculate that the differences could be related to our intratracheal challenge with live bacteria (*M. haemolytica*), giving residential macrophages the opportunity to respond before bacteria gets into the bloodstream. Also, time is needed for bacteria to replicate inside the lungs, giving a longer-term response. In contrast, most studies used parenteral administration of LPS, resulting in an acute response. We presume that LPS via parenteral administration would be rapidly inactivated locally or in the bloodstream with less chance to reach other tissues (muscle and fat) that might respond with cytokine production (IL-6 and TNFα).

Gene Expression in LM and SCF

TLR4. In the battle against infection, the host recruits components of both the innate and adaptive arms of the immune system. Toll-like receptors mediate the activation of cells of the innate immune system, leading to dynamic functions including direct antimicrobial activity, induction of cytokine secretion, triggering dendritic cell maturation, and triggering apoptosis. Furthermore, Krutzik and Modlin (2004) suggested that TLR activation is capable of modulating the adaptive immune response with a bias

towards a Th1 T-cell response. Krutzik and Modlin (2004) used ncp BVDV or cp BVDV to infect bovine monocytes resulting in upregulation of TLR3 1 hour after inoculation with ncp BVDV, but not with cp BVDV-infected monocytes, TLR7 expression dominated at 24 hours of infection with both BVDV strains. Gabler and Spurlock (2007) investigated the response to LPS administration in pigs, two sequential injections with 10 and 2.5 μg of LPS/kg of BW 23 hours apart resulted in a marked down regulation of TLR4 at the protein level in adipose tissue of the challenge group compared with control pigs. The results of our study showed upregulation of TLR4 in muscle (Figure 2a) and fat (Figure 3a) for challenged steers compared with CON steers. Upregulation of TLR4 in muscle was greater for EarlyCh than LateCh steers. Due to our experimental design, we were unable to elucidate whether the upregulation effect was produced by BVDV or MH challenge or both. Due to timing of *M. haemolytica* challenge following PI BVDV exposure we assume we missed the time effect produced by BVDV on gene expression of TLR4 in muscle for the LateCh steers. However, immunosuppression caused by BVDV could have depressed muscle's ability to produce an immune response against *M. haemolytica* challenge through upregulation of TLR4. In addition, fat may response slower than muscle to a BVDV insult, which might explain the lack of upregulation of TLR4 in fat observed for the EarlyCh group, while LateCh showed significant upregulation at 12 hours.

Innate immunity is typically considered to involve macrophages and neutrophils. However, recent findings implicate adipocytes and myofibers as participants in innate immunity. Studies with cell culture of adipocytes and myoblasts indicate expression of Toll-like receptors in response to bacterial LPS by producing TNFα and IL-6, classical

pro-inflammatory cytokines (Lin et al., 2000; Frost et al., 2003). Pasare and Medzhitov (2004) mentioned that the importance of activation of resident macrophages through TLRs allows the production of various cytokines (IL-1, IL-6, TNF, etc.) and chemokines [(kemokine C-1 (KC-1) and monocyte chemoattractant protein-1 (MCP-1)], which collectively orchestrate the acute inflammatory response to infection. Matsumura et al (2000) showed that a single intraperitoneal administration of LPS in mice resulted in TLR2 mRNA upregulation in brain, heart, lung, liver, and kidney tissues, and down regulation in spleen. In the same experiment, TLR4 mRNA was decreased in brain, increased in heart and lungs, and not changed in the liver, kidney, or spleen. In agreement with these previous studies, we also showed that TLR4 is expressed in somatic tissues (muscle and fat) in addition to macrophages and neutrophils.

NFκB. Nuclear transcriptional factor kappa B (NFκB) is an ubiquitous rapid response transcription factor in cells involved in immune and inflammatory reactions, and exerts its effect by expressing cytokines, chemokines, cell adhesion molecules, growth factors, and immunoreceptors (Lee and Burckart, 1998). The induction of the immune cascade and NFκB by LPS requires initiation of TLR4. Adipocytes are an important source of proinflammatory cytokine production (Ajuwon et al., 2004) by the effect of stimulation of NFκB that consequently would induce IL-6 and TNFα production (Berg et al., 2004). Our results for NFκB in muscle (Fig 2b) showed a similar pattern as TLR4, with significant expression across time from 12 to 72 h and greater expression at 72 hours after *M. haemolytica* challenge. Based on our results and results reported in the literature, we could assume that initial activation of TLR4 functions as a pivotal activation of NFκB in muscle tissue. Lack of increased expression of NFκB in muscle for LateCh steers

could be related to the lack of expression of TLR4 in this tissue. Although TLR4 was upregulated in fat tissue for both EarlyCh and LateCh steers (Fig 3a and 3b, respectivately) compared to the CON, there were no significant differences in expression of NFκB.

TNFα.. Tumor necrosis factor α is a biologically active, pleiotropic cytokine produced mainly by macrophages. TNFα plays a physiologically important role in the activation of the immune response by modulating the production and activity of an array of cytokines (Adler et al., 1996). TNFα has been hypothesized to be an important mediator of the septic response (Cooney et al., 1999). Borge et al. (2009) used qtRT-PCR to measure TNFα and IL-6 gene expression in skeletal muscle and showed upregulation of these cytokines in animals with LPS administration compared with controls. Their experiment supports the theory that myocytes play an important role in the immune defense mechanisms of the host against pathogens by producing proinflammatory cytokines. Our results showed a significant upregulation of TNFα in muscle for EarlyCh steers. In contrast, significant down regulation for LateCh steers was observed from 12 to 24 h and at 72 h. As mentioned above, activation of TLR4 is needed to begin an enzymatic cytoplasmic cascade that will activate NFκB, which in turn would trigger TNFα and IL-6 production. This may explain why, when upregulation of TLR4 and NFκB in muscle was present in EarlyCh steers, TNFα was upregulated in the same group following the same pattern of TLR4 and NFκB. There was no significant upregulation of TNFα in fat for EarlyCh or LateCh treament groups compared with CON steers.

IL-6. Interleukin-6 is considered a pleiotropic cytokine with multiple functions in both immune and non immune cells. This interleukin is a proinflammatory cytokine that plays a pivotal role during pathogenic infection, injury and trauma. Initially, it was believed that IL-6 was only synthesized by macrophages and lymphocytes; more recently skeletal muscle (Frost et al., 2003) and fat (Weisberg et al., 2003) have become recognized as sources of this cytokine. Weisberg et al. (2003) showed that adipocytes are a source and target of proinflammatory cytokines (TNFα and IL-6). In addition, Ross et al. (2002) reported that proinflammatory cytokines (IL-1b, PG-E2, TNFα and IL-6) are generated by vascular cells. Leibel et al. (1997) reported that macrophage cells isolated from fat expressed TNFα and IL-6 in greater quantities than the non-macrophage cell population. Our data agree with these previous reports where IL-6 was expressed in muscle and fat tissues in response to pathogen insults (BVDV and *M. haemolytica*). We observed that upregulation of IL-6 in muscle (Fig 2d) for the EarlyCh steers following the same pattern of TLR4 and NFκB. In contrast, no upregulation of IL-6 in muscle for the LateCh steers was present. As previously mentioned, activation of TLR4 would influence the upregulation of proinflammatory cytokines (TNFα and IL-6). We also observed upregulation of IL-6 in fat tissue (Fig 3b) for both EarlyCh and LateCh groups. In agreement with our results, Frost et al. (2003) demonstrated that LPS via intraperitoneal injection in mice was able to significantly increase serum concentrations of IL-6, and IL-6 mRNA 100-fold in muscle. Frost et al. (2003) also observed that IL-6 protein was increased by direct stimulus of LPS to C2C12 myoblasts (6- to 8-fold) and IL-6 mRNA (5- to 10-fold). Borge et al. (2009) concluded that muscle cells appear to be

an important source of these cytokines during endotoxemia, and are likely to contribute significantly to the cytokine concentrations in plasma.

We conclude that BRD pathogens (BVDV and *M. haemolytica*) involved in this study are playing an important role in gene expression of cytokines expressed in muscle and fat tissues. Differences in gene expression profiles in LM and SCF and serum cytokine concentrations in response to pathogens commonly associated with BRD could ultimately decrease animal growth and carcass quality in growing beef steers.

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Table 1. Primers and probes used in this study for determination of TLR4, NFκB, TNFα, and IL-6

Target gene	Acc. No.	Classification	Sequence 5' – 3'/ABI commercial assay[1]
Primers used in LM[2]			
TLR4	NM_174198	Forward	GCT GGA CCT GAG CTT TAA CTA CCT
		Reverse	GTG GTT TAG GCC CTG AAA TGT GTC
NFκB	TC244499	Forward	CTG ATG ATT TGC TGG CAC AAG GAG
		Reverse	CCT CGT AGT TGT CCA TGA GGG TTT
TNFα	AF011926	Forward	ACG TTG TAG CCG ACA TCA ACT CTC
		Reverse	AGT AGA TGA GGT AAA GCC CGT CAG
IL-6	X57317	Assay[1]	Bt03211903_m1
Primers and probes used in SCF[2]			
TLR4	NM_174198	Forward	GGG AGC CTT TTC TGG GCT ATC
		Reverse	GCC ACA TTA AGC TCT TTC AAG TTT T
		Probe	AAG CTG GTG GCC GTG GAG ACA AAC
TNFα	AF011926	Forward	TCT ACC AGG GAG GAG TCT TCC A
		Reverse	CTG CCC AGA CTC GGC ATA GT
		Probe	CAG TGC TGA GAT CAA CCT GCC GGA
NFκB	TC244499	Assay[1]	Bt03272779_m1
IL-6	X57317	Assay[1]	Bt03211903_m1

[1]ABI, Applied Biosystems

[2]LM, *Longissimus dorsi* muscle; SCF, subcutaneous fat

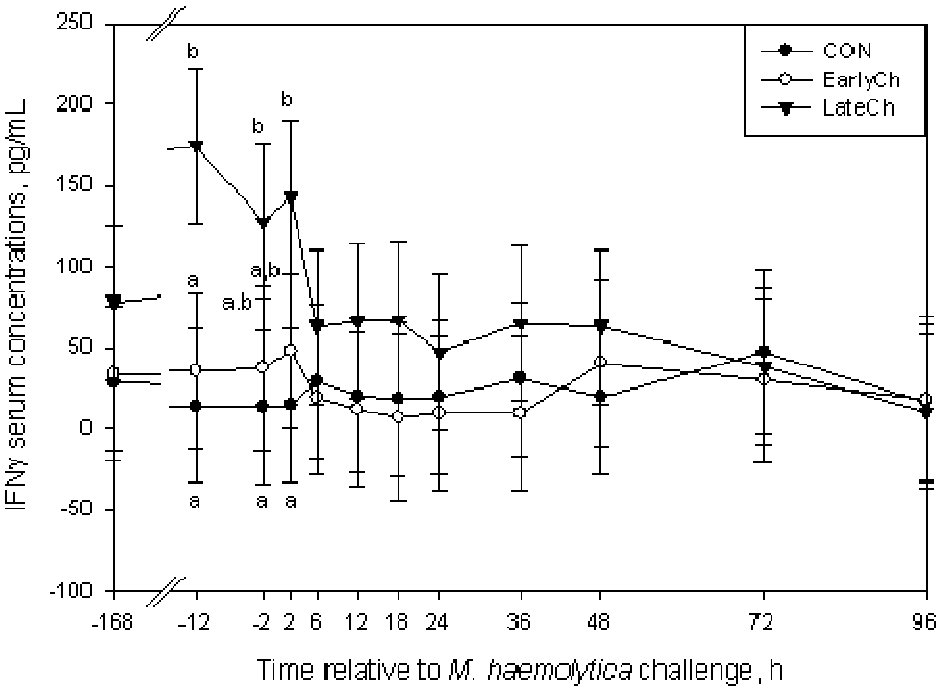

Fig 1a. Serum concentrations of IFNγ in calves exposed to steers PI with BVDV for 72 hours followed by an intratracheal challenge on d 0 with 6×10^9 CFU of *M. haemolytica* serotype 1 (MH) 12 (EarlyCh) or 84 (LateCh) hours after BVDV exposure compared with non-exposed or challenged steers (CON). There was a significant treatment × time interaction ($P < 0.0001$; SEM = 17.19). Values plotted represent Least squares means ± standard error of the mean, calculated for 8 animals per experimental group. [a,b]Within day, Least squares means with different letters are different ($P < 0.05$).

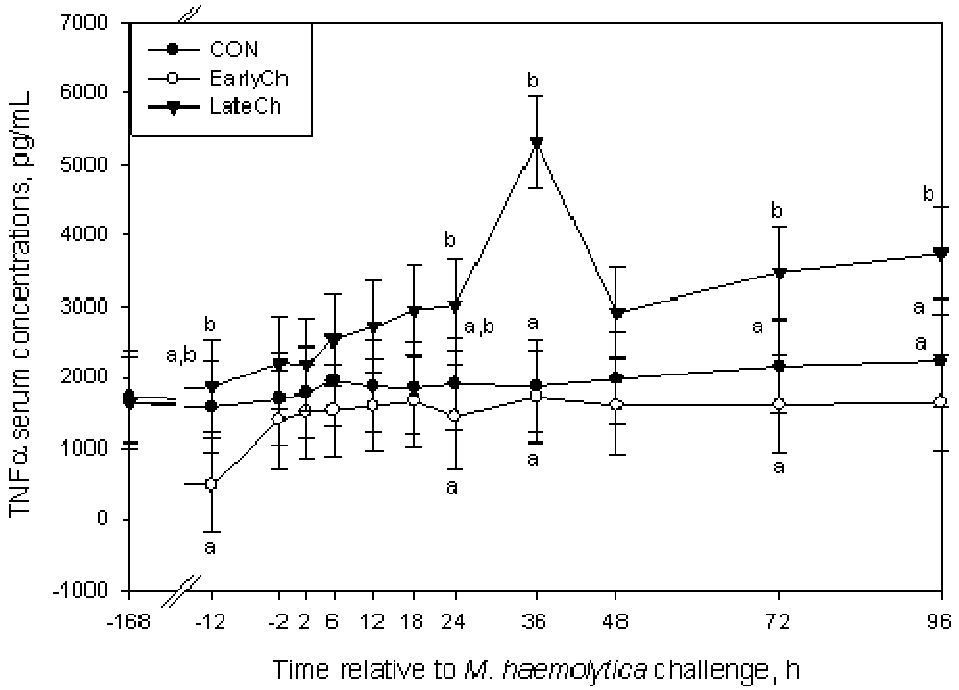

Fig 1b. Serum concentrations of TNFα in calves exposed to steers PI with BVDV for 72 hours followed by an intratracheal challenge on d 0 with 6×10^9 CFU of *M. haemolytica* serotype 1 (MH) 12 (EarlyCh) or 84 (LateCh) hours after BVDV exposure compared with non-exposed or challenged steers (CON). There was a significant treatment × time interaction ($P < 0.0001$; SEM = 358.05). Values plotted represent Least squares means ± standard error of the mean, calculated for 8 animals per experimental group. [a,b]Within day, Least squares means with different letters are different ($P < 0.05$).

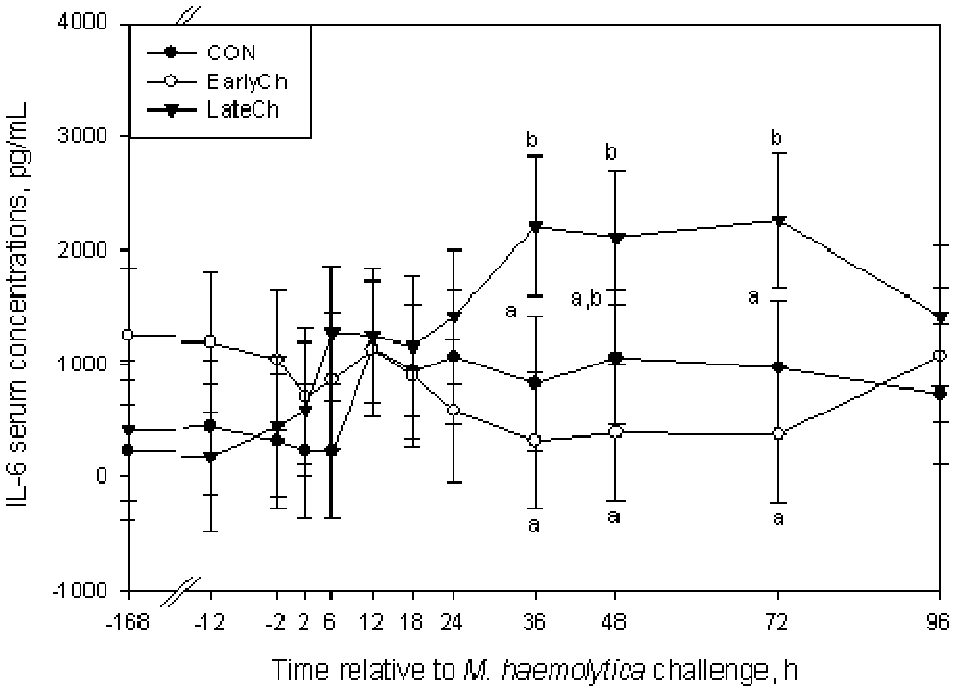

Fig 1c. Serum concentrations of IL-6 in calves exposed to steers PI with BVDV for 72 hours followed by an intratracheal challenge on d 0 with 6×10^9 CFU of *M. haemolytica* serotype 1 (MH) 12 (EarlyCh) or 84 (LateCh) hours after BVDV exposure compared with non-exposed or challenged steers (CON). There was a significant treatment × time interaction ($P < 0.0001$; SEM = 403.36). Values plotted represent Least squares means ± standard error of the mean, calculated for 8 animals per experimental group. [a,b]Within day, Least squares means with different letters are different ($P < 0.05$).

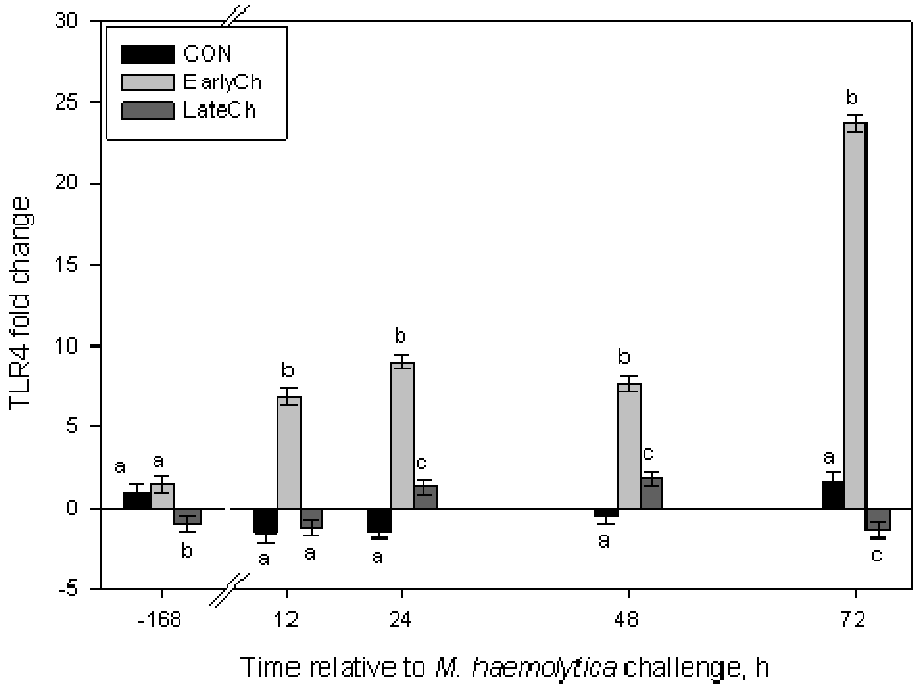

Fig 2a. Gene expression of Toll-like receptor-4 (TLR4) in *longissimus dorsi* muscle (LM) in calves exposed to steers PI with BVDV for 72 hours followed by an intratracheal challenge on d 0 with 6×10^9 CFU of *M. haemolytica* serotype 1 (MH) 12 (EarlyCh) or 84 (LateCh) hours after BVDV exposure compared with non-exposed or challenged steers (CON). There was a significant treatment × time interaction ($P < 0.0001$; SEM = 0.28). Values plotted represent Least squares means ± standard error of the mean, calculated for 6 animals per experimental group. [a,b,c]Within day, Least squares means with different letters are different ($P < 0.05$).

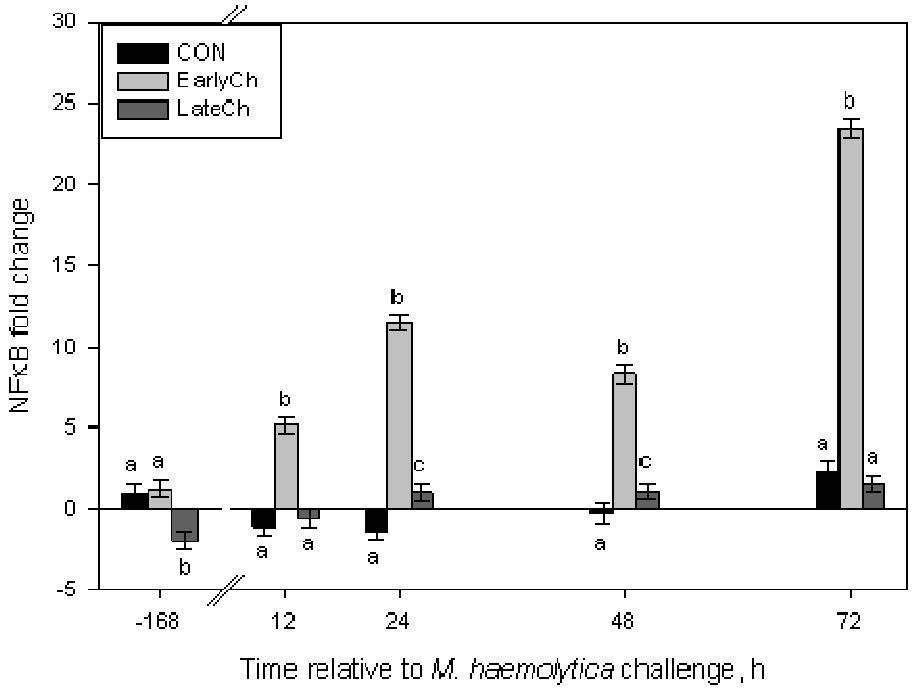

Fig 2b. Gene expression of NFκB in *longisimus dorsi* muscle (LM) in calves exposed to steers PI with BVDV for 72 hours followed by an intratracheal challenge on d 0 with 6×10^9 CFU of *M. haemolytica* serotype 1 (MH) 12 (EarlyCh) or 84 (LateCh) hours after BVDV exposure compared with non-exposed or challenged steers (CON). There was a significant treatment × time interaction ($P < 0.003$; SEM = 0.36). Values plotted represent Least squares means ± standard error of the mean, calculated for 6 animals per experimental group. [a,b,c]Within day, Least squares means with different letters are different ($P < 0.05$).

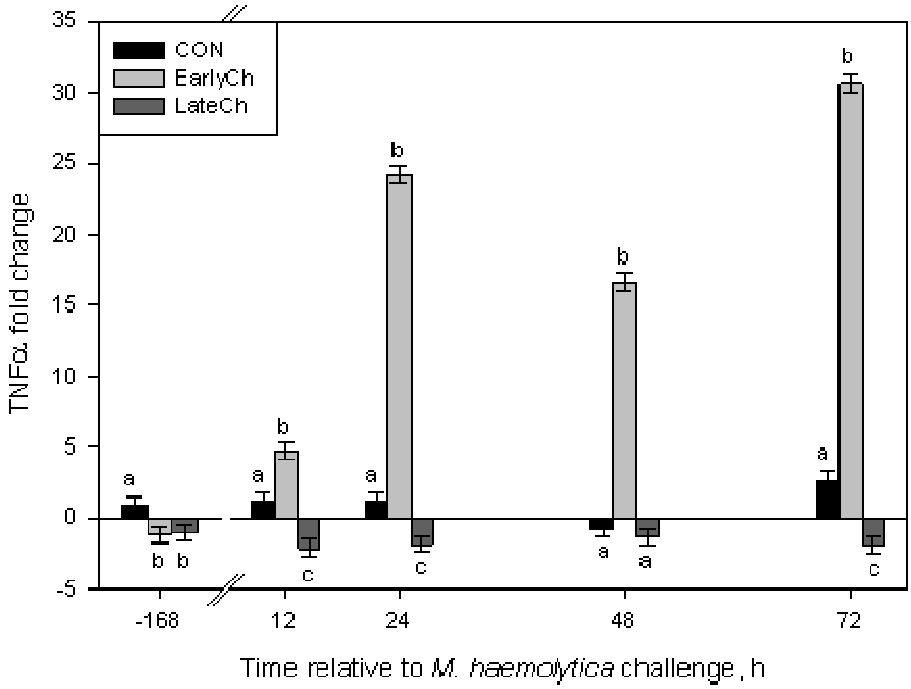

Fig 2c. Gene expression of TNFα in *longissimus dorsi* muscle (LM) in calves exposed to steers PI with BVDV for 72 hours followed by an intratracheal challenge on d 0 with 6×10^9 CFU of *M. haemolytica* serotype 1 (MH) 12 (EarlyCh) or 84 (LateCh) hours after BVDV exposure compared with non-exposed or challenged steers (CON). There was a significant treatment × time interaction ($P < 0.0001$; SEM = 0.33). Values plotted represent Least squares means ± standard error of the mean, calculated for 6 animals per experimental group. [a,b,c]Within day, Least squares means with different letters are different ($P < 0.05$).

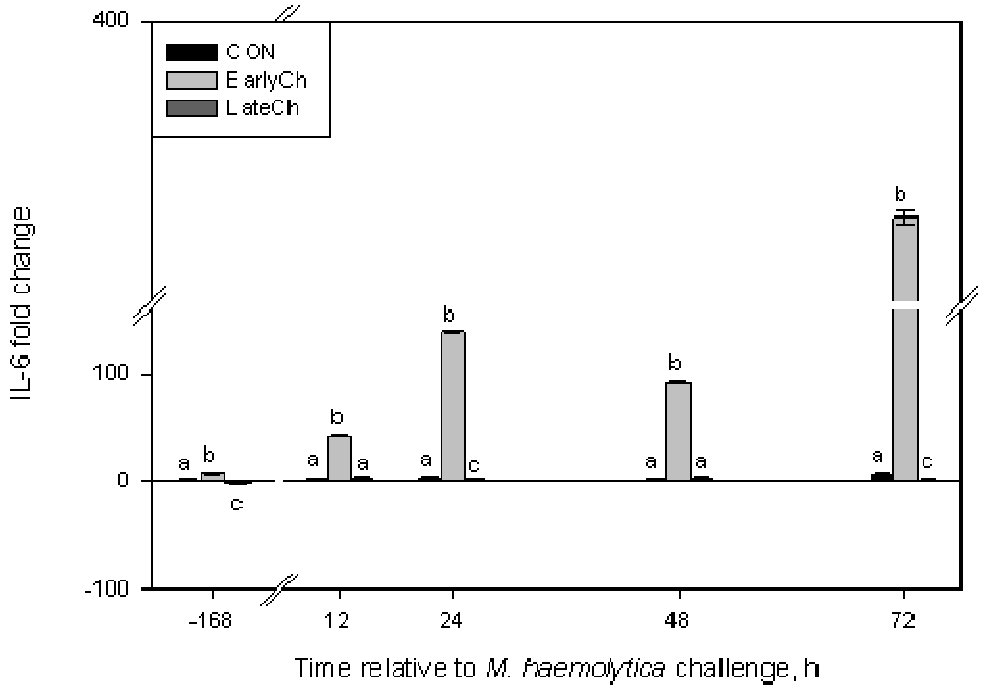

Fig 2d. Gene expression of interleukin-6 (IL-6) in *longissimus dorsi* muscle (LM) in calves exposed to steers PI with BVDV for 72 hours followed by an intratracheal challenge on d 0 with 6×10^9 CFU of *M. haemolytica* serotype 1 (MH) 12 (EarlyCh) or 84 (LateCh) hours after BVDV exposure compared with non-exposed or challenged steers (CON). There was a significant treatment × time interaction ($P < 0.009$; SEM = 0.63). Values plotted represent Least squares means ± standard error of the mean, calculated for 6 animals per experimental group. [a,b,c]Within day, Least squares means with different letters are different ($P < 0.05$).

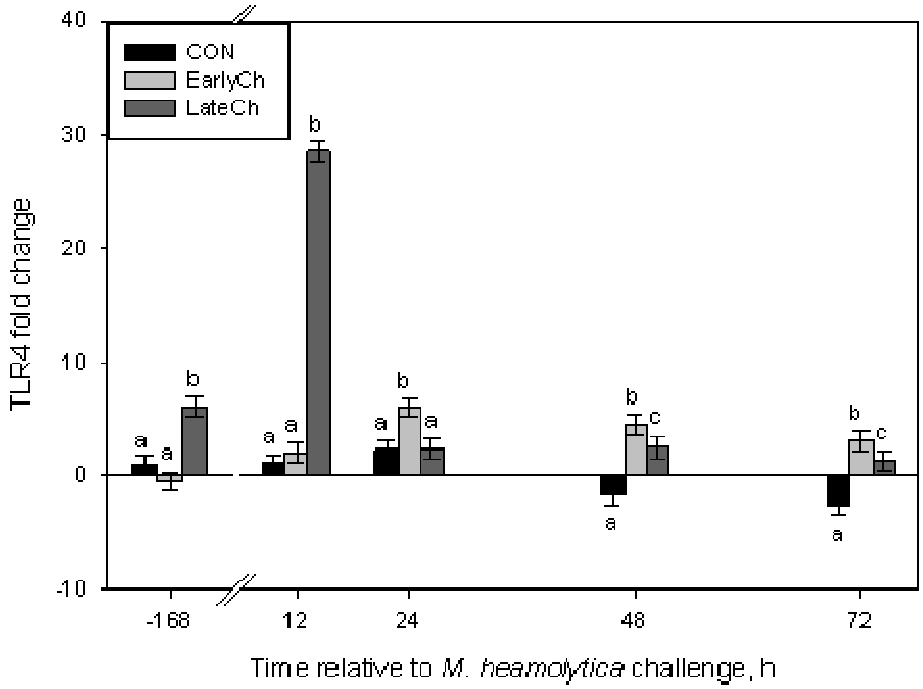

Fig 3a. Gene expression of Toll-like receptor-4 (TLR4) in adipose tissue of calves exposed to steers PI with BVDV for 72 hours followed by an intratracheal challenge on d 0 with 6×10^9 CFU of *M. haemolytica* serotype 1 (MH) 12 (EarlyCh) or 84 (LateCh) hours after BVDV exposure compared with non-exposed or challenged steers (CON). There was a significant treatment × time interaction ($P = 0.02$; SEM= 0.62). Values plotted represent Least squares means ± standard error of the mean, calculated for 6 animals per experimental group. [a,b,c]Within day, Least squares means with different letters are different ($P < 0.05$).

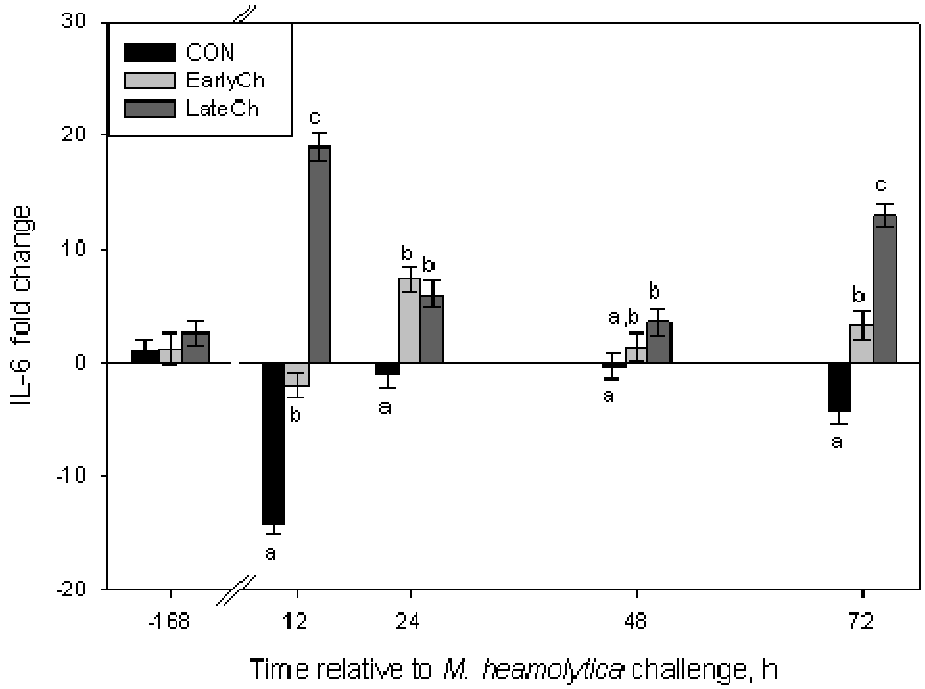

Fig 3b. Gene expression of interleukin-6 (IL-6) in adipose tissue of calves exposed to steers PI with BVDV for 72 hours followed by an intratracheal challenge on d 0 with 6×10^9 CFU of *M. haemolytica* serotype 1 (MH) 12 (EarlyCh) or 84 (LateCh) hours after BVDV exposure compared with non-exposed or challenged steers (CON). There was a significant treatment × time interaction ($P = 0.02$; SEM= 0.70). Values plotted represent Least squares means ± standard error of the mean, calculated for 6 animals per experimental group. [a,b,c]Within day, Least squares means with different letters are different ($P < 0.05$).

VITA

Leonardo Carlos Valdez

Candidate for the Degree of

Doctor of Philosophy

Dissertation: BOVINE RESPIRATORY DISEASE: EFFECTS ON PERFORMANCE, IMMUNE RESPONSE, CYTOKINES AND GENE EXPRESSION

Major Field: Animal Nutrition

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Professional Memberships: America Society of Animal Science

Name: Leonardo Carlos Valdez Date of Degree: July, 2009

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: BOVINE RESPIRATORY DISEASE: EFFECTS ON PERFORMANCE, IMMUNE RESPONSE, CYTOKINES AND GENE EXPRESSION

Pages in Study: 103 Candidate for the Degree of Doctor in Philosophy

Major Field: Animal Nutrition

Scope and Method of Study: The Objective was to determine the effects of timing of an intratracheal *Mannheimia haemolytica* challenge relative to 72-hour exposure to bovine viral diarrhea type 1b (BVDV1b) persistently infected calves (PI) on serum antibody production, total and differential white blood cell count (WBC), clinical signs, and performance of feedlot steers. Twenty-four steers (initial BW = 276 ± 31 kg) were randomly allocated to one of three treatments (eight animals/treatment) in a randomized complete block design. Treatments were: 1) steers not challenged with BVDV or *M. haemolytica* (**CON**); 2) steers intratracheally challenged with *M. haemolytica* 84 hours after being exposed to calves PI with BVDV1b for 72 hours (**LateCh**); and 3) steers intratracheally challenged with *M. haemolytica* 12 hours after being exposed to calves PI with BVDV1b for 72 hours (**EarlyCh**). In the second experiment we used 18 steers of the original 24. Our objective was to determine the effects of two different timings of an intratracheal *M. haemolytica* (**MH**) serotype 1 challenge following short-term exposure (72 hours) to BVDV type 1b on serum concentrations of cytokines and gene expression of cytokines in subcutaneous fat (**SCF**) and *longissimus dorsi* muscle (**LM**) in growing beef steers.

Findings and Conclusions: Delaying the *M. haemolytica* challenge for 84 hours after exposure to calves PI with BVDV increased clinical attitude of BRD and the acute phase response compared with delaying *M. haemolytica* challenge for 12 hours after BVDV exposure. The increased clinical and acute phase response did not affect short-term performance, which appeared to be driven by the greater decrease in dry matter intake for EarlyCh steers. For the second experiment we concluded that the differences in gene expression profiles in LM and SCF and an increment in serum cytokine concentrations during induction of the acute phase protein response to pathogens commonly associated with BRD could ultimately decrease animal growth and carcass quality in growing beef steers.

ADVISER'S APPROVAL: Dr. Clint R. Krehbiel