Immunological impacts following an in-utero BVDV exposure to post-weaning calves

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ABSTRACT

Maternal viral infection has profound impacts on fetal immune development and neonatal susceptibility of disease(s) in humans and livestock. Calves infected in-utero with bovine viral diarrhea virus (BVDV) during gestation are reported to have an increased susceptibility to early life infections such as bovine respiratory disease. The purpose of this study will elucidate mechanisms of the long-term immunological impact of in-utero BVDV infection. Meeting this objective, at gestation day 200 pregnant dams were exposed to BVDV or sham, resulting in the births of transiently infected (TI) and control calves. These calves were then inoculated with *Mannheimia haemolytica* (MH) or media via endoscopic broncho-alveolar lavage (BAL) post weaning. Calves were distributed in a two-by-two study design based on infection status. Control calves, not exposed to BVDV in-utero, inoculated with media (CRTL/CRTL, n=4), control calves inoculated with MH (CRTL/MH, n=3), TI calves, exposed to BVDV in-utero with media inoculation (TI/CRTL, n=3), and TI calves with MH inoculation (TI/MH, n=5). All groups were assigned clinical illness scores (CIS) daily with thoracic ultrasound scoring (TUS) throughout the study. BAL fluid (BALF) was collected for biomarker concentration and cytology analysis. Serum samples were collected to analyze biomarker concentrations and serology. Results found that thoracic ultrasound scores and rectal temperature correlate with MH infection status and study day supported by serology results confirming a successful experimental MH infection. However, the difference between TI calves and control calves infected with MH yielded significant correlations when comparing CIS and TUS. Significant differences were found in mean concentrations of white blood cells (WBC) and lymphocyte between study days. Treatment by study day effects from linear regression was observed in IL-1β, IL-36Ra, and IP-10 from BALF samples and VEGF-A from blood serum. Linear regression between treatment groups was statistically significant in IL-1β, IFNγ, IL-17A, IL-8, IL-36Ra, IP-10, and TNFα from BALF as well as IL-1α and VEGF-A in serum. Post-hoc multiple comparisons yielded statistical significance between study days in VEGF-A and MCP-1 from BALF, MCP-1 and IP-10 in blood serum and serological analysis. These findings hint at a dysfunctional immune response caused by the late gestational in-utero BVDV.

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CHAPTER ONE
INTRODUCTION AND GENERAL INFORMATION

Calf health and production are some of the most important aspects of the cattle industry. This production can be hindered by diseases found in the surrounding environment. Specifically in beef calves, the time of weaning can make them extremely vulnerable to infection due to the stress they experience. Not only does the act of weaning cause stress, but also the common events that occur at this time in their life such as extended periods of transportation, pain experienced from castration and/or banding, dehorning, and co-mingling if taken to sale or feedlot. Additionally, many calves are vaccinated upon arrival to a sale or feedlot which stimulates immune responses to prepare the animal's body for future encounters with pathogens. However, at the moment this may do more harm than good as this adds to the internal stress experienced by the animal. In combination with environmental stressors, the calves' immune response is not as effective and thus making them more susceptible to infection (Hudson et al., 2020). These risks can be amplified if the calf encounters a viral infection during the early stages of development still in the womb.

The maternal environment can have profound impacts on the development of the fetus. If the mother is chronically stressed mentally or physically, or undergoes an extremely traumatic event while pregnant, the fetus's neurodevelopment and immune system will be compromised after birth into adulthood (Kinsella & Monk, 2009). In humans prolonged stress experienced by the mother is correlated with low birthweight, shortened gestation periods or miscarriage, and issues with fetal brain development (Graignic-Philippe et al., 2014). Physical stressors such as chronic illness, anemia, and malnutrition also negatively affect the development of the fetus (Mahajan et al., 2009). Included in the category of physical stress endured by pregnant females are viral infections. The placenta plays a key role in the protection of the fetus from viral infections by mounting its own immune response against invasive pathogens (Zaga-Clavellina et al., 2021). Impacts to fetal development can occur if viral pathogens can cross the placental barrier. This phenomenon has been seen in human cases that connects the viral infection during gestation to defects in fetal development. Outcomes range from compromised immune systems to increased risk of mental illness later into adulthood (Racicot & Mor, 2017). Similarly in cattle, calves are at risk of infection as soon as the early stages of development in the case of bovine viral diarrhea virus (BVDV).

BVDV is a pestivirus in the Flaviviridae family that can cross the placental barrier when infecting pregnant dams and is spread primarily by contact with persistently infected (PI) animals (Falkenberg et al., 2018; Moennig & Liess, 1995). This virus can lead to the decrease in productivity of those infected and can result in long-term consequences to the income of beef cattle producers unaware of a persistently infected animal spreading the virus to the rest of their stock (Damman et al., 2015). A persistently infected calf is produced when the fetus is infected under 150 days of gestation (Brock, 2003). At this point of gestation the fetal calf has not established immunocompetence and thus becomes immunotolerant to the virus (Fredriksen et al., 1999). If the fetus survives to birth this animal will continue to shed virus for the entirety of their life (Houe, 1995). After 150 days of gestation the fetus has established immunocompetence and will clear the virus, falling into the classification of a transiently infected (TI) calf. However, these calves are born with specific antibodies after surviving a virus during fetal development (McDougall, 2021) to later test antibody-negative via ELISA or immunohistochemistry (Fulton et al., 2006). Whether the calf is born persistently or transiently infected, the animal will experience a negative impact on their overall performance and will see an increase in susceptibility to future infections. This impact makes TI calves vulnerable to infection by other pathogens later in life (Hessman et al., 2012). What is currently unknown is the mechanism behind TI BVDV calves having greater susceptibility to infection of respiratory pathogens post-weaning. A recent study found that TI calves showed a shift in epigenetic expression in white blood cells that were collected at the time of birth which may be predictive of the negatively impacted immune response seen in TI calves later in life (Campen et al., 2024). This negative impact on the immune response foreshadows the topic of this study's investigation. By identifying the biological components behind this negative impact, affective measures can be put into place to better combat this immune dysfunction to restore the productivity and welfare of TI BVDV calves.

One of these pathogens being *Mannheimia haemolytica* (MH). While this bacterium is normally found commensally in the tonsils of cattle it can lead to acute pathology and pneumonia when it infiltrates the lungs triggering the production of various virulence factors. MH infection can occur when the calf experiences external stressors such as weaning, long transportation periods, or presence of a viral infection (Melendez et al., 2022). These factors can weaken the first defenses of the innate immune system that would prevent the infiltration of the

pathogen under less extraneous circumstances (Burciaga-Robles et al., 2010). The primary pathology causing virulence factors include leukotoxin, adhesins, proteases, outer membrane proteins, and lipopolysaccharides (Confer & Ayalew, 2019). These virulence factors aid MH in inflicting damage to the lung lobes by evading the immune system inducing the destruction of leukocytes and triggering the production of proinflammatory cytokines (Singh et al., 2011). As a result, MH infiltration can lead to severe pathology in the lungs (such as fibrinous pleuropneumonia and bronchopneumonia) as well as other clinical symptoms that are commonly seen in Bovine Respiratory Disease (BRD) which include fever, inappetence, ocular/nasal discharge, and presence of a cough (Rice et al., 2008). In events of BRD outbreaks caused by pathogenic MH, the pattern in which the bacteria spreads horizontally from one individual to another is suggestive that the characteristics of the immune systems of the calves in the immediate area contributed more to the risk of infection rather than exposure to the pathogen alone (Rice et al., 2008). One of these uninvestigated characteristics is the infection susceptibility of TI calves that had been exposed to BVDV in the later stages of gestation. This characteristic calls for further investigation due to the economic impact on the cattle industry caused by increased morbidity seen in TI BVDV calves, resulting in a decrease in productivity due to their increase in infection susceptibility.

CHAPTER TWO: LITERATURE REVIEW

Introduction

Bovine Respiratory Disease (BRD) affects nearly all branches of the cattle industry including dairy and beef production, cow-calf operations, feedlots, transporters and stockers. Costs of treatment, loss of income from cattle mortality, decreased milk productivity and decreased average daily gain of weaned calves makes BRD the most economically burdening disease in the cattle industry (Koziol et al., 2021). As of 2015, the estimated cost to treat a single individual for BRD is approximately $23.60, leading to even greater losses in profit with herd-wide infections (Grissett et al., 2015). As of 2020, the average cost of treatment per calf increased to range between $29-$50 (Overton, 2021). Between the years of 2011 and 2015, this disease cost beef cow-calf operations $165 million annually (Wang et al., 2018). Nationwide, BRD is estimated to cost the feedlot industry between $800 million and $900 million per year (Blakebrough-Hall et al., 2020; Chirase & Greene, 2001). These expenses include but are not limited to cost of antimicrobials, increase in abortion rates, mortality, metaphylaxis practices, reduced milk production, and decrease of calf weight at sale (Peel, 2020). Prices on which calves are sold depend primarily on the weight of the animal at the time of sale. This means that even though calves eventually grow to their expected weight prior to resolution of infection, calves with decreased weight at the time of sale will have a lower value when sold (Cuevas-Gomez et al., 2020). Many BRD pathogens can also have reproductive consequences. Abortions can occur when the pathogen in an infected dam crosses the placental barrier causing placental damage, alterations in the microbiome of the reproductive tract, and/or fetal death (Oliveira et al., 2022). Not only does the loss of the calf from abortion decrease overall profit, but rebreeding may not be possible if the abortion occurred later in gestation. If the abortion occurs in early gestation, the dam may be able to be rebred to calve that same year. However, this is still an expenditure if the owner uses the method of artificial insemination (Van der Fels-Klerx et al., 2001). To lower the incidence of BRD within cow-calf operations, effective vaccinations against common BRD pathogens are commercially available. However, this is yet another addition to the cost of management practices and preventative measures taken to prevent infection and the spread of respiratory disease. While administration of a vaccine against respiratory pathogens has shown to assist in the prevention of BRD, it does not completely prevent the chances of infection, especially when calves are put in high stress environments (Dubrovsky et al., 2020). Combining

environmental stressors along with the many different factors that result in BRD is what makes this disease so difficult to manage. This is what makes BRD a complex. Attributes from the host, the causing agent, and environmental factors all play a role in the outcome of a BRD case.

Risk Factors of Bovine Respiratory Disease

There are many bacterial and viral pathogens that can result in respiratory disease. However, what gives these pathogens the chance to cause disease in cattle are environmental stressors that weaken the immune system. At the time of birth, calves are especially vulnerable to diseases due to being agammaglobulinemic at birth. Cattle have an epitheliochorial placenta with the uterine epithelium intact throughout the duration of the pregnancy which does not allow for the transfer of maternal antibodies or exposure to pathogens that would lead to a further developed immune system at birth (Mallard et al., 1998). This means that calves need to ingest colostrum at an IgG concentration of 25g/L within 24 to 48 hours after birth (Lombard et al., 2020). Any delays in this initial intake of colostrum, such as a dystocia event, could result in failure of passive immunity, giving BRD causing pathogens a chance to infect the calf (Filteau et al., 2003). Furthermore, even when passive immunity is successfully transferred, absorption of maternal antibodies obtained from colostrum decreases over time making calves 3-5 months of age especially susceptible to BRD (Blodörn et al., 2015). Dairy calves are at greater risk of disease pre-weaning as compared to beef calves due to the nature of beef cattle operations where calves are born in relatively low stress and unconfined environments. In dairy operations, calves spend much of their lives in close quarters with other cattle in buildings and shelters with poor ventilation due to the geographical climate and management practices. Lack of ventilation, used bedding and increased calf-to-calf contact puts dairy calves at an increased risk of BRD infection (G. U. Maier et al., 2019). Additionally, colostrum from young dams or first-time heifers do not contain as many antibodies as colostrum from mature, multiparous dams (Odde, 1988; Schumann et al., 1990; Smith, 2021). This increases the risk of pneumonia in the pre-weaning period of calves born to nulliparous dams. Neonatal calves must also rely on the development of their upper respiratory tract to provide protection against respiratory pathogens via the mucosal epithelium (Hill et al., 2012). The mucosal epithelium is the first physical and innate barrier of the immune system that a respiratory pathogen encounters. This development of the physical layer of the immune system is also dependent upon the transfer of passive immunity at the time of birth (Osman et al., 2018).

By the time calves are weaned, and maternal antibodies have expired, they are relying on their own immune systems for protection. The act of weaning can be a stressful time for calves especially if it occurs at the same time of transportation to another location either for sale or backgrounding (Duff & Galyean, 2006). Calves will often ball excessively when suddenly separated from their dam (Whalin et al., 2021). The action of this vocalization involves large inhalations which may cause the calf to inhale a BRD causing pathogen. These vocalizations along with excessive coughing results in damage to the protective mucosal lining of the nasopharynx region which can allow easy passage for BRD pathogens to enter the lower airways (Chernitsky & Safonov, 2021). Additionally, labored breathing from physical excursion or extreme fluctuations of environmental temperature can compromise the physical barrier of the nasopharynx region that is the mucosal layer (Pratelli et al., 2021). When the mucosal layer is damaged, it gives respiratory pathogens a direct path into the lower airways. During transport, calves are placed into close quarters with others for extended periods of time, making the spread of infectious respiratory pathogens easier as many are spread through aerosolization (Grissett et al., 2015). Calves also experience other stressors such as dehydration, exhaustion, and lowered rumen fill during transportation which put them at an increased risk of infection (Goetz et al., 2021). In dairy calves experiencing heightened stress during transportation, fecal cortisol metabolites have been seen to increase regardless of weaning status which acts as biological evidence of the stress experienced during transport (Vogt et al., 2023).

Upon arrival at a new location, calves continue to experience similar environmental stressors including being comingled with cattle from different locations. Firstly, this adds stress to calves in a behavioral sense as the animals are re-establishing herd hierarchy which sometimes involves aggression towards one another (Landsberg & Denenberg, 2024). Secondly, the stress of transport negatively affects B cell activity making the calf more vulnerable to novel pathogens that could be present or transferred from other animals at the new location (Zhao et al., 2021). While vaccinations against BRD pathogens are available, a low-percentage of cow-calf operations vaccinate against BRD prior to the sale of their stock and thus many of the animals are vaccinated upon arrival at a sale or feedlot (USDA, 2009; Wilhelm et al., 2023). This often is not a guaranteed prevention method as the vaccination needs time to establish effective protection. Additionally, increased cortisol levels resulting in immunosuppression in response to the calf being under stress can lower the efficacy of a vaccine (Richeson, 2015). Administration

of vaccinations alone can be a stressor to a calf as its body is attempting to create humoral immunity to the pathogens presented on top of the events happening around the calf (Richeson & Falkner, 2020). By the time humoral immunity has been established, the calf may have already been infected and is actively spreading disease. These combined stressors are amplified if the calves are malnourished or have co-morbidities such as other infections (Pratelli et al., 2021). Once a calf arrives at a new location, there is a chance for it to spread disease upon introduction to a new herd if there is not ample quarantine time, or if the calf is experiencing a subclinical illness that cannot be identified by visual observation alone. Subclinical BRD cases are becoming more common with the increasing investigations being done on the matter. Cattle that are subclinical often go without treatment and are found to have lung lesions present along with decreased average daily gain as well as decreased carcass values (Griffin, 2014). These cases must be identified using special diagnostic methods such as thoracic ultrasound or other invasive diagnostic methods that do not rely strictly on visual observation alone (Fernandez et al., 2020; Kamel et al., 2024; Mahmoud et al., 2022).

Bovine Respiratory Disease-Causing Pathogens

Multiple viral and bacterial pathogens contribute to the BRD complex upon infection of the respiratory system, and many times BRD cases are not from a single causative agent. Bacterial pathogens that result in BRD include: *Mannheimia haemolytica, Mycoplasma bovis, Histophilus somni and Pasturella multocida.* The viral pathogens that result in BRD include bovine viral diarrhea virus (BVDV1 and 2), bovine herpesvirus type 1 (BoHV-1), parainfluenza-3 virus (PI3), bovine corona virus (BCoV), bovine respiratory syncytial virus (BRSV) (Grissett et al., 2015; Hause et al., 2015; Kiser & Neibergs, 2021), and bovine adenovirus (BAdVs) (Werid et al., 2025). Environmental stressors as discussed previously allow these pathogens easy access to the body to cause disease. Due to the various factors that contribute to the complex of BRD, it is necessary to have a well-rounded approach to diagnosis and management (Kamel et al., 2024). Subclinical cases and the complex factors of BRD make early diagnosis and prevention challenging, as decisions to pursue invasive investigations often depend on visible clinical signs. In most cases, a respiratory virus sets the stage for infection of a bacterial pathogen. A virus will infect the animal causing clinical illness including damage of the mucosal layer lining the respiratory tract allowing bacterial pathogens easy entry into the lower airways to cause further infection (Gaudino et al., 2022). It is also in these cases of coinfections that

mortality is most likely to occur due to the dysfunction of immune cells caused by the primary viral infection (Taylor, Fulton, Lehenbauer, Step, & Anthony, 2010).

Mannheimia haemolytica

Mannheimia haemolytica is the most common respiratory bacterial pathogen that results in BRD among United States cattle industries (Crosby et al., 2018; Griffin et al., 2010). It is gram-negative, non-spore forming and a member of the Pastuerellaceae (Confer & Ayalew, 2019). *M. haemolytica* is a part of the natural microbiota within mucosal biofilms of the upper airways including the nasal passages, nasopharynx region, and tonsils of cattle (Gandhi et al., 2023). When *M. haemolytica* becomes pathogenic and results in clinical disease, it has traveled from the nasal passage into the lower airways resulting in lesions and lung consolidation (Cozens et al., 2019). Clinical manifestation of infection includes common respiratory signs such as cough, depression, decrease in appetite, labored breathing, ocular and nasal discharge, and presence of a fever. Internal symptoms can include acute fibrinous pleuropneumonia, fibrinous bronchopneumonia, lung consolidation, and mediastinal enlarged lymph nodes that can be seen through thoracic ultrasonography (Grissett et al., 2015). *M. haemolytica* can also be spread horizontally from animal to animal through aerosolization of particles. It is most prevalent in calves that have not been previously immunized or exposed to the pathogen prior to transportation and introduction to new individuals (Woolums et al., 2018). If a BRD case caused by *M. haemolytica* is suspected, a sample is taken from the respiratory tract via transtracheal wash or deep nasopharyngeal swab. Upon confirmation of *M. haemolytica* being the causative agent, a treatment plan is made as a bacterial infection can be treated using antimicrobials. Many antibiotics are labeled for the treatment of BRD pathogens including tilmicosin and tulathromycin for prevention, i.e. metaphylaxis or active therapeutic treatment (Zaheer et al., 2013).

The onset of disease can occur as early as 24-hours post infection of the lungs. Peak clinical signs ranging between the first 24 to 48 hours with resolution occurring around 8 days post infection (Grissett et al., 2015). Under normal circumstances, *M. haemolytica* would remain in the upper airways. However, during times of high stress such as travel, sale, weaning, failure of passive immunity, viral infection or a combination of such factors, the mucosal layer of the respiratory tract is degraded thus allowing *M. haemolytica* particles to be released from their biofilms and infect the lower airways (Crosby et al., 2018; Griffin et al., 2010). In-vitro studies

have tested this phenomenon by challenging *M. haemolytica* biofilms with epinephrine, norepinephrine, and Substance P which simulates the chemicals released in a cow's body during times of stress. These studies showed that after being challenged with these chemicals, *M. haemolytica* is released from their respective nasal biofilms and proliferate (Pillai et al., 2018). Substance P is a common chemical seen in calves infected with *M. haemolytica* that acts as a blood biomarker indicating the experience of pain which may be associated with common management procedures such as castration (Theurer et al., 2013a). When *M. haemolytica* enters the lungs, the bacteria proliferates and begins secreting leukotoxins, and lipopolysaccharides. Leukotoxins attack white blood cells that aid in the body's immune response and lipopolysaccharides protect the bacteria. These two factors allow *M. haemolytica* to cause severe pathology in the lungs of infected cattle (Cozens et al., 2019).

Using PCR analysis, *M. haemolytica* was found to have various virulence genes including LKT, which reduces neutrophils ability to form NETs, putative adhesion (adhs), O-sialogycoprotease (gcp) that removes glycoproteins from macrophages and white blood cells, and Transferrin binding proteins (tbpB) that allow the bacteria to acquire iron from the host (Aulik et al., 2010; Lo, 2001). These genes code for virulence factors such as the secretion of leukotoxins and lipopolysaccharides, adhesins, capsules, outer membrane proteins, and a variety of proteases. These genes are consistent across all serotypes. Adhesins allow bacteria particles to adhere to epithelial cells (Kisiela & Czuprynski, 2009). This adhesion is aided by neuraminidase, also produced by *M. haemolytica*. Neuraminidase lowers the viscosity of the mucus membrane in the respiratory tract which results in easy access for the bacteria to the epithelium. Another function of neuraminidase is the cleavage of sialic acid residues on the epithelial surface (Rogers, 2024b). This allows bacteria cells to get closer proximity to epithelial cells. Leukotoxins attack white blood cells produced by the innate immune system of the host to protect the invading bacteria by degrading the cell membranes of bovine alveolar macrophages and neutrophils, resulting in cytolysis of these immune cells (Jeyaseelan et al., 2002). Following cytolysis, these immune cells release proinflammatory substances and proteolytic enzymes that cause further damage to alveolar linings. Specific to neutrophils, which are non-specific innate immune cells that are first to the site of infection, leukotoxins inhibit their ability to release reactive oxygen intermediates that would be harmful to the bacteria (Czuprynski & Noel, 1990). This reaction is caused by the surge of calcium into bovine alveolar macrophages and neutrophils

(Jeyaseelan et al., 2002). Bovine neutrophils are also seen to have less ability to travel from the bloodstream to the site of infection in the presence of leukotoxins (Clinkenbeard et al., 1989). With the help of transferrin binding proteins that allow the bacteria to acquire iron, *M. haemolytica* can cause severe pathology within the lower airways. These virulence factors allow the pathogen to avoid elimination by the innate immune cells. The event of infection further induces the stimulation of proinflammatory cytokines. While this is the body's way of enhancing inflammation in attempt to eradicate an infection, it increases the amount of immunopathologic damage to the lungs resulting from the infection in addition to the endotoxins secreted by *M. haemolytica*.

M. haemolytica can be classified into different serotypes. Both A1 and A2 can be found in the normal microflora of the tonsils and nasal passage. Serotype A1 and A6 are the strains that result in disease when introduced to the lower airways (Rice et al., 2008). After the bacteria proliferates in the lungs, *M. haemolytica* uses transcytosis to invade differentiated bovine bronchial epithelial cells. Within these epithelial cells, *M. haemolytica* undergoes rapid replication to allow increased spread (Cozens et al., 2019). With the increase of antibiotic resistance in *M. haemolytica* serotypes, other treatment approaches are being analyzed. Resistance has been seen in the use of broad-spectrum antibiotics such as fluoroquinolones. *M. haemolytica* resistance to fluoroquinolones has been seen specifically in the serotype A6, creating obstacles during treatment in the face of an active infection (Katsuda et al., 2009). Prevention methods common against *M. haemolytica* include commercially available vaccinations that have shown to induce effective immune responses (Aubry et al., 2001). Other methods target inflammation caused by *M. haemolytica* virulence factors. LPS endotoxin specifically can lead to pathological damage to the bronchial epithelial layer which can be targeted through treatment rather than targeting the bacteria itself (McClenahan et al., 2008). A recent study found that when virulence factors induce the release of pro-inflammatory molecules (IL-6, IL-8, and TNF-α) and increase toll-like receptors 5 and 4 (TLR5 and TLR4) expression that is activated by MAPK proteins and NF-kB p65. This weakens the epithelial layer, allowing pathogens access into respiratory cells to cause further pathology. The conclusion of this study suggests that further research into targeting TLR5 and 4 expressions in the face of an *M. haemolytica* infection may bring more treatment options to the surface aside from antimicrobial therapy (Cai et al., 2020). Identifying causative and prevalent serotypes is also important when

analyzing clinical disease along with planning prevention and treatment. The serotype A6 is normally found in clinically diseased cattle while A2 is commensally present. However, these serotypes differ greatly in genotype which can dictate the resulting outcome and if there will be resulting pathology (Lawrence et al., 2010). In a recent study, 96 *M. haemolytica* isolates were genome sequenced to identify trends in antibiotic resistance and resulting pathology upon necropsy exam. Serotypes A1 and A6 were found in cattle with acute fibrinous pleuropneumonia while serotype A2 was found in cattle with fibrinous polyserositis. Serotypes A6 and A1 were shown to have lower amounts of antibiotic resistant genes (Het Lam et al., 2023). By being able to identify the infecting *M. haemolytica* serotype, practitioners can make treatment plans according to which serotypes are more likely to be resistant to antibiotics.

Other BRD Causing Bacterial Pathogens

Many other bacterial pathogens can result in clinical BRD. Similar to *M. haemolytica*, *Histophilus somni* is also found in the natural microflora of cattle respiratory tracts and it can also be spread via aerosolation. *H. somni* becomes pathogenic in the upper airways when the animal's immune system is compromised from environmental stressors or a previous viral infection (Compiani et al., 2023; Janosi et al., 2009; Meyer, 2025). If the pathogen invades the circulation, it then has the ability to cause pathology in other systems including the respiratory system (Meyer, 2025). In other systems, this pathogen can result in thrombotic meningoencephalitis, synovitis, myocarditis, arthritis, and future reproductive issues (Confer et al., 2016; Margineda et al., 2019). Clinically this pathogen displays respiratory disease symptoms such as coughing, labored breathing, and fever (Shirbroun, 2020).

Another gram-negative BRD causing bacteria that can be found commensally in the nasopharyngeal region is *Pasteurella multocida*. After infection of the lungs, the bacteria will express virulence factors that allow it to cause pathology in the lungs that may lead to bronchopneumonia, pleuritis, and fibrinous pleuritis (Dabo et al., 2008; McGavin & Zachary, 2006). Clinical manifestation of the infection includes depression, nasal discharge, inappetence, cough and fever. This pathogen is more common in calves. A distinct feature of *Pasteurella multocida* is that when it does result in bronchopneumonia, it does not occur as suddenly as bronchopneumonia caused by *M. haemolytica* (Dabo et al., 2008).

A major BRD causing bacteria is *Mycoplasma bovis* which is an important pathogen as it can cause chronic pneumonia in cattle despite antibiotic treatments which eventually becomes

fatal (Caswell et al., 2010). The reason for this phenomenon is because *Mycoplasma* has virulence factors that allow it to adhere to host cells, effectively avoiding immune responses mounted against it (Bürki et al., 2015). Resulting pathology of *Mycoplasma bovis* includes the development of pulmonary lesions of caseonecrotic bronchopneumonia where areas of lung consolidation develop nodes of dry fibrin. *Mycoplasma* is spread horizontally from cattle encountering the nasal secretions of an infected individual (Arcangioli et al., 2021). Calves still in the pre-weaning stages of life can also become infected if the milk they ingest is contaminated. The clinical manifestations that are characteristic of *Mycoplasma* are recurring respiratory infections, lameness, and inner ear infections. This pathogen also induces common respiratory clinical signs such as fever, cough, nasal discharge, and depression (Caswell et al., 2010).

Bovine Viral Diarrhea Virus

One of the most complex viral pathogens that results in BRD is the *Pestivirus* bovine viral diarrhea virus (BVDV). BVDV is a single stranded RNA virus which gives it the ability to easily mutate (Bolin, 2002). DNA viruses invade host cells to perform their own replication using the metabolism of the host cell. Whereas RNA viruses use the machinery of a host cell to make microbial proteins that exit the host cell to continue the spread of the virus (Durmus & Ulgen, 2017). Not only is this infection a danger to susceptible adult cattle and calves, but it is also a danger to calves still in fetal development. The effect of this virus depends greatly on the genotype and biotype of the virus, the stage of life of the animal being infected which in-turn affects the type of infection, and the presence of other respiratory pathogens (Ridpath, 2010). The origin of most outbreaks from BVDV occurs from an interaction with an animal that is persistently infected with the virus (Moennig et al., 2005). The virus spreads when an infected animal sheds the virus and comes into contact with others, transmitting via aerosolized droplets, shared feed, water, space, and specific vectors (Khodakaram-Tafti & Farjanikish, 2017). In healthy, non-pregnant animals an acute infection of BVDV will likely result in a subclinical or a mild clinical case. The animal will then be viremic and display symptoms such as fever, depression, loss of appetite, ocular/nasal discharge, and diarrhea up to 15 days after initial infection (Khodakaram-Tafti & Farjanikish, 2017). Both the cytopathic and non-cytopathic biotypes of the virus can result in an acute infection. The cytopathic biotype is less common and is found when the animal is displaying symptoms of mucosal disease which in most cases is a

result of a persistent superinfection (Kehrli et al., 2009). An in-vitro study found that the cytopathic strain results in mitochondrial breakdown of infected cells leading to the increase of reactive oxygen species (ROS) finalizing in apoptosis of the cell (Grummer et al., 2002). However, it is unclear whether this molecular mechanism of the cytopathic biotype pathogenesis reflects the observation of epithelial cell apoptosis and in-vivo immunosuppression (Ridpath, 2010). The non-cytopathic strain is the most common in acute infections and is the only type that can result in a persistent infection (Kehrli et al., 2009; Walz et al., 2020). A transient infection of the non-cytopathic strain can result in immunosuppression by targeting lymphoid cells and inhibiting the functions of neutrophils (Kehrli et al., 2009; Walz et al., 2020). Immunosuppression caused by acute infections of BVDV sets the stage for a secondary infection of a bacterial respiratory pathogen and, with the innate immune system shut down by the immunosuppressive effects of BVDV, the development of BRD from a viral-bacterial co-infection is inevitable (Basqueira et al., 2017; Perino, 1989).

Infection of mature animals' results in a decrease in fertility and reproductive success. When a breeding bull is infected, not only is the semen produced contaminated, but the virus can sometimes be correlated to the occurrence of testicular lesions. However, bulls that are born as persistently infected animals that survive to maturity can produce clinically healthy offspring (Fray et al., 2000). The significance of this is the virus cannot be spread to fetal calves paternally through semen, horizontal transmission can only occur from a maternal infection (Meyling & Jensen, 1988). Cows and heifers that are used for breeding experience fewer ovulations and a decrease in the quality of embryos when infected with BVDV (Fray et al., 2000). Before ovulation, a BVDV infection can reduce the rate in which a follicle can mature (Grooms et al., 1998). Not only do these occurrences make a breeding animal less productive, but it can also be difficult for the producer if they use artificial insemination to breed their stock which relies heavily on the timing of the animal's estrous cycle. In operations that utilize artificial insemination, semen from a PI bull can pass on the virus to the cow that would birth a PI calf (Meyling & Jensen, 1988). The mechanisms behind how BVDV negatively affects the ovarian reproduction system are unknown but suggest unnatural hormone imbalances caused by the viral infection (Fray et al., 2000; Grooms, 2004). If a pregnant animal is infected with BVDV the virus has the capability to cross the placental barrier and infect the fetus (Swasdipan et al., 2002). In some cases, a fetal infection can result in the fetus not surviving to birth from the virus

causing the dam to abort the pregnancy or trigger a premature birth (Cornell University, 2025; Grünberg, 2024). In the case of fetal death, mummification of the fetus is also a possibility which can be harmful to the dam (Grünberg, 2024). If the fetus survives to birth, the calf can be born with multiple malformations such as cerebellar degeneration, ocular lesions, and malformations to the central nervous system (Moennig & Liess, 1995). Other outcomes of a successful birth of a fetal infection depend on the biotype of the virus and the stage of gestation infection occurs. A transient infection occurs when a fetal calf is infected at gestation day 150 to birth (Knapek et al., 2020). By then the fetus's immune system is established enough to recognize the virus as foreign and will mount an immune response, clearing the virus before birth (Peterhans et al., 2003). If the infection of a non-cytopathic BVDV strain occurs less than 150 days of gestation when the immune system of the fetus is not developed enough to recognize a virus as foreign, the result will be the birth of a persistently infected calf if the pregnancy is successful (Brock, 2003). This means that the calf will continually shed virus and infect others it encounters for the length of its life (Brock, 2003). Some calves will succumb to the virus before they reach adulthood due to their compromised immune system and other complications such as cerebral hyperplasia, cataracts, and stunted growth (Grooms, 1998). Others will survive to maturity and be less productive while others will grow completely normal for production and/or breeding (Khodakaram-Tafti & Farjanikish, 2017; USDA, 2023; Xue et al., 2009).

Other BRD-Causing Viral Pathogens

Similar to the many bacterial pathogens that cause BRD, there are many BRD causing viral pathogens. Bovine respiratory syncytial virus (BRSV) begins showing respiratory clinical signs on average 3 days post infection which is also when the infected animal becomes viremic (Grissett et al., 2015). A discerning feature of BRSV is the severe pathology in the lungs induced by the virus attacking cells in the mucosal ciliary layer creating the ideal environment for respiratory pathogens (Zoetis, 2024). Another identifying result of this virus is the formation of epithelial syncytia and intracytoplasmic viral inclusions (Chien et al., 2022). The virus will stay inside the syncytial cell and continue to replicate (Jessie & Dobrovolny, 2021). It results in emphysema, bronchopneumonia, and severe lungs lesions. Parainfluenza-3 (PI3) shares the same respiratory symptoms as BRSV and BVDV and is seen in most cases to resolve around 7 days post infection. Without the presence of a co-infection, PI3 is a less aggressive virus in terms of physical pathology. This virus results in damage of the mucosal layer in the nasopharynx region,

mild to severe bronchopneumonia and infection of alveolar macrophages (Jolly & Ditchfield, 1965; Probert et al., 1977). Instances of a fatal infection are usually in the presence of another infection in addition to PI3. Bovine herpes virus-1 (BHV-1) (or infectious bovine rhinotracheitis) can be delayed in its clinical manifestation by 2-3 days (Grissett et al., 2015). When it does manifest, it appears as lesions in the nasal passage, inflammation of the trachea and conjunctivitis (Jones & Chowdhury, 2007). This virus also affects the reproductive system as it causes infectious pustular vulvovaginitis, abortions, and inflammation of the external genitalia of bulls (Jones & Chowdhury, 2007). In calves it can cause neurological issues such as encephalitis (Schudel et al., 1986). Another discerning factor of this disease is after initial infection, "flare-ups" can continue to occur when the animal experiences an excess of environmental or internal stressors. In this case vaccines only offer short-term protection. Fatalities from this virus alone rarely occur unless the immune system is already previously compromised from another respiratory bacterial pathogen (Campbell, 2023; Drivers, 2008). Bovine coronavirus (BCoV) appears clinically as yellow diarrhea, infection of the intestines and respiratory symptoms such as cough, nasal discharge, and labored breathing (Clark, 1993; Vlasova & Saif, 2021). This virus is also responsible for winter dysentery in adult cattle characterized by the presence of blood in diarrhea (Vlasova & Saif, 2021). However, when sampled, coronaviruses can be found in both clinically ill and healthy animals. The virus itself is of low virulence, as it rarely results in moralities without the help of other present respiratory pathogens (Ellis, 2019). Some strains of the virus do not result in clinical illness and only shed virus through the body of the host. Horizontal spread of bovine coronavirus can occur through aerosolization or fecal to oral transfer (Frucchi et al., 2022). Other than treating for symptoms, there is no treatment specifically for the virus (Hodnik et al., 2020). Bovine adenovirus also has clinical respiratory symptoms similar to other respiratory viral pathogens in cattle excluding an increase in body temperature. Other symptoms include loss of appetite, conjunctivitis, bronchopneumonia, and lesions in the lung lobes. Molecularly, the virus causes an increase in neutrophil granulocytes and a decrease in leukocytes. Horizontal transfer occurs through aerosolization (Belák et al., 1977; Ide et al., 1969).

Methods in Bovine Respiratory Disease Diagnosis

One of the biggest issues with BRD is the lack of reliable diagnosis methods available for in-field use. This is due to the absence of a definitive pattern of symptoms that explicitly reveals

whether an animal or herd is experiencing a BRD outbreak. What makes diagnosis an even more difficult task is the occurrence of sub-clinical cases where the animal is infected with a BRD causing pathogen yet will not display any clinical symptoms. This then leads to herd-wide outbreaks as the sub-clinically infected animal does not receive treatment and continues to spread disease to herd mates. When an animal does display clinical illness, the symptoms are commonly seen among other diseases which makes determination of treatment difficult. As of now, the most reliable methods of diagnosis are postmortem necropsy and transtracheal wash (Cummings et al., 2022; Pardon & Buczinski, 2020). The downside to postmortem necropsy is it comes at the cost of an animal and is time-consuming, allowing for an outbreak to occur in the time it takes for results to be reported back (Cummings et al., 2022).

Clinical Scoring Systems

Visual examinations of prolonged abnormal behavior from an animal are usually the first indicators of an illness. In the cases of clinical BRD, there are validated guidelines and scoring systems used by caretakers and practitioners to make a diagnosis before moving to more invasive measures. Currently, there are two different scoring systems. The Wisconsin system and the UC Davis Scoring System. Both look at similar clinical symptoms such as rectal temperature, nasal and ocular discharge, presence of cough, and ear position with further evaluation of respiratory quality in the UC Davis system (Aly et al., 2014). However, how the symptoms are scored are different depending on the system used (Gabriele U. Maier et al., 2019). The Wisconsin Scoring System uses a range of 0 to 3 based on the severity of each individual symptom. If the combined respiratory scores reach 4, the animal is monitored to see if symptoms worsen. If the combined score reaches 5 or more, the animal is to begin treatment as recommended by a veterinarian (McGuirk & Peek, 2014). The UC Davis Scoring System assigns the severity of the scores based on the symptoms seen. Normal ranges are given a score of zero. The presence of ocular discharge, cough, labored breathing, and rectal temperature of 102.5°F or higher are each given a score of two regardless of the severity. The presence of nasal discharge is given a score of 4 and the presence of an ear droop or head tilt is given a score of 5. If these combined scores reach a total of 5 or more, the animal is considered a positive BRD case (Aly et al., 2014; Decaris et al., 2022; Love et al., 2014). However, these scoring systems alone are not efficacious in the event of a sub-clinical BRD case (Cuevas-Gomez et al., 2020).

Thoracic Ultrasound

Since most pathogens of BRD affect the respiratory tract and lower airways, another method of diagnosis would be thoracic ultrasonography (TU) to search for lesions or other evidence of pathology in the lungs (Buczinski, Forté, & Bélanger, 2013). While this method may not be practical to perform on every individual animal in a herd, it can be used to identify individual BRD cases or as a screening tool to make timely treatment decisions and minimize morbidity and mortality. When performing a TU on a cow or calf, a transrectal probe is used over the cranial thorax and axillary region to view the inside of the lung lobes (Ollivett & Buczinski, 2016). Prior to examination, the area between the fifth and twelfth intercostal space is shaved (Babkine & Blond, 2009). When looking at an ultrasound, evidence of lung pathology will show up as defects in the pleural surface, comet tails, and "pockets" of lung consolidation (Porter et al., 2021). One can also use a TU to evaluate the severity of present lung pathology (Buczinski, Forté, Francoz, et al., 2013). This method can identify subclinical cases of BRD as well as early onset of disease even before an inflammatory response can be triggered by the innate immune system (Cramer et al., 2019). Like clinical illness scoring systems, a thoracic ultrasound scoring (TUS) system was developed to aid in-field diagnosis of pneumonia in cattle. In a range from 0 to 5, increased evidence of consolidation as well as amount of lung lobes consolidated is aligned with increasing scores (Ollivett & Buczinski, 2016). Unfortunately, this method alone is not always guaranteed to definitively allow veterinarians to diagnose a BRD case. In a recent study to compare the agreement and alignment of clinical respiratory scores and TUS, it was found that clinical illness had to reach severe levels to be an indicator of lung consolidation (Hinnant et al., 2023). Cattle can have a large percentage of their lungs damaged and still not show any clinical symptoms. *Mannhiemia haemolytica* for example can cause pathology in the lungs very quickly after infection before clinical signs appear. Furthermore, lung lesions seen on a TUS image does not always indicate an active BRD case (Cuevas-Gomez et al., 2021).

Nasal and Nasopharyngeal Swab

To identify which exact pathogen is causing an infection, a sample from the respiratory tract must be taken for analysis. The least invasive method is a nasal swab or a nasopharyngeal swab (NPS). A nasal swab is a simple sample collection of the inner naris. The issue with this method is since many of the bacterial BRD causing pathogens are commensal in the nasal passage without causing disease, it is difficult to identify an invasive and disease-causing

pathogen from this method alone. The NPS is a more effective method of sampling the nasal passage (Pohjanvirta et al., 2021). However, the risk of sample contamination remains from other commensal pathogens of the tonsils and respiratory tract. It is also a more expensive method comparative to the nasal swab (Doyle et al., 2017). When these methods are paired with TUS, researchers can compare the change in the microbiome of the nasopharyngeal region to the presence of lungs lesions. Depending on the change to the nasopharyngeal region microbiome can indicate a BRD case with evidence of lung pathology since lung lesions alone does not always indicate a BRD case (Raabis et al., 2021).

Bronchoalveolar Lavage

A more invasive method of BRD diagnosis is bronchoalveolar lavage (BAL). BALs allow the extraction of samples directly from the pulmonary fluid of the lungs (Cornell University, 2024). An endoscope can also be used to extract samples from any desired lung lobe. This method can be done quickly without the sedation of the animal. A disadvantage to this method is the BAL catheter must pass through the nasal passage into the trachea to reach the lungs. During this process, contamination of the pulmonary fluid sample can occur when going through the nasal passage (Capik et al., 2017). By analyzing pulmonary fluid from BALs, veterinarians are able to identify specific pathogens present in the lungs and thus be able to report if the pathogens are BRD causing (Doyle et al., 2017). In addition, pulmonary fluid from BALs can also be used to analyze immune cell concentrations that appear in the presence of an infection. Immune cells that are indicative of a respiratory infection include neutrophils, macrophages, eosinophils, and lymphocytes in the respiratory tract (Van Leenen et al., 2019). The exception to this occurrence is BVDV which results in the disfunction of neutrophils depending on the biotype of the virus causing the disease (Thakur et al., 2020). BALs can accurately identify if an animal is infected with a BRD causing pathogen in both clinical and subclinical cases when analyzing neutrophil concentrations (Ollivett et al., 2015). The most invasive method of diagnosis is a transtracheal wash (TTW). This method is similar to BAL as it involves extraction of pulmonary fluid for cytology analysis and bacteriology. However, TTW, which is the “gold-standard” method of identifying the causative pathogen, takes away the risk of sample contamination as involves inserting a catheter directly into the trachea as opposed to entering the airway through the nasal passage. A disadvantage to this method is not only is it invasive, but it is also time-consuming (Doyle et al., 2017; Pardon & Buczinski, 2020).

Choosing a Sample Collection Method

The preference of a sample collection method is dependent on situational factors such as desired area of sampling, extent of restraint needed, skill needed to obtain the sample, and materials needed for the collection. The "gold standard" method of pathogen identification used in the field is transtracheal wash (TTW) (Pardon & Buczinski, 2020). This method allows for a representation of the entire lung in a short amount of time. Endoscopic BAL is a less invasive and is an economically conscious method if a sample of the lower respiratory tract is needed. It is important to note that samples collected through BAL will not always match microorganisms found in an NPS sample and vice versa (Capik et al., 2017). The significance of this is that bacteria found using an NPS collection method cannot 100% accurately predict disease causing pathogens in the lower respiratory tract as the microbiota of the nasopharyngeal region shift during times of stress experienced by the animal (Zeineldin et al., 2017). BAL and TTW agree when comparing sample results and a viral respiratory pathogen is involved. However, in the case of a virus, BAL and TTW do not agree well with upper respiratory sample collection methods such as NS and NPS, this is especially true with BoCOV infections. In the case of a bacterial infection, TTW agrees with BAL and upper respiratory tract collection methods (Doyle et al., 2017). Timing is also an important factor to consider when choosing a method of diagnosis. Bacteria may be present in the lungs of cattle arriving to a new location within the first few weeks. If a clinical illness is present after that time in which bacterial pathogens have been cleared from the lungs, one may hypothesize that an infection is viral in the lower airways (Allen et al., 1991). Choosing a sampling method is also dependent on the number of animals one needs to investigate. If an entire herd needs to be sampled for a BRD causing pathogen, fast and cost-effective methods such as an NPS is an ideal method of sampling (Godinho et al., 2007).

Treatment Methods of Bovine Respiratory Disease

Once a diagnosis has been made that an animal is infected with a BRD causing pathogen and the pathogen has been identified, an appropriate quarantine and treatment plan can be made. In some cases, treatment is needed before the causing pathogen can be identified. In these situations, medications labeled for metaphylactic use can be administered to the animal by discretion of the veterinarian on-site. For bacterial pathogens, treatment involves antimicrobials to combat the infection. These antimicrobials can be single injections or therapy through multiple injections depending on the drug used and the pathogen it is treating. Common antimicrobials

that can be used to treat BRD caused by bacteria include oxytetracycline, enroflaxcin, florfenicol, ceftiofur, tilmicosin, and tulathromycin (Draxxin) (Campbell, 2023). A debate has been studied whether mortality rates caused by BRD will decrease if antimicrobials were used in intervals at smaller concentrations as opposed to single injection treatments (Apley, 2015). This may result in fewer relapses and faster recovery. However, the issue of antimicrobial resistance calls for more research on this matter (Cuevas-Gomez et al., 2020; Gorden & Plummer, 2010). Prophylactic antimicrobial treatments have become common practice as a method of prevention. An antimicrobial can be given to calves before the event of transport where a calf becomes at higher risk of BRD infection which, in some studies, have shown be an effective method (Rerat et al., 2012). Feedlots have adopted the method of antimicrobial prophylaxis which involves the administration of an antimicrobial to a high-risk, stressed individual or herd of cattle to decrease the likelihood of BRD incidence (Van Donkersgoed, 1992). This method has been shown to decrease mortality and morbidity in newly arrived feedlot calves (Ives & Richeson, 2015). The metaphylaxis/prophylaxis methods that proved most effective were broad-spectrum antimicrobials (Baptiste & Kyvsgaard, 2017). These methods are meant to be highly effective for short time periods to prevent immediate mortality and/or morbidity (Baptiste & Kyvsgaard, 2017). A downfall to this is the emergence of antimicrobial resistance in BRD causing pathogens. While data covering this is limited, resistance in *P. multocida* and *M. haemolytica* against penicillin, tetracyclines, and tulathromycin is occurring at an increasing rate (Schonecker et al., 2020). These antimicrobials can be paired with anti-inflammatory medications such as meloxicam to improve the patient's behavior and encourage physical recovery (Lockwood et al., 2003). With this encouraged recovery, one can see a decrease in lung lesions after administration of an NSAID (Francoz et al., 2012). Unless paired with a bacterial co-infection, respiratory viruses can only be vaccinated against and cannot be treated. It is important to note that resolution of the disease without medication will occur if the animal survives the entirety of the infection excluding persistently infected BVDV animals and those infected with BHV-1.

Prevention of Bovine Respiratory Disease

Taking into consideration the risk factors and nature of the causal pathogens, methods of prevention come in a wide range. Since calves are born agammaglobulinemic and are at the greatest risk of infection at birth, ensuring the transfer of passive immunity that comes from colostrum after birth is essential in management practices (Stokka, 2010; Windeyer et al., 2017).

Calves need to intake at least 8 percent of their body weight in colostrum within at least 5 hours post-partum (Morter, 2010). Colostrum given to the calf needs to have an IgG concentration of no less than 10 mg/mL (Godden, 2008). Adequate levels of IgG concentration in colostrum is classified at 50 g/L (McGuirk & Collins, 2004). This is especially important in bottle calves. Directly after ensuring transfer of passive immunity, naval dipping in iodine is another method of preventing invasive pathogens from entering through the umbilicus (Grover & Godden, 2011).

As BRD affects the respiratory tract, having adequate ventilation in calving units can decrease the spread of infectious pathogens as well as decrease the amount of stress a calf experiences in crowded environments. If calf hutches are being utilized, they need to be placed at least 4 feet apart and sanitized before the next use (Gorden & Plummer, 2010). Ventilation is a key component when analyzing methods of BRD prevention especially in calves as this decreases the prevalence of airborne pathogens and the chances of a calf inhaling a BRD causing pathogen (Brscic et al., 2012). Selection of dams can also be a prevention strategy. First-time heifers and 2-year-old dams have been found to not have the volume of maternal antibodies as multiparous cows have (Smith, 2021) This is important to note when considering when ensuring transfer of passive immunity to calves.

A more common method of BRD prevention is calf vaccinations. Vaccinating calves perinatally directly after birth when transfer of passive immunity is successful and maternal antibodies are present in the calf's blood stream does not often provide the desired protective effect. This is due to the maternal antibodies present in their system neutralizing the antigens injected into the calf before it can induce antibody production, making the vaccine ineffective (Chase et al., 2008). However, vaccinating calves post birth can prime B and T memory cells to be more efficient after maternal antibodies degrade (Chamorro et al., 2016). Vaccinating pre-weaned calves between the ages of two and five weeks old also show little affect in decreasing the incidence of BRD in this age group (G. U. Maier et al., 2019). Other efforts to increase the efficacy of the immune system of a pre-weaned calf against BRD causing pathogens include the enhancement of fluids and the mucosal layer of the respiratory tract using oral administrations of sodium iodide. Iodine plays a part in the production of hypoiodous acid which has antiviral and bacterial attributes (Shoemake et al., 2018). However, this hypothesis has only been found to be effective during in-vitro studies and has not produced results that would prove this method to be a reliable prevention strategy without further investigation (Gamsjäger et al., 2020). The strategy

to enhance the immunity of the mucosal layer of the nasal passage is utilized in the administration of intranasal vaccines. This has also been found to be an effective vaccine in calves directly after birth as maternal antibodies have little effect against the induced immunity of an intranasal vaccine. This effect, however, has a short duration and thus call for the calf to have booster vaccinations upon weaning (Ellis et al., 2013). While studies have found that an intranasal vaccine decreases the level of viral shedding of an infected animal, the effectiveness of this class of vaccine has not been studied extensively in the field and thus the true effectiveness of an intranasal vaccination against BRD has not been fully evaluated (Masset et al., 2020). One study found that calves intranasally vaccinated prior to transportation to a finishing unit had improved weight gain (Sandelin et al., 2020). After weaning, calves receiving parenteral vaccines against the BRD causing pathogens, effectively reduce viral shedding postinfection (Salt et al., 2007). These vaccines protect against most BRD causing pathogens but, this effect is not guaranteed as not all vaccines are of similar efficacy. The most common vaccine practice is the combination of a modified-live and a bacterin (Stokka, 2010). The timing of these vaccines is also important. Depending on the vaccine and the immunocompetence of the animal, it can take 1-3 weeks for protective immunity to be induced (Urban-Chmiel & Grooms, 2012). However, many times calves are vaccinated upon arrival to a new location, thus not giving the vaccine enough time to induce humoral immunity and exposing them to environmental stressors (Pratelli et al., 2021). With correct timing of vaccination administration, antibody titers can be increased against BRD causing pathogens which decreases an individual's risk of infection (Chamorro & Palomares, 2020). When comparing vaccination administration upon arrival to a new location versus administration after two weeks acclimation to new location, a delayed administration of a modified-live BRD vaccine was shown to induce an improved immune response (Richeson et al., 2008). Pre-conditioning can also be used as a preventative measure. This method involves giving ample healing time after dehorning and castration, weaning before sale, and time to learn to eat and drink from a trough before transportation or relocation (Taylor, Fulton, Lehenbauer, Step, & Confer, 2010). Vaccinating calves that have been weaned 30-45 days prior to transportation is another attribute of pre-conditioning. These animals show to have lower mortality and morbidity rates and are sold for higher prices to feedlots (Urban-Chmiel & Grooms, 2012).

Immune System Development of the Fetal Calf

Consistent with all pregnancies, development of the immune system of the fetus is greatly dependent on the dam. Firstly, female cattle must be in good health before conception. Even in the first days of gestation, the dam needs to intake to correct amount of dietary nutrients to ensure a balanced endocrine system for successful impregnation (Robinson et al., 1995). Dams that are malnourished during pregnancy give birth to calves with higher cortisol levels and produced colostrum with reduced IgG concentrations (Hough et al., 1990). Adequate nutrition is also important for the immune system of the dam as it supports the production of immune cells and inflammatory responses (Content, 2020). Cortisol can only be passed from cow to calf through the placenta and has been seen to have a correlation with the decrease of cellular immune function in calves when cortisol is in excess (Arfuso et al., 2023). Excessive heat can also negatively affect the developing fetus. Heat stress during late gestation is associated with reduced fetal growth, dysfunctional immune tissue development, and intestinal apoptosis within days after birth (Ahmed et al., 2021). The main events during the first stages of bovine gestation are growth, vascularization and differentiation of the placenta as well as fetal organogenesis (Funston et al., 2010). Interferon induction in the fetus can occur within the first trimester of the pregnancy (Charleston et al., 2001). As pregnancy progresses, the innate immune system of the calf also continues to develop. As the fetus develops, humoral immunity components continue to increase however, they remain at lower levels compared to adult cattle (Chase et al., 2008). At 90 days of gestation, complement activity in the blood can be detected (Barrington & Parish, 2001). B lymphocytes in the calf remain low throughout the entire pregnancy (Senogles et al., 1979). These innate immune cell levels do not increase until approximately 130 days into gestation. Specifically in cattle, B cells are produced from the ileal Peyer's Path in the intestines which develops between the later stages of gestation and juvenile age (Ekman et al., 2010). The thymus is the primary organ where T-cells are produced. Development of fetal bone marrow and thymus occurs within 27 to 30 days gestation (Cortese, 2009). From there, T-cells disperse into the peripheral system of the animal that can later be stimulated by cytokines and antigens (Isashiki et al., 2022). After stimulation, T-cells can differentiate into T-helper or T-regulatory cells based on the specifics of the present infection (Zhu et al., 2010). Ileal Peyer's patches serve as a reservoir for B-cells in cattle and small ruminants (Yasuda et al., 2006). The placenta plays a large role in the development of the fetal calf immune system. Cattle and small ruminants have an

epitheliochorial placenta which does not allow for the transfer of maternal antibodies until birth where they are attained by the calf ingesting colostrum (Davenport et al., 2023). The placenta produces progesterone, prostaglandins, and cytokines that suppress adaptive and innate immunity upon completion of gestation. Progesterone causes increased levels of amino acids, cytokines, glucose, and growth factors to support the fetus in development (Chase et al., 2008). While transfer of passive immunity does not occur until after birth, nutrients are still supplied to the fetus through vascularized placentomes formed from integrated cotyledons in endometrial caruncles (Davenport et al., 2023).

BVDV results in different outcomes if infection occurs at certain stages of bovine gestation due to the fetus's progress of development. If the virus is present in contaminated semen during breeding, fertilization could fail or the embryo will not survive to the fetal stages (Vanroose et al., 2000). However, if infection occurs between 30 and 110 days of gestation, the result could be the birth of a persistently infected (PI) calf (Lanyon et al., 2014). The result of a live PI calf birth is due to infection of the non-cytopathic strain of BVDV infection before the immune system is competent enough to identify between "self" and "foreign" antigens, later developing an immunotolerance to the virus (Yitagesu et al., 2021). A potential mechanism for the development of this immunotolerance is that B and T lymphocytes that would "combat" the virus are not selected and produced and thus do not neutralize the virus (Grooms, 2004). Internal physical evidence of a PI BVDV infection has been found observing pathology slides of epithelial layers of the digestive tract that have been disrupted (Bielefeldt Ohmann, 1988).

Calf births that result in a transient infected calf occur when BVDV infects a fetus after 150 days of gestation (Knapek et al., 2020). At this point of development, the calf's innate immune system is functional enough to recognize the virus as "foreign" and can thus mount a defense against it. These infections do not result in physical defects as the infection is usually cleared by the time of birth (Kelling & Topliff, 2013). However, while normal, healthy calves are born agammaglobulinemic, calves that are born BVDV transient infected are immunocompetent with systemic antibodies present (Fray et al., 2000). These calves however are seen to have higher morbidity rates than healthy born calves due to congenital infections that occasionally result in abnormalities (Munoz-Zanzi et al., 2003). Non-cytopathic transient infections result in strong IFN expressions which promote antiproliferation and apoptosis of host cells, effectively decreasing the virus's ability to spread (Shoemaker et al., 2009). This serves as molecular

evidence that the immune system of the calf recognizes the virus as invasive and mounts an immune response.

Another important aspect to calf immunity is the development of the gut microbiome. This acts as a bridge between innate immunity of the calf and acquired nutrient levels. Upon reaching the stage of gestation where the gut of the fetus is developed, it is quickly colonized by microbial metabolites. Each metabolite interacts with the innate immune system uniquely to ensure proper function and health of the gut. A major metabolite needed in the gut is acetate as its purpose is to stimulate interaction between the epithelial layer and innate immune cells. Acetate does this by activating B cells to create IgA which changes localization of bacteria found in the colon of the fetus (Takeuchi et al., 2021). Secretory IgA can differentiate between harmful and beneficial bacteria in the intestines (Thurnheer et al., 2003). However, IgA can only be attained by the calf ingesting colostrum directly after birth (Du et al., 2023).

Innate Immunity

To vary degrees, all organisms are born with an innate immune system. This level of the immune system acts as the base layer of protection that is relied upon most when exposed to a new pathogen. Cells that the innate immune system relies on are neutrophils, macrophages, mast cells, and dendritic cells (Nurnberger et al., 2004). Neutrophils are the innate immune systems' first line of defense that are non-specific cells that destroy pathogenic cells through phagocytosis using Neutrophil Extracellular Traps (NETs) and releasing Reactive Oxygen Species (ROS) that result in an inflammatory response of the surrounding tissue (Kumar & Sharma, 2010). These cells also play a large role in recruiting the assistance of other immune cells by releasing chemotactic signals that attract monocytes and dendritic cells (Bennouna et al., 2003). This is followed by the production of tumor-necrosis factors that dictate macrophage and dendritic cell activation and differentiation (Van Gisbergen et al., 2005). To leave the bloodstream to attack an invasive pathogen, neutrophils depend on surface markers such as CD-14, CD18, and L and P-selectin which can be prime targets for viruses (Thakur et al., 2020). Macrophages are the most abundant leukocyte found in the body and are also a key part of the innate immune response in eliminating infectious agents. Like neutrophils, macrophages are also phagocytic cells that ingest and digest pathogenic particles as well as releasing ROS (de Oliveira-Junior et al., 2011). Depending on the stage of the infection, macrophages can differentiate into two different types of macrophages. M_1 macrophages phagocytize pathogenic particles and are pro-inflammatory

which results in tissue damage much like the effect of neutrophils. M_2 macrophages are anti-inflammatory and clean the area of dead and dying cells as well as repairs the surrounding tissue (Mills & Ley, 2014). Mast cells are present in all body tissues, but they are primarily located in the mucosal layers of tracts that come into contact with the external environment (Reber et al., 2015). Rather than phagocytizing infectious pathogens, mast cells release histamines, proteases, cytokines, and proteoglycans that mediate the attack (Mekori & Metcalfe, 2002). Cytokines are the bridge to immune cell communication and behavior. They can bind to target cells and produce signaling molecules that recruit the presence of immune cells such as neutrophils and macrophages (Strieter et al., 2002). Multiple types of cytokines also play their own parts in the function of the immune system. Chemokines are a cytokine that is particularly important for the immune cells to travel to the site of infection. CXC chemokines bind to receptors on inflammatory cells and drag them out of circulation (Strieter et al., 2002). Interferons are a type of cytokine that is particularly important when battling against viral infections. These cells interfere with viral replication and thus stop the virus from spreading further (Decker et al., 2002). Recently it has been discovered that interferons can be produced by macrophages and dendritic cells in the presence of a bacterial or protozoal infection (Bogdan, 2000). Dendritic cells act as the bridge between the innate and adaptive arms of the immune system. By taking samples of a disease-causing pathogen, dendritic cells present the samples to other immune cells. This results in the activation and differentiation of these cells depending on the antigen presented to them (Liu, 2016). These innate immune cells are important for the survival of the host organism. Without them, the disease-causing pathogen would replicate and become unmanageable by the time the adaptive immune system could act against it. For the innate immune system to know how to reach a pathogen, it must first be stimulated through pattern recognition receptors (PRRs). Once PRRs recognize the presence of an infectious agent, phagocytosis and inflammatory responses can occur (Santoni et al., 2015). PRRs recognize Damage-Associated Molecular Patterns (DAMPs) and Pathogen-Associated Molecular Patterns (PAMPs) that come from the pathogen itself or the damage and cellular debris the pathogen causes (Roh & Sohn, 2018).

Adaptive Immunity

When innate immunity falls short, the adaptive immune system steps up to assist in the battle against infection. Viruses and bacteria have developed ways to avoid the innate immune

system or at least delay its effects and thus the adaptive immune system is called to the situation. Classic identifiers of this system are antibodies produced by B and T lymphocytes. These cells travel from the bone marrow where they originate to lymph nodes and the spleen where they detect and capture infectious pathogens. They can also be stimulated by antigen-presenting cells (APCs) that notify B and T cells of the presence of an infection in which results in lymphocytes leaving to travel to the site of infection (Bonilla & Oettgen, 2010). To be stimulated directly by pathogens, B and T cells have receptors called B cell receptors and T cell receptors (BCRs and TCRs) that bind to major histocompatibility complex (MHC) molecules on pathogens or APCs. MHC class one receptors are found on pathogens while MHC class two receptors are found on APCs (Bonilla & Oettgen, 2010). Before B and T cells can perform any defensive measures against a pathogen, they must be exposed to the pathogen. Otherwise they remain non-specified naïve cells (O'Leary et al., 2006). From there, T cells are activated by B cells to differentiate in accordance with what is needed to eliminate the pathogen. T cells are then converted into effector cells until the infection is eliminated (Matzinger, 1994). B cells are an integral part of antibody production. T-helper cells interact and activate B cells to differentiate into plasma cells whose main purpose is to rapidly create and secrete antibodies (Alberts et al., 2002). Antibodies attach themselves to pathogenic antigen receptors and neutralize them where they cannot bind and invade a healthy host cell to replicate and spread further. Antibody covered viruses are then vulnerable to phagocytic cells (Rogers, 2024a). Upon exposure to a novel pathogen, the response of antibody neutralization is slow due to the adaptive immune system being naïve to the pathogen. Exposures following the initial will see a quicker antibody response (Giesker & Hensel, 2014). It is this process that is the target of developing efficient vaccinations. Vaccines use differing components of a pathogen and eliminate the virulence factors that would cause disease upon injection. From there the vaccine can be administered to induce an immune response to pre-expose the immune system to a pathogen. Upon the body’s exposure to a wild-type pathogen, antibodies are already developed in the system for rapid neutralization. Some vaccines require adjuvants to assist in the stimulation of cytokines and chemokines to activate the desired immune response (Garlapati et al., 2009). Other immune responses induced by vaccines are short-lived and thus the individual will need to acquire regular booster shots to retain the acquired immunization (AVMA, 2024).

Immunity of the Respiratory Tract

The respiratory tract is incredibly vulnerable to infectious pathogens since it is the body's way of intaking gases from the outside environment. It is due to this fact that primary physical barriers play a large part in preventing respiratory disease. Specialized respiratory dendritic cells (rDCs) monitor the respiratory mucosal layers and epithelium for foreign pathogens. When encountered with a pathogen, rDCs induce T cells to proliferate and respond accordingly (Langlois & Legge, 2007). Before a pathogen can enter the lower airways, it must first overcome the defenses of the nose or cause disease at that location. The epithelium of the nasal passage is lined with cilia that are finger-like structures that catch and push pathogens out of the body through the behavioral action of sneezing and coughing (Bustamante-Marin & Ostrowski, 2017). Respiratory epithelial cells also serve as part of the physical immune barrier as they can recognize foreign pathogens through PRRs to induce an immune response, primarily activating IgA found in mucosal surfaces (Wang et al., 2015). These attributes of respiratory immunity cover the nasal passage, trachea and upper airways. Larger particles tend to stop at this level of the respiratory tract. Smaller pathogens are sometimes able to slip past into the lower airways. The lower airways have similar physical defense mechanisms such as the epiglottal reflex and the mucociliary layer to prevent the initial infiltration of a pathogen (Chase & Kaushik, 2019; NHS, 2024). In the alveoli on the cellular level, pulmonary macrophages and lymphoid cells in the bronchi act as the primary defense mechanism of the lungs (Quinn, 1981). A protective lipoprotein film can be found in the alveolar-bronchi area that contains protective proteins produced by B lymphocytes and plasma proteins from the adjacent capillary bed (Canto et al., 1994). The primary objective of the respiratory tract is to perform the gas exchange of oxygen delivery to the lungs and blood stream, then to expel carbon dioxide. This is done in the alveoli. Disease results when invasive particles get passed the primary and secondary defenses of the respiratory tract and thus cause dysfunction in the essential exchange of gases. Primary defenses included physical barriers, such as the cilia layer, and phagocytic immune cells. Secondary defenses include antiviral cells such as interferons and antibodies (Tonozzi, 2021). Intranasal vaccines take advantage of these processes. By instilling a nonvirulent pathogen into the nasal passage in which immune cells, such as DCs and B lymphocytes, uptake the particles and induce an immune response that prepares the immune system of the respiratory tract for future infections (Schneider, 2024). Intranasal vaccination can also create nonspecific immunity by

enhancing IgA concentrations in the mucosal lining of the upper and lower airways (Midla et al., 2021).

Immune Response to Viruses (BVDV)

A unique characteristic to viruses is their need for a host to cause disease and replicate after initial entry into the body. Viruses use binding surface receptors that can attach to a host cell's surface receptor to do one of two things that allow the virus to take advantage of the host cell's replicating ability. The first possibility, depending on the virus, is the virus can enter the cell through endocytosis that would allow it access to the nucleus or cytoplasm. The second possibility is that the virus can translocate across the cell membrane or cause the vesicle the break apart (Marsh & Helenius, 2006). After replication, viruses will emerge from the cell either by inducing cytolysis of the cell or emerging from the cell membrane, releasing virions to infect more host cells (Mueller & Rouse, 2008). The body's innate immune system will be the first to respond sending phagocytic cells such as neutrophils and macrophages as well as natural killer cells (Klimpel, 1996). To identify an invasive virus cell that has infected a host cell, class 1 major histocompatibility complex (MHC-1) proteins will contain pieces of the virus protein that can be detected by T cells. Specifically, cytotoxic T cells have receptors on their surface that bind to MHC-1. When a cytotoxic T cell detects a viral peptide on the MHC-1, it releases cytotoxic factors into the infected cell. These factors include perforin that allows granzymes to enter the virus infected cell and trigger apoptosis. Natural killer cells have a similar process (Laing & Hutchinson, 2024). The infected cell itself also has a method of its own defense against viruses. Within these cells, interferons are released that inhibit the virus's ability to replicate. This also sends a signal to nearby host cells that there is a virus present. Cells that have not been infected yet will then begin to increase interferon genes that code for proteins to better equip themselves to combat the virus (Grandvaux et al., 2002). After initial the response from the innate immune system, the adaptive immune system responds by sending antibodies to neutralize the virus. The antibodies can be obtained naturally from previous infections, or artificially through vaccine administration (Rouse & Sehrawat, 2010).

In the case of a BVDV infection, the immune response depends on the life stage the animal is in when infected and the biotype in which the animal is infected with as well as the result of a transient or persistent infection. The biotype also determines the result pathology caused by the disease. The main tools that this virus uses to continue causing infection is

immunosuppression and immunotolerance (Peterhans et al., 2003). Cytopathic biotype of BVDV originates from a mutated non-cytopathic strain of BVDV. Non-cytopathic strains being the only biotype of the two to result in a persistent infection, causes a reduction in interferon activation as well as inhibiting the host cell's ability to perform apoptosis (Adler et al., 1995; Schweizer & Peterhans, 1999). This allows the virus to spread to surrounding cells without interference of immune cells or defenses from other host cells. For cell-to-cell transmission of the BVDV virus, the proposed mechanism is from an infected cell, vesical containing virus particles transport to the extracellular space where cells make contact. From there the virus displays the glycoprotein E2 on its surface that attaches to coreceptors on the surface of local uninfected cells. Once the binding of the receptors is successful, the cell engulfs the virus via clathrin-dependent endocytosis (Merwaiss et al., 2019; Riedel et al., 2019). By replicating in an animal during the first stages of gestation, the virus can be seen as non-foreign to the fetal developing immune system by avoiding and resisting interferons. Resulting in the immunotolerance of the virus replicating within the host (Peterhans & Schweizer, 2013). This biotype, however, sees a larger onslaught of T-cells and T-helper cells being the primary respondents. Lymphocyte production has been found to be consistent in response the either of the biotypes (Lambot et al., 1997). Considering the adaptive arm of the immune system, cases that result in a PI animal see minimal antibody response due to the virus not being seen as a foreign antigen (Chase, 2013). Since transient infected animals have a functional immune system in the later stages of gestation when infection occurs, the fetal body can mount an immune response against it which results in the birth of calves with systemic antibodies (Knapek et al., 2020).

Immune Response to a Bacteria (*Mannhiemia haemolytica*)

Similar to how the innate arm of the immune system detects a viral infection, when a pathogenic bacterium is present, it is detected by PRRs that sense PAMPs that indicate the presence of an invasive bacteria. This then promotes the production of proinflammatory cytokines and type 1 interferons as an initial response (Kumar et al., 2013). A specific PRR that is responsible for the recognition of bacteria lipopolysaccharides (LPS) is Toll-like receptor 4 (TLR 4) which initiates the innate immune response (Poltorak et al., 1998). This is critical in the face of an *M. haemolytica* infection as LPS is unique to gram-negative bacteria (Farhana & Khan, 2023). TLRs reside on the surface of epithelial cells and leukocytes that, when introduced to a bacterium, release signals that cause a cascade of stimulated innate immune cells including

pro-inflammatory cytokines, chemokines that recruit more leukocytes, natural killer cells and phagocytes to destroy the bacteria (Tosi, 2005). TLR5 recognizes flagellin monomer on the flagella of gram-negative bacteria which is the primary tool used by gram-negative bacteria that are responsible for virulence factors, chemotaxis, and attachment and entrance of a host cell (Hayashi et al., 2001). TLRs that detect the bacterial LPS activates macrophages that the TLRs are found on which secrete the cytokine, tumor necrosis factor-α (TNF-α) (C. A. Janeway et al., 2001). When activated, this cytokine stimulates endothelial cells which induces further inflammatory response (Hunt & Jurd, 1998). However, TLR detection is not enough to fully signal the innate immune system. LPS must also bind to an LPS binding protein that also attaches to the coreceptor CD14 that transfers the LPS to an accessory soluble protein to assist the TLR functions (Albiger et al., 2007).The adaptive immune system is slower to activate using T-cell and B-cell receptors on the surface of lymphocytes. Dendritic cells (DCs) are antigen presenting cells that take samples of the pathogen to local lymphoid tissues to stimulate T-cell activation and differentiation (Iwasaki & Medzhitov, 2004).

In the situation of an *M. haemolytica* infection, neutrophils release neutrophil extracellular traps (NETs) to destroy the bacteria. Macrophages have also been found to have a similar tool in the destruction of invasive pathogens. This tool is called a macrophage extracellular trap (MET) which is produced in response to the detection of leukotoxins (Aulik et al., 2012). Vaccines to protect against *M. haemolytica* are administered intranasally to enhance the adaptive immunity of the immune cells in the mucosal layer of the nasopharynx region and the epithelial cells in the same area. One study found that by injecting Lactobacillus spp., which has antimicrobial properties, limits *M. haemolytica* proliferation (Amat et al., 2019). *M. haemolytica* intranasal vaccines can also be modified-live type or bacterin-leukotoxoid through systemic injection. Both were found to successfully stimulate B-cell memory when used separately (Stoltenow et al., 2011).

Acute vs Chronic Infections

The initial difference between chronic and acute infections is that a chronic infection prevails over long periods of time or never resolves where host and virus maintain a state of equilibrium. An acute infection shows a rapid onset but resolve quickly (Deigendesch & Stenzel, 2018). Immune response to an acute BVDV infection involves the proliferation of CD8 lymphocytes which produces interleukin-2 (IL-2) and IFN-γ (Chase, 2013). These cytokines

assist in the stimulation and differentiation of both pro- and anti-inflammatory T-cells which is a major process that works towards resolving the infection (Ross & Cantrell, 2018). This process is identified as a type 1 cytokine response (Lucey et al., 1996). A type 2 cytokine response assists in the repair of previously infected and injured tissue, whereas type 1 cytokine responses assist in the defense against foreign pathogens (Gieseck III et al., 2018). Acute infections of BVDV can only occur in the case of transient infection as the animal eventually clears the disease. During this, viral particles can be found in parts of the body that are responsible for the production of immune cells such as the bone marrow, lymphoid tissues, and bloodstream. This contributes to the virus's ability to cause immunosuppression making the animal vulnerable to co-infections (Kelling & Topliff, 2013). Chronic infections of BVDV can occur in persistently infected calves that were infected in-utero, or in the reproductive system of adult cattle. BVDV molecules that result in chronic infection can be found in ovarian tissue of cows and seminiferous tubules of bulls (Givens & Marley, 2013). In the development of a PI calf, consistent exposure to type 1 IFN causes stunted intrauterine growth which may be the reason for PI calves to be born at lower weights compared to healthy calves. Since the virus is not seen as foreign, the infection never resolves and this growth suppressive interferon continues to be upregulated (Hansen et al., 2010). Type 1 IFNs are more rapid in response time compared to type 2 and type 3 IFNs (Lazear et al., 2019). Type 1 IFNs are also protective against the effects of viruses by activating interferon stimulated genes (ISGs) in uninfected host cells, which prepares these cells for a defense against the spreading virus (Katze et al., 2002). Type 2 IFNs are made by natural killer cells to enhance the innate immune response by increasing the function of antigen presenting cells and upregulating the mechanisms needed to induce the release of ROS from macrophages (Lee & Ashkar, 2018). Type 3 IFNs reduce viral replication in epithelial barrier cells exclusively (Manivasagam & Klein, 2021). *M. haemolytica* is only an acute infection that clinically manifests withing 24-48 hours of infection in the form of pneumonia and lung lesions (Jiao et al., 2024).

Conclusion

As mentioned previously, BRD negatively affects the cattle industry with such severity that it calls for the need to investigate viable solutions that not only benefit those in industry economically but will also contribute to the improvement of animal welfare. Since BVDV is easily transmitted from one animal to another and negatively affects the health and productivity

of both beef and dairy calves, finding the cause of this phenomenon is of great interest. With the focus on the immunological impacts of an in-utero transient BVDV infection, this project first elucidates the connection between physical symptoms and internal pathogenesis of the development of bronchopneumonia in calves. Secondly, the experiment will investigate the biological mechanisms behind the morbidity increase seen in transiently infected BVDV calves when exposed to other respiratory disease-causing pathogens after the weaning period. Prior to the study, it was hypothesized that TI BVDV calves will display more severe clinical illness and lung pathology compared to healthy control calves. It was also hypothesized that TI BVDV calves will present a dysfunctional immune response that can be observed on a molecular level. To test these hypotheses, TI BVDV calves and healthy control calves were intranasally inoculated with *Mannheimia haemolytica* directly into the lower airways to simulate respiratory disease which induces an immune response. Clinical illness was monitored and recorded using the Wisconsin-Madison calf health scoring system that ranks the severity of clinical symptoms. A thoracic ultrasound scoring system was also used to compare lung pathology between individuals which will supplement the clinical manifestation of disease caused by bacterial infection by allowing a view into the lungs of the subjects to see the development of pathology. Endoscopic BAL fluid and serum from whole blood samples were collected for molecular analysis of biomarker concentrations, cytology analysis, and antibody concentrations. Information gathered from this project will guide future investigators researching the biological mechanisms behind the immune dysfunction seen in TI BVDV calves to a greater extent as well as inform those in the cattle industry about the impacts of BVDV specifically to cow-calf operations.

CHAPTER THREE: THE EXPERIMENT

Materials and Methods

Cell Culture and viral propagation

Madin-Darby Bovine Kidney (MDBK) cells were used for viral propagation. Cells were first maintained Dulbecco's Modified Eagle Medium (Gibco DMEM) supplemented with 5% FBS (Gibco HI FBS) and 1% penicillin-streptomycin (Gibco Anti-Anti 100x) and incubated at 37°C with 5% CO_2. Once cells were at 80-90% confluency, they were collected for propagation into new flasks using trypsin activity. Media was removed and cells were washed cells with 5 mL of D-PBS. Two milliliters of 0.25% Trypsin-EDTA (Gibco 0.5%, no phenol red, diluted 1:1 with D-PBS) was applied to the cell layer until cell detachment. Trypsin activity was then stopped with an equal volume of 5% FBS DMEM and the cell suspension was split into new flasks. RT-qPCR in subsequent steps was used to confirm cells were BVDV negative.

At 60% confluency, cells were inoculated with BVDV viral stock of non-cytopathic strain BJ. Media was removed and cells were washed with sterile 1x PBS then recovered with 1 mL of DMEM without additives. The virus was then applied to the cell layer through a sterile syringe filter (Pall Laboratory, 5 um) and incubated at 37°C with 5% CO_2 for 2 hours. Cells were then maintained for 72 hours in conditions previously described. Viral inoculated media and cells were then collected into 1-mL aliquots and frozen at -80°C to serve as viral stock for animal inoculation.

Real-time quantitative PCR

Real-time quantitative PCR (RT-qPCR) was used for the detection of BVDV RNA in naive MDBK cells prior to inoculation and in cells and media to verify infection. A viral RNA/DNA isolation kit (ThermoFisher PureLink Viral RNA/DNA Mini Kit) was used for RNA isolation per manufacturer's instructions. A microplate reader (Take-3, Biotek Epoch) was used for RNA quality and quantity assessment. PCR reactions were performed in triplicate using 100 ng of RNA with RNAse free water serving as negative control and Singer BVDV stock as the postive control. One-step real-time PCR was performed with the (Bio Rad CFX96 Real-Time System) using the Superscript™ III One-Step RT-PCR System with Platinum™ *Taq*DNA Polymerase kit (Invitrogen).

BVDV Primers:

Reverse: GYGTCGAACCAYTGACGACT

Forward: CCATRCCCDTAGTAGGACTAGC

Probe: [6~FAM] TGGATGGCYRAABCCCTGA

Immuno-peroxidase assay for determination of viral titers

Immuno-peroxidase assay (IPA) was performed to determine the TCID50 (50% tissue culture infectious dose) of viral stocks from media and infected cell aliquots. The following reagents were prepared: fixative (80 mL 0.85% saline, 20µL 1% BSA, 20 mL acetone), diluent (100 mL D-PBS, 20µL 1% BSA, 50µL Tween 20), wash media (500 mL D-PBS, 250µL Tween 20), and preservation media (96 mL D-PBS, 4 mL formaldehyde). A 96-well plate was inoculated with the viral samples using a 1:10 dilution of viral stock. Each well received 90µL of DMEM with 10% FBS, followed by 10µL of viral stock in triplicate in the first column. Serial dilutions were performed by transferring 10µL across the columns. MDBK cells were harvested from culture and resuspended, with 50µL of the cell suspension added to each well. The plate was incubated for 72 hours at 37°C with 5% CO2. After incubation, the media was removed, and the plate was left to air dry for at least 2 hours to prevent detachment of the cell monolayer. Once dry, 50µL of fixative was added to each well, and the plate was incubated at room temperature for approximately 10 minutes. The fixative was discarded, and the plate was air-dried again for at least 2 hours. A diluted anti-BVDV antibody (1:1000, D89, VMRD) was added to each well at 50µL per well and incubated for 1 hour at 37°C. After incubation, the plate was washed three times with 100µL of wash media. Next, diluted conjugated rabbit anti-mouse IgG (1:1000, Jackson ImmunoResearch Laboratories, Peroxidase AffiniPure Rabbit Anti-Mouse IgG) was added and incubated under the same conditions as before, followed by the same washing procedure. A staining substrate (Enzo AEC Peroxidase Kit) was prepared and applied to the wells (300 µl per well). The plate was incubated in the dark at room temperature until red staining appeared, indicating a positive result, which was then examined under a light microscope.

Dam selection and management

All animal work was performed in compliance with the University of Tennessee, Knoxville Institutional Animal Care and Use Committee (IACUC protocols 3022-1223 A1). A total of 26 yearling beef heifers, predominantly of Angus breed, were selected from multiple farms across Tennessee, USA. Selected heifers were confirmed to be seronegative for BVDV exposure and free of persistent infection (PI) status. To ensure they were negative for PI, an ear notch sample was taken and stored at -80°C before being sent to an external lab (Kord Animal

Health Diagnostic Laboratory - KAHDL) for antigen capture ELISA testing. Additionally, 10 milliliters of blood were collected via the coccygeal vein into vacutainer tubes without additives, then immediately chilled on ice for transport to the laboratory within 3 hours. The blood was centrifuged at 3,000 x g for 10 minutes at 4°C to separate the serum, which was then transferred to cryovials and stored at -80°C until testing for serum neutralizing antibodies at KAHDL. Upon arrival, the heifers were rectally examined to ensure normal reproductive anatomy, vaccinated for clostridial diseases (Merck Animal Health, Bovilis Vision 7), and treated with a topical anthelmintic (Zoetis, Dectomax Pour-On). The heifers were synchronized using the CO-Synch plus CIDR program (Johnson et al., 2013) for timed artificial insemination (TAI). In addition, two Angus bulls were placed with the heifers to provide further breeding opportunities. Sixty days after TAI, pregnancy was diagnosed via transrectal ultrasound (E.I. Medical Imaging IBEX) to assess gestational age, and a follow-up pregnancy check was done 77 days post-TAI to confirm pregnancy maintenance. Non-pregnant heifers were removed from the study, while pregnant ones were randomly assigned to three treatment groups: persistent infection-inoculation (PI, n=8), transient infection-inoculation (TI, n=7), and control (CTRL, n=5). A larger number of heifers were assigned to the inoculation groups to account for potential pregnancy loss due to viral infection. These groups were then separated into three mixed-grass pastures with no fence-line contact, and approximately 20-foot lanes were maintained between each group.

Dam inoculation and verification

Viral aliquots were carefully thawed on ice and mixed with 5 mL of DMEM (without supplements) at room temperature in 12 mL conical tubes to achieve a final inoculum with a $TCID_{50}$/mL of $1x10^6$ for the dam inoculation. For the control dams, a sham inoculum was prepared under identical conditions using 6 mL of DMEM. Heifers were restrained in a chute, and the inoculum was administered through an aerosolized spray system (Preval Sprayer) into both nostrils. The inoculum was sprayed over the course of approximately 60 seconds to allow for better nasal contact and inhalation of the solution. The pregnant-infected (PI) and control dams were inoculated at 85 days post-timed artificial insemination, while the treatment-infected (TI) and control groups were inoculated at 216 days post-breeding. To confirm pregnancy status, all dams were rectally palpated on the second inoculation day. Blood samples (10 mL) were collected from the coccygeal vein 30 days post-inoculation for PI dams and 50 days post-inoculation for TI and control dams. The blood was kept on ice until processed within 3 hours.

After centrifugation at 3,000 g for 10 minutes at 4°C, the serum was separated, transferred into cryovials, and stored at -80°C until it was sent to KAHDL for the assessment of serum-neutralizing antibodies. This testing confirmed the presence of antibodies in BVDV-infected animals and ensured no exposure in control groups.

Calves Enrolled

15 weaned beef calves sourced from a previous BVDV study were born and housed at the Northeastern Tennessee Research and Education Center for the duration of the study. During calving, research staff and farm personnel monitored the dams every two hours for signs of parturition. If no intervention was necessary, the cows were allowed to calve naturally in the pasture. Before the calves ingested colostrum, 30 milliliters of whole blood were drawn from their jugular veins. Of this, 20 milliliters were placed into EDTA vacutainer tubes, and 10 milliliters were placed into serum vacutainer tubes without additives. Each calf was assigned a numbered tag in sequential order. An ear notch sample was taken from each calf for BVDV antigen capture ELISA testing to assess PI status. Blood was stored at 4°C until subsequent processing. Prior to the study, calves were assigned MH or sham inoculation which determined how the calves were separated physically into outdoor paddocks immediately after weaning. In these paddocks the calves were given hay, and water ab libitum. A two-by-two study design was used classifying the calves into four categories: control calves (not exposed to BVDV in-utero) inoculated with media (CTRL/CTRL, n=4), control calves inoculated with MH (CTRL/MH, n=3), TI calves (exposed to BVDV in-utero) with media inoculation (TI/CTRL, n=3), and TI calves with MH inoculation (TI/MH, n=5). The paddocks in which the calves were housed were 3 acres of mixed warm season and fescue grass separated by an alley way approximately 10 meters apart, restricting the calves from any fence line contact. The catch chute system in the CTRL group was setup up in the corner furthest from the alley way separating the CTRL and MH paddocks. Each day of sample collection calves were gathered in a catch pen and herded individually into the chute. In the MH group the chute was set up in a similar manner in a barn adjacent to the paddock. All procedures were done starting with the CTRL group and ending with the MH group to reduce the risk of contamination of the CTRL group. On the day of infection investigators wore Tyvek PPE that was exchanged for clean PPE between groups. For all procedures excluding Clinical Illness Scoring were performed with the animals restrained in a head chute. Prior to beginning the experiment, all calves were vaccinated for the first time at an

average of 4.5 months of age with a combination respiratory and Leptospira inactivated product (Boehringer Ingelheim, Triangle 10 HB) and 7-way clostrial vaccine (Merck Animal Health, Bovilis Vision 7). Boosters were administered of each vaccine 4 weeks later. The calves were also tested for any prior MH infection by submitting deep nasopharyngeal swab samples for PCR analysis (Kansas State Veterinary Diagnostic Laboratory). After the experiment calves were treated subcutaneously with Draxxin (Tulathromycin 2.50 mg/kg).

Preparation of Mannheimia haemolytica

The inoculum used for the experiment was a clinical isolate obtained from a primary lung culture of a calf that succumbed to bronchopneumonia and was submitted to the UT CVM for necropsy. The isolate was reconstituted in sterile PBS and allowed to grow in a CO_2 incubator at 37°C for 24 hours on a brain heart infusion (BHI) agar supplemented with 5% bovine blood. Colonies were then selected and resuspended in BHI broth and incubated for approximately 7 hours at 37°C and 5% CO^2. Previous growth curve analysis demonstrated a consistently measured bacterial density of 3-5 x 10^9 CFUs/ml at 7 hours incubation. Therefore at 7 hours the bacterial suspension was withdrawn and centrifuged at 1,600 x g for 15 minutes then vortexed and washed in sterile PBS for three repetitions, then resuspended in 5 ml sterile PBS at an approximate density of 5-6 x10^9 CFUs total inoculum. Post-inoculation concentration was confirmed in triplicate via 24-hour at 37°C and 5% CO_2 of serial dilutions onto blood agar plates.

Mannheimia haemolytica Inoculation

Inoculation was performed via bronchoscopy on study day 0 after baseline samples were collected. An inoculum of 5x10^9 CFUs MH in 5 ml sterile PBS was deposited at the main stem bifurcation of the trachea followed by approximately 120 ml or sterile 0.9% saline to ensure dispersion into the lungs. This was done by placing two halters on the calf to elevate the head and neck. After cleaning the external nares with a clean paper towel a 2m polyethylene catheter was inserted through the nasal passage, past the larynx and into the trachea. Calves in the CTRL group received 5 ml sterile PBS sham inoculum and 120 ml sterile 0.9% saline in the same manner (Eberhart et al., 2017).

Clinical Illness Scoring

Calves were observed at approximately 7:30am from a distance by the same observer (AW) prior to any manipulation on study days -1, 0, 1, 2, 3, 4, 5, 6, and 7 to be assigned a Clinical Illness Score (CIS) using an abbreviated version of the University of Wisconsin Calf

Health Scoring Criteria (McGuirk & Peek, 2014). Briefly, scores of 0-3 for the categories of cough, nasal discharge, ocular discharge, and ear position. In addition, once calves were handled for sample collection, rectal temperature was measured and a score of 0-3 was added to the total score. The combined total score was used for progression and statistical analysis described mentioned in the *Statistical Analysis* section. If elevated CIS (5 or above) and rectal temperatures persisted for 48 hours or longer the animal would be removed from the study to receive appropriate veterinary care.

Thoracic Ultrasound Scoring (TUS)

Thoracic ultrasounds were performed on study days -1, 1, 3, 5, and 7 by the same operator (MC). Prior to the procedure, 70% isopropyl alcohol was applied to the calf's thorax. Each evaluation was performed with portable ultrasound equipped with a 2.5-5 MHz convex probe set at a depth of 7cm and gain of 16dB, (N 10 dB, F 36 dB; Ibex Pro, E.I. Medical Loveland CO). Both left and right lung fields were evaluated with the same technique as described for dairy calves (Ollivett & Buczinski, 2016) starting with the probe positioned dorsally and parallel to the ribs in the 6^{th} intercostal space and moving ventrally then cranially to the most cranial intercostal space permitted by each calf's anatomy. A thoracic ultrasound score (TUS) of 0 – 5 was assigned based on the previously refenced system where severity based on the thickness of consolidation and the amount of lung lobes consolidated.

Endoscopic Bronchoalveolar Lavage (BAL)

All groups were subject to BALs on study days 0, 3, 5, 7, and 14 for the collection of pulmonary fluid. Two halters were applied, and the head and neck of the calf were elevated. After cleaning the external nares with a clean paper towel a 2m polyethylene catheter was inserted through the nasal passage, past the larynx and into the trachea. The catheter was advanced until approximately near the tracheal bifurcation and up to 120mL of 0.9% saline in 60mL syringes was lavaged until an adequate sample of a foamy, opaque BALF was recovered (Eberhart et al., 2017). BALF samples were stored on ice for transportation. Samples that were submitted to the UTCVM Clinical Pathology department were transferred into 2mL microcentrifuge tubes for cytology analysis. For biomarker concentration analysis, samples were stored in 1.8mL cryovials at -80°C.

Serum Collection and Analysis

Whole blood samples were collected from all groups on study days -11, -1, 1, 3, 7, 14, 21, and 28 via venipuncture from the jugular vein alternating sides between each collection. Up to 15mL of blood was collected at each timepoint and stored on ice for transportation. Whole blood samples were centrifuged at 3000 x g at 4℃ for 20 minutes. Serum was extracted and stored in 1.8mL cryovials at -80℃.

Serum samples were tested for *Mannheimia haemolytica*-specific antibodies at the Washington Animal Disease Diagnostic Laboratory (WADDL) using an *M. haemolytica* IgG-specific indirect enzyme-linked immunosorbent assay (ELISA) as described elsewhere (Poonsuk et al., 2023).

In brief, the M. haemolytica A1 strain P1148 (10^8 CFU/100 µL) was diluted 1:50 in phosphate-buffered saline (PBS) containing 0.05% sodium azide. A 100-µL aliquot of the prepared coating solution was dispensed into each well of a 96-well polystyrene microplate (Thermo Fisher Scientific™, Waltham, MA, USA). The plates were then sealed and incubated at 4°C. After 18 hours incubation, the plates were washed three times with deionized water and subsequently blocked using ChonBlock blocking/sample dilution ELISA buffer (Chondrex Inc., Woodinville, WA, USA). After blocking, the plates were washed with Tris-buffered saline containing 2% Tween 20 (TBS-Tween20), air-dried, and stored at 4°C in resealable foil pouches with desiccants until further use.

Serum samples were diluted in ChonBlock blocking/sample dilution ELISA buffer at a ratio of 1:100. The diluted samples were first transferred to 96-well polystyrene pre-dilution microplates and subsequently added to the ELISA plates. The plates were then sealed and incubated at 37°C for one hour. Following incubation, the plates were washed thoroughly with TBS-Tween20 to remove unbound components. For detection, IgG-specific secondary antibodies conjugated to horseradish peroxidase (Bethyl Laboratories, Inc.) were added to the plates and incubated for one hour at room temperature. Reactions were developed using 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (Alpha Diagnostic Intl Inc., San Antonio, TX, USA) for 10 minutes. The reaction was stopped using a stop solution, and the absorbance was measured at 450 nm using a microplate spectrophotometer.

The results were represented as sample-to-positive (S/P) ratios calculated using the following formula:

$$S/P\ ratio = \frac{(sample\ OD - blank\ well\ control\ mean\ OD)}{(positive\ control\ mean\ OD - blank\ well\ control\ mean\ OD)}$$

Biomarker concentration analysis

Select cytokine and chemokine concentrations were analyzed in pulmonary fluid collected from the BAL samples on study days 0, 3, and 7. A MILLIPLEX® Bovine Cytokine/Chemokine Magnetic Bead Panel was used measuring for concentrations of IFNγ, IL-1α, IL-1β, IL-4, IL-6, IL-8, IL-10, IL-17A, MIP-1α, IL-36RA, IP-10, MCP-1, MIP-1β, TNFα, and VEGF-A following manufacturer's instructions. Briefly, in each well of a 96-well plate 200uL of wash buffer (prepared by mixing 60mL of 10X wash buffer and 540 mL of deionized water stored at 2-8°C) was added. The plate was then sealed and placed on a plate shaker at room temperature for 10 minutes. After decanting the wash buffer and removing any residual liquid (done by inverting the plating and tapping it) 25uL of Standard or Control was added into the assigned wells. The same amount of Assay Buffer was added to the assigned background wells. 25uL of Assay Buffer was also added to the remaining sample wells. Following this step 25uL of pulmonary fluid sample was added in duplicate into their pre-assigned wells. Premixed antibody-immobilized beads were sonicated for 30 seconds and vortexed for 1 minute prior to adding 25uL to each well. The plate was then sealed and wrapped in foil to incubated for 16-18 hours on a plate shaker at 2-8°C. The contents were then removed, and the plate was washed 3 times followed by the addition of 25uL of detection antibodies to each well. The plate was sealed, covered, and allowed to incubate on a plate shaker for 1 hour at room temperature. After which 25uL of Streptavidin-Phycoerythrin was added to each well prior to 30-minute incubation at room temperature. Once undergoing the washing procedure 3 times, 150uL of Sheath Fluid PLUS was added to each well and the plate was placed on a shaker for 5 minutes. The plate was run on a Luminex® 200™ with xPONENT software analyzing median fluorescent intensity (MFI).

BALF Cytology Analysis

BALF samples were processed within 6 hours of sample collection. Total nucleated cell counts (TNCC) were performed with hyaluronidase-treated aliquots of BALF fluid on a Horiba ABX Micros ESV 60 using bovine settings. Two concentrated specimens per sample were prepared for microscopic analysis by using 100uL BALF fluid and centrifuging on a Wescor Aerospray® 7120 Hematology Cytocentrifuge at 1,000 RPM for 3 minutes. Cytocentrifuged

reparations were air-dried then stained with an Eli Tech Aerospray®PRO and Modified-Wright stain.

Both stained cytocentrifuged preparations were observed by an ACVP-boarded clinical pathologist (NS) who was blinded to treatment group. Leukocyte differential cell counts were performed on each preparation by counting 100 cells in four quadrants (12, 3, 6, and 9 o'clock) of the preparation. For samples where cellularity was too low to count 400 cells, either a 100 or 200 cell differential count was performed across the entirety of the preparation. The differential cell counts from preparation 1 and preparation 2 were averaged for statistical analysis. The presence of respiratory epithelium, erythrocytes, bacteria, yeast, and pollen were semiquantified as none, rare, few, moderate, or many.

Statistical Analysis

The effects of treatment and time on variables of interest were examined using mixed model analysis for repeated measures with treatment as the between-subject effect while time as the within-subject effect. Ranked transformation was applied when diagnostic analysis on residuals exhibited violation of normality and equal variance assumptions using Shapiro–Wilk test and Levene's test. Post hoc multiple comparisons were performed with Tukey's adjustment. Statistical significance was identified at $p < 0.05$. Analyses were conducted in SAS 9.4 TS1M8 (SAS institute Inc., Cary, NC).

CHAPTER FOUR: RESULTS AND DISSCUSSION

Clinical Illness Scoring indicated the presence of MH infection

Upon analysis of clinical illness score progression of all animals enrolled in the study, significance was found between study days (p-value = 0.03), treatment groups (p-value 0.0988), and a study day by treatment effect was observed (p-value = 0.05). Significance in the treatment by study effect is displayed in **Figure 1**. Statistical difference was found in the CTRL/MH group (p-value 0.0238) and the TI/MH group (p-value 0.0556) when compared to the CTRL/CTRL treatment group. However, no significant difference was found when comparing the CTRL/MH group and the TI/MH group until day 6 (p-value 0.0665).

Rectal Temperature progression indicating presence of MH infection

While rectal temperature scores were included in the overall mean clinical scores, significance was found when analyzing individual rectal temperature scores and their progression through the study (**Figure 2**). Significance was found between treatment groups (p-value 0.0367) and between study days (p-value $\leq$ 0.0001). A treatment by study day effect was also observed (p-value 0.0524). MH infected groups show elevated temperatures compared to non-MH infected control groups.

Thoracic Ultrasound scores and images indicating successful experimental disease induction

Statistically significant difference in TUS was among treatment groups (p-value 0.007), study days (p-value $\leq$ 0.0001) as well as an interaction in treatment by study day (p-value 0.0017) (**Figure 3**). Similar to CIS, TUS was significantly higher in MH infected groups vs. non-MH infected. Elevated scores were observed on day 3 and 5 with resolution occurring by day 7. However, no significant difference was found between CTRL/MH and TI/MH groups. Ultrasound images representing the varying TUS from the study population are displayed in **figure 4**.

Cytology using BALF

Cytological analysis of BALF did not find any statistical difference in treatment by study day effect or between treatment groups. However, significant statistical differences were found between study days (p-value 0.03). White blood cell (WBC) count reported a statistically significant difference between study day 3 and study day 14 (p-value 0.04). All groups observed a decrease from day 3 to day 14 except for the CTRL/MH which increased from day 3 to day 14.

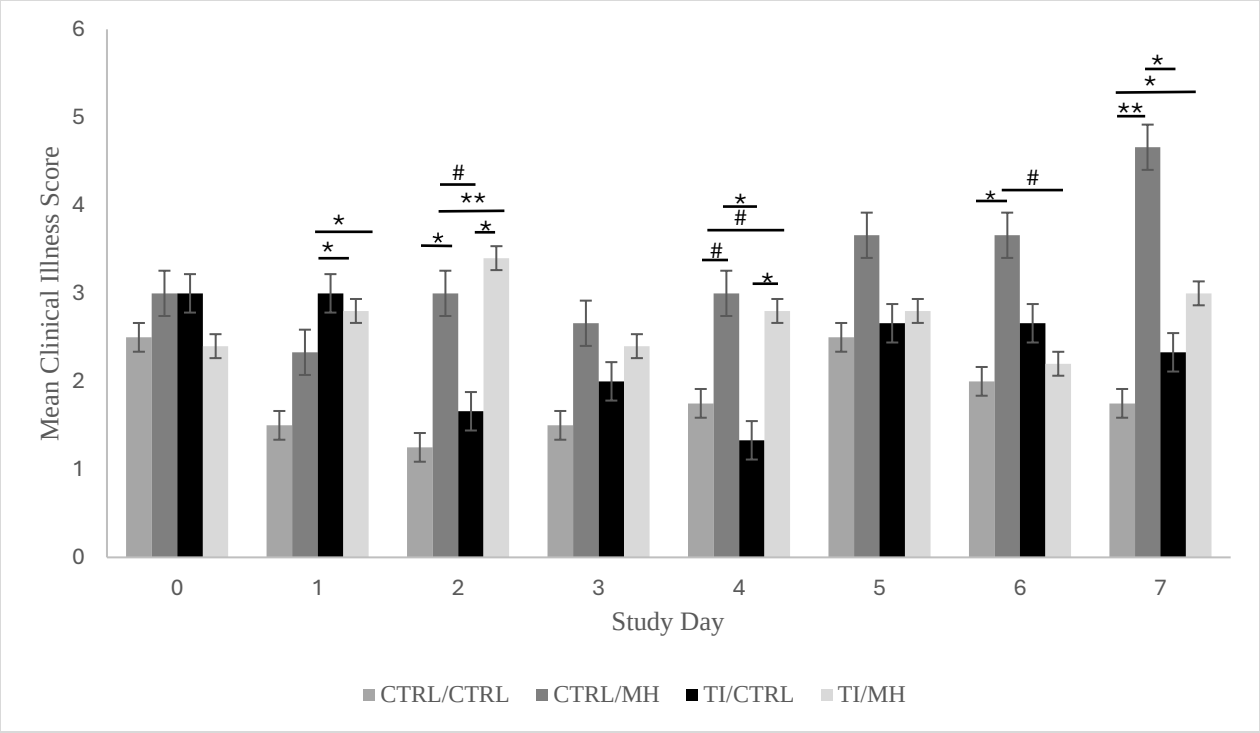


Figure 1: Mean clinical illness score progression. A significant correlation in treatment by day was indicated with (*) if the p-value ≤0.05, (**) if the p-value ≤0.01, and (#) if the p-value was ≤0.10.

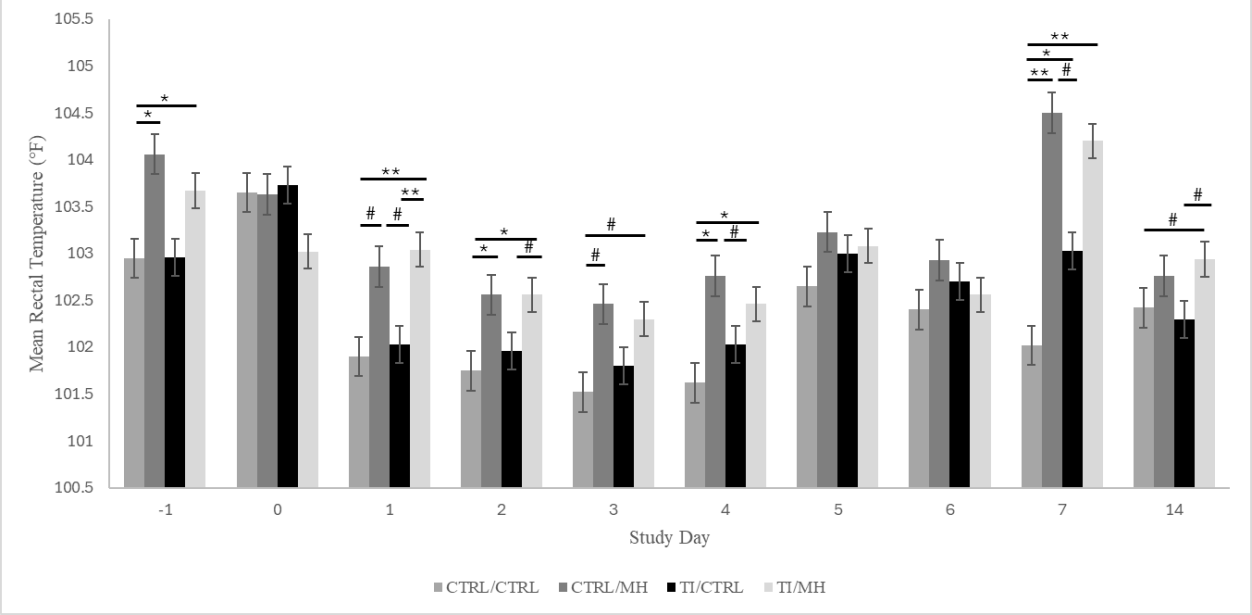


Figure 2: Mean rectal temperatures outlined by treatment group and study day. A significant correlation in treatment by day was indicated with (*) if the p-value ≤0.05, (**) if the p-value ≤0.01, and (#) if the p-value was ≤0.10.

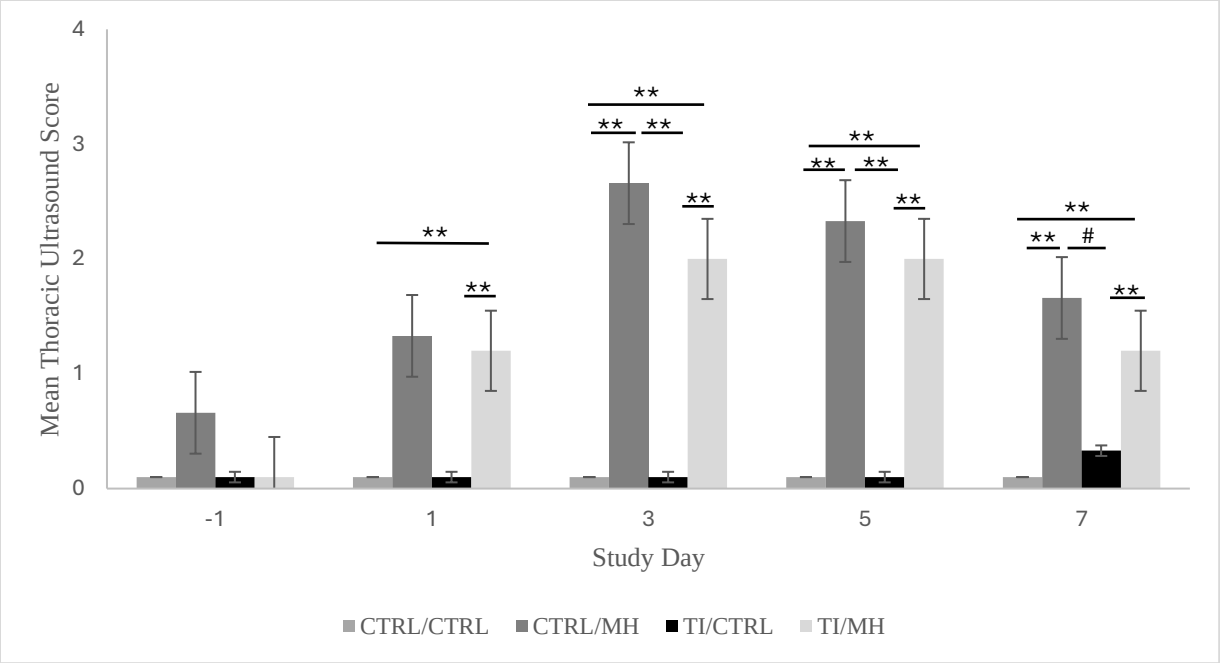


Figure 3: Mean thoracic ultrasound scores outlined by treatment group and study day. A significant difference in treatment by study day was indicated with (*) if the p-value ≤0.05, (**) if the p-value ≤0.01, and (#) if the p-value was ≤0.10.

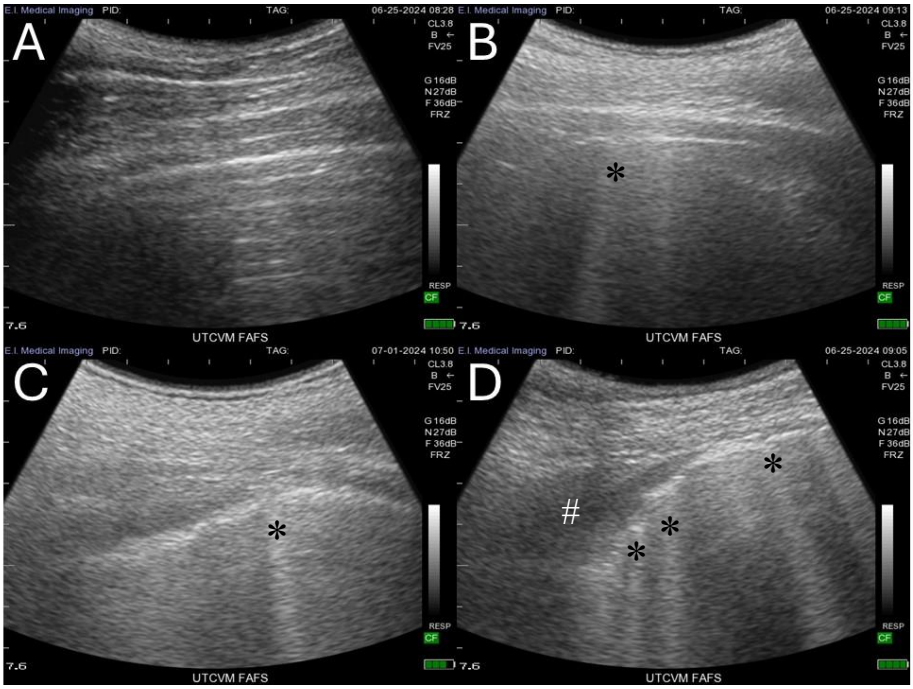


Figure 4: Representative thoracic ultrasound images corresponding with the thoracic ultrasound criteria used by (Ollivett & Buczinski, 2016): **(A)** TUS of 0 representing the criteria of normal layers of the lung in a healthy calf; **(B)** TUS of 1 showing the presence of diffuse comet tails on the pleural surface; **(C)** TUS of 2 showing the presence of consolidation of ≥1cm and comet tails; **(D)** TUS of 3-5 showing the presence of lobular pneumonia and severe comet tails (*).

Similarly, neutrophils were only found to have statistical difference between study days 0 and 3 (p-value 0.06), day 0 and day 7 (0.06), 3 and 14 (p-value 0.08), and days 7 and 14 (0.06). Increases in neutrophil concentrations occur from day 0 to day 3 and 7 in all groups with levels decreasing by day 14. Average lymphocyte concentration also saw no statistical difference in treatment group or in treatment by study day effect. Between study days, however, was found to be statistically significant (p-value 0.02). Except for the TI/MH group, lymphocyte concentrations increased from day 0 to day 14 (p-value 0.07). The TI/MH group experienced a decrease in lymphocyte concentration from day 0 to day 14. Apart from the TI/CTRL group that experienced a decrease from day 3 to day 14, all other groups experienced an increase in lymphocyte concentrations (p-value 0.007). All groups experience an increase in lymphocytes from day 7 to day 14 (p-value 0.01). Cytology results that show significance and numerical trends are presented in **Table 1** with a complete list of all cytology means and standard errors listed in **Table S1**. Macrophages, basophils, and eosinophils were also measured and included on **Table S1** but no statistical significance or trend was found.

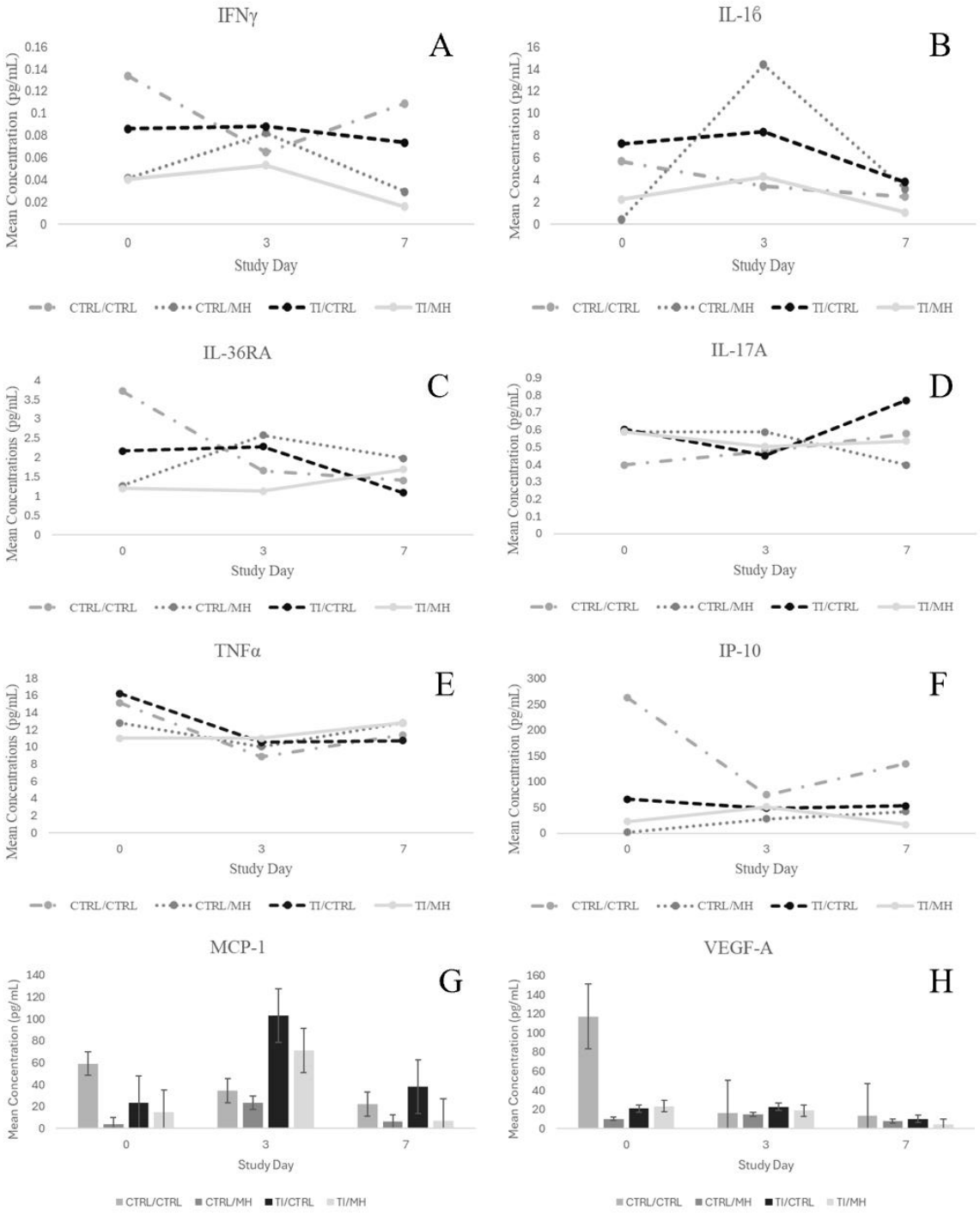
Biomarker concentration analysis from BALF

BALF biomarker concentration analysis found statistical differences in IL-1δ, IFNγ, IL-17A, IL-36RA, IP-10, and TNFα **(Figure 5)**. Linear regression was used to test if the treatment groups changed significantly overtime as well as how that change compared to the change overtime among the other treatment groups. IL-1δ saw a treatment by study day effect in the CTRL/MH group (p-value 0.0707). A significant trend was also seen when comparing linear regression between groups CTRL/CTRL vs. CTRL/MH (p-value 0.0646), CTRL/MH vs. TI/CTRL (p-value 0.0748) and CTRL/MH vs. TI/MH (p-value 0.0707). IFNγ displayed a significant regression with a negative slope in the TI/MH group (p-value 0.0407). In IL-17A a significant regression was found in the treatment groups CTRL/CTRL (p-value 0.0879). The CTRL/MH group saw a significant negative regression in IL-17A (p-value 0.0936) and a significant difference was found when comparing the regression between groups CTRL/CTRL and CTRL/MH (p-value 0.0584). Significant regression was found in the TI/MH group for the cytokine IL-8 (p-value 0.0476). IL-36RA saw a significant treatment by study day effect in the TI/CTRL group (p-value 0.060) as well as a significant regression within the same treatment group (p-value 0.0754). When comparing regression slopes, significance was found when

Table 1: Mean (standard deviation) cell concentration cytology results from BALF samples collected on study days 0, 3, 7, and 14.

	Study Day	CTRL/CTRL	CTRL/MH	TI/CTRL	TI/MH
WBC Count	0[ab]	650.000 (635.085)	133.333 (115.470)	400.000 (529.150)	200.000 (122.474)
	3[a]	375.000 (330.403)	400.000 (458.257)	300.000 (0)	280.000 (148.323)
	7[ab]	125.000 (250.000)	266.666 (115.470)	100.000 (100.000)	200.000 (158.113)
	14[b]	100.000 (81.649)	566.666 (723.417)	100.000 (100.000)	120.000 (44.721)
Neutrophil Avg.	0[a]	17.750 (23.117)	34.666 (22.673)	39.000 (42.755)	15.100 (18.136)
	3[a]	32.375 (26.132)	59.500 (35.815)	45.750 (44.194)	36.800 (30.523)
	7[a]	22.125 (44.250)	40.333 (43.336)	45.166 (46.830)	28.000 (30.120)
	14[a]	16.000 (21.051)	34.666 (51.393)	29.833 (37.551)	17.600 (30.824)
Lymphocyte Avg.	0[ab]	5.875 (5.088)	6.500 (3.905)	4.166 (3.253)	12.300 (16.076)
	3[a]	3.875 (2.719)	3.166 (1.258)	8.000 (7.778)	2.200 (1.303)
	7[ab]	0.625 (1.250)	3.666 (3.329)	2.500 (2.500)	7.500 (5.419)
	14[ab]	8.625 (4.130)	8.500 (4.769)	4.000 (4.000)	9.300 (7.546)

Figure 5: Mean biomarker concentrations from BALF samples that yielded statistical significance. **(A)** Mean IFNγ BALF concentrations overtime between treatment groups. **(B)** Mean IL-1ϐ concentrations in BALF overtime between treatment groups. **(C)** Mean IL-36RA concentrations in BALF overtime between treatment groups. **(D)** Mean IL-17A concentrations in BALF overtime between treatment groups. **(E)** Mean TNFα concentrations in BALF overtime between treatment groups. **(F)** Mean IP-10 concentrations in BALF overtime between treatment groups. **(G)** Mean MCP-1 concentrations between treatment groups. **(H)** Mean VEGF-A concentrations between treatment groups. A significant difference in treatment by study day was indicated with (*) if the p-value ≤0.05, (**) if the p-value ≤0.01, and (#) if the p-value was ≤0.10.


IFNγ
A
Mean Concentration (pg/mL)
Study Day
IL-1ẟ
B
Mean Concentration (pg/mL)
Study Day
IL-36RA
C
Mean Concentrations (pg/mL)
Study Day
IL-17A
D
Mean Concentration (pg/mL)
Study Day
TNFα
E
Mean Concentrations (pg/mL)
Study Day
IP-10
F
Mean Concentration (pg/mL)
Study Day
MCP-1
G
Mean Concentration (pg/mL)
Study Day
VEGF-A
H
Mean Concentration (pg/mL)
Study Day
CTRL/CTRL
CTRL/MH
TI/CTRL
TI/MH

comparing groups CTRL/MH vs TI/CTRL (p-value 0.0804) and groups TI/CTRL vs. TI/MH (p-value 0.0601). IP-10 upon statistical analysis of pair-wise comparisons, statistical significance was found between treatment groups (p-value 0.0423) as well as a treatment by study day effect (p-value 0.0584). This biomarker also displayed a significant regression in treatment groups CTRL/CTRL (p-value 0.0244) and CTRL/MH (p-value 0.0758). When comparing the linear regression of IP-10 concentration between treatment groups, significance was found between the groups CTRL/CTRL vs. CTRL/MH (p-value 0.006), CTRL/CTRL vs. TI/CTRL (p-value 0.0569), and CTRL/MH vs. TI/MH (p-value 0.0867). TNFα saw a treatment by study day effect when performing pair-wise comparisons in the TI/CTRL group (p-value 0.0878). Significance was also found when comparing the linear regression of this biomarker's concentration between groups TI/CTRL vs. TI/MH (p-value 0.0878).

In the biomarkers VEGF-A and MCP-1, statistical significance was found when analyzing multiple comparisons (**figure 5**). It was observed that VEGF-A was statistically difference among study days (p-value 0.0379) specifically between study days 0 and 7 (p-value 0.0115). MCP-1 was also observed to display significance between study days (p-value 0.0344) specifically between study days 0 and 3 (p-value 0.0331) and days 3 and 7 (p-value 0.0176). A complete list of all BALF biomarker concentration means between treatment group and study day are included in the supplementary **Table S2**.

Biomarker concentration analysis from blood serum

Biomarker concentration analysis from blood serum samples yielded statistical significance in IL-1α, IP-10, VEGF-A, and MCP-1 (**figure 6**). A positive relationship was observed in the TI/MH group with IL-1α concentrations in serum (p-value 0.0162). Concentrations of IP-10 in serum were not statistically difference between treatment groups but did yield statistical difference between study days (p-value 0.0025) specifically when comparing study days 3 and 7 and study days -1 and 3. Similarly, MCP-1 concentrations in serum also observed a significant difference between study days (p-value 0.0089) but not between treatment groups. Serum VEGF-A concentrations displayed a treatment by study day effect with a negative linear relationship in treatment groups TI/MH (p-value 0.0451) and CTRL/MH (p-value 0.0422). Significance was also observed when analyzing linear regression in the TI/MH group (p-value 0.0692). When comparing linear regression of VEGF-A concentrations between treatment groups, significance was found when comparing groups CTRL/MH vs. TI/MH (p-value 0.0422)

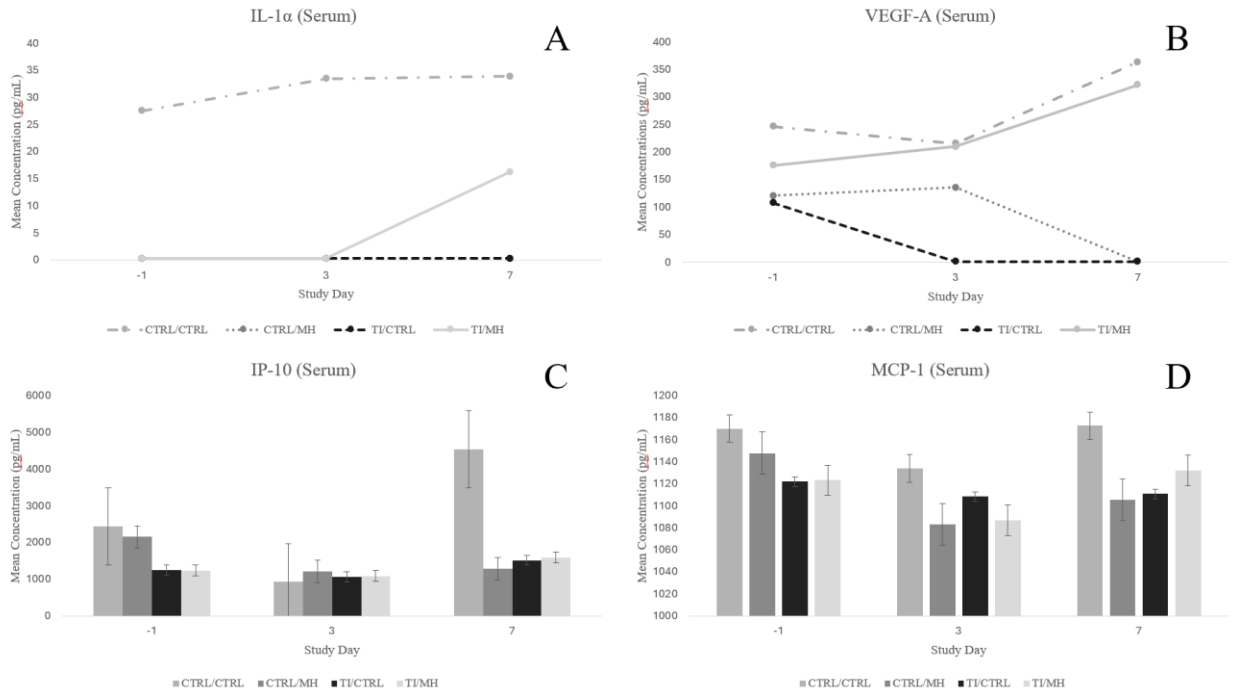


Figure 6: Mean concentrations of biomarkers from serum samples displaying statistical significance. **(A)** IL-1α concentrations found in serum samples over time within each group. **(B)** VEGF-A concentrations found in serum samples over time within each group. **(C)** IP-10 concentrations found in serum samples compared between treatment groups. **(D)** MCP-1 concentrations found in serum samples compared between treatment groups. A significant difference in treatment by study day was indicated with (*) if the p-value ≤0.05, (**) if the p-value ≤0.01, and (#) if the p-value was ≤0.10.

and between groups TI/CTRL vs. TI/MH (p-value 0.0452) both with negative linear relationships. A complete list of biomarker concentrations from serum samples can be found in the supplementary **Table S3**.

Serology from serum samples

Serology analysis from serum samples were measured for optical density (OD). OD differed among treatment groups (p-value 0.0056) and between study days (<0.0001). A treatment by study day effect was also observed when measuring OD (0.0486). Significance and effects yielded by serological analysis are displayed in **figure 7**.

Discussion

In reference to the study day pattern of peak clinical illness seen throughout the study period, our results reflect those found in previous studies that report days of clinical illness appearing as early as 12 hours post infection with peak occurring at approximately 24-48 hours post infection with resolution occurring approximately at day 5 post inoculation (Eberhart et al., 2017; Hanzlicek et al., 2010; Porter et al., 2021). Prior to the study it was hypothesized that TI calves will display higher clinical illness compared to control calves in the face of a bacterial respiratory infection. While statistically there is no significant difference between TI/MH and CTRL/MH except for day 6, numerically there is a pattern in which the CTRL/MH group CIS is higher than the TI/MH group CIS on study days 3-7. We found that MH-infected calves displayed higher CIS compared to non-MH infected which aligns with previous MH-infection

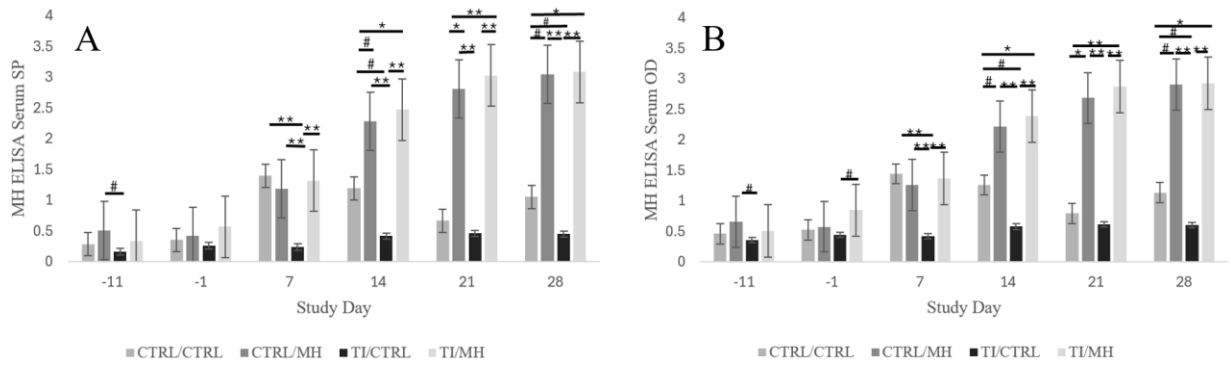


Figure 7: Mean SP and OD from serology of serum samples. **(A)** Mean SP from ELISA analysis from serum samples compared between treatment groups. **(B)** Mean OD from ELISA analysis from serum samples comparing treatment groups. A significant difference in treatment by study day was indicated with (*) if the p-value ≤0.05, (**) if the p-value ≤0.01, and (#) if the p-value was ≤0.10.

studies (Theurer et al., 2013b). However unexpectedly, elevated rectal temperatures were recorded on study day -1 prior to infection in both MH infected and non-MH infected groups. While elevated rectal temperatures has been reported as a symptom of a MH infection, this elevation prior to inoculation may have been due to the elevated ambient temperatures as the procedures were performed outdoors during the summer months (Theurer et al., 2013b). Rectal temperatures in the MH infected groups remained elevated throughout the study with initial elevation directly after inoculation followed by a decrease by day 3. Temperatures then re-elevated on days 4-7 with resolution occurring by day 14. This pattern has also been seen in a previous study analyzing similar parameters (Theurer et al., 2013b). The initial increase that is seen the day prior to observing lung pathology on day 3 may be due to the immediate molecular response of LPS produced by MH as soon as it enters the lungs and encounters the pulmonary epithelium. Fever is an expected symptom of an MH infection as the bacteria releases the virulence factor LPS that induces the activation of proinflammatory cytokines and other inflammatory factors that result in the increase of body temperature (Jeyaseelan et al., 2002). Recruitment of neutrophils is also a side effect of LPS toxicity which is known to cause collateral damage to the surrounding tissue. Phospholipid interaction with LPS in pulmonary surfactant of the lungs also allows the LPS to persist resulting in increased damage leading to prolonged lesions in the lungs (Singh et al., 2011). Elevated rectal temperatures may have also been a result of working the calves one at a time starting with the non-MH infected group and finishing with the MH infected group starting in the morning when temperatures are lower but increasing as the day went on. Furthermore, this explains the unexpected elevation of rectal temperatures on day 7 when normal resolution would occur. Increased CIS and rectal temperature paired with resulting lung pathology confirms a successful experimental infection using MH. Previous research using thoracic ultrasound as a technique for detection reports considerable lung consolidation within 24 hours post infection and presence of pleural fluid and fibrin by 48 hours post infection (Bassel et al., 2019). Other studies that involve MH infection implementing necropsy examination find extensive pathology in the lungs that were not observable in thoracic ultrasound. These lesions were found within 24 hours post infection which matches with the times points in which lesions were detected on thoracic ultrasound in this study (Hanzlicek et al., 2010). Peak TUS occurred on day 3 which we predict is due to the length of time it takes for the development of lung pathology to result from molecular damage caused by

the leukotoxin produced by MH. In connection with this prediction, peak CIS occurs on day 2 as previous literature has shown that the lungs need to have at least ≥1cm of consolidation before clinical symptoms manifest (Buczinski, Forté, Francoz, et al., 2013; Lowie et al., 2022).

Antibody concentration analysis from blood serum samples yielded a treatment by study day effect. Within the MH-infected groups, an increase in antibodies titers overtime was observed. A previous study utilized concentrations of IgG, IgA, and IgM derived from serum samples as a way to confirm MH-infection (Poonsuk et al., 2023). In **figure 7**, antibody titers increase gradually peaking on day 28. This follows the normal behavior of these antibodies of interest. IgM is produced quickly with a strong ability to bind to multiple antigens (Charles A. Janeway et al., 2001; Poonsuk et al., 2023). The production of this immunoglobulin explains the initial increase seen in the MH-infected animals 7-day post MH-infection. IgG is a more specific antibody that remains in tissue for a longer period of time (Charles A. Janeway et al., 2001). IgA is an antibody found on mucosal and epithelial surfaces that is not as potent as IgM and IgG but is still important as MH proliferates on mucosal surfaces leading to infiltrations of the lungs (Cozens et al., 2019; Charles A. Janeway et al., 2001). The progression of these antibodies in this study supports the confirmation that experimental infection using MH was successful.

Based on another study done to show the importance of neutrophils against an MH infection, it was expected that neutrophil levels increased directly after inoculation. This same study also described that neutrophils were essential for acute lung damage caused by the experimental bacterial infection (Slocombe et al., 1985). Another study of similar nature saw increased circulatory neutrophils within 6 hours post inoculation with MH which aligned with our results of seeing increase neutrophils concentrations in pulmonary fluid as they are the body's initial defense against foreign pathogens (Breider et al., 1988). Literature also shows that MH leukotoxin can result in the apoptosis of lymphocytes and white blood cells (DiFranco et al., 2012; Sun et al., 1999). The initial increase in WBCs seen in some of the treatment groups inoculated with MH may be the delayed reaction caused by the leukotoxin produced by MH.

An unexpected outcome presented itself when analyzing biomarker concentrations from BALF. Prior to the study it was hypothesized that proinflammatory or anti-inflammatory biomarkers would show a trend of dysregulation between the treatment groups; specifically, between the CTRL/MH group and the TI/MH group in which the TI/MH group would display elevated biomarker levels. Proinflammatory biomarkers IFNγ, IL-1β, IL-17A, TNFα, IP-10,

MCP-1, and VEGF-A analyzed from BALF samples showed statistical significance. IFNγ and IL-1ϐ numerically showed lower mean concentrations in the TI/MH group compared to the CTRL/MH group that was also presented statistically. IFNγ resulted in a negative linear association overtime in the TI/MH group. This proinflammatory biomarker functions as an activator for macrophages and other antigen presenting immune cells. This biomarker also regulates T-helper 1 cells (Bradley et al., 1996). IL-1ϐ is a proinflammatory cytokine that is produced in response to tissue damage and/or LPS from the bacteria, signaling to the body of a present infection while acting as a co-stimulate for T-cell activation (Eeckhout et al., 2021; Singh et al., 2011). In response to the bacterial infection, statistical significance was that those concentrations were greater in the CTRL/MH group compared to the TI/MH group. This occurrence would explain why more lung pathology was seen in the CTRL/MH group compared to the TI/MH group. IP-10 is a chemokine that is produced in response to IFNγ and assists in directing T cells to areas of infection (Khan et al., 2000; Torvinen et al., 2007). From BALF fluid, IP-10 was elevated in the TI/MH group compared to the CTRL/MH group until study day 7 in which the TI/MH IP-10 concentrations fell below the CTRL/MH group's concentrations. Since the production of IP-10 is connected to the production of IFNγ, it is unexpected that IP-10 concentrations in the CTRL/MH group increase on study day 7 while IFNγ concentrations decreased on study day 7. Serum IP-10 concentrations statistically only saw a difference between study days in which day -1 differed from day 3 and day 3 differed from day 7. MCP-1 (monocyte chemoattractant protein-1) is a chemokine that increases the expression of inflammatory cells by directing the travel of monocytes, microglia, and memory T cells to the site of infection (Singh et al., 2021). In BALF samples for the CTRL/MH, TI/CTRL, and the TI/MH groups it is expected to see that the mean MCP-1 concentrations would increase at study day 3 as this is where peak lung pathology occurred. In the CTRL/CTRL group, however the MCP-1 mean concentrations decreased with each measurement. It is unknown why this group did not follow the same pattern as the CTRL/MH group or the TI/CTRL group but considering that the CTRL/CTRL group was not exposed to any pathogen throughout the study, it is expected that MCP-1 concentrations would not elevate. Concentrations of MCP-1 from serum samples yielded only statistical difference between study days. VEGF-A (vascular endothelial growth factor A) is a protein that moderates blood vessel formation in fetal development and during disease (Holmes & Zachary, 2005). In our study, this protein concentration decreased significantly from day 0 to day 7 in all

treatment groups. This may have been because of the bacterial infection that halts the angiogenic effects of this protein. However, this would not explain why this also occurred in the non-MH infected groups. From serum samples, VEGF-A concentrations did show a difference between CTRL/MH calves and TI/MH calves in which concentration in the CTRL/MH animals are greater than in the TI/MH group. While this does align with the study's initial hypothesis, it does not follow the behavior of the other biomarkers that yielded statistical significance where the concentrations are higher in the CTRL/MH group compared to the TI/MH group. IL-1α concentrations in serum showed a linear trend over study day in the TI/MH group. When activated, IL-1α allows neutrophils and monocytes to infiltrate damaged tissue by stimulating chemokine production and promoting adhesin molecule on endothelial cells (Dinarello, 2017). This is another biomarker that follows the initial expectation of cytokine behavior in the TI/MH groups, it does not align with the other proinflammatory biomarker behaviors. BALF IL-17A concentrations yielded a linear trend in which CTRL/MH calves had higher concentrations than TI/MH. This cytokine also displayed a linear significance comparing the CTRL/CTRL group to the CTRL/MH group. This was to be expected in response to the bacterial infection. This proinflammatory cytokine functions as a neutrophil recruiter and induces other proinflammatory immune cells as well as activating T cells (Iwakura et al., 2008). One anti-inflammatory cytokine, IL-36RA, showed linear significance when comparing the slope of the MH-infected groups compared to the non-MH infected groups. However, numerically, the TI/MH group shows the lowest concentrations out of all the treatment groups until study day 7. In reference to the other proinflammatory cytokines and their behavior throughout the study, one would infer that a pro-inflammatory cytokine would display an opposite trend. IL-36RA acts as an immune response regulator that prevents the activation of proinflammatory pathways (Zhou & Todorovic, 2021). Another study used similar multiplex cytokine and chemokine assay techniques using serum collected from calves inoculated with LPS and were able to find statistical significance in majority of the biomarkers (Sullivan et al., 2022). In a study that used the same multiplex cytokine/chemokine assay that was used in this study, biomarker concentrations were also measured using serum samples collected from calves inoculated with bovine coronavirus. The panel was able to detect a majority of the biomarkers while finding statistical significance in 6 of the 10 biomarkers detected (Cho et al., 2024). The goal of analyzing BALF for biomarker concentration analysis is to gain understanding of what was occurring during a respiratory

pathogen infection at the molecular level locally in the lungs. While serum does show differences in biomarker concentrations in a diseased animal, the results are from a systematic point of view and not at the site of active pathology caused by the infection.

CHAPTER FIVE: CONCLUSIONS

The abnormal behavior of select biomarker concentrations found in pulmonary fluid and blood serum indicates that a late gestational BVDV exposure results in a dysfunctional immune response to other respiratory bacterial pathogens later in a calf's life. Another recent study investigating the impacts of late gestational BVDV exposure to the productivity of calves found that TI calves had lower body weights post-weaning after a feeding period. The study links the phenomenon to increased inflammation of the gastrointestinal tract found in BVDV exposed cattle (Engle et al., 2024). While the patterns of proinflammatory biomarkers in this study discussed were subdued in the TI/MH group, it does show that there is an abnormal immune response because of TI BVDV status of the calves within the TI groups. Furthermore, while the results of this study may appear to show a protective effect from late gestational BVDV exposure, this would not reflect all the stressors a calf would encounter outside of an isolated experimental study. The calves of this study were under little stress when exposed to the bacteria compared to calves in a production setting where they experience many stressors such as weaning, banding, long transport, and co-mingling simultaneously. Our results imply that in addition to TI status and exposure to a respiratory bacterial pathogen, a calf undergoing these stressful events would not be as protected by their internal immune system due to their TI status and thus their productivity would be negatively impacted as well as their overall health.

Limitations to this study include a small sample size due to the experiment being a pilot study decreased the power for statistical analysis. With a larger sample size statistical significance may have been found between TI and CTRL calves and would further support the findings of immune dysfunction in TI BVDV calves as well as elucidate the mechanism behind the dysfunction. While all procedures were consistently performed by the same investigators, thoracic ultrasounds, BALs, and clinical illness scoring procedures were not blinded due to the nature of the housing of the animals. In future studies of similar nature, it would be beneficial to ensure blinding of all procedures among as many investigators as possible to exclude bias. To further investigate the lung pathology between TI and CTRL calves, conducting necropsy reports of the lungs at the peak of clinical illness of the bacterial pathogen would allow more comparison of the impacts a late gestational BVDV infection would have on the development of pneumonia. By comparing lung consolidation to weight ratios among a representative animal from each treatment group, one could link the biomarker concentration to physical evidence of disease.

These findings may also counteract the protective effect seen in the current study by the suppression of select proinflammatory biomarker if more lung pathology is found among TI calves. In-field clinical applications of the knowledge gained from this study would be the increased awareness of the benefits from preconditioning stock prior to sale and/or long transportation as well as continued diligence in the detection of BVDV within susceptible herds.

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APPENDIX

Table S1: Mean cytology results outlined by treatment and study day.

	Study Day	CTRL/CTRL	CTRL/MH	TI/CTRL	TI/MH
WBC Count	0[ab]	650.000 (635.085)	133.333 (115.470)	400.000 (529.150)	200.000 (122.474)
	3[a]	375.000 (330.403)	400.000 (458.257)	300.000 (0)	280.000 (148.323)
	7[ab]	125.000 (250.000)	266.666 (115.470)	100.000 (100.000)	200.000 (158.113)
	14[b]	100.000 (81.649)	566.666 (723.417)	100.000 (100.000)	120.000 (44.721)
Number of Cells	0	400.000 (0)	300.000 (173.205)	400.000 (0)	360.000 (89.442)
	3	300.000 (200.000)	300.000 (173.205)	400.000 (0)	320.000 (109.544)
	7	100.000 (200.000)	400.000 (0)	166.666 (208.166)	260.000 (194.935)
	14	275.000 (150.000)	400.000 (0)	166.666 (208.166)	240.000 (167.332)
Neutrophil Avg.	0[a]	17.750 (23.117)	34.666 (22.673)	39.000 (42.755)	15.100 (18.136)
	3[a]	32.375 (26.132)	59.500 (35.815)	45.750 (44.194)	36.800 (30.523)
	7[a]	22.125 (44.250)	40.333 (43.336)	45.166 (46.830)	28.000 (30.120)
	14[a]	16.000 (21.051)	34.666 (51.393)	29.833 (37.551)	17.600 (30.824)
Lymphocyte Avg.	0[ab]	5.875 (5.088)	6.500 (3.905)	4.166 (3.253)	12.300 (16.076)
	3[a]	3.875 (2.719)	3.166 (1.258)	8.000 (7.778)	2.200 (1.303)
	7[ab]	0.625 (1.250)	3.666 (3.329)	2.500 (2.500)	7.500 (5.419)
	14[ab]	8.625 (4.130)	8.500 (4.769)	4.000 (4.000)	9.300 (7.546)

Table S1: Continued

	Study Day	CTRL/CTRL	CTRL/MH	TI/CTRL	TI/MH
Macrophage Avg.	0	76.500 (20.988)	58.833 (18.790)	56.833 (40.565)	72.100 (25.454)
	3	38.750 (29.341)	37.333 (37.055)	46.500 (36.062)	61.000 (30.563)
	7	2.250 (4.500)	56.000 (40.841)	19.000 (29.512)	44.200 (37.912)
	14	75.500 (24.859)	56.833 (47.149)	32.833 (38.027)	53.100 (39.430)
Eosinophil Avg.	0	0 (0)	0 (0)	0 (0)	0 (0)
	3	0 (0)	0 (0)	0 (0)	0 (0)
	7	0 (0)	0 (0)	0 (0)	0.25 (0.50)
	14	0 (0)	0 (0)	0 (0)	0 (0)
Basophil Avg.	0	0 (0)	0 (0)	0 (0)	0.10 (0.22)
	3	0 (0)	0 (0)	0 (0)	0 (0)
	7	0 (0)	0 (0)	0 (0)	0 (0)
	14	0 (0)	0 (0)	0 (0)	0 (0)

Table S2: Mean biomarker concentrations with standard deviations analyzed from BALF samples.

Cytokine	Study Day	CTRL/CTRL	CTRL/MH	TI/CTRL	TI/MH
IFNγ	0	0.134 (0.049)	0.041 (0.051)	0.086 (0.109)	0.040 (0.011)
	3	0.065 (0.038)	0.082 (0.016)	0.088 (0.066)	0.053 (0.047)
	7	0.109 (0.143)	0.029 (0.009)	0.073 (0.057)	0.015 (0.015)
IL-1α	0	6.862 (7.905)	1.049 (1.315)	11.599 (18.675)	2.282 (4.361)
	3	2.284 (2.829)	8.661 (13.716)	11.430 (10.525)	3.776 (5.695)
	7	8.001 (13.355)	5.134 (8.390)	10.437 (17.569)	0.254 (0.080)
IL-1β	0	5.692 (5.029)	0.467 (0.148)	7.253 (10.680)	2.242 (2.293)
	3	3.426 (3.210)	14.380 (21.228)	8.348 (7.246)	4.295 (3.677)
	7	2.498 (2.226)	3.179 (2.971)	3.799 (5.923)	1.094 (0.986)

Table S2: Continued

	Study Day	CTRL/CTRL	CTRL/MH	TI/CTRL	TI/MH
IL-4	0	2.964 (0.737)	3.024 (1.239)	3.076 (0.997)	2.920 (0.632)
	3	2.471 (0.581)	3.042 (0.292)	2.715 (0.142)	3.092 (0.390)
	7	2.707 (0.746)	3.133 (0.481)	3.228 (0.321)	2.339 (0.416)
IL-6	0	2.590 (0)	2.590 (0)	2.590 (0)	2.590 (0)
	3	2.590 (0)	2.590 (0)	2.590 (0)	2.590 (0)
	7	2.590 (0)	2.590 (0)	2.590 (0)	2.590 (0)
IL-8	0	8.170 (5.693)	2.190 (0)	14.920 (15.221)	1.314 (1.199)
	3	12.301 (17.513)	14.880 (21.980)	87.084 (112.255)	15.602 (2.190)
	7	8.034 (10.122)	8.034 (10.122)	16.389 (24.594)	2.190 (0)
IL-10	0	2.068 (2.000)	1.184 (0.406)	2.654 (2.951)	1.163 (0.379)
	3	1.068 (0.205)	1.133 (1.051)	3.118 (1.879)	1.112 (0.219)
	7	0.950 (0)	1.301 (0.608)	1.536 (1.015)	0.950 (0)
IL-17A	0	0.396 (0.265)	0.590 (0)	0.603 (0.022)	0.590 (0)
	3	0.477 (0.194)	0.587 (0.004)	0.451 (0.240)	0.503 (0.193)
	7	0.577 (0.022)	0.396 (0.185)	0.770 (0.311)	0.535 (0.122)
MIP-1A	0	7.633 (4.225)	4.066 (0.542)	9.773 (8.770)	5.092 (1.831)
	3	5.527 (4.402)	6.416 (4.732)	26.071 (29.761)	6.189 (3.026)
	7	8.598 (9.302)	6.852 (5.579)	14.714 (19.874)	3.554 (1.264)
IL-36RA	0	3.717 (3.292)	1.270 (0)	2.170 (0.901)	1.203 (0.741)
	3	1.659 (1.105)	2.577 (2.264)	2.281 (2.098)	1.135 (0.301)
	7	1.410 (1.430)	1.982 (1.233)	1.087 (0.315)	1.697 (0.956)
IP-10	0	263.238 (104.201)	1.682 (1.177)	65.790 (102.509)	22.207 (14.177)
	3	74.741 (97.932)	27.766 (29.570)	48.266 (29.272)	50.921 (67.870)
	7	134.631 (232.166)	42.265 (33.300)	53.027 (66.734)	16.985 (14.001)
MCP-1	0[ab]	59.245 (67.989)	3.931 (3.500)	23.701 (38.517)	15.102 (20.501)
	3[a]	34.480 (32.026)	23.255 (27.737)	103.290 (84.304)	71.409 (130.782)
	7[b]	22.374 (35.444)	6.106 (7.333)	38.325 (63.097)	7.180 (7.461)

Table S2: Continued

	Study Day	CTRL/CTRL	CTRL/MH	TI/CTRL	TI/MH
MIP-1B	0	14.407 (13.925)	2.531 (0.591)	9.370 (12.254)	2.201 (1.278)
	3	3.478 (1.505)	6.682 (9.720)	21.679 (24.920)	7.532 (7.029)
	7	21.041 (32.652)	9.944 (13.604)	13.971 (23.025)	1.583 (0.633)
TNFα	0	15.150 (4.131)	12.790 (0)	16.219 (5.939)	11.019 (3.930)
	3	8.872 (6.785)	10.023 (4.792)	10. 534 (3.864)	11.025 (3.945)
	7	11.403 (2.402)	12.790 (0)	10.726 (3.574)	12.790 (0)
VEGF-A	0[a]	177.321 (91.327)	10.188 (11.495)	20.745 (21.854)	23.393 (14.615)
	3[ab]	16.002 (23.981)	14.755 (12.365)	22.540 (24.012)	18.696 (13.291)
	7[b]	13.034 (20.377)	7.898 (6.099)	10.078 (15.257)	4.163 (4.207)

Table S3: Mean biomarker concentrations with standard deviations analyzed from serum samples.

Treatment	Study day	IFNγ	IL-1α	IL-8	IP-10	MCP-1	TNFα	VEGF-A
CTRL/CTRL	-1	9.463 (16.217)	27.598 (47.281)	18158.94 (23816.86)	2439.16 (1494.87)	1170.28 (77.15)	1815.35 (3122.11)	246.92 (227.28)
	3	0.100 (0.00)	33.496 (57.498)	12624.21 (19378.47)	929.63 (90.851)	1134.11 (126.54)	2316.70 (3990.46)	215.69 (218.14)
	7	10.588 (14.832)	33.940 (58.267)	12631.63 (19372.31)	4546.40 (4794.50)	1172.89 (90.41)	3016.97 (3972.37)	363.81 (131.88)
CTRL/MH	-1	11.687 (20.069)	0.300 (0.00)	23696.43 (19578.35)	2159.31 (1857.39)	1148.00 (53.41)	12.800 (0.00)	121.15 (169.53)
	3	0.100 (0.00)	0.300 (0.00)	1279.36 (60.610)	1219.21 (439.96)	1083.24 (13.72)	12.800 (0.00)	136.01 (190.54)
	7	0.100 (0.00)	0.300 (0.00)	18229.47 (23717.11)	1292.67 (336.46)	1105.69 (17.83)	12.800 (0.00)	1.280 (0.00)

Table S3: Continued

Treatment	Study Day	IFNγ	IL-1α	IL-8	IP-10	MCP-1	TNFα	VEGF-A
TI/CTRL	-1	0.100 (0.00)	0.300 (0.00)	35000.00 (0.00)	1255.22 (366.61)	1122.11 (14.67)	12.800 (0.00)	108.35 (151.42)
	3	0.100 (0.00)	0.300 (0.00)	35000.00 (0.00)	1067.99 (121.17)	1108.46 (22.56)	12.800 (0.00)	1.280 (0.00)
	7	0.100 (0.00)	0.300 (0.00)	35000.00 (0.00)	1520.67 (225.16)	1110.95 (20.11)	12.800 (0.00)	1.280 (0.00)
TI/MH	-1	6.159 (12.118)	0.300 (0.00)	14901.32 (18348.08)	1243.01 (366.25)	1123.32 (36.91)	623.36 (1365.27)	176.15 (239.76)
	3	0.100 (0.00)	0.300 (0.00)	26534.40 (16931.20)	1091.19 (251.50)	1087.09 (30.20)	807.82 (1777.74)	210.03 (184.89)
	7	0.100 (0.00)	16.290 (31.981)	26664.22 (16671.56)	1597.79 (591.96)	1132.46 (46.86)	1112.63 (2199.67)	322.66 (98.69)

VITA

Born and raised in Southeastern Ohio, Alyssa Wilson attended Ohio University in Athens, Ohio, graduating with a Bachelor of Science in Biological Sciences. With the intention of attending veterinary medicine school, she decided to first supplement her scientific education by obtaining a Master of Science degree at the University of Tennessee Knoxville in Comparative and Experimental Medicine. Her research project focuses on the long-term effects of an in-utero Bovine Viral Diarrhea Virus infection which is a highly impactful disease in the cattle industry. With this extension to her knowledge, not only will she utilize what she has learned from her mentors and experiences obtaining this degree but will also apply this knowledge going forward with her veterinary medicine education. Following graduation, she will attend Michigan State University to begin in their Doctor of Veterinary Medicine program. She is beyond grateful to her mentors who have given her the opportunity for this unique experience as well as her friends and family who never falter in providing support and giving her confidence to reach her goals.