Determining differences in growth, behavior, and serotonin in dairy calves affected by respiratory disease as diagnosed by lung ultrasound and a clinical respiratory score

By

Mary Caitlin Cramer

A dissertation submitted in partial fulfillment of

the requirements for the degree of

Doctor of Philosophy

(Dairy Science)

at the

UNIVERSITY OF WISCONSIN-MADISON

2018

Date of final oral examination: 4/30/2018

This dissertation is approved by the following members of the Final Oral Committee:

Theresa L. Ollivett, Assistant Professor, School of Veterinary Medicine

David K. Combs, Professor, Dairy Science

Laura L. Hernandez, Associate Professor, Dairy Science

Kathryn L. Proudfoot, Assistant Professor, Preventative Veterinary Medicine

Kent A. Weigel, Professor, Dairy Science

DEDICATION

This thesis is dedicated to my parents, Bob and Lisa Cramer. I have been able to take risks because I know you will be there to pick me up if I fail. Your unconditional love and support have made this achievement and everything else in my life possible. You are genuinely interested in my life and research. You are proud of me and never hesitate to say so. You both lead by example and instilled in me the values of hard work, kindness, drive, and resiliency- all of which were integral to my success in graduate school. I am proud of the hard work that went into this thesis, but I am most proud to be your daughter.

To my beloved Grandma and Grandpa: I wish you could be here to celebrate this achievement. I hope you know that your love and support helped me get to where I am today.

To Nancy and Pam: You are the best aunts I could ever ask for. Thank you for all the support and encouragement, and for being two fierce role models.

To Logan and Raygan: Being your Aunt has, and will continue to be, one of the greatest adventures of my life. Thank you for reminding me what life is all about.

ACKNOWLEDGEMENTS

First and foremost, I want to thank my advisor, Dr. Terri Ollivett. Terri, thank you for caring about me as a person, rather than just a graduate student. Your willingness to talk about and advocate for the wellbeing of your students is refreshing and appreciated. You taught me to write and speak with intention and precision. You asked me to think about the details and the big picture, because both matter equally. You pushed me to consider the 'why' in everything I did, and I am a better scientist because of this. Your passion for teaching, calf health, working with producers, and research is unmatched. Thank you for your patience, kindness, compassion, steadfast attitude, enthusiasm, and encouragement. It is a true pleasure working with you.

To my committee members:

Dr. Laura Hernandez: Thank you for encouraging me to pursue the work with serotonin in calves and for your help with this aspect of my thesis. I am also very grateful for the kindness and generosity you and your family have shown me.

Dr. Kent Weigel: Thank you for working out the research agreement that made this thesis possible and for encouraging me to stay at UW. I also appreciate all the feedback you've given for this project.

Dr. Dave Combs: Thank you for all your suggestions for this research. You always push me to consider the practical application of my research and I am very grateful.

Dr. Katy Proudfoot: Thank you for all your guidance and support. Your knowledge of animal welfare, standards for research, and passion for teaching are unparalleled. I learned so much from you about animal welfare, behavior, and teaching. I sincerely appreciate all your feedback

and our many discussions about animal welfare. Thank you for being patient, kind, and generous with your time and knowledge.

Next, I want to thank STGenetics and Mr. Juan Moreno for their generous financial support of my PhD program. This research would not have been possible without their funding or willingness to let us work on their farm. The year I spent at the Ohio Heifer Center taught me invaluable lessons about calf health and personnel management that will serve me well going forward. I also want to thank Paul Detweiler, Liz Grim, Sue Sifrit, Emily Dudash, the farm veterinarians, and the rest of the staff at the Ohio Heifer Facility for accommodating this research project. I especially want to thank Liz Grim whose flexibility, help, and support during a busy data collection period were much appreciated.

Thank you to the students that helped with data collection: Amel Hamed, Marisa Joldrichsen, Hannah Donley, and Ella Jackson. I sincerely appreciate all your help. I especially want to thank Hannah and Ella whose upbeat attitude, sense of humor, hard work, and attention to detail made data collection much more enjoyable.

I also need to thank the members of the Hernandez laboratory: Samantha Weaver, Adrienne Cheng, Faye Nashold, and Meghan Connelly for and answering all my questions about lab work. I especially want to thank Samantha Weaver who taught me how to run the serotonin assays and for her thoughtful contributions to the serotonin manuscript. I can say, without a doubt, that she taught me everything I know about lab work. Her patience and knowledge are much appreciated and this research is better because of her involvement.

Thank you to my fellow graduate students, Elizabeth Binversie and Dr. Sarah Raabis. I appreciate your friendship, support, and willingness to share ideas.

I also want to thank the wonderful people in the Food Animal Production Medicine Department and the School of Veterinary Medicine. I appreciate everyone's help and feedback. Mr. Tom Bennett, thank you for your help with SAS and all of my computer problems. Mr. Chris Eisle, thank you for including me in all the FAPM activities and for keeping things running smoothly.

Thank you to Mr. Peter Crump for your help with the statistical analyses. Your knowledge and patience were much appreciated.

My graduate school experience wasn't just shaped by my research. The people I met here were integral to my wonderful time in Madison and will be lifelong friends. There is not enough space here to express my sincere gratitude and appreciation for their friendship.

To Sam Weaver: Thank you for being a rare find. You are smart, bold, funny, compassionate, and loyal. Your willingness to go above and beyond for your friends is incredible. You have cheered me on during the good times and supported me through the more difficult times. I think the theme song of our favorite show says it best: 'Thank you for being a friend. We travelled down the road and back again. Your heart is true- you're a pal and a confidante.'

To John and Kym Penry: thank you for your generosity, kindness, and welcoming me into your family. We shared a lot of memories and I cherish them all. Your optimistic attitude, even during difficult times, is inspiring.

To Callyssa Cronick, Laura Amundson, Jimena Laporta, Jess Pempek, Meghan Bergman, Adrienne Cheng, Meghan Connelly, Aine O'Connell, and Steph Metzger: Thank you for your wonderful friendships. The laughs, memories, and drinks we've shared made this journey a pretty great one. I cherish you all.

To my high school Agricultural Education teacher, Mr. Carl Beeman: Thank you for sharing your passion for animal agriculture and getting me interested in food animals.

To my friend and mentor, Dr. Jason Lombard: Thank you for your guidance, friendship, and encouragement. I sincerely appreciate your continued support and kindness.

To my favorite undergraduate professor and the person who got me hooked on dairy cattle, Dr. Noa Roman-Muniz. Thank you for your passion and hard work to make the dairy industry better for the animals and the people who care for them.

To Dr. Sheila McGuirk: I consider myself incredibly lucky to know you. Thank you for your kindness and guidance over the last five years. I enjoyed the opportunity to learn from you. I truly value your wealth of knowledge and the kind-hearted way you interact with everyone.

Last, but certainly not least, I want to thank all of the calves. Thank you for teaching me patience and compassion and reminding me to never get too full of myself. I hope this research makes some difference in your quality of life.

ABSTRACT

This dissertation sought to determine how bovine respiratory disease (**BRD**), diagnosed using lung ultrasound (**US**) and a clinical respiratory score (**CRS**), affects calf growth, behavior, and non-neuronal serotonin in dairy calves.

Chapter 1 determined how the amount of lung consolidation and the clinical status affected calf growth. Lung consolidation ($\geq$ 1cm^2) decreased average daily gain (ADG) by 0.11 kg/d in preweaned dairy calves. We also discovered that clinical status impacted ADG whereby calves that had clinical BRD had a decrease in ADG of 0.10 kg/d.

Chapters 3 through 5 combined the lung status and the clinical respiratory status of the calf to define three BRD categories: clinical BRD, subclinical BRD, and no BRD. Chapter 3 compared automated calf feeding behaviors between the three categories of BRD. Calves with subclinical BRD drank more milk than calves with clinical BRD (10.9 vs. 9.9 L/d) and calves with no BRD (10.9 vs. 8.9 L/d). Calves with subclinical BRD drank faster than calves with clinical BRD (768 vs. 664 mL/min); calves with clinical BRD tended to drink slower than unaffected herdmates (664 vs. 772 mL/min). Drinking speed was not different between calves with subclinical BRD and unaffected herdmates (768 vs. 772 mL/min). Chapter 4 compared a behavioral attitude score ('normal' versus 'depressed') on the day of BRD diagnosis between three BRD categories. Calves with subclinical BRD had similar attitude scores to unaffected herdmates. However, calves with clinical BRD were 5 times more likely to have a depressed attitude compared to both calves with subclinical BRD and unaffected herdmates. Producers should not use feeding behavior or attitude score as the sole means of BRD detection as these tools failed to identify calves with subclinical BRD and did not detect every calf with clinical BRD.

Finally, Chapter 5 examined the relationship between non-neuronal serotonin and BRD. For every 1-unit increase in serum serotonin at 3-5 days of age, the odds of a calf developing subclinical BRD versus no BRD increased by 1.0. At the time of BRD diagnosis, serotonin was 779 ng/mL higher in calves without BRD compared to both calves with clinical and subclinical BRD.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1 CHAPTER 1: LITERATURE REVIEW

1.1 INTRODUCTION

In young dairy calves, bovine respiratory disease (**BRD**) affects performance (Stanton et al., 2012, Dunn et al., 2018) and welfare, which necessitates improvements in early disease detection on farm. Despite the simplistic name, bovine respiratory disease (BRD) is not one disease. Rather, BRD is an umbrella term that describes three separate categories of respiratory illnesses: enzootic pneumonia of dairy calves, shipping fever of feedlot cattle, and atypical interstitial pneumonia (Lillie, 1974). In this literature review and throughout the thesis, BRD will refer to enzootic pneumonia, the primary concern in young dairy calves.

Respiratory disease can be classified into clinical and subclinical BRD by combining clinical respiratory scoring and lung ultrasound (Ollivett and Buczinski, 2016). 'Clinical' refers to the presence of visible signs of BRD and includes any combination of the following: depressed attitude, increased respiratory rate, cough, droopy ears, and purulent ocular or nasal discharge (Van Donkersgoed et al., 1993, McGuirk, 2008). Clinical BRD reflects inflammation of the upper airway, the lower airway, or a combination of both (Ollivett and Buczinski, 2016). Subclinical BRD refers to a calf that does not have visible signs of respiratory disease despite the presence of lower airway disease. Any interventions that seek to improve BRD must include lung health, not just clinical signs.

Mortality attributable to BRD has remained relatively unchanged over the last 20 to 30 years (Gorden and Plummer, 2010, USDA, 2010). Prevalence estimates range from 2.5% (Gulliksen et al., 2009) to 39% (Van Donkersgoed et al., 1993). The manner in which we raise calves contributes to the persistently high morbidity and mortality. More farms are housing calves in large groups or indoors (USDA, 2010), which contribute to higher BRD incidence (Svensson and Liberg, 2006, Cobb et al., 2014). To address the high morbidity and mortality associated with BRD, some researchers and veterinarians advocated for feeding calves more

milk (Appleby et al., 2001), vaccinating calves for respiratory pathogens (Poulsen and McGuirk, 2009), and using and developing different classes of antibiotics (Berge et al., 2005, McGuirk, 2008). However, despite these management changes, little progress has been made in reducing morbidity and morality due to BRD. This might be because the methods researchers, producers, and veterinarians have used to detect BRD do not measure overall lung health. Clinical signs are transient (White et al., 2012) and have discrepancies among observers (Buczinski et al., 2016). Furthermore, producers fail to identify a large proportion of calves that are sick with BRD (Sivula et al., 1996, Cramer et al., 2016). These challenges are compounded by the presence of subclinical BRD (Ollivett and Buczinski, 2016), which is nearly impossible to identify using visual observations. As such, a large proportion of calves with clinical or subclinical BRD likely go undiagnosed, leading to the misclassification of calves on farm or in research studies. Furthermore, research that examined BRD interventions detected BRD using clinical signs, which are poorly correlated with lung consolidation (Buczinski et al., 2014), leading to management recommendations or interventions (e.g., vaccines or antibiotics) that might not improve lung health.

There is a need to study management practices or interventions that improve overall lung health, therefore leading to a true improvement in respiratory health. This can be accomplished using lung ultrasound, which measures lung health, combined with the Wisconsin calf health scoring chart, which captures the clinical picture of the calf. These are currently the most sensitive tools available to assess lung health (Buczinski et al., 2014) and can be easily performed calf-side. This literature review and subsequent chapters will focus on our current knowledge of BRD, identify gaps in our understanding of BRD, and use highly sensitive BRD diagnostic tools to validate on farm tools for disease detection.

1.2 IMPACT OF BRD

Long-term effects of BRD

The effects of clinical BRD in young calves can last into adulthood (e.g., Stanton et al., 2012; Dunn et al., 2018). A study in Ontario found that calves treated for clinical pneumonia in the first three months of life were 2.5 times more likely to die before their first calving compared to calves not treated for clinical pneumonia in the first 3 months of life (Waltner-Toews et al., 1986). Stanton et al. (2012) found that calves identified with clinical BRD between 8 to 10 weeks of age were less likely to survive to first calving, and if they did survive to calving, were more likely to calve with greater calving difficulty compared to unaffected herdmates. Heifers identified with four or more clinical BRD cases between birth and first calving were half as likely to complete the first lactation compared to heifers not identified with clinical BRD (Bach, 2011).

Subclinical BRD can also affect future performance. Adams et al. (2016) observed that calves with extensive lung consolidation (≥ 6 cm in one or more areas) had a greater risk of dying or being culled compared to calves with less severe or no lung consolidation. Additionally, calves identified with ≥ 3 cm of lung consolidation during the first 56 days of life had a 525 kg decrease in first-lactation 305-day milk production (Dunn et al., 2018). Researchers in a recent New York study found that calves identified with lung consolidation (any amount of consolidation) at weaning (60 d of age) were almost 5 times more likely to be removed from the herd between weaning and the age of first insemination (350 d of age) compared to calves without lung consolidation at weaning (Teixeira et al., 2017). Additionally, heifers with lung consolidation at weaning were 0.7 times less likely to become pregnant compared to calves without consolidation at weaning (Teixeira et al., 2017). The long-term

effects of BRD incentivize the need to reduce BRD prevalence.

Impact of BRD on calf growth

BRD has variable impacts on calf growth, which is of particular concern to dairy producers because of long-term impacts of higher average daily gain (**ADG**) during the preweaned period (Soberon et al., 2012). Donovan et al. (1998) performed a prospective cohort study and reported that the number of days treated for producer-identified pneumonia before 6 months of age slightly decreased ADG during 6 - 14 months of life and pneumonia accounted for a 0.002 kg per d reduction in growth; this accounts for a 0.48 kg difference in weight gain, which is not biologically significant. In a New York study, pneumonia that was identified by veterinarians was associated with a 0.07 kg/d decrease in ADG during the first month of life; in the 3rd month of life, each additional week of diagnosed pneumonia reduced ADG by 0.8 kg/d (Virtala et al., 1996).

In a randomized clinical trial that utilized the Wisconsin calf clinical health scoring system, no difference in ADG was observed between calves that were positive on the clinical scoring system and calves that were negative (Heins et al., 2014). However, the authors suggested that a high treatment incidence on some of the study herds might have indicated a high presence of subclinical disease. The misclassification of subclinical calves might have contributed to similar ADG between calves clinically sick and clinically healthy.

Bach et al., (2011) observed a tendency for decreased ADG in calves that were identified with 3 or more BRD cases. Stanton et al. (2010) reported that BRD identified by farm staff during the first 8 weeks of life reduced body weight when calves exited the weaning barn by 2.9 kg and BRD from 8 - 14 weeks of life reduced body weight when calves exited the weaning barn by 7.9 kg. In a subset of calves from the 2010 study, calves treated for BRD had a reduction of

0.17 kg, 0.07 kg, and 0.04 kg compared to calves not treated for BRD between 2 and 3 months, 3 and 6 months, and 6 and 9 months, respectively (Stanton et al., 2012).

To date, research that examined the effect of BRD on calf growth used clinical signs to detect BRD (e.g., Bach et al., 2011, Stanton et al., 2012), which has poor accuracy for detecting subclinical BRD (Buczinski et al., 2014). Therefore, the impact of subclinical BRD on growth has not been well studied.

Impact of BRD on producers

The financial impact of BRD is difficult to calculate. Costs include treatment expenses, labor, replacement of removed animals, as well as future production losses (e.g., Dunn et al., 2018). A Dutch computer-based economic model, accounting for treatment costs and costs associated with increased mortality and culling, and reduced growth, fertility and milk production, estimated that BRD in calves less than three months of age costs between $16 and $49 USD (18.4-57.1 Euro), with a median of $38 USD (31 Euro) per calf (Van der Fels-Klerx et al., 2001). Studies in the United States estimate BRD costs range from ~$10 to $16 per calf (reviewed by Gorden and Plummer, 2010), however these estimates do not include the lower productivity associated with BRD and likely underestimate the true cost of BRD.

In addition to the financial losses associated with BRD, there is also a potential psychological cost to the people caring for sick calves. Employees on dairy farms with higher disease incidence rates reported experiencing more psychosocial symptoms (irritation, fatigue, insomnia, headache, nervousness, and abdominal pain) compared to employees on farms with lower disease incidence rates (Kolstrup and Hultgren, 2011). Thus, if we improve calf health, we have the potential to also improve the working conditions for farm employees. It is recognized in other industries that improving working conditions for employees leads to less

turnover, and greater worker productivity (Cohen et al., 2009). In turn, retaining highly motivated farm employees might improve the level of care calves receive.

1.3 RISK FACTORS FOR BRD

Failure of passive transfer and plane of nutrition can affect BRD susceptibility. Failure of passive transfer, which occurs when adequate absorption of maternal antibodies across the small intestine does not occur (Godden, 2008), can increase the risk of BRD later in life (Windeyer et al., 2014). In addition to successful transfer of antibodies, plane of nutrition may reduce the risk of BRD. In a challenge study using *Cryptosporidium* parvum, researchers found that calves on a higher plane of nutrition maintained hydration and had faster resolution of diarrhea compared to calves on a low plane of nutrition (Ollivett et al., 2012). There is no research regarding plane of nutrition and the risk of BRD. However, both BRD and gastrointestinal disease involve immune responses (Ackermann et al., 2010, Blanchard, 2012) and improved nutrition is thought to improve calf immunity (Bach, 2012). Therefore, we and infer we can infer that an increased plane of nutrition would be beneficial for reduced susceptibility of BRD

Ventilation, ability of the calf to nest, and group size can all impact the likelihood of calf BRD. Poorly ventilated housing is recognized as a risk factor for BRD (Callan and Garry, 2002). Furthermore, a large cross-sectional study in naturally ventilated barns found increased pen bacterial counts, lack of a barrier between individual calves, and an inability of the calf to nest (measured using a nesting score; inability to nest determined if calf's legs were fully visible when laying down) in deep bedding were associated with an increased prevalence of BRD (Lago et al., 2006). Calves housed in groups of 6 to 9 animals had lower incidence of respiratory disease compared to calves housed in groups of 12 to 18 (Svensson and Liberg, 2006). A Danish

study observed an increased risk of BRD was associated with the following: previous calf history of diarrhea compared to no diarrhea (hazard ratio = 3.9), an age range of more than 8 weeks between calves housed together in the same group pen compared to having pen mare of a more similar age, and prolonged (> 24 hours) separation from the dam compared to earlier separatation (Gulliksen et al., 2009).

1.4 DEFINING BRD

Our knowledge of BRD impacts and risk factors are based on a wide range of definitions (Ollivett, 2014). Variations in these definitions might contribute to discrepancies in results among studies. For example, the definition for clinician-diagnosed pneumonia in Virtala et al. (1996) included inducible cough, abnormal auscultation of respiratory tract, evidence of body temperature >39.5°C, depression, and lack of involvement of other body systems that could contribute to a fever. Moreover, Borderas et al. (2009) described the health examinations performed by a veterinarian, which included rectal temperature, respiratory and cardiac rates and sounds, presence of diarrhea, presence of nasal and ocular discharges, general state of the coat, and assessment of dehydration (including tent test and muzzle humidity), however they do not provide the criteria for BRD. In contrast, some studies did not describe the screening methods or the definition for BRD at all (Waltner-Toews et al., 1986, Sivula et al., 1996).

Comparing results and translating research data to practical farm solutions is difficult due to the variability in BRD definitions. Furthermore, using methods of BRD detection with poor accuracy, such as clinical signs or thoracic auscultation, impede our ability to truly understand the impact of BRD on calf performance, economics, calf behavior, and welfare. For example, thoracic auscultation has an estimated sensitivity of 6% (Buczinski et al., 2015); this means that if 100 calves in a study are truly sick with BRD, auscultation will only identify 6 calves, whereas

the remaining 94 calves will be misclassified as healthy. Using diagnostic tools with poor sensitivity makes it difficult to measure the true difference between calves with BRD and unaffected herdmates.

1.5 ETIOLOGY, PATHOPHYSIOLOGY, AND IMMUNOLOGY OF BRD

Overview

Environmental stressors can decrease the efficacy of the calf's immune system, leading to increased BRD susceptibility (Yates, 1982, Taylor et al., 2010). Therefore, it is it easier for ubiquitous bacteria residing in the nasopharynx or inhaled particulate matter to infect the respiratory tract (Yates, 1982, Hodgins et al., 2002, Moeller Jr et al., 2013).

Etiology

Common viral pathogens associated with BRD include bovine herpesvirus 1, bovine parainfluenza 3, and bovine respiratory syncytial virus (Hodgins et al., 2002). Common bacterial respiratory pathogens include *Pasteurella multocida*, *Mycoplasma bovis, Trueperella pyogenes, Histophilus somni,* and *Mannheimia haemolytica* (Hodgins et al., 2002, Welsh et al., 2004, Gagea et al., 2006). Bacterial pneumonia is the primary concern in young dairy calves (Poulsen and McGuirk, 2009).

Immunology of BRD

The bovine respiratory tract contains several innate immune defenses to prevent infection. Commensal microflora in the upper respiratory tract occupy receptor sites resulting in reduced colonization by pathogens, hairs along the external nares act as barriers to inhalation of large particles, and the stratified squamous epithelium along the anterior nares is resistant to microbial adhesions (Ackermann et al., 2010). The mucociliary apparatus in the bovine respiratory tract is lined with an air-surface liquid which acts as a protective layer against inhaled

particulate matter, aerosols, vapors, and microbial pathogens (Ackermann et al., 2010). Molecules within the air-surface liquid mediate antimicrobial and pro- and anti- inflammatory activity and immunomodulation (Ackermann et al., 2010).

Despite the innate immune defenses of the upper respiratory tract, sometime pathogens are inhaled into the lungs. Alveolar macrophages respond initially to engulf inhaled pathogens and release cytokines to stimulate an immune response (Ackermann et al., 2010). Pathogen-associated molecular patterns (**PAMPs**) are present on microbial agents and are recognized by the host immune system. Gram-negative bacteria, such as *P. multocida* and *M. haemolytica*, contain LPS as their PAMP (Ackermann et al., 2010). All bacterial respiratory pathogens produce some type of PAMP, which are, in turn, recognized by the lung epithelia, alveolar macrophages, macrophages within small capillaries of the alveolar walls, and neutrophils (Ackerman et al., 2010). The lung also contains numerous receptors that recognize PAMPs, including toll-like receptors, on the cell surface of respiratory epithelia. Toll-like receptors initiate a host immune response (Ackerman et al., 2010).

During an acute inflammatory response, inflammatory mediators activate vascular endothelial cells, which allows immune cells, such as neutrophils, complement, hydrolytic enzymes, IgG, IgM, IgE, and acute phase proteins, to enter the alveolar lumen (Hodgins et al., 2002, Ackermann et al., 2010). This inflammatory response results in local tissue damage (Hodgins et al., 2002), sometimes leading to the development of lung consolidation. Lung consolidation is visible on ultrasound within 2 hours after inoculation with 10^9 cfu/mL of *M. haemolytica*, with severe consolidation visible 6 hours post-inoculation (Ollivett, 2014). Another study observed ultrasonographic changes in lung consolidation 24 hours after inoculation with 10^9 cfu/mL of *P. multocida* (Reinhold et al., 2002).

1.6 ROLE OF SEROTONIN IN PULMONARY FUNCTION, THE IMMUNE SYSTEM, AND BRD

Serotonin and pulmonary function

Serotonin is a monoamine derived from L-tryptophan (Wang et al., 2002) and outside the brain (non-neuronal), serotonin is involved with the cardiovascular, pulmonary, gastrointestinal, central nervous, and genitourinary systems in humans (Berger et al., 2009). Serotonin helps regulate breathing through effects on the brainstem (Berger et al., 2009). Serotonin acts on the baroreceptors in the carotid artery, which in turn send feedback to the brain medulla to regulate breathing (Hilaire et al., 2010).

Serotonin and the immune system

In addition to serotonin's role in pulmonary function, serotonin has important roles in the immune system (reviewed by Ahern, 2011). Mast cells express the serotonin transporter and are able to accumulate serotonin, which is released in response to inflammation signaled through complement, platelet activating factor, or IgE complexes (Matsuda et al., 1997, Gordon and Barnes, 2003). Dendritic cells take up serotonin from their microenvironment and from activated T-cells (O'Connell et al., 2006). Furthermore, dendritic cells modulate T-cell proliferation and differentiation through the uptake of serotonin at inflammatory sites in the body and shuttle serotonin to naïve T-cells (O'Connell et al., 2006). The expression of serotonin transporter is increased in B- lymphocytes upon activation (Meredith et al., 2005). In summary, mast cells (Kushnir-Sukhov et al., 2008), natural killer cells (Evans et al., 2008), macrophages (Nakamura et al., 2008, Mikulski et al., 2010), dendritic cells (Idzko et al., 2004, Müller et al., 2009), T-cells (Yin et al., 2006, Nakamura et al., 2008), and B-cells (Ek et al., 2002, Rinaldi et al., 2010) contain serotonin receptors (reviewed by Ahern, 2011), thus demonstrating a presumptive role of serotonin in the modulation of the immune system.

In calves specifically, supplementation of colostrum and milk with 5-hydroxy-L-tryptophan, which is the precursor to serotonin, increased blood mRNA abundance of haptoglobin, IL-1β, cyclooxygenase 2, chemokine C-C, serum amyloid A, and NF-kB (Hernández-Castellano et al., 2018). Hernández-Castellano et al. (2018) summarized the relationship between immune factors and serotonin. Serotonin stimulates macrophage secretion of IL-1β through activation of NF-kB, which in turn enhances the phagocytic activity of those macrophages (Freire-Garabal et al., 2003, Ghia et al., 2009). Activated macrophages secrete cyclooxygenase 2, which is an enzyme involved in prostaglandin synthesis which generates the inflammatory response (Smith et al., 2000, Ricciotti and FitzGerald, 2011). Macrophages also secrete IL-1β, which stimulates the release of haptoglobin and serum amyloid A from hepatocytes (Moshage, 1997) and chemokine C-C from endothelial cells (Selvan et al., 1998). Haptoglobin and serum amyloid A are acute phase proteins which help limit the availability of iron for bacterial growth and attract other immune cells to the inflammation cite and limit (Hernández-Castellano et al., 2014, Sikora et al., 2015). In summary, haptoglobin, serum amyloid A, and chemokine C-C are inflammatory chemoattractants for monocytes, lymphocytes, and granulocytes (Donlon et al., 1990, Maffei et al., 2009, Gouwy et al., 2015). Although supplementation of the precursor to serotonin increases immune factors, it is unknown if naturally-occurring levels of serum serotonin are associated with disease.

Serotonin and BRD

The relationship between serotonin and BRD is complex and not well understood. Serotonin is derived from tryptophan, which has been used to induce bovine atypical interstitial pneumonia (Schiefer et al., 1974) (Çevikkalp et al., 2016). Two metabolic pathways can breakdown tryptophan; tryptophan can be converted to kynurenine through the indoleamine 2,3-

dioxygenase (**IDO**) pathway or tryptophan can be converted to serotonin via the tryptophan hydroxylase pathway (Gál and Sherman, 1980). Tryptophan is required for the growth of microorganisms and IDO is thought to inhibit microbial growth by converting tryptophan to kynurenine (Meier et al., 2017). Recently, researchers identified IDO as an inhibitor of T-cell proliferation (Yeung et al., 2015). Furthermore, tryptophan, IDO, and kynurenine were implicated in community-acquired pneumonia in humans (Meier et al., 2017); researchers observed increased activation of IDO, leading to a shift in metabolism from tryptophan to kynurenine in patients with pneumonia. In the same study, tryptophan and serotonin were negatively correlated with the severity of pneumonia, suggesting that metabolism of tryptophan is shifted away from serotonin and converted into kynurenine.

Plasma serotonin concentrations remained unaltered in calves inoculated with bovine herpes virus-1 (3×10^7 tissue culture infectious dose/ nostril), followed 3 days later by challenge with *Pasteurella hemolytica* (15×10^9 cfu intratracheally; (Emau et al., 1987)). Hare et al. (1996) experimentally infected feedlot calves with *P. haemolytica* and found reduced rectal temperature in calves given metrenperone (a serotonin receptor antagonist) compared to calves given a placebo; however, there was no effect of metrenperone on lung consolidation observed at necropsy. These studies suggest that the metabolism of tryptophan plays an important role in the modulation of the immune response and potentially BRD. An increased understanding of the relationship among tryptophan, serotonin, and BRD, may lead to the development of management interventions that can improve calf health. Furthermore, the investigation of a biomarker that is associated with BRD would be useful to target management strategies on an individual calf basis. However, previous studies have only investigated BRD in a challenge model, rather than in a large-scale study with naturally occurring disease.

1.7 SICKNESS BEHAVIOR

Overview

Sickness behavior is a term used to describe the behavioral changes that accompany illness and centers around helps conserve heat and energy in order to facilitate the febrile response to infection (Hart, 1988). Sickness behaviors, which are common across species and often non-specific for disease, include depression, anorexia, reduced water intake, decreased grooming, and decreased exploratory behavior (Hart, 1988, Haba et al., 2012). Sickness behavior is thought to be an adaptive response to illness and evolved to increase an animal's potential for survival (Hart, 1988). The expression of sickness behaviors is thought to enhance the ability of the immune system to fight infection and inhibit pathogen proliferation (Hart, 1988, Johnson, 2002).

Mechanism of sickness behavior

The expression of sickness behaviors is the result of a highly coordinated response to illness, arising from the immune cells. Macrophages express toll-like receptors, which recognize PAMPs on infectious pathogens (Konsman et al., 2002). In response to pathogen recognition, macrophages release pro-inflammatory cytokines, such as interleukin (IL)-1, IL- 6, and tumor necrosis factor- α (reviewed byJohnson, 2002). These pro-inflammatory cytokines induce fever by activating the release of cyclooxygenase-2 (COX-2) and prostaglandin- E2 synthase from the endothelial cells of brain vessels (Konsman et al., 2002). COX-2 and prostaglandin-E2 synthase diffuse into the brain and act on neuronal receptors in the brainstem and hypothalamus that are sensitive to prostaglandin-E2. The action of prostaglandin-E2 inhibits the firing rate of warm-sensitive neurons in the hypothalamus (Kluger, 1991, Johnson, 2002). This increases the hypothalamic set point such that animals will now try to maintain a higher body temperature than normal. In order to achieve this higher (febrile) body temperature, mammals begin to increase

heat production and increase heat conservation (Johnson, 2002), through behaviors such as increased lying time (e.g., Borderas et al., 2008).

Pro-inflammatory cytokines, especially IL-1β, facilitate anorexia by reducing animals' motivation for food. These cytokines have receptors throughout the endocrine system and in the brain; the receptors help convey messages from the immune system to the endocrine system and CNS to induce anorexia (Johnson, 1998). Macrophages produce IL-1, which mediates the depressed attitude that often accompanies illness through activation of the limbic structures in the brain directly or indirectly via a neuronal pathway (Konsman et al., 2002). The depression that accompanies illness is induced via the vagus nerve. The vagus nerve contains macrophages that produce IL-1 in the periphery in response to infection. This nerve sends an afferent signal to the brainstem, hypothalamus, and limbic system, which are responsible for regulating behavior (Konsman et al., 2002).

Sickness behavior as a motivational state

Aubert (1999) suggested that sickness behavior is motivational and that animals will re-prioritize the expression of sickness behaviors, depending on external factors and the consequences that accompany them. This motivational state has been demonstrated in species other than cattle; external factors that change behavior include breeding status (Owen-Ashley and Wingfield, 2006), environmental temperature (Aubert et al., 1997), social environment (Lopes et al., 2012), dominance status (Cohn and Sa- Rocha (2006), and gender (Avitsur et al., 1997). In calves specifically, milk allowance can change the expression of sickness behavior. Sick calves fed high amounts of milk had decreased milk intake, visits to the feeders, and duration of visits for seven days after showing clinical signs of disease compared to healthy calves on the same milk allowance (Borderas et al., 2009). In contrast, calves fed a low milk

allowance decreased only the duration of visits to the feeder (Borderas et al., 2009).

Sickness behavior in calves

In comparison to cows or other species, there is very little research regarding sickness behavior in dairy calves. Over the past 10 years, most of the literature regarding sickness behavior in calves has centered around changes in feeding behavior on automated milk feeders. Svensson and Jensen (2007) reported that the number of unrewarded visits to the milk feeder decreased for diseased calves, but milk consumption was not affected when calves were fed 5.6 to 8.1L of milk. In a series of experiments, Borderas et al. (2009) found that illness was associated with changes in feeding behavior, but these changes differed between calves offered high and low amounts of milk. Automated feeding behaviors associated with illness are described in detail under the 'detection' section of this literature review.

Dairy calves challenged with lipopolysaccharide (LPS) showed a decreased duration of rumination and hay eating, a decreased frequency of self-grooming, and an increased duration of lying inactive compared to control calves challenged with saline (Borderas et al., 2008). Calves challenged with LPS also had increased duration of lying inactive, and an increased bout frequency and mean bout duration of standing inactive (Borderas et al., 2008). Fever, but not clinical respiratory score or lung consolidation, was associated with total lying time in preweaned dairy calves (Ollivett, 2014).

Male Holstein calves aged 5-9 weeks were inoculated with *Mycoplasma* bovis; calves with severe clinical disease spent less time at the fed bunk and more time in the shelter compared to calves with less severe disease (White et al., 2012). Additionally, distance traveled was negatively associated with extent of lung consolidation identified at necropsy.

Dairy calves with clinical signs of bovine respiratory disease (BRD) or fever were less likely to approach a novel object or stationary human compared to healthy calves (Cramer and Stanton, 2015). Calves with clinical signs of BRD were more likely to score positive on a behavior score that included the following behaviors: abnormal posture, isolation from the group, and not approaching a stationary human (Cramer et al., 2016).

1.8 IMPACT OF BRD ON CALF WELFARE

Dr. David Fraser proposed three ethical frameworks for animal welfare: functional, affective state, and natural behavior (Fraser et al., 1997). In the functional perspective, health, growth, and production are emphasized. The affective states viewpoint highlights the prevention of pain and suffering of animals. Finally, the natural behavior view focuses on the ability of animals live in a manner that corresponds to their behavioral adaptations. The World Health Organization also emphasizes the three aspects of animal welfare and defines good animal welfare as: "healthy, comfortable, well nourished, safe, able to express innate behavior, and . . . is not suffering from unpleasant states such as pain, fear, and distress" (OIE, 2016). When all three ethical frameworks are considered and in particular when the viewpoints overlap, we have a more holistic understanding of animal welfare. We can use the three ethical frameworks to discuss the impact of BRD on dairy calf welfare.

Good health is central to good welfare (Mellor and Stafford, 2004, von Keyserlingk et al., 2009). The functional impacts associated with BRD have received the most research attention and includes reduced growth (e.g., Stanton et al., 2012) and long term effects such as decreased milk production in adulthood (Dunn et al., 2018). Production factors (e.g. growth and milk production later in life) are important to the producer, and it is often assumed that poor production indicates poor welfare and high production indicates good welfare (von Keyserlingk

et al., 2009). However, poor growth in calves might be due to factors (such as genetic) that are welfare neutral, whereas pushes for higher production in cattle can put extra demands on the animal, which may lead to higher disease incidence (Fleischer et al., 2001, von Keyserlingk et al., 2009). Therefore, high production does not equate to good welfare, nor is poor production always indicative of poor welfare (von Keyserlingk et al., 2009).

The affective state, or how the animal feels, is fundamental to animal welfare (von Keyserlingk et al., 2009). Experiencing pain, fear, or distress can negatively affect welfare (Fraser et al., 1997). Substance P, a neuropeptide involved with nociception, was found to be higher in calves inoculated with *M. haemolytica* compared to control calves (Theurer et al., 2013). Human patients report chest pain during pneumonia (Melbye et al., 1992) and taken together with the Substance P results reported by Theurer et al. (2013) suggest that pneumonia may be a painful condition in calves. Although most research in affective state has focused on the avoidance of pain, the ability of the animal to experience pleasurable activities is also important. For example, play behavior has been suggested to reflect a young animal's physical and emotional welfare in nature (Held and Špinka, 2011). Although the relationship between play behavior and BRD has not been investigated specifically, play behavior was not observed in lambs after a painful procedure (Thornton and Waterman-Pearson, 2002). More research is needed to determine how BRD impacts the affective state of the calf so that interventions, such as pain relief, can be provided during disease to minimize the negative impact on welfare.

The impact of BRD on natural behavior or natural living is less straightforward. The expression of sickness behavior could be considered natural; after all, this behavioral response to illness has evolved over time and exists in a variety of species (Hart, 1988). However, BRD may impede the ability of the calf to perform behaviors that are important to healthy calves. Previous

work indicates that calves are highly motivated to obtain full social contact with another calf (Holm et al., 2002), but self-isolation has been reported in calves with BRD (Cramer et al., 2016). Play behavior, as mentioned previously, is thought to contribute positively to animal welfare and some suggest that play behavior does not occur during illness (Held and Špinka, 2011). Another way to consider the effect of BRD on natural living is the calf's ability to express sickness behaviors. During illness, sickness behaviors might be highly important to the calf. However, most pens on dairy farms are designed for healthy animals (Millman, 2007) and may not be conducive to the expression of sickness behavior.

Although there is little research investigating the impact of BRD on animal welfare, existing works suggests that BRD is detrimental to calf welfare. The primary focus of calf management should be BRD prevention. However, more research is needed to determine the best ways to care for calves with BRD so that the negative welfare implications can be mitigated as much as possible.

1.9 DETECTION OF BRD

Early detection and therefore early intervention may help alleviate some of the negative consequences of BRD, particularly the impacts on calf welfare. However, accurate and early detection of BRD is difficult due to the inconsistent clinical presentations of BRD (Apley, 2006). On-farm personnel are primarily responsible for the diagnosis and treatment decisions regarding calves (Gordon and Plummer, 2010). Unfortunately, producers have been shown to only identify 56% (Sivula et al., 1996), 41% (Buczinski et al., 2014), or 25% (Cramer et al., 2016) of ill calves, depending on the study. This presents a challenge, as late detection of illness can reduce treatment success and increase the rate of recurrence (McGuirk, 2008). Therefore, there is a need to investigate detection methods that can identify BRD accurately, early, and in a manner

that is practical for daily use on-farm.

Clinical scoring system

A well-known clinical scoring system is the Wisconsin Calf Respiratory Score (**CRS**), which assigns scores from 0 (normal) to 3 (severely abnormal) for each of the following categories: nose, eyes, ears, cough, and rectal temperature (McGuirk, 2008). Treatment is recommended for a calf with 2 categories with a score ≥ 2, which is based on unpublished comparison between the clinical respiratory score and bronchoalveolar lavage fluid culture and cytology (McGuirk, 2008). This score was designed to facilitate early disease detection on farm and to improve upon vague definitions of BRD in previous studies and in farm treatment protocols (McGuirk, 2008).

Using behavior to detect disease

The focus of disease detection in calves has primarily centered on using data collected from automated milk feeders, which automatically collect drinking speed, milk consumption, and number of visits to the feeder. Borderas et al. (2009) observed that sick calves fed a high milk allowance drank 2.6 L/d less and had 2.4 fewer visits per day compared to healthy calves. In calves on a restricted milk allowance, Svensson and Jensen (2007) observed a 25% decrease in the number of unrewarded (without milk) visits in diseased calves compared to unaffected herdmates. A recent study observed that sick calves drank 183 mL/min slower, drank 1.2 L/d less, and had 3.1 fewer unrewarded visits compared to healthy calves (Knauer et al., 2017).

Recently, a few studies have investigated behaviors other than changes in feeding behavior to determine their usefulness for disease detection. Dairy calves with clinical BRD were less likely to approach both a novel object and stationary person on the day of BRD diagnosis (Cramer and Stanton, 2015). Additionally, a score that included 5 behaviors was

developed for use on farms who may not have automated feeder data; dairy calves with BRD were more likely to have an abnormal behavior score compared to calves without BRD (Cramer et al., 2016). In beef heifers, no difference was observed between heifers with and without BRD for time spent at the feed bunk and using a brush prior to BRD onset (Toaff-Rosenstein and Tucker, 2018).

Ultrasound

During bronchopneumonia, the lung is infiltrated with neutrophils and other cellular debris, which displace air from the bronchioles. These pathological changes allow for the pre-mortem identification of pneumonic lung using ultrasound (Blond and Buczinski, 2009). Normal lung is characterized by a bright, highly reflective (hyperechoic) pleural surface with attenuating reverberation artifact (Babkine and Blond, 2009). In contrast, consolidated lung appears similar to liver-like parenchyma with mixed echogenicity. Consolidated lung can be lobular (focal regions of consolidation surrounded by normal lung) or lobar (the full thickness of the lung lobe is consolidated).

Recently, lung ultrasound has been validated in dairy calves as a rapid, on-farm diagnostic tool for the identification of the lung consolidation associated with BRD (Buczinski et al., 2013, Buczinski et al., 2014, Ollivett, 2014). Accurately detecting pneumonia and determining the extent of abnormal lung is possible when using lung ultrasound (Ollivett and Buczinski, 2016). A 0 to 5 scoring system for ultrasound has been proposed, based on the amount of lung consolidated (Ollivett and Buczinski, 2016).

Comparison of BRD detection methods

Detection methods for BRD should have high sensitivity so a large proportion of truly sick calves are identified, as well as a moderate specificity to avoid false-positives. When

comparing sensitivity and specificity among studies, it is important to consider the study population, the 'gold-standard' or reference method, and the technique used.

The CRS was poorly correlated with lung consolidation ≥ 1 cm identified using lung ultrasound (Buczinski et al., 2014), suggesting that the CRS is not ideal to identify lung consolidation. However, lung ultrasound as the reference method is imperfect; misclassification can occur if consolidation does not extend to the lung surface, and therefore cannot be observed on ultrasound (Babkine and Blond, 2009). Additionally, although rare, animals with consolidation not due to BRD might be misclassified as positive (Babkine and Blond, 2009). In order to adjust for the pitfalls of lung ultrasound, a recent study used Bayesian estimates to compare the CRS and lung ultrasound (Buczinski et al., 2015). Researchers compared lung ultrasound (cutoff of ≥ 1 cm) and the CRS (cutoff of a score of ≥ 5) and found the CRS had a sensitivity of 62% (95% CI: 48-76) and a specificity of 74% (95%CI: 65-83). Lung ultrasound had a sensitivity of 80% (95% CI: 66-91) and a specificity of 95% (95%CI: 88-98).

An additional aspect to consider with the CRS is the inter-observer agreement, which is important if multiple people are diagnosing illness either on a farm or in a research setting. In a 2016 study, the Kappas for inter-observer agreement were 0.35, 0.06, and 0.13 for CRS cutoffs of ≥ 4, ≥ 5, and ≥ 6, respectively, indicating slight to fair agreement (Buczinski et al., 2016). Although the CRS underperforms lung ultrasound, the CRS might improve the ability of producers to detect disease early and represents a low cost (other than labor) method of disease detection. Furthermore, the examination of fecal consistency and joint and navel palpation can be performed at the same time as the CRS in order to identify non-BRD health issues.

Test characteristics are often not reported for behavioral studies, in part because no research has addressed cutoffs for feeding alarms to indicate BRD. Two behavioral studies

reported sensitivities and specificities, although their reference method was CRS, which is imperfect. The sensitivity and specificity of a novel object approach test to identify BRD were 64% and 43%, respectively (Cramer and Stanton, 2015). The sensitivity and specificity of a stationary human to identify BRD were 68% and 43%, respectively (Cramer and Stanton, 2015). A behavior score, which included abnormal lying or standing posture, isolation from the group, approach tests, and lethargy, had a sensitivity of 48% and a specificity of 79% for the identification of calves with BRD (Cramer et al., 2016). One challenge with using behavior to detect disease is that because sickness behavior is motivational (Aubert, 1999), calves might change their behavior depending on external factors, potentially making it more difficult to detect disease.

Lung ultrasound does require training and an ultrasound machine, which can be expensive. However, many veterinarians that perform reproductive work in cattle likely have a machine that will suffice for calf lung ultrasound. Another important aspect to consider is that ultrasound for disease diagnosis is covered under the Veterinary Practice Act in several states, including Wisconsin (Wisconsin Standards of Practice and Unprofessional Conduct for Veterinarians, 1989). Therefore, only veterinarians can perform lung ultrasound when used to diagnose disease. However, it does represent a billable service veterinarians can provide to their clients. Lung ultrasound outperforms the CRS and thoracic auscultation (Buczinski et al., 2014) and the sensitivity and specificity are high (94% and 100%, respectively) for detecting subclinical lung consolidation (Ollivett et al., 2015). Lung ultrasound can be performed rapidly (less than 1 minute per calf) and represents a non-invasive technique to measure BRD (Ollivett and Buczinski, 2016). There is a need to investigate methods of BRD detection using accurate BRD detection tools as the reference methods.

1.10 CONCLUSIONS AND THESIS OBJECTIVES

This persistently high BRD mortality and morbidity may be due to our inability to promptly and accurately diagnose BRD. Our poor detection of BRD is partially driven by the use of diagnostic tools with poor accuracy. Lung ultrasound, combined with clinical respiratory scoring system, provides a highly sensitive method of BRD detection. The use of these scoring systems will allow us to investigate the full effect of BRD, including subclinical BRD, on growth, behavior, and serotonin, which previously have not been investigated using these detection methods.

Therefore, the aim of this thesis was to determine differences in growth, behavior, and serotonin concentrations among novel categories of respiratory disease diagnosed using lung ultrasound.

Specifically, the research objectives were to:

1) Identify a cut point on a previously described six-level ultrasound score at which average daily gain is affected and to determine if there is any added benefit of using a clinical respiratory score
2) Determine if calves with subclinical BRD exhibit different feeding behaviors compared to calves with clinical BRD or without BRD during the 3 d before until the d of diagnosis.
3) Determine if calves with subclinical BRD, clinical BRD, or without BRD exhibit differences in behavioral attitude scores on the day of disease
4) Determine if serum serotonin concentrations in 3-5 d old dairy calves were associated with the development of subclinical BRD, clinical BRD, or without BRD; and describe differences in serotonin concentrations between calves with and without BRD at the time of diagnosis.

1.11 REFERENCES

Ackermann, M. R., R. Derscheid, and J. A. Roth. 2010. Innate immunology of bovine respiratory disease. Veterinary Clinics: Food Animal Practice 26(2):215-228.

Adams, E. and S. Buczinski. 2016. Short communication: Ultrasonographic assessment of lung consolidation postweaning and survival to the first lactation in dairy heifers. J. Dairy Sci. 99(2):1465-1470.

Ahern, G. P. 2011. 5-HT and the immune system. Curr. Opin. Pharm. 11(1):29-33.

Apley, M. 2006. Bovine Respiratory Disease: Pathogenesis, Clinical Signs, and Treatment in Lightweight Calves. Vet. Clin. North Am. Food Anim. Pract. 22(2):399-411.

Appleby, M. C., D. M. Weary, and B. Chua. 2001. Performance and feeding behaviour of calves on ad libitum milk from artificial teats. Appl. Anim. Behav. Sci. 74(3):191-201.

Aubert, A. 1999. Sickness behaviour in animals: a motivational perspective. Neurosci. Biobehav. Rev. 23:1029-1036.

Babkine, M. and L. Blond. 2009. Ultrasonography of the bovine respiratory system and its practical application. Veterinary Clinics: Food Animal Practice 25(3):633-649.

Bach, A. 2011. Associations between several aspects of heifer development and dairy cow survivability to second lactation. J. Dairy Sci. 94:1052-1057.

Bach, A. 2012. Ruminant nutrition symposium: Optimizing performance of the offspring: Nourishing and managing the dam and postnatal calf for optimal lactation, reproduction, and immunity. J. Anim. Sci. 90(6):1835-1845.

Berge, A. C. B., P. Lindeque, D. A. Moore, and W. M. Sischo. 2005. A clinical trial evaluating prophylactic and therapeutic antibiotic use on health and performance of preweaned calves. J. Dairy Sci. 88(6):2166-2177.

Berger, M., J. A. Gray, and B. L. Roth. 2009. The expanded biology of serotonin. Annu. Rev. Med. 60:355-366.

Blanchard, P. C. 2012. Diagnostics of dairy and beef cattle diarrhea. Vet. Clin. North. Am. Food. Anim. Pract. 28(3):443-464.

Blond, L. and S. Buczinski. 2009. Basis of ultrasound imaging and the main artifacts in bovine medicine. Vet. Clin. North Am. Food Anim. Pract. 25(3):553-565.

Borderas, T. F., A. M. d. Passille, and J. Rushen. 2008. Behavior of dairy calves after a low dose of bacterial endotoxin. J. Anim. Sci. 86(11).

Borderas, T. F., J. Rushen, M. A. G. von Keyserlingk, and A. M. B. de Passillé. 2009. Automated measurement of changes in feeding behavior of milk-fed calves associated with illness. J. Dairy Sci. 92:4549-4554.

Buczinski, S., C. Faure, S. Jolivet, and A. Abdallah. 2016. Evaluation of inter-observer agreement when using a clinical respiratory scoring system in pre-weaned dairy calves. N. Z. Vet. J.:1-5.

Buczinski, S., G. Forté, and A. Bélanger. 2013. Short communication: Ultrasonographic assessment of the thorax as a fast technique to assess pulmonary lesions in dairy calves with bovine respiratory disease. J. Dairy Sci. 96(7):4523-4528.

Buczinski, S., G. Forté, D. Francoz, and A. M. Bélanger. 2014. Comparison of thoracic auscultation, clinical score, and ultrasonography as indicators of bovine respiratory disease in preweaned dairy calves. J. Vet. Intern. Med. 28(1):234-242.

Buczinski, S., T. L. Ollivett, and N. Dendukuri. 2015. Bayesian estimation of the accuracy of the calf respiratory scoring chart and ultrasonography for the diagnosis of bovine respiratory disease in pre-weaned dairy calves. Prev. Vet. Med. 119(3):227-231.

Callan, R. J. and F. B. Garry. 2002. Biosecurity and bovine respiratory disease. Vet. Clin. North Am. Food Anim. Pract. 18(1):57-77.

Çevikkalp, S. A., G. B. Löker, M. Yaman, and B. Amoutzopoulos. 2016. A simplified HPLC method for determination of tryptophan in some cereals and legumes. Food Chem. 193:26-29.

Cobb, C. J., B. S. Obeidat, M. D. Sellers, A. R. Pepper-Yowell, and M. A. Ballou. 2014. Group housing of Holstein calves in a poor indoor environment increases respiratory disease but does not influence performance or leukocyte responses. J. Dairy Sci. 97(5):3099-3109.

Cohen, J., D. Stuenkel, and Q. Nguyen. 2009. Providing a healthy work environment for nurses: the influence on retention. J. Nurs. Care Qua. 24(4):308-315.

Cramer, M., T. Ollivett, and A. Stanton. 2016. Associations of behavior-based measurements and clinical disease in preweaned, group-housed dairy calves. J. Dairy Sci. 99(9):7434-7443.

Cramer, M. and A. Stanton. 2015. Associations between health status and the probability of approaching a novel object or stationary human in preweaned group-housed dairy calves. J. Dairy Sci. 98(10):7298-7308.

Donlon, T., A. Krensky, M. R. Wallace, F. S. Collins, M. Lovett, and C. Clayberger. 1990. Localization of a human T-cell-specific gene, RANTES (D17S136E), to chromosome 17q11. 2-q12. Genomics 6(3):548-553.

Donovan, G. A., I. R. Dohoo, D. M. Montgomery, and F. L. Bennett. 1998. Calf and disease factors affecting growth in female Holstein calves in Florida, USA. Prev. Vet. Med. 33(1-4):1-10.

Dunn, T. R., T. L. Ollivett, D. L. Renaud, K. E. Leslie, S. J. LeBlanc, T. F. Duffield, and D. F. Kelton. 2018. The effect of lung consolidation, as determined by ultrasonography, on first-lactation milk production in Holstein dairy calves. J. Dairy Sci.

Ek, S., C.-M. Högerkorp, M. Dictor, M. Ehinger, and C. A. Borrebaeck. 2002. Mantle cell lymphomas express a distinct genetic signature affecting lymphocyte trafficking and growth regulation as compared with subpopulations of normal human B cells. Cancer Res. 62(15):4398-4405.

Emau, P., S. Giri, and M. Bruss. 1987. Viral-bacterial pneumonia in calves: effects on plasma eicosanoids and long chain fatty acids. Int. J. Tiss. React. 9(3):199-214.

Evans, D. L., K. G. Lynch, T. Benton, B. Dubé, D. R. Gettes, N. B. Tustin, J. P. Lai, D. Metzger, and S. D. Douglas. 2008. Selective serotonin reuptake inhibitor and substance P antagonist enhancement of natural killer cell innate immunity in human

immunodeficiency virus/acquired immunodeficiency syndrome. Biol. Psychiatry 63(9):899-905.

Fleischer, P., M. Metzner, M. Beyerbach, M. Hoedemaker, and W. Klee. 2001. The relationship between milk yield and the incidence of some diseases in dairy cows. J. Dairy Sci. 84(9):2025-2035.

Fraser, D., D. M. Weary, E. A. Pajor, and B. N. Milligan. 1997. A scientific conception of animal welfare that reflects ethical concerns. Anim. Welfare 6(3):187-205.

Freire-Garabal, M., M. Nunez, J. Balboa, P. López-Delgado, R. Gallego, T. García-Caballero, M. Fernández-Roel, J. Brenlla, and M. Rey-Méndez. 2003. Serotonin upregulates the activity of phagocytosis through 5-HT1A receptors. Br. J. Pharmacol. 139(2):457-463.

Gagea, M. I., K. G. Bateman, T. van Dreumel, B. J. McEwen, S. Carman, M. Archambault, R. A. Shanahan, and J. L. Caswell. 2006. Diseases and pathogens associated with mortality in Ontario beef feedlots. J. Vet. Diagn. Invest. 18(1):18-28.

Gál, E. M. and A. D. Sherman. 1980. l-Kynurenine Its synthesis and possible regulatory function in brain. Neurochem. Res. 5(3):223-239.

Ghia, J. E., N. Li, H. Wang, M. Collins, Y. Deng, R. T. El–Sharkawy, F. Côté, J. Mallet, and W. I. Khan. 2009. Serotonin has a key role in pathogenesis of experimental colitis. Gastroenterology 137(5):1649-1660.

Godden, S. 2008. Colostrum management for dairy calves. Vet. Clin. North Am. Food Anim. Pract. 24(1):19-39.

Gorden, P. J. and P. Plummer. 2010. Control, Management, and Prevention of Bovine Respiratory Disease in Dairy Calves and Cows. Veterinary Clinics of North America-Food Animal Practice 26(2):243-+.

Gordon, J. and N. M. Barnes. 2003. Lymphocytes transport serotonin and dopamine: agony or ecstasy? Trends Immunol. 24(8):438-443.

Gouwy, M., M. Buck, N. Pörtner, G. Opdenakker, P. Proost, S. Struyf, and J. Damme. 2015. Serum amyloid A chemoattracts immature dendritic cells and indirectly provokes monocyte chemotaxis by induction of cooperating CC and CXC chemokines. Eur. J. Immunol. 45(1):101-112.

Gulliksen, S. M., E. Jor, K. I. Lie, T. Løken, J. Åkerstedt, and O. Østerås. 2009. Respiratory infections in Norwegian dairy calves. J. Dairy Sci. 92(10):5139-5146.

Hare, J., D. Tesarowski, G. Dawe, K. Vlaminck, P. Shewen, and L. Viel. 1996. Effect of 5-hydroxytryptamine type-2 receptor blockade on pulmonary function in calves with experimentally induced Pasteurella haemolytica pneumonia. Am. J. Vet. Res. 57(7):1034-1039.

Hart, B. L. 1988. Biological basis of the behavior of sick animals. Neurosci. Biobehav. Rev. 12(2):123-137.

Heins, B., D. Nydam, A. Woolums, R. Berghaus, and M. Overton. 2014. Comparative efficacy of enrofloxacin and tulathromycin for treatment of preweaning respiratory disease in dairy heifers. J. Dairy Sci. 97(1):372-382.

Held, S. D. and M. Špinka. 2011. Animal play and animal welfare. Anim. Behav. 81(5):891-899.

Hernández-Castellano, L. E., A. M. Almeida, M. Ventosa, A. V. Coelho, N. Castro, and A. Argüello. 2014. The effect of colostrum intake on blood plasma proteome profile in newborn lambs: low abundance proteins. BMC Vet. Res. 10(1):85.

Hernández-Castellano, L. E., R. Özçelik, L. L. Hernandez, and R. M. Bruckmaier. 2018. Supplementation of colostrum and milk with 5-hydroxy-l-tryptophan affects immune factors but not growth performance in newborn calves. J. Dairy Sci. 101(1):794-800.

Hilaire, G., N. Voituron, C. Menuet, R. M. Ichiyama, H. H. Subramanian, and M. Dutschmann. 2010. The role of serotonin in respiratory function and dysfunction. Respir. Physiol. Neurobiol. 174(1-2):76-88.

Hodgins, D. C., J. A. Conlon, and P. E. Shewen. 2002. Respiratory viruses and bacteria in cattle.

Holm, L., M. B. Jensen, and L. L. Jeppesen. 2002. Calves' motivation for access to two different types of social contact measured by operant conditioning. Appl. Anim. Behav. Sci. 79(3):175-194.

Idzko, M., E. Panther, C. Stratz, T. Müller, H. Bayer, G. Zissel, T. Dürk, S. Sorichter, F. Di Virgilio, and M. Geissler. 2004. The serotoninergic receptors of human dendritic cells: identification and coupling to cytokine release. The Journal of Immunology 172(10):6011-6019.

Johnson, R. 1998. Immune and endocrine regulation of food intake in sick animals. Domest. Anim. Endocrinol. 15(5):309-319.

Johnson, R. W. 2002. The concept of sickness behavior: a brief chronological account of four key discoveries. Vet. Immunol. Immunopathol. 87:443-450.

Kluger, M. J. 1991. Fever: role of pyrogens and cryogens. Physiol Rev. 71(1):93-117.

Knauer, W. A., S. M. Godden, A. Dietrich, and R. E. James. 2017. The association between daily average feeding behaviors and morbidity in automatically fed group-housed preweaned dairy calves. J. Dairy Sci. 100(7):5642-5652.

Kolstrup, C. L. and J. Hultgren. 2011. Perceived physical and psychosocial exposure and health symptoms of dairy farm staff and possible associations with dairy cow health. J. Agric. Saf. Health 17(2):111-125.

Konsman, J. P., P. Parnet, and R. Dantzer. 2002. Cytokine-induced sickness behaviour: mechanisms and implications. Trends Neurosci. 25(3):154- 159.

Kushnir-Sukhov, N., E. Brittain, L. Scott, and D. Metcalfe. 2008. Clinical correlates of blood serotonin levels in patients with mastocytosis. Eur. J. Clin. Invest. 38(12):953-958.

Lago, A., S. M. McGuirk, T. B. Bennett, N. B. Cook, and K. V. Nordlund. 2006. Calf respiratory disease and pen microenvironments in naturally ventilated calf barns in winter. J. Dairy Sci. 89:4014-4025.

Lillie, L. 1974. The bovine respiratory disease complex. The Canadian Veterinary Journal 15(9):233.

Maffei, M., M. Funicello, T. Vottari, O. Gamucci, M. Costa, S. Lisi, A. Viegi, O. Ciampi, G. Bardi, and P. Vitti. 2009. The obesity and inflammatory marker haptoglobin attracts

monocytes via interaction with chemokine (CC motif) receptor 2 (CCR2). BMC Biol. 7(1):87.

Matsuda, H., H. Ushio, G. P. Geba, and P. W. Askenase. 1997. Human platelets can initiate T cell-dependent contact sensitivity through local serotonin release mediated by IgE antibodies. The Journal of Immunology 158(6):2891-2897.

McGuirk, S. M. 2008. Disease management of dairy calves and heifers. Vet. Clin. of North Am. Food Anim. Pract. 24(1):139-153.

Meier, M. A., M. Ottiger, A. Vögeli, C. Steuer, L. Bernasconi, R. Thomann, M. Christ-Crain, C. Henzen, C. Hoess, and W. Zimmerli. 2017. Activation of the tryptophan/serotonin pathway is associated with severity and predicts outcomes in pneumonia: results of a long-term cohort study. Clinical Chemistry and Laboratory Medicine (CCLM) 55(7):1060-1069.

Melbye, H., B. Straume, U. Aasebøs, and K. Dale. 1992. Diagnosis of Pneumonia in Adults in General Practice Relative Importance of Typical Symptoms and Abnormal Chest Signs Evaluated Against a Radiographic Reference Standard. Scand. J. Prim. Health Care 10(3):226-233.

Mellor, D. J. and K. J. Stafford. 2004. Animal welfare implications of neonatal mortality and morbidity in farm animals. Vet. J. 168(2):118-133.

Meredith, E. J., M. J. Holder, A. Chamba, A. Challa, A. Drake-Lee, C. M. Bunce, M. T. Drayson, G. Pilkington, R. D. Blakely, and M. J. Dyer. 2005. The serotonin transporter (SLC6A4) is present in B-cell clones of diverse malignant origin: probing a potential anti-tumor target for psychotropics. The FASEB journal 19(9):1187-1189.

Mikulski, Z., Z. Zasłona, L. Cakarova, P. Hartmann, J. Wilhelm, L. H. Tecott, J. Lohmeyer, and W. Kummer. 2010. Serotonin activates murine alveolar macrophages through 5-HT2C receptors. American Journal of Physiology-Lung Cellular and Molecular Physiology 299(2):L272-L280.

Millman, S. T. 2007. Sickness behaviour and its relevance to animal welfare assessment at the group level. Anim. Welfare 16(2):123-125.

Moeller Jr, R. B., J. Adaska, J. Reynolds, and P. C. Blanchard. 2013. Systemic bovine herpesvirus 1 infections in neonatal dairy calves. J. Vet. Diagn. Invest. 25(1):136-141.

Moshage, H. 1997. Cytokines and the hepatic acute phase response. The Journal of pathology 181(3):257-266.

Müller, T., T. Dürk, B. Blumenthal, M. Grimm, S. Cicko, E. Panther, S. Sorichter, Y. Herouy, F. Di Virgilio, and D. Ferrari. 2009. 5-hydroxytryptamine modulates migration, cytokine and chemokine release and T-cell priming capacity of dendritic cells in vitro and in vivo. PLoS One 4(7):e6453.

Nakamura, K., T. Sato, A. Ohashi, H. Tsurui, and H. Hasegawa. 2008. Role of a serotonin precursor in development of gut microvilli. The American journal of pathology 172(2):333-344.

O'Connell, P. J., X. Wang, M. Leon-Ponte, C. Griffiths, S. C. Pingle, and G. P. Ahern. 2006. A novel form of immune signaling revealed by transmission of the inflammatory mediator serotonin between dendritic cells and T cells. Blood 107(3):1010-1017.

OIE. 2016. World Organisation for Animal Health. Introduction to Animal Welfare 2017.

Ollivett, T. 2014. Understanding the diagnosis and risk factors for respiratory disease in dairy calves. in Department of Population Medicine. Vol. PhD. University of Guelph, Guelph, Ontario, Canada.

Ollivett, T., J. Caswell, D. Nydam, T. Duffield, K. Leslie, J. Hewson, and D. Kelton. 2015. Thoracic Ultrasonography and Bronchoalveolar Lavage Fluid Analysis in Holstein Calves with Subclinical Lung Lesions. J. Vet. Intern. Med. 29(6):1728-1734.

Ollivett, T. L. and S. Buczinski. 2016. On-Farm Use of Ultrasonography for Bovine Respiratory Disease. Vet. Clin. North Am. Food Anim. Pract. 32(1):19-35.

Ollivett, T. L., D. V. Nydam, T. C. Linden, D. D. Bowman, and M. E. Van Amburgh. 2012. Effect of nutritional plane on health and performance in dairy calves after experimental infection with Cryptosporidium parvum. J. Am. Vet. Med. Assoc. 241(11):1514-1520.

Poulsen, K. P. and S. M. McGuirk. 2009. Respiratory disease of the bovine neonate. Veterinary Clinics of North America, Food Animal Practice 25(1).

Reinhold, P., B. Rabeling, H. Günther, and D. Schimmel. 2002. Comparative evaluation of ultrasonography and lung function testing with the clinical signs and pathology of calves inoculated experimentally with Pasteurella multocida. The Veterinary record 150(4):109-114.

Ricciotti, E. and G. A. FitzGerald. 2011. Prostaglandins and inflammation. Atertio. Thromb. Vasc. Biol. 31(5):986-1000.

Rinaldi, A., A. M. Chiaravalli, M. Mian, E. Zucca, M. G. Tibiletti, C. Capella, and F. Bertoni. 2010. Serotonin receptor 3A expression in normal and neoplastic B cells. Pathobiology 77(3):129-135.

Schiefer, B., M. Jayasekara, and J. Mills. 1974. Comparison of naturally occurring and tryptophan-induced bovine atypical interstitial pneumonia. Vet. Pathol. 11(4):327-339.

Selvan, R. S., H. B. Kapadia, and J. L. Platt. 1998. Complement-induced expression of chemokine genes in endothelium: regulation by IL-1-dependent and-independent mechanisms. The Journal of Immunology 161(8):4388-4395.

Sikora, M., J. Król, M. Nowak, T. Stefaniak, G. Aubertsson, and R. Kozdrowski. 2015. The usefulness of uterine lavage and acute phase protein levels as a diagnostic tool for subclinical endometritis in Icelandic mares. Acta Vet. Scand. 58(1):50.

Sivula, N. J., T. R. Ames, W. E. Marsh, and R. E. Werdin. 1996. Descriptive epidemiology of morbidity and mortality in Minnesota dairy heifer calves. Prev. Vet. Med. 27:155-171.

Smith, W. L., D. L. DeWitt, and R. M. Garavito. 2000. Cyclooxygenases: structural, cellular, and molecular biology. Annu. Rev. Biochem. 69(1):145-182.

Soberon, F., E. Raffrenato, R. W. Everett, and M. E. v. Amburgh. 2012. Preweaning milk replacer intake and effects on long-term productivity of dairy calves. J. Dairy Sci. 95(2).

Stanton, A. L., D. F. Kelton, S. J. LeBlanc, S. T. Millman, J. Wormuth, R. T. Dingwell, and K. E. Leslie. 2010. The effect of treatment with long-acting antibiotic at postweaning movement on respiratory disease and on growth in commercial dairy calves. J. Dairy Sci. 93(2).

Stanton, A. L., D. F. Kelton, S. J. LeBlanc, J. Wormuth, and K. E. Leslie. 2012. The effect of respiratory disease and a preventative antibiotic treatment on growth, survival, age at first calving, and milk production of dairy heifers. J. Dairy Sci. 95(9):4950-4960.

Svensson, C. and M. B. Jensen. 2007. Short communication: Identification of diseased calves by use of data from automatic milk feeders. J. Dairy Sci. 90(2):994-997.

Svensson, C. and P. Liberg. 2006. The effect of group size on health and growth rate of Swedish dairy calves housed in pens with automatic milk-feeders. Prev. Vet. Med. 73(1):43-53.

Taylor, J. D., R. W. Fulton, T. W. Lehenbauer, D. L. Step, and A. W. Confer. 2010. The epidemiology of bovine respiratory disease: What is the evidence for preventive measures? Can. Vet. J. 51(12):1351-1359.

Teixeira, A., J. McArt, and R. Bicalho. 2017. Thoracic ultrasound assessment of lung consolidation at weaning in Holstein dairy heifers: Reproductive performance and survival. J. Dairy Sci. 100(4):2985-2991.

Theurer, M., D. Anderson, B. White, M. Miesner, D. Mosier, J. Coetzee, J. Lakritz, and D. Amrine. 2013. Effect of Mannheimia haemolytica pneumonia on behavior and physiologic responses of calves during high ambient environmental temperatures. J. Anim. Sci. 91(8):3917-3929.

Thornton, P. D. and A. E. Waterman-Pearson. 2002. Behavioural responses to castration in lambs. Anim. Welfare 11(2):203-212.

Toaff-Rosenstein, R. L. and C. B. Tucker. 2018. The sickness response at and before clinical diagnosis of spontaneous bovine respiratory disease. Appl. Anim. Behav. Sci.

USDA. 2010. Heifer calf health and management practices on U.S. dairy operations, 2007. USDA: APHIS: VS,CEAH, Fort Collins, CO.

Van der Fels-Klerx, H., J. T. Sørensen, A. Jalvingh, and R. Huirne. 2001. An economic model to calculate farm-specific losses due to bovine respiratory disease in dairy heifers. Prev. Vet. Med. 51(1):75-94.

Van Donkersgoed, J., C. S. Ribble, L. Boyer, and H. Townsend. 1993. Epidemiological study of enzootic pneumonia in dairy calves in Saskatchewan. Can. J. Vet. Res. 57(4):247.

Virtala, A. M. K., G. D. Mechor, Y. T. Grohn, and H. N. Erb. 1996. The effect of calfhood diseases on growth of female dairy calves during the first 3 months of life in New York state. J. Dairy Sci. 79(6):1040-1049.

von Keyserlingk, M. A. G., J. Rushen, A. M. de Passille, and D. M. Weary. 2009. The welfare of dairy cattle-Key concepts and the role of science. J. Dairy Sci. 92(9):4101-4111.

Waltner-Toews, D., S. W. Martin, and A. H. Meek. 1986. The effect of early calfhood health status on surviviorship and age at first calving. Can. J. Vet. Res. 50:314-317.

Wang, L., H. Erlandsen, J. Haavik, P. M. Knappskog, and R. C. Stevens. 2002. Three-Dimensional Structure of Human Tryptophan Hydroxylase and Its Implications for the Biosynthesis of the Neurotransmitters Serotonin and Melatonin. Biochemistry 41(42):12569-12574.

Welsh, R. D., L. B. Dye, M. E. Payton, and A. W. Confer. 2004. Isolation and antimicrobial susceptibilities of bacterial pathogens from bovine pneumonia: 1994–2002. J. Vet. Diagn. Invest. 16(5):426-431.

White, B. J., D. E. Anderson, D. G. Renter, R. L. Larson, D. A. Mosier, L. L. Kelly, M. E. Theurer, B. D. Robert, and M. L. Walz. 2012. Clinical, behavioral, and pulmonary changes in calves following inoculation with *Mycoplasma bovis*. Am. J. Vet. Res. 73(4):490-497.

Windeyer, M. C., K. E. Leslie, S. M. Godden, D. C. Hodgins, K. D. Lissemore, and S. J. LeBlanc. 2014. Factors associated with morbidity, mortality, and growth of dairy heifer calves up to 3 months of age. Prev. Vet. Med. 113(2):231-240.

Wisconsin Standards of Practice and Unprofessional Conduct for Veterinarians. 1989. Pages 13-15. United States.

Yates, W. 1982. A review of infectious bovine rhinotracheitis, shipping fever pneumonia and viral-bacterial synergism in respiratory disease of cattle. Can. J. Comp. Med. 46(3):225.

Yeung, A. W., A. C. Terentis, N. J. King, and S. R. Thomas. 2015. Role of indoleamine 2, 3-dioxygenase in health and disease. Clin. Sci. 129(7):601-672.

Yin, J., R. H. Albert, A. P. Tretiakova, and B. A. Jameson. 2006. 5-HT1B receptors play a prominent role in the proliferation of T-lymphocytes. J. Neuroimmunol. 181(1):68-81.

2 CHAPTER 2: GROWTH OF PREWEANED, GROUP HOUSED DAIRY CALVES DIAGNOSED WITH RESPIRATORY DISEASE USING CLINICAL RESPIRATORY SCORING AND THORACIC ULTRASOUND - A COHORT STUDY

M. C. Cramer* and T. L. Ollivett†[1]

*Department of Dairy Science, and

†Department of Medical Sciences, School of Veterinary Medicine, University of Wisconsin, Madison 53706

2.1 ABSTRACT

The objectives of this cohort study were to identify a cut point on a previously described 6 - level ultrasound score (USS6) at which ADG is affected and to determine if there is any added benefit of using a clinical respiratory score. Calves from a commercial herd in Ohio, USA were enrolled at entry to an automated calf feeder barn at 21 ± 6 (mean ± standard deviation) d of age (n = 308). Calves that survived until 50 d (n = 233) were included in the analyses. Twice weekly health exams included a clinical respiratory score (CRS), USS6 (0 – 5, based on lung mass involved), and body weight. For the CRS, the nose, eyes, ears, cough, and rectal temperature were assigned a score (0 - 3) and calves were considered positive (CRS+) when at least 2 areas scored ≥ 2. For analysis, USS6 and CRS status were based on a calf's first BRD event identified during the study period. The first multivariable linear model was fit to determine if USS6 was associated with ADG and controlled for CRS. There was no significant difference among calves with USS6 scores of 2, 3, 4, and 5. Based on this finding, we proposed a simplified 2 - level ultrasound score (USS2; without lung consolidation or with lung consolidation ≥ 1cm^2). A second multivariable model was fit to assess the association between USS2 and ADG; this model controlled for CRS, birth weight category, breed, and cohort. Calves with lung consolidation (n = 169) had lower ADG compared to calves without lung consolidation (n = 64; 0.73 vs. 0.85 kg/d, respectively). Calves that were CRS+ (n = 61) had lower ADG compared to calves that were CRS- (n = 172; 0.74 vs. 0.84 kg/d, respectively). However, CRS did not affect the relationship between USS2 and ADG, but both CRS and USS2 are necessary to explain variation in ADG. We simplified USS6 and proposed a USS2, based on how lung consolidation affected ADG. A simplified 2 - level ultrasound score may be more practical for veterinarians to identify calves that may be at risk for poor growth. The effect on ADG was similar between calves with lung consolidation and calves identified as CRS+.

Therefore, both thoracic ultrasonography and CRS should be used to identify calves with all types of respiratory disease that affect growth. However, this study represents calves in group housing from 21 to 50 d of age on 1 farm with high disease incidence. We encourage studies that investigate the effects of lung consolidation and CRS on ADG in different management systems.

Key words: automated calf feeder, average daily gain, bovine, pneumonia,

2.2 INTRODUCTION

As reported by dairy producers, bovine respiratory disease (**BRD**) accounts for approximately 23% of heifer deaths during the preweaning period (USDA, 2010). Aside from mortality, other short - term consequences of preweaning BRD include increased treatment costs (Van Donkersgoed et al., 2007) and reduced growth (e.g. Virtala et al., 1996). The effects of preweaning BRD can last into adulthood and include decreased survival to first calving (Adams and Buczinski, 2016, Teixeira et al., 2017) and decreased milk production (Dunn et al., 2017).

Early life growth is of interest to dairy producers because of the long - term associations between preweaning growth and first lactation milk production (Moallem et al., 2010, Soberon et al., 2012). In a New York study, BRD was associated with a 0.07 kg/d reduction in ADG during the first month of life (Virtala et al., 1996). Another study reported that calves treated for BRD had lower ADG between 2 and 3 months, 3 and 6 months, and 6 and 9 months of age (0.17 kg/d, 0.07 kg/d, and 0.04 kg/d respectively) compared to healthy calves (Stanton et al., 2012). In contrast, a recent study that used the Wisconsin calf clinical respiratory scoring system (**CRS**; McGuirk, 2008) to detect sick calves, did not find an association between CRS status and ADG (Heins et al., 2014, Ollivett et al., 2014).

The variable impact of BRD on calf growth might be due to different BRD definitions, which in general are highly variable (Ollivett, 2014). Furthermore, a limitation of studies that rely on producer - based diagnoses and CRS is that both producers and the CRS lack sensitivity for the identification of pneumonia (Sivula et al., 1996, Buczinski et al., 2015). Recently, thoracic ultrasonography (**TUS**) was validated as a rapid, on - farm diagnostic tool for the identification of lung consolidation associated with BRD in preweaned dairy calves (Buczinski et al., 2013, Buczinski et al., 2014, Ollivett, 2014). Thoracic ultrasonography can accurately detect pneumonia and determine the extent of abnormal lung (Ollivett and Buczinski, 2016). Buczinski

et al. (2015) reported Bayesian estimates for TUS sensitivity (79.4%, 95% CI: 66.4 - 90.9) and specificity (93.9%; 95%CI: 88.0 - 97.6), which outperform the CRS. The within herd prevalence of subclinical pneumonia can range from 23% to 67% (Ollivett and Buczinski, 2016), which represents a large source of unidentified disease. Thoracic ultrasonography, which can detect pneumonia regardless of clinical status (Buczinski et al., 2014), might improve our ability to accurately assess the impact of BRD on calf growth and assist with early management decisions.

Early disease detection can be achieved through systematic disease detection procedures, which include the CRS and TUS. However, a potential concern with performing systematic scoring on all calves is that it is time consuming and labor intensive. Because TUS has superior sensitivity and specificity compared to CRS (Buczinski et al., 2015), the use of only TUS may be more expedient and accurate for the detection of BRD. A 6 - level thoracic ultrasound score (**USS6**) ranging from 0 to 5 based on the mass of lung tissue involved has been proposed (Jericho, 1982; Ollivett and Buczinski, 2016). Thoracic ultrasound has a sensitivity and specificity of 94% and 100%, respectively, for the identification of subclinical lung consolidation (Ollivett et al., 2015). However, it is unknown which levels of the USS6 affect calf performance and a simplified version may be more practical for veterinarians interested in identifying calves who may have poor ADG. Therefore, the objectives of this cohort study were to identify a cut point on a previously described 6 - level ultrasound score at which ADG is affected and to determine if there is any added benefit of using a clinical respiratory score.

2.3 MATERIALS AND METHODS

Data collection for this cohort study took place in Ohio, USA between February and August 2016 on a dairy cattle facility that raised heifer and bull calves and freshened only primiparous heifers. The Institutional Animal Care and Use Committee at the University of Wisconsin - Madison (A005049 - A03) approved this study.

Animal management prior to study enrollment

Calves were separated from the dam within 30 minutes of birth and received 4L of pasteurized maternal colostrum within 3 hours of birth or colostrum replacer when adequate quality colostrum (BRIX ≥ 22%) was unavailable. Farm staff recorded birth weight and calving ease score for all calves. The calving ease score ranged from 1 - 5 (1 = no assistance, 2 = minor assistance, 3 = hard pull, 4 = mechanical assistance and hard pull, 5 = surgical delivery). Calves were housed in 1 of 2 identical barns that contained straw bedded individual pens for approximately 3 weeks prior to entering an automated calf feeder barn (**ACFB**). Calves were fed 5L whole milk or milk replacer twice per d by bucket while housed individually.

During the pre - enrollment phase of the study, calves were monitored daily and all treatments were recorded by farm staff on paper treatment records. Farm staff defined a diarrhea event prior to enrollment as extremely watery feces during at least 2 consecutive feedings. Farm staff defined a respiratory event prior to enrollment as a calf with labored breathing, cough, and droopy ears. During the pre - enrollment phase of the study, research staff collected jugular venous blood from 3 – 5 d old calves using a 20 g x 1 inch vacutainer needle and glass tube without anticoagulant (BD Vacutainer Precision Glide, Becton, Dickinson, and Co., Franklin Lakes, NJ) to measure serum total protein (**STP**) as an indicator of passive transfer of maternal antibodies. Blood tubes were stored on ice for up to 2 hours and then centrifuged at 3000 rpm for 20 minutes at 20°C. Serum total protein was measured using a digital refractometer (Misco PA202, Solon, OH, USA). The STP values were stored in farm management software and collected digitally at the end of the study.

Enrollment and animal management

Enrollment for this study occurred from February to July 2016. Calves were formally enrolled into the study upon entry to the ACFB at approximately 21d of age. Calves were eligible for study enrollment if they entered the ACFB by 30 d of age and remained in the ACFB until the end of follow- up, which was 50 d of age. The study period was defined as the time a calf was enrolled until 50 d of age. The ACFB had 2 identical sides with 4 pens on each side for a total of 8 pens; each pen measured 3.4m x 18.3m. Pens were bedded with straw from February to March and a combination of shavings and sand from April through August. The ACFB was naturally ventilated with curtain sidewalls and supplemental positive pressure tube ventilation. Calves had nose - to - nose contact between pens.

Four automated calf feeders (Lely Calm calf feeder, Lely North America, Pella, IA, USA) served the 8 pens with 1 nipple provided per pen. Calves were allowed *ad libitum* milk access until 40 d of age, from 41 - 46 d of age calves were stepped up from 8 to 9.7 L per d, from 47 - 56 d of age calves were stepped down from 9.7 to 9 L per d, from 57 - 70 d of age calves were gradually weaned from 9 to 2 L per d, and weaned completely at 71 d of age. Feeder correction days were used to adjust for calf age. Whole milk from early lactation cows, purified using ultraviolet light (UVPure calf milk purifier, GEA Group Aktiengesellschaft, Düsseldorf, Germany), was the predominate source of liquid feed in the ACFB. The farm chose to supplement this whole milk with milk replacer (32 g milk replacer per 1 L whole milk). More specifically, from February to April, calves were supplemented with medicated 22:20 milk replacer that contained 1600 g/ton neomycin sulfate and 1600 g/ton oxytetracycline (Renaissance Nutrition Inc., Roaring Spring, PA, USA). From May through August, the farm supplemented calves with a 22:20 milk replacer (Renaissance Nutrition Inc., Roaring Spring, PA, USA) that contained Celmanax (Arm & Hammer Animal Nutrition).

Clinical scoring system, ultrasonographic data collection, and weighing during study period

Research staff performed health examinations on enrolled calves twice weekly until 50 d of age. Health examinations were recorded using the Wisconsin Calf Health Scorer App (https://www.vetmed.wisc.edu/dms/fapm/apps/chs.htm) and included a CRS (Lago et al., 2006, McGuirk, 2008), a 6 level USS (**USS6**; Ollivett and Buczinski, 2016), and fecal, navel, and joint scores (McGuirk, 2008). Briefly, the CRS assigned 0 (normal) to 3 (severely abnormal) points for each of the following categories: nasal discharge, eye discharge, ear position, cough, and rectal temperature. Calves were considered positive for clinical respiratory disease (CRS+) when 2 categories with a score of 2 or greater were observed.

For the USS6, a portable linear rectal ultrasound set at a depth of 9 cm, frequency of 6.2 MHz, and gain of 23 dB (Near 13 dB; Far 36 dB; Ibex Pro, E.I. Medical, Loveland, CO, USA) was used. Approximately 150mL 70% isopropyl alcohol was applied to the hair as a transducing agent. One researcher (MCC) performed all USS6 exams. The left and right lungs were scanned in a systematic manner using a technique previously described in dairy calves (Ollivett et al., 2015, Ollivett and Buczinski, 2016). Briefly, the USS6 exam began on the right side of the calf dorsally at the level of the scapula in the right 6th intercostal space (**ICS**) and moved cranially to the right 1st ICS. This allowed for examination of the right middle, caudal aspect of the right cranial lung lobe, and cranial aspect of the right cranial lung lobe (Ollivett and Buczinski, 2016). On the left side of the calf, the USS6 exam began dorsally at the level of the scapula in the left 6th ICS and moved cranially to the left 2nd ICS. This allowed for examination of the caudal aspect of the left cranial lung lobe and cranial aspect of the left cranial lung lobe (Ollivett and Buczinski, 2016). Within each ICS, the probe was held parallel to the ribs and scanned ventrally until specific ultrasonographic anatomical landmarks were identified prior to moving cranially

into the next ICS as described previously (Ollivett and Buczinski, 2016). Normal lung was characterized by observation of a hyperechoic line with reverberation artifact, indicative of the normal pleural interface (Blond and Buczinski, 2009). Consolidated lung appeared hypoechoic and lacked both the hyperechoic line of the pleural surface and reverberation artifact. For the USS6, 5 lung lobes in total were examined and considered separate lung lobes for the scoring system (Ollivett and Buczinski., 2016). The USS6 ranged from 0 to 5 (0 = normal or $\leq$ 1cm^2 consolidation; 1 = diffuse comet tails; 2 = lobular pneumonia: consolidation $\geq$ 1 cm^2; 3 = lobar pneumonia, 1 entire lung lobe consolidated; 4 = lobar pneumonia, 2 entire lung lobes consolidated; 5 = lobar pneumonia, $\geq$ 3 entire lung lobes consolidated). Lung characteristics such as necrosis, abscessation, or pleural effusion were noted. Fecal scores ranged from 0 to 3 (0 = normal, formed feces; 1 = semi - formed feces; 2 = loose feces but stays on top of bedding; 3 = watery feces that sifts through bedding; McGuirk, 2008).

One researcher (MCC) weighed calves at each health examination using a calf weigh tape (Nasco, Fort Atkinson, WI, USA). The tape was placed around the heart girth and pulled snug. Average daily gain was calculated between enrollment and 50 d of age by performing a simple linear regression (SAS Institute Inc., Cary, NC) for each calf using all weights.

BRD definitions and treatment criteria

For analysis, USS6 scores (Table 2.1) and CRS status were based on the first BRD event identified at health exams during the study period. When CRS+, calves were treated according to a standardized treatment protocol developed in cooperation with the farm management and farm veterinarians. A 48 h treatment moratorium was included in the treatment protocol. Research staff treated the first 2 respiratory events detected to ensure consistency and accuracy of treatment records. Treatment for the first CRS+ event was ceftiofur crystalline free acid

(6.6 mg/ kg subcutaneous at the base of the ear once; Excede; Zoetis Services LLC., Parsippany, NJ, USA). Treatment for the second CRS+ event was gamithromycin (6 mg/ kg subcutaneous once; Zactran; 2017 Merial, Inc., Duluth, GA, USA). For any subsequent CRS+ events, the farm staff was responsible for administering treatments as defined by the farm protocol. Flunixin meglamine (2.2 mg/ kg once intravenous; Vetameg; Aspen Veterinary Resources, LTD, Greeley, CO, USA) was administered to calves with both a rectal temperature ≥ 39.4°C and when the calf was depressed, slow to stand, or reluctant to lie down. Calves with diarrhea (fecal score = 3), infected navel (navel score ≥ 2), or infected joints (joint score ≥ 2) were reported to the farm staff and treated by farm staff according to a standardized treatment protocol.

Statistical analysis

Calves with missing data, lost to follow – up (due to death or leaving the ACFB prior to 50 d of age), or inadvertently enrolled (without meeting eligibility requirement), were not included in final analyses. Data were stored, cleaned, and analyzed using Microsoft Excel (Microsoft, Redmond, WA) and SAS (SAS Institute Inc., Cary, NC). Sample size calculations performed *a priori* determined the necessary sample size to be 115 calves per group using an alpha of 0.8 and a mean ± SD difference of 0.06 ± 0.6 kg/d ADG between calves with and without any category of BRD (Ollivett, 2014).

The experimental unit was the calf. The outcome of interest was ADG (kg/d) between entry to ACFB and 50 d of age and was analyzed as a continuous variable. As a method to control for time, cohort was defined as a group of calves that were enrolled into the study within 1 week of each other. Categorical explanatory variables included USS6 (0 - 5), cohort (1 - 8), and birth weight category (low = birth weight less than or equal to 29.1 kg; medium = birth weight > 29.1 but less than 40 kg; high = birth weight ≥ 40 kg; categories were based on the 1st

quartile, median, and 3rd quartile). Dichotomous explanatory variables included CRS (0 = CRS-; 1 = CRS+), sex (male = 0; female = 1), breed (Holstein = 0; Jersey = 1), dystocia (no assistance or easy pull = 0; hard pull or surgical delivery = 1), failure of passive transfer (STP ≥ 5.5 g/ dL = 0; STP < 5.5 g/dL = 1; (Weaver et al., 2000)), diarrhea (fecal score always < 3 in ACFB = 0 ; fecal score of 3 at least once in ACFB = 1), farm diagnosed diarrhea prior to study enrollment (no = 0; yes = 1), and farm diagnosed respiratory disease prior to study enrollment (no = 0; yes = 1). All continuous explanatory variables were assessed for normality using the Shapiro Wilk test and visual inspection of graphical data. Measures of central tendency for raw data are presented as mean ± SD or median (1st quartile, 3rd quartile). Estimates from multivariable models are presented as mean ± SE.

Associations between explanatory variables and the outcome of interest were evaluated using univariable analyses (PROC MIXED). A variable was considered a potential confounder at the univariable level if it met criteria as described by Dohoo et al., (2003); significant association at the univariable level was defined as alpha < 0.20 level. Backwards stepwise elimination was used to select variables for the final model to include only variables that were significant at alpha ≤ 0.05 level. Prior to the final elimination of a variable, a change in estimate criterion of ≥ 30% for the predictor of interest was used to detect confounding. All potential interactions with the predictor of interest and the remaining variables in the model were assessed and interactions with $P > 0.05$ were removed. Predicted means for ADG were assessed using the LSMEANS statement and was adjusted using the DIFF option. Type 3 tests of fixed effects were used to determine significance at the alpha ≤ 0.05 level. P - values in multivariable models were adjusted for multiple comparisons using Tukey's method for multiple comparisons. Residuals were plotted and used to check model assumptions.

The first multivariable linear model assessed the association between USS6 (predictor of interest; Table 2.4) and ADG (outcome). The full model included USS6, CRS, dystocia, birth weight category, breed, and cohort. The final model included USS6, CRS, birth weight category, breed, and cohort. Results from the first multivariable model were used to determine a cut point on the USS6 that was associated with ADG. Differences in LSMEANS estimates for ADG were compared between all scores to determine a cut point on the USS6 at which ADG was affected. Categories of USS6 were collapsed to form a simplified 2 - level ultrasound score (USS2; 0 = without lung consolidation, USS6 scores ≤ 1; 1 = with lung consolidation ≥ 1 cm^2, USS6 scores ≥ 2; Table 1). The second model assessed the association between the USS2 (predictor of interest) and ADG (outcome). The full model included USS2, CRS, dystocia, birth weight category, breed, and cohort. The final model included USS2, CRS, breed, and cohort. Post hoc power calculations (PROC POWER; SAS) were performed when no significant difference was observed.

2.4 RESULTS

Of the 835 calves that were born on the facility during the study period, farm records indicated that 259 calves were removed prior to 30 d of age and therefore prior to the age criteria for study enrollment (n = 211 were sold or sent off - site; n = 48 died). The farm elected not to send 268 calves through the ACFB. A total of 308 calves entered the ACFB and were examined for eligibility. However, 24% (75/308) of calves were excluded from analysis because they were lost to follow – up (n = 11 sold; n = 26 died), were missing birth weights (n = 10), or did not meet the study eligibility enrollment age criteria (cohort 4; n = 28; entered ACFB at 40 d of age). Therefore, 233 calves were confirmed eligible and included in final analyses. Calves were 21 ± 6 d at entry to ACFB and housed in groups of 13 ± 3. For calves that were lost to follow - up,

27% (10/37), 0, 30% (11/37), 8% (3/37), 14% (5/37), and 22% (8/37) had USS6 scores of 0, 1, 2, 3, 4, or 5 at their last health exam, respectively.

Calf descriptive information is presented in Table 2.2. Overall, no calves were identified with USS6 = 1 at their first BRD event and 27% (64/233) of calves had USS6 = 0 for the entire study period (Table 2.1). Seventy - four percent (172/233) and 26% (61/233) of calves were identified as CRS – and CRS+, respectively (Table 2.3). All calves (61/61) that were CRS+ at their first BRD event received an antibiotic. Forty - seven percent (30/64) and 64% (109/169) of calves were identified without and with lung consolidation, respectively, received an antibiotic at a subsequent health score because they were later identified as CRS+. Raw mean ± SD ADG stratified by USS6 were: 0 (0.99 ± 0.41 kg/d), 2 (0.87 ± 0.30 kg/d), 3 (0.91 ± 0.31 kg/d), 4 (0.93 ± 0.31 kg/d), and 5 (0.97 ± 0.28 kg/d). Calves that were CRS- and CRS+ had raw mean ± SD ADG of 0.94 ± 0.34 kg/d and 0.89 ± 0.36 kg/d, respectively. The overall ADG for all calves in this study was 0.93 ± 0.34 kg/d.

We observed a tendency for USS6 to be associated with ADG ($P = 0.07$) using the multivariable model. We did not observe a significant difference in ADG (predicted mean ± SE) among calves with USS6 scores of 2 (0.71 ± 0.04 kg/d), 3 (0.74 ± 0.04 kg/d), 4 (0.71 ± 0.07 kg/d), or 5 (0.87 ± 0.10 kg/d); $P > 0.60$). Average daily gain was also not different for calves with USS6 score of 0 (0.85 ± 0.04 kg/d) versus 3, 4, or 5 ($P > 0.39$). Average daily gain tended to be different between calves with USS6 scores of 0 and 2 ($P = 0.08$). The power to detect a difference in ADG between USS6 score of 2 and USS6 scores of 3, 4, and 5 was ≤ 0.28.

Using the multivariable model that assessed the association between USS6 and ADG, we defined the cut point on the USS6 scoring system at a score of 2, leading to a simplified 2 - level scoring system (USS2; Table 2.1). Calves with lung consolidation (defined as a consolidation

≥1cm^2) at their first BRD event were compared to calves without lung consolidation (defined as no lung consolidation or consolidation ≤ 1cm^2) at their first BRD event. Seventy - three percent of calves (169/233) were identified with lung consolidation (Tables 2.1 and 2.3). Calves without and with lung consolidation had raw ADG (mean ± SD) of 1.0 ± 0.41 and 0.90 ± 0.30 kg/d, respectively. Using a multivariable linear model that assessed the association between USS2 and ADG, we found that calves with lung consolidation had lower ADG (predicted mean ± SE) compared to calves without lung consolidation (0.73 ± 0.03 vs. 0.84 ± 0.04 kg/d, respectively; *P* = 0.01; Table 2.4; Figure 2.1). Calves that were CRS+ had lower ADG compared to calves that were CRS- (0.74 ± 0.04 vs. 0.84 ± 0.03 kg/d, respectively; *P* = 0.04; Table 2.4). Clinical respiratory score did not affect the relationship between USS2 and ADG (interaction *P* = 0.2873).

2.5 DISCUSSION

To the authors' knowledge, this is the first cohort study to determine an appropriate cut point on a previously described USS6 (Ollivett and Buczinski, 2016) based on how scores affected ADG. Our first objective was to identify a cut point that predicts ADG using a previously described USS6. We simplified the USS6 to form USS2 and discovered that calves with lung consolidation ≥1cm^2 at their first BRD had a 0.11 kg/d reduction in ADG compared to calves without lung consolidation. The USS2 we propose will likely be faster to perform on - farm because it requires the person scanning to identify the presence or absence of lung consolidation, rather than differentiating between the extent of lung consolidation as in USS6. Furthermore, we observed separate, but not interactive, effects of USS2 and CRS on ADG, which demonstrates the importance of incorporating both scoring systems into calf management in order to identify sick calves that are at risk for poor growth.

Research regarding the effects of lung consolidation on ADG is limited in dairy calves. We observed a more marked effect of lung consolidation on ADG compared to Ollivett et al. (2014) who found that calves identified with lung consolidation between 1 week and 8 to 12 weeks of age had a 0.04 kg/d decrease in ADG. One potential explanation for the different ADG estimates for lung consolidation is that our study defined consolidation as ≥ 1cm, whereas Ollivett et al. (2014) defined consolidation as > $3cm^2$. As a result, it is possible that the estimates for ADG from Ollivett et al. (2014) were biased towards the null, as their normal calves could have included calves with consolidation < $3cm^2$. Our study and Ollivett et al. (2014) both observed an association between lung consolidation and growth, however, the present study focused on a calf's first BRD event whereas Ollivett et al. (2014) examined the effect of lung consolidation at any point during preweaning on ADG. Future studies should examine a calf's worst or average ultrasound score, or duration of lung consolidation to give a more accurate estimate of the effect of BRD on performance.

We proposed that because TUS outperformed CRS in sensitivity and specificity (Ollivett et al., 2015, Buczinski et al., 2015) for the identification of lung consolidation, it would be more efficient for veterinarians to focus on using TUS, and not CRS. However, in the present study both USS2 and CRS were associated with ADG. Furthermore, USS2 and CRS identify different populations of animals: those with lung consolidation and those with clinical BRD. Therefore, veterinarians should consider using both TUS and CRS in calf programs. Our simplified scoring system (USS2) is most useful for veterinarians interested in identifying calves at risk for poor growth and measuring lung consolidation prevalence.

Interestingly, despite a large proportion of calves with BRD, the mean ADG in the present study population was 0.93 kg/d, which is numerically higher than Soberon et al. (2012)

who reported ADG in preweaned calves to be 0.82 kg or 0.66 kg, depending on the herd. Bateman et al. (2012) reported ADG of 0.52 kg for all calves through 8 weeks of age, which is also lower than the ADG for all calves in the present study. It is possible that because calves in the present study were on a high plane of nutrition, the effect of disease on ADG was somewhat ameliorated similar to previous work (Ollivett et al., 2012). Furthermore, calves in the present study were treated with an antibiotic immediately after they were identified as CRS+ and underwent rigorous health examinations by research staff. It is likely that the disease detection methods used in the present study are more sensitive than previous studies that used producer - reported disease definitions, as producers often have poor sensitivity for overall disease detection (Sivula et al., 1996). It is possible that the effects of BRD categories on ADG would be more pronounced if the producer performed disease identification and treatment. Additionally, calves were enrolled in the present study by 30 d of age, thus excluding ADG from the first 2 – 3 weeks of life, when gain in calves is slower than later in life (Davis and Drackley, 1998).

We attempted to enroll a larger number of calves, however fewer calves entered the ACFB than expected and due to time constraints, we were unable to enroll the a priori calculated sample size. We performed analysis on fewer calves than originally intended to determine if we observed a significant association between USS6 and ADG. Although we proposed a simplified USS, we were unable to differentiate between USS6 of 2, 3, 4, and 5 due to inadequate power. We would expect that there is a difference in growth due to a difference in the amount of lung mass (Jericho and Langford, 1982); greater amounts of affected lung might result in less energy available for growth. We encourage future studies to investigate if a multi - level ultrasound scoring system would better describe the impact on gain, which may be useful to veterinarians or producers making treatment or culling decisions based on the extent of lung consolidation.

Moreover, USS and CRS statuses in the present study were based on a calf's first BRD event after study enrollment. However we did not enroll calves until 21 d of age, but clinical BRD (Cramer and Stanton, 2015) and lung consolidation (Binversie, 2017) have been reported in calves younger than 3 weeks and at 7 d of age, respectively. Therefore, it is possible that calves in the present study had a first BRD event prior to study enrollment, which may have resulted in a decrease in ADG (Virtala et al., 1996) and could have lasted several months (Stanton et al., 2012). However, if calves with BRD prior to study enrollment recovered before entry to the ACFB, we would have identified these calves as normal, thus biasing our findings towards the null.

Finally, analysis for the present study only included calves that entered the ACFB by 30 d of age and survived to 50 d of age, effectively excluding calves that died. The exclusion of calves that died may have biased our results towards the null, as calves that died were likely most affected by disease and therefore may have been the poorest growing. Additionally, we only included observations until 50 d of age, despite weaning completion at 70 d of age. Many calves left the ACFB between 50 and 70 d of age due to space concerns and transporting calves to other facilities. In order to include as many calves as possible in analysis, we chose to observe calves until 50 d of age. However, BRD that occurs around weaning has been associated with reduced ADG (Stanton et al., 2012). As a result of our study design, it is possible that calves may have had BRD after the study ended, but before weaning was completed. Therefore, we may not have accurately estimated the impact of all preweaned BRD on ADG.

2.6 CONCLUSIONS

This was the first study to offer a simplified lung ultrasound score based on the associations between ultrasound score and ADG. Calves with lung consolidation at their first BRD event had lower ADG compared to calves without lung consolidation. We also found that

calves with clinical signs at their first BRD event had lower ADG compared to calves without clinical signs. Therefore, both ultrasound scoring and clinical scoring systems should be implemented in calf programs to identify calves all with BRD who are at risk for poor growth. Our simplified 2 - level lung ultrasound scoring system may be more practical for veterinarians compared to a multi - level scoring system. Furthermore, veterinarians can use our simplified scoring system to identify calves that may be at risk for poor growth, determine herd level prevalence of lung consolidation, and measure herd level lung consolidation before and after interventions. Additionally, BRD researchers should consider using both ultrasound and clinical respiratory scoring in order to correctly classify all calves affected with BRD. Our findings are most generalizable for calves from 21- 50 d of age, in group housing, and on a high plane of nutrition. Therefore, we suggest similar studies in a variety of management systems. We encourage future work to understand the impact of duration of lung consolidation on ADG and if a multi level ultrasound scoring system better describes the impact of lung consolidation on gain.

2.7 ACKNOWLEDGEMENTS

This material is based upon work supported by funding from STGenetics (Navasota, Texas, United State, 77868). Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the view of STGenetics. The authors wish to thank the staff and owners of the calf facility for allowing us to perform research and Ella Jackson and Hannah Donley for their assistance with data collection.

2.8 REFERENCES

Adams, E. A and S. Buczinski. 2016. Short communication: Ultrasonographic assessment of lung consolidation postweaning and survival to the first lactation in dairy heifers. J. Dairy Sci. 99:1465-1470.

Bateman, H. G., T. M. Hill, J. M. Aldrich, R. L. Schlotterbeck, and J. L. Firkins. 2012. Meta-analysis of the effect of initial serum protein concentration and empirical prediction model for growth of neonatal Holstein calves through 8 weeks of age. J. Dairy Sci. 95:363-369.

Binversie, E. 2017. Using visual observations and thoracic ultrasonography to evaluate treatment success in preweaned dairy calves affected by respiratory disease. MS Thesis. University of Wisconsin- Madison, Madison, WI.

Blond, L. and S. Buczinski. 2009. Basis of ultrasound imaging and the main artifacts in bovine medicine. Vet. Clin. North Am. Food Anim. Pract. 25:553-565.

Buczinski, S., T.L. Ollivett, and N. Dendukuri. 2015. Bayesian estimation of the accuracy of the calf respiratory scoring chart and ultrasonography for the diagnosis of bovine respiratory disease in pre-weaned dairy calves. Prev. Vet. Med. 119:227-231.

Buczinski, S., G. Forté, and A. Bélanger. 2013. Short communication: Ultrasonographic assessment of the thorax as a fast technique to assess pulmonary lesions in dairy calves with bovine respiratory disease. J. Dairy Sci. 96:4523-4528.

Buczinski, S., G. Forté, D. Francoz, and A. M. Bélanger. 2014. Comparison of thoracic auscultation, clinical score, and ultrasonography as indicators of bovine respiratory disease in preweaned dairy calves. J. Vet. Intern. Med. 28:234-242.

Cramer, M. C. and A. L. Stanton. 2015. Associations between health status and the probability of approaching a novel object or stationary human in preweaned group-housed dairy calves. J. Dairy Sci. 98:7298-7308.

Davis, C. L. and J. K. Drackley. 1998. The development, nutrition, and management of the young calf. Iowa State University Press, Ames, IA.

Dohoo, I., W. Martin, and H. Stryhn. 2003. Veterinary Epidemiologic Research. 2nd ed. AVC Inc., Prince Edward Island, Canada.

Dunn, T.R., T.L. Ollivett, D.L. Renaud, and D.F. Kelton. 2017. The effect of lung consolidation, as determined by ultrasonography, on first lactation milk production in Holstein dairy calves. Page 194 in Proc. American Dairy Science Assn. Annual Mtg., Pittsburgh, PA.

Heins, B. D. , D. V. Nydam, A. R. Woolums, R. D. Berghaus, and M. W. Overton. 2014. Comparative efficacy of enrofloxacin and tulathromycin for treatment of preweaning respiratory disease in dairy heifers. J. Dairy Sci. 97:372-382.

Jericho, K. W. and E. V. Langford. 1982. Aerosol vaccination of calves with pasteurella haemolytica against experimental respiratory disease. Can. J. Comp. Med. 46:287-292.

Lago, A., S. M. McGuirk, T. B. Bennett, N. B. Cook, and K. V. Nordlund. 2006. Calf respiratory disease and pen microenvironments in naturally ventilated calf barns in winter. J. Dairy Sci. 89:4014-4025.

McGuirk, S. M. 2008. Disease management of dairy calves and heifers. Vet. Clin. of North Am. Food Anim. Pract. 24:139-153.

Moallem, U., D. Werner, H. Lehrer, M. Zachut, L. Livshitz, S. Yakoby, and A. Shamay. 2010. Long-term effects of ad libitum whole milk prior to weaning and prepubertal protein supplementation on skeletal growth rate and first-lactation milk production. J. Dairy Sci. 93:2639-2650.

Ollivett, T. L. 2014. Understanding the diagnosis and risk factors for respiratory disease in dairy calves. PhD Thesis. University of Guelph, Guelph, Ontario, Canada.

Ollivett, T. L., D. F. Kelton, and T. F. Duffield. 2014. A randomized controlled clinical trial to evaluate the effect of an intranasal respiratory vaccince on calf health, ultrasonographic lung consoldiation, and growth in Holstein dairy calves. Page 113-144 in Proc. American Assn. of Bovine Practitioners, Albuquerque, NM.

Ollivett, T. L. , J. L. Caswell, D. V. Nydam, T. Duffield, K. E. Leslie, J. Hewson, and D. Kelton. 2015. Thoracic Ultrasonography and Bronchoalveolar Lavage Fluid Analysis in Holstein Calves with Subclinical Lung Lesions. J. Vet. Intern. Med. 29:1728-1734.

Ollivett, T. L. and S. Buczinski. 2016. On-farm use of ultrasonography for bovine respiratory disease. Vet. Clin. North Am. Food Anim. Pract. 32:19-35.

Ollivett, T. L., D. V. Nydam, T. C. Linden, D. D. Bowman, and M. E. Van Amburgh. 2012. Effect of nutritional plane on health and performance in dairy calves after experimental infection with Cryptosporidium parvum. J. Am. Vet. Med. Assoc. 241:1514-1520.

Sivula, N. J., T. R. Ames, W. E. Marsh, and R. E. Werdin. 1996. Descriptive epidemiology of morbidity and mortality in Minnesota dairy heifer calves. Prev. Vet. Med. 27:155-171.

Soberon, F., E. Raffrenato, R. W. Everett, and M. E. Van Amburgh. 2012. Preweaning milk replacer intake and effects on long-term productivity of dairy calves. J. Dairy Sci. 95:783-793.

Stanton, A. L., D. F. Kelton, S. J. LeBlanc, J. Wormuth, and K. E. Leslie. 2012. The effect of respiratory disease and a preventative antibiotic treatment on growth, survival, age at first calving, and milk production of dairy heifers. J. Dairy Sci. 95:4950-4960.

Teixeira, A. G. V., J. A. A McArt, and R. C. Bicalho. 2017. Thoracic ultrasound assessment of lung consolidation at weaning in Holstein dairy heifers: Reproductive performance and survival. J. Dairy Sci. 100:2985-2991.

USDA. 2010. Heifer calf health and management practices on U.S. dairy operations, 2007. USDA: APHIS: VS,CEAH, Fort Collins, CO.

Van Donkersgoed, J., J. Merrill, and S. Hendrick. 2007. Comparative efficacy of tilmicosin versus tulathromycin as a metaphylactic antimicrobial in feedlot calves at moderate risk for respiratory disease. Veterinary therapeutics: research in applied veterinary medicine 9:291-297.

Virtala, A. M. K., G. D. Mechor, Y. T. Grohn, and H. N. Erb. 1996. The effect of calfhood diseases on growth of female dairy calves during the first 3 months of life in New York state. J. Dairy Sci. 79:1040-1049.

Weaver, D. M., J. W. Tyler, D. C. VanMetre, D. E. Hostetler, and G. M. Barrington. 2000. Passive transfer of colostral immunoglobulins in calves. J. Vet. Intern. Med. 14:569-577.

2.9 Tables and Figures

Table 2.1. Classifications used for defining lung consolidation at the first bovine respiratory disease (BRD) event in 233[1] preweaned dairy calves observed between 21 – 50 d of age (%, no./no.)

Ultrasound Score	Levels	Definition	Proportion of calves
USS6[2]			
	0	Normal or ≤ 1cm^2 consolidation	27% (64/233)
	1	Diffuse comet tails	0
	2	Lobular pneumonia: consolidation ≥ 1 cm^2	31% (73/233)
	3	Lobar pneumonia, 1 entire lung lobe consolidated	29% (68/233)
	4	Lobar pneumonia, 2 entire lung lobes consolidated	8% (19/233)
	5	Lobar pneumonia, ≥ 3 entire lung lobes consolidated	4% (9/233)
USS2[3]			
	0	Normal or consolidation < 1cm^2	27% (64/233)
	1	Consolidation ≥ 1 cm^2	73% (169/233)

1- Calves who were not identified with lung consolidation were assigned a score of 0 (n = 64)
2- Six - level thoracic ultrasound score (Ollivett and Buczinski, 2016); Refers to ultrasound score at a calf's first BRD event
3- Two - level thoracic ultrasound score; cut point determined from analysis testing the association between a USS6 and average daily gain, Categories that were statistically ($P >$ 0.10) and biologically similar were collapsed.

Table 2.2. Proportion (n) of preweaned dairy calves with failure of passive transfer, dystocia, and proportion of calves for each sex, breed, and birth weight category, by cohort

Cohort[1]	Failure of passive transfer[2]	Dystocia[3]	Sex	Breed		Birth weight category (kg)		
			Male	Holstein	Jersey	≤ 29.1	29.2 – 39.9	≥ 40
1 (n = 31)	58% (18)	13% (4)	81% (25)	90% (28)	10% (3)	19% (6)	58% (18)	23% (7)
2 (n = 48)	50% (24)	23% (11)	85% (41)	81% (39)	19% (9)	15% (7)	44% (21)	42% (20)
3 (n = 35)	11% (4)	14% (5)	83% (29)	63% (22)	37% (13)	31% (11)	49% (17)	20% (7)
5 (n = 15)	27% (4)	13% (2)	53% (8)	53% (8)	47% (7)	47% (7)	33% (5)	20% (3)
6 (n = 26)	12% (3)	8% (2)	54% (14)	85% (22)	15% (4)	19% (5)	58% (15)	23% (6)
7 (n = 26)	19% (5)	15% (4)	46% (12)	92% (24)	8% (2)	23% (6)	54% (14)	23% (6)
8 (n = 26)	19% (5)	12% (3)	81% (21)	92% (24)	8% (2)	23% (6)	42% (11)	35% (9)
9 (n = 26)	15% (4)	19% (5)	100% (26)	100% (26)	0% (0)	4% (1)	62% (16)	35% (9)
Overall (n = 233)	28% (65)	15% (36)	75% (176)	83% (193)	17% (40)	21% (48)	51% (118)	29% (67)

1- A cohort is a group of calves that were enrolled into the study within 1 week of each other. Calves in cohort 4 were excluded from final analysis because they were not enrolled by 30 d of age.
2- Serum protein refractometry value < 5.5 g/dL
3- Calf was delivered by hard pull or surgery.

Table 2.3. A 2x2 contingency table comparing the distribution of CRS[1] and USS2[2] at the first Bovine Respiratory Disease event in preweaned dairy calves (n 233)

	CRS	
USS2	+	-
Consolidation	30	139
No Consolidation	31	33

1- Clinical respiratory score adapted from McGuirk (2008); calves were considered CRS+ when 2 categories with a score of 2 or greater were observed.

2- Simplified ultrasound score adapted from Ollivett et al., 2016; lung consolidation was defined as $\geq 1 cm^2$ on ultrasound.

Table 2.4. Multivariable linear regression model for ADG in 233 preweaned dairy calves for calves that survived to 50 d of age. Calves were enrolled in the study at 21±6 (mean ± SD) d of age and underwent twice weekly health exams. This model contains USS2[1] as the predictor of interest. Estimates for ADG and reported *P* – values are from solutions for fixed effects.

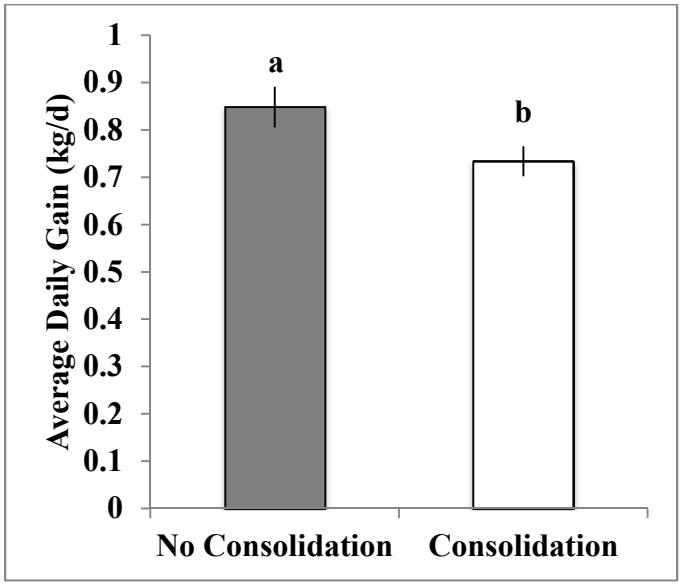
Variable	ADG Estimate (kg/d)	Standard Error	*P* - value
Intercept	0.4157	0.0889	< 0.0001
USS2			
No consolidation	0.1147	0.0459	0.0132
Consolidation	Referent		
CRS[2]			
-	0.0975	0.0471	0.0398
+	Referent		
Breed			
Holstein	0.3674	0.0536	< 0.0001
Jersey	Referent		
Cohort[3]			
1	0.1992	0.0783	0.0116
2	0.2505	0.0715	0.0006
3	-0.0159	0.0771	0.8366
5	0.0348	0.0977	0.7218
6	0.1599	0.0816	0.0511
7	0.0786	0.0804	0.3295
8	-0.0235	0.0805	0.7706
9	Referent		

1- Simplified ultrasound score adapted from Ollivett et al., 2016; lung consolidation was defined as ≥1cm^2 on ultrasound

2- Clinical respiratory score adapted from McGuirk (2008); calves were considered CRS+ when 2 categories with a score of 2 or greater were observed.

3- A cohort is a group of calves that were enrolled into the study within 1 week of each other. Calves in cohort 4 were excluded from final analysis because they were not enrolled by 30 d of age.

Figure 2.1 Least squares means (± SE) estimates for average daily gain (kg/d) for 233 preweaned group housed calves with (≥ 1 cm of consolidation for at least one ultrasound exam) or without lung consolidation (< 1 cm of consolidation at all ultrasound exams). This simplified ultrasound score was adapted from Ollivett et al., 2016; lung consolidation was defined as ≥ 1cm^2 on ultrasound. Estimates obtained from a multivariable linear model that controlled for clinical respiratory disease status of the calf, cohort, and breed. Different letters denote significant differences in mean ADG ($P < 0.05$).



3 CHAPTER 3: AUTOMATED FEEDING BEHAVIORS ASSOCIATED WITH SUBCLINICAL RESPIRATORY DISEASE IN PREWEANED DAIRY CALVES- A COHORT STUDY

M. C. Cramer*, K. L. Proudfoot†, and T. L. Ollivett‡[1]

*Department of Dairy Science, University of Wisconsin, Madison, 53706

†Department of Veterinary Preventive Medicine, The Ohio State University, Columbus 43210

‡Department of Medical Sciences, School of Veterinary Medicine, University of Wisconsin, Madison 53706

3.1 ABSTRACT

Bovine respiratory disease (**BRD**) can occur without clinical signs, however little is known about feeding behaviors of calves with subclinical BRD (**SBRD**). The objective of this cohort study was to determine if calves with SBRD exhibit different feeding behaviors compared to calves with clinical BRD (**CBRD**) or without BRD (**NOBRD**) during the 3 d before until the d of diagnosis. Preweaned dairy calves (n = 133; 21 ± 6 d) were enrolled at entry into a group-housed automated milk feeder barn. Twice weekly health exams included a clinical respiratory score (CRS; - or +) and a lung ultrasound to identify consolidation. BRD status for each calf was defined as SBRD (calves with lung consolidation ≥ 1cm^2 and CRS -; n = 81) or CBRD (CRS+ with or without lung consolidation; n = 39), based on the first BRD event. Calves with NOBRD (n = 13) never had lung consolidation ≥ 1cm^2 or CRS+. Feeding behavior data (drinking speed, daily milk intake, average meal size, number of rewarded and unrewarded visits) were collected automatically during the 3 d before and the d of diagnosis for SBRD and CBRD calves; for calves with NOBRD, d 0 was the mean age of all calves with SBRD and CBRD (31 ± 8 d). Three mixed linear models for continuous outcomes and 2 logistic models for discrete outcomes were used to determine if BRD status was associated with feeding behaviors. Models included day as a repeated measure, and sex, breed (Holstein or Jersey), supplemental milk replacer type (probiotic or medicated), BRD status, and a day by BRD status interaction as fixed effects; calf was included as a random effect. Calves with SBRD drank more milk than calves with CBRD (10.9 vs. 9.9 L/d) and calves with NOBRD (10.9 vs. 8.9 L/d) across the 4 d period. There was no difference in milk intake between calves with CBRD and calves with NOBRD (9.9 vs. 8.9 L/d). Calves with SBRD drank faster than calves with CBRD (768 vs. 664 mL/min), and calves with CBRD tended to drink slower than calves with NOBRD (664 vs. 772 mL/min). There was no difference in drinking speed between calves with SBRD and calves with NOBRD (768 vs.

772 mL/min). There was no effect of BRD status on any other behavior, no effects of day, and no interactions between day and BRD status for any behavior. Feeding behavior did not adequately differentiate between calves with SBRD, CBRD, and NOBRD. Thus, dairy producers should be cautious when using automated feeder data as the primary means of detecting calves with BRD.

Key words: automated calf feeder, bovine, calf lung ultrasound, pneumonia

3.2 INTRODUCTION

Bovine respiratory disease (**BRD**) is reported to affect approximately 12% of preweaned calves in the United States and accounts of 22% of all preweaned calf deaths (USDA, 2010). Early disease detection, and therefore early intervention, likely ameliorates the negative consequences of BRD. However, producers and veterinarians struggle to identify all calves with BRD (Sivula et al., 1996, Buczinski et al., 2015, Cramer et al., 2016). Detecting sick calves in group housing is challenging due to the difficulty of observing individual animals and our limited knowledge of how sick calves behave in a social environment. These challenges are compounded by the prevalence of subclinical BRD (lung consolidation, but no outward signs of disease), which can range from 23 to 67% (Ollivett and Buczinski, 2016).

Data collected from automated milk feeders, which collect drinking speed, milk intake, and number of visits to the feeder, might be useful to detect calves with BRD. Borderas et al. (2009) observed that calves with gastrointestinal illness, respiratory illness, or a combination fed *ad libitum* drank 2.6 L/d less and had 2.4 fewer visits per day compared to calves without illness. A recent study observed that sick calves (defined as diarrhea, respiratory disease, or ill thrift) drank 183 mL/min slower, drank 1.2 L/d less, and had 3.1 fewer unrewarded visits compared to healthy calves (Knauer et al., 2017). Previous work indicates that feeding behaviors change around the time of illness, but disease was defined broadly and used clinical (outward signs of disease) detection tools.

One gap in knowledge for calf feeding behavior centers on methods used for BRD detection. Previous feeding behavior studies used clinical detection tools which include visual observations and lung auscultation (Svensson and Jensen, 2007, Borderas et al., 2009), or the Wisconsin Calf Respiratory Scoring chart (McGuirk, 2008, Knauer et al., 2017) to define BRD. However, lung auscultation and BRD scoring systems that rely solely on clinical signs lack

sensitivity (Buczinski et al., 2014). The limitations of clinical BRD detection methods preclude our ability to accurately identify all calves with BRD, and therefore we are unable to fully grasp the behavioral changes associated with BRD.

Recently, calf lung ultrasonography has been validated in dairy calves as a rapid, on-farm diagnostic tool for the identification of lung consolidation associated with BRD (Buczinski et al., 2013, Ollivett, 2014). Calf lung ultrasonography can accurately detect lung consolidation, regardless of the calf's clinical status (Ollivett and Buczinski, 2016) and outperforms clinical respiratory scoring and lung auscultation (Buczinski et al., 2014). The sensitivity and specificity of ultrasound are high (94% and 100%, respectively) for detecting subclinical lung consolidation (Ollivett et al., 2015). Within herd prevalence of subclinical lung consolidation can range from 23% to 67% (Ollivett and Buczinski, 2016), representing a large source of unidentified disease. The advantages of calf lung ultrasonography, combined with a population of unidentified calves with BRD, provide impetus to use ultrasonography as a tool to detect BRD and determine if feeding behavior is useful to differentiate between types of BRD.

Feeding behavior data collected from automated milk feeders have shown promise to identify sick calves identified using clinical detection tools. Furthermore, automated feeders offer an additional way to find sick calves, are available on more dairies, require less calf handling, and provide daily data on calves. Therefore, the objective of this cohort study was to determine if calves with subclinical BRD (lung consolidation, but no outward signs of disease) exhibit different feeding behaviors compared to calves with clinical BRD or calves without BRD during the 3 d before and the d of BRD diagnosis. We hypothesized that calves with subclinical BRD would have deviations in feeding behaviors prior to disease compared to calves without BRD, but to a lesser degree than calves with clinical BRD.

3.3 MATERIALS AND METHODS

Data collection for this cohort study took place in Ohio, USA between February and August 2016 on a commercial dairy cattle facility. The Institutional Animal Care and Use Committee at the University of Wisconsin - Madison (A005049 - A03) approved this study. Prior to study enrollment, calves were housed individually until they entered the automated calf feeder barn.

Animal enrollment and management

Calves were enrolled into the study upon entry to the automated calf feeder barn at 21 ± 6 d. Calves were recruited for enrollment from February to July 2016 and were eligible for study enrollment if they entered the automated calf feeder barn by 30 d of age. Calves were followed until 50 d of age.

The automated calf feeder barn had 2 identical sides with 4 pens on each side for a total of 8 pens; each pen measured 3.4m x 18.3m. Pens were bedded with straw from February to March and a combination of shavings and sand from April through August. The automated calf feeder barn was naturally ventilated with curtain sidewalls and supplemental positive pressure tube ventilation. Calves had nose-to-nose contact between pens.

Milk feeding and behavioral data collection

Four automated calf feeders (Lely Calm calf feeder, Lely North America, Pella, IA, USA) served 8 pens with 1 nipple per pen. Calves had *ad libitum* milk access until 40 d of age, from 41 to 46 d of age calves were stepped up from 8 to 9.7 L per d, from 47 to 56 d of age calves were stepped down from 9.7 to 9 L per d. The age of the calf was entered into the automated feeding program to ensure calves were on the correct feeding stage based on their age. Milk meal size offered by the feeder ranged from 1- 2.5 L/meal and depended on the feeding stage of the calf.

Whole milk from early lactation cows, purified using an ultraviolet light (UVPure calf milk purifier, GEA Group Aktiengesellschaft, Düsseldorf, Germany) was the predominate source of liquid feed in the calf feeders. The farm added milk replacer (32 g milk replacer per 1 L whole milk) in the calf feeders to supplement the whole milk. From February to April, calves were supplemented with milk replacer that contained 1600 g/ton neomycin sulfate and 1600 g/ton oxytetracycline (Renaissance Nutrition Inc., Roaring Spring, PA, USA), whereas from May through August, Celmanax (Arm & Hammer Animal Nutrition; Ewing, NJ) was added.

The following feeding behaviors were automatically generated by the milk feeder: average daily drinking speed (mL/min), total daily milk intake (L/d), number of unrewarded visits per day (number of visits to the feeder without milk), and number of rewarded visits per day (number of visits to the feeder with milk). Average meal size consumed by the calf across one day was calculated by the following: daily milk intake/rewarded visits. Research staff exported feeder data weekly.

Health data collection

Research staff performed health examinations on all enrolled calves twice weekly between 0900 and 1300h until 50 d of age. Health examinations were recorded using the Wisconsin Calf Health Scorer App (https://www.vetmed.wisc.edu/dms/fapm/apps/chs.htm) and included a clinical respiratory score (**CRS**; Lago et al., 2006, McGuirk, 2008), a 6 level ultrasound score (Ollivett and Buczinski, 2016), and a fecal score (0 = normal fecal consistency; 1 = semi-formed, pasty; 2 = loose but stays on top of bedding; 3 = watery, sifts though bedding; Mcguirk, 2008). Briefly, the CRS assigned 0 (normal) to 3 (severely abnormal) points for each of the following categories: nasal discharge, eye discharge, ear position, cough, and rectal temperature. Calves were considered positive for clinical respiratory disease (**CRS+**) when 2

categories with a score of 2 or greater were observed. Diarrhea status was defined as at least one fecal score ≥ 2 during the 4 d of feeding behavior observations.

Ultrasound examinations were performed using a portable linear rectal ultrasound set at a depth of 9 cm, frequency of 6.2 MHz, and gain of 23 dB (Near 13 dB; Far 36 dB; Ibex Pro, E.I. Medical, Loveland, CO, USA). The left and right lungs were scanned in a systematic manner using a technique previously described in dairy calves (Ollivett et al., 2015, Ollivett and Buczinski, 2016). During the ultrasound exam, 5 lung lobes (right middle lung lobe, caudal and cranial aspects of the right cranial lung lobe, and the caudal and cranial aspects of the left cranial lung lobe) were examined and considered separate lung lobes for the scoring system (Ollivett and Buczinski., 2016). The ultrasound scoring system ranged from 0 to 5 (0 = normal or ≤ 1cm^2 consolidation; 1 = diffuse comet tails; 2 = lobular pneumonia: consolidation ≥ 1 cm^2; 3 = lobar pneumonia, 1 entire lung lobe consolidated; 4 = lobar pneumonia, 2 entire lung lobes consolidated; 5 = lobar pneumonia, ≥ 3 entire lung lobes consolidated). Lung characteristics such as necrosis, abscessation, or pleural effusion were also noted.

Three research staff members performed the CRS; one experienced researcher trained two research staff members on how to properly score calves using the CRS prior to the study. Inter-observer agreement for CRS status (CRS+ versus CRS-) was determined prior to the study (kappa = 0.6) and halfway through the study (kappa = 0.7). One researcher performed all ultrasound exams.

BRD status definitions

Three levels of BRD status were defined and were based on a calf's first BRD event: 1) clinical BRD (**CBRD**): CRS+ at their first BRD event, regardless of ultrasound score, 2) subclinical BRD (**SBRD**): lung consolidation ≥ 1cm^2 and CRS– at their first BRD event, or 3)

without BRD (**NOBRD**): never CRS+ and never had lung consolidation ≥ 1cm^2 throughout the study.

All CRS+ calves were treated with an antibiotic according to a standardized treatment protocol developed in cooperation with the farm management and farm veterinarians. Calves identified with SBRD at their first BRD event were not treated with an antibiotic; these calves may have later received an antibiotic if they were subsequently identified as CRS+. Research staff administered treatments for the first 2 CRS+ events to ensure consistency and accuracy of treatment records. Ceftiofur crystalline free acid (6.6 mg/ kg subcutaneous at the base of the ear once; Excede; Zoetis Services LLC., Parsippany, NJ, USA) and gamithromycin (6 mg/ kg subcutaneous once; Zactran; ©2017 Merial, Inc., Duluth, GA, USA) were the first and second line treatments, respectively. Calves were not re-treated with an antibiotic until after 48 h; they were retreated if they were identified as CRS+. For any subsequent CRS+ events, the farm staff was responsible for administering treatments as defined by the farm protocol. Flunixin meglamine (1.1 mg/ kg once intravenous; Vetameg; Aspen Veterinary Resources, LTD, Greeley, CO, USA) was administered to calves with a rectal temperature ≥ 39.4°C and when the calf was depressed, slow to stand, or reluctant to lie down.

Statistical analysis

Feeding behavior data were summarized by day for the 3 d before (d -3, d -2, d -1) and the day of diagnosis (d 0) for CBRD and SBRD calves. To minimize any effects of age on feeding behavior, d 0 for NOBRD calves was set using the mean age of all calves with SBRD and CBRD (31 ± 8 d old). A one-way ANOVA was used to ensure that age was not different on d 0 between BRD categories. Calves with missing feeder data were not included in the final analysis. Data were stored, cleaned, and analyzed using Microsoft Excel (Microsoft, Redmond,

WA) and SAS (version 9.3; SAS Institute Inc., Cary, NC). Sample size calculations performed *a priori* determined the necessary sample size to be 14 calves per group using an alpha of 0.8 and a mean ± SD difference of 2.4 ± 2.1 total visit duration to the automated feeder (minutes) between sick and healthy calves (Borderas et al., 2009).

The experimental unit was the calf for all analyses. Data were screened for outliers using visual assessment and for normality using PROC UNIVARIATE in SAS. The outcomes of interest were daily average drinking speed (mL/min), milk intake (L/d), average meal size (L/visit), number of unrewarded visits (no./d), and number of rewarded visits (no/d); each outcome was modeled separately. The predictor of interest was BRD status (CBRD, normal, and SBRD). Day relative to BRD was considered a categorical variable (d -3 to 0). Dichotomous explanatory variables included supplemented form of milk replacer (Celmanax = 0; Medicated = 1), sex (male = 0; female = 1), breed (Holstein = 0; Jersey = 1). Measures of central tendency for raw data are presented as mean ± SD or median (1^{st} quartile, 3^{rd} quartile). Estimates from multivariable models are presented as mean ± SE.

Associations between explanatory variables and the outcome of interest were evaluated using univariable analyses (PROC MIXED in SAS). A variable was considered a potential confounder if it met criteria as described by Dohoo et al. (2003); significant association at the univariable level was defined as alpha < 0.20 level. Backwards stepwise elimination was used to select variables for the final model to include only variables that were significant at alpha ≤ 0.05 level. Prior to the final elimination of a variable, a change in estimate criterion of ≥ 30% for the predictor of interest was used to detect confounding. All potential interactions with the predictor of interest and the remaining variables in the model were assessed and interactions with $P > 0.05$ were removed.

All full models included BRD status, day relative to BRD diagnosis, sex, breed, milk replacer type, diarrhea, and the interaction between day relative to BRD diagnosis and BRD status as fixed effects. Day relative to BRD was controlled for as a repeated measure and calf was a random effect. Predicted means for feeding behaviors were assessed using the LSMEANS statement and differences between BRD statuses were assessed using differences in LSMEANS. Type 3 tests of fixed effects were used to determine significance at the alpha ≤ 0.05 level. *P*-values in multivariable models were adjusted for multiple comparisons using Tukey's method for multiple comparisons. Residuals were plotted and used to check model assumptions. Covariance structures were selected based on the lowest AIC.

Three separate multivariable linear mixed models (PROC MIXED in SAS) were used to determine if BRD status was associated with drinking speed, milk intake, and average meal size. Two separate multivariable logistic mixed models (PROC GLIMMIX in SAS) with poisson distributions were used to determine if BRD status was associated with number of unrewarded and rewarded visits to the feeder. The final model for drinking speed included BRD status, sex, and breed. The final model for milk intake included BRD status, breed, and milk replacer type. The final model for average meal size included BRD status, sex, breed, and milk replacer type. The final model for number of unrewarded visits included BRD status, breed, and milk replacer type. The final model for rewarded visits included BRD status, day relative to BRD diagnosis, sex, breed, and milk replacer type.

3.4 RESULTS

Of the 835 calves that were born on the facility during the study period, farm records indicated that 259 calves were removed prior to 30 d of age and therefore prior to the age criteria for study enrollment (n = 211 were sold or sent off - site; n = 48 died). The farm elected not to send 268 calves through the automated calf feeder barn due to limited space in the barn. A total

of 308 calves entered the automated calf feeder barn and were examined for study eligibility. However, 9% (28/308) of calves did not meet the enrollment age criteria for study eligibility, as they entered automated calf feeder barn at 40 d of age. Therefore, 280 calves were confirmed eligible for enrollment and were carried forward in the analysis of the automated feeder data. Data from 147 calves were excluded due to incorrect entry of feeder correction days (n = 35) or calves that did not have complete feeder data for the 3 d prior to and the d of BRD diagnosis (n = 112). As a result, final analysis included 133 calves, representing 532 calf days.

Calves were 21 ± 6 d (mean ± SD) at entry to the automated calf feeder barn and were housed in groups of 13 ± 3. Calf descriptive information and the proportion of calves with diarrhea and fever during the 4 d of feeding behavior observations are presented in Table 3.1. Twenty-nine percent (39/133) and 61% (81/133), of calves were identified with CBRD and SBRD at their first BRD event, respectively. Ten percent (13/133) of calves were never identified with CBRD or SBRD and were therefore categorized as NOBRD. The age (mean ± SD) of calves with NOBRD (33 ± 5 d), calves with SBRD (32 ± 7 d), and calves with CBRD (30 ± 8 d) on d 0 was not different between BRD groups ($P = 0.45$). Raw values for feeding behaviors are presented in Table 3.2.

Model estimates for feeding behaviors are written as predicted mean ± SE. There was an effect of BRD status on drinking speed (P=0.01; Table 3.3), whereby calves with SBRD drank faster than calves with CBRD (768 ± 27 vs. 664 ± 35 mL/min; P=0.0004); calves with CBRD tended to drink slower than calves with NOBRD (664 ± 35 vs. 772 ± 52 mL/ min; P=0.07). There was no difference in drinking speed between calves with SBRD and calves with NOBRD (768 ± 27 vs. 772 ± 52 mL/min; P=0.9). Day relative to BRD diagnosis ($P = 0.38$) and the

interaction between day relative to BRD diagnosis and BRD status ($P = 0.68$) were not significantly associated with drinking speed.

There was an effect of BRD status on intake (P=0.01; Table 3.4), which was driven by calves with SBRD drinking more milk than both calves with CBRD (10.9 ± 0.4 vs. 9.9 ± 0.5 L/d; P=0.04) and calves with NOBRD (10.9 ± 0.4 vs. 8.9 ± 0.7 L/d; P=0.04). There was no difference in intake between calves with CBRD and calves with NOBRD (9.8 ± 0.5 vs. 8.9 ± 0.7 L/d; P=0.31). Day relative to BRD diagnosis ($P = 0.89$) and the interaction between day relative to BRD diagnosis and BRD status ($P = 0.96$) were not significantly associated with intake. We did not observe an association between BRD status and average meal size ($P = 0.23$), rewarded visits ($P = 0.80$), or unrewarded visits ($P = 0.21$; model results not shown). We did not observe an association between day relative to BRD diagnosis and average meal size ($P = 0.70$), rewarded visits ($P = 0.39$), or unrewarded visits ($P = 0.49$; model results not shown).

3.5 DISCUSSION

To our knowledge, this cohort study was the first to investigate if feeding behavior in calves with subclinical BRD differed from calves with clinical BRD or calves without BRD. We expected that calves with subclinical BRD would have deviations in feeding behaviors prior to disease compared to calves without BRD, but to a lesser degree than calves with clinical BRD. However, calves with subclinical BRD did not differ from calves without BRD in any behavior except intake, and subclinical BRD calves drank more than normal calves. Additionally, we did not observe an effect of BRD status on average meal size, rewarded visits, or unrewarded visits, nor did we observe an effect of d relative to BRD diagnosis for any behavior.

In the present study, calves with subclinical BRD drank more milk than calves without BRD, which was unexpected. The reason for the lower intake in calves without BRD is unclear. Although 10% of calves without BRD had diarrhea at least once during the 4 day feeding

behavior observations, diarrhea was not associated with any feeding behavior in multivariable models. Furthermore, fever was not identified in any of the calves without BRD during the 4 day feeding behavior observations. It is possible that calves without BRD consumed more starter grain instead of milk; healthy adult cows have increased DMI compared to cows with metritis (Huzzey et al., 2007). We did not observe a difference in intake between calves with clinical BRD and calves without BRD. Similarly, Knauer et al. (2017) did not observe a difference in milk intake between calves with clinical BRD and healthy calves, although calves were limit-fed on many farms and the intakes for all calves were lower compared to calves in the present study. In contrast, Borderas et al. (2009) observed a trend for ill calves fed a high milk allowance to reduce their milk intake 2 d prior and the d of illness detection. However, Borderas et al. (2009) combined gastrointestinal and respiratory illness into one overall category and compared ill calves to healthy, making it difficult to compare with the present study. Alarms on automated calf feeders are designed to detect reductions in milk intakes. Therefore, because calves with clinical BRD and calves with subclinical BRD did not reduce their milk intake compared to calves without BRD, it is unlikely that a feeder alarm would identify calves with clinical or subclinical BRD. Our results suggest that milk intake is not a useful measure to identify calves with clinical BRD, nor subclinical BRD.

We observed that calves with subclinical BRD drank faster than calves with clinical BRD and similar to calves without BRD. Lethargy has been previously associated with clinical BRD (Cramer et al., 2016). Lethargic calves might not have the strength or desire to drink at faster speeds and, although we don't have data on lethargy in the present study, it is possible that calves with subclinical BRD had faster drinking speeds because they were not lethargic. Furthermore, sickness behaviors are thought to conserve energy for the febrile response to

illness, thus increasing chances of survival (Hart, 1988). It is possible that calves with subclinical BRD did not exhibit sickness behaviors, such as slower drinking speeds, because a smaller proportion of calves with subclinical BRD had a fever, compared to calves with clinical BRD. Similar drinking speeds between calves with subclinical BRD and calves without BRD suggest that subclinical BRD does not affect the calf's ability to drink at faster speeds.

Calves with clinical BRD in the present study tended to drink slower than calves without BRD over the 4 d period. Similarly, Knauer et al. (2017) reported that calves with clinical BRD drank slower than calves without clinical BRD on the day of illness detection. The drinking speeds reported by Knauer et al. (2017) were similar to our results for calves with clinical BRD, but calves without clinical BRD in the Knauer et al. (2017) study drank faster than both calves with subclinical BRD and calves without BRD in our study. Knauer et al. (2017) excluded calves with any treatment event (including treatments for diarrhea) from the control group, which is one possible reason for the faster drinking speeds compared to our study. Based on our results, drinking speed may be useful to identify calves with clinical BRD, but it may not be a suitable measure to identify calves with subclinical BRD.

We did not observe an effect of BRD status on rewarded visits, similar to previous studies (Johnston et al., 2016, Knauer et al., 2017). However, Knauer et al. (2017) found that calves with clinical BRD had fewer unrewarded visits on the d of treatment for BRD compared to healthy calves, whereas we observed no effect of BRD status on unrewarded visits. Calves in the present study had a median of 0 unrewarded visits, which was lower compared to Knauer et al. (2017; mean unrewarded visits: 7.5); the dissimilarity in the number of unrewarded visits is likely due to differences in milk allowance. High milk allowance has been shown to reduce the number of unrewarded visits (Jensen, 2006). Calves in the present study were offered *ad libitum*

milk access until 40 d of age, whereas Knauer et al. (2017) reported an average full feeding level of 9.4 L/d (range = 7 – 16). Therefore, varied milk allowances between studies might contribute to different findings between the relationship between BRD and the number of rewarded and unrewarded visits. Results from the present study suggest that rewarded and unrewarded visits are not ideal to identify calves with clinical or subclinical BRD.

The interpretation of results from the present study should consider the potential biases of our study design. We based BRD status on a calf's first BRD event after study enrollment. However we did not enroll calves until 21 d of age, but clinical BRD (Cramer and Stanton, 2015) and lung consolidation (Binversie, 2017) have been reported in calves younger than 3 weeks and at 7 d of age, respectively. Therefore, it is possible that calves we identified as normal were actually recovered from a BRD event prior to study enrollment. It is unlikely that changes in feeding behaviors from a BRD event prior to study enrollment would last through the study period, as Knauer et al. (2017) did not observe differences in feeding behaviors between calves with clinical BRD and controls after the d of BRD identification. However, we may have introduced selection bias as only the calves that survived until 21 d of age were eligible for study enrollment. The exclusion of calves that died prior to 21 d of age may have biased our results towards the null, as calves that died were likely most affected by disease. The exclusion of feeding behavior from calves < 21 d in general may also have biased our results towards the null. Young calves are less explorative than older calves (Færevik et al., 2010) and may not cope as well with illness compared to older calves; age may affect how calves express sickness behavior. A previous study observed an interaction between age and treatment (bacterial endotoxin versus control) for time spent inactive in dairy calves (Borderas et al., 2008), thus age can affect the

expression of sickness behavior. Additionally, we were unable to measure starter grain intake and therefore did not fully understand all feeding behavior.

Our results are most applicable to calves aged 21 to 40 d of age in group housing fed large quantities of milk. We encourage similar studies to explore changes in feeding behavior among calves with subclinical lung consolidation, clinical BRD, and without BRD in calves < 21 d of age. More research is needed to investigate if changes in feeding behavior occur in the period of time after BRD diagnosis, particularly for calves with subclinical lung consolidation, who are the most likely group of calves with BRD to go unidentified, and therefore untreated. Further research is also needed to determine if the severity of lung consolidation affects feeding behavior. More generally, research is needed to determine if and how subclinical lung consolidation affect calf welfare.

3.6 CONCLUSIONS

This was the first study to our knowledge to investigate if calves with subclinical lung consolidation, identified with lung ultrasound and clinical respiratory scoring, exhibit differences in feeding behavior from calves with clinical BRD or calves without BRD. Feeding behavior did not adequately differentiate between calves with SBRD, CBRD, and NOBRD. As such, dairy producers should be cautious when using automated feeder data as the primary means of detecting calves with BRD. Veterinarians and producers should consider implementing lung ultrasonography and clinical respiratory scoring systems in calf management programs to identify calves with all types of BRD.

3.7 ACKNOWLEDGEMENTS

This material is based upon work supported by funding from STGenetics (Navasota, Texas, United State, 77868). Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the view of

STGenetics. The authors wish to thank the staff and owners of the calf facility for allowing us to perform research and Ella Jackson and Hannah Donley for their assistance with data collection.

3.8 REFERENCES

Borderas, T. F., A. M. d. Passille, and J. Rushen. 2008. Behavior of dairy calves after a low dose of bacterial endotoxin. J. Anim. Sci. 86(11).

Borderas, T. F., J. Rushen, M. A. G. von Keyserlingk, and A. M. B. de Passillé. 2009. Automated measurement of changes in feeding behavior of milk-fed calves associated with illness. J. Dairy Sci. 92:4549-4554.

Buczinski, S., G. Forté, and A. Bélanger. 2013. Short communication: Ultrasonographic assessment of the thorax as a fast technique to assess pulmonary lesions in dairy calves with bovine respiratory disease. J. Dairy Sci. 96(7):4523-4528.

Buczinski, S., G. Forté, D. Francoz, and A. M. Bélanger. 2014. Comparison of thoracic auscultation, clinical score, and ultrasonography as indicators of bovine respiratory disease in preweaned dairy calves. J. Vet. Intern. Med. 28(1):234-242.

Buczinski, S., T. L. Ollivett, and N. Dendukuri. 2015. Bayesian estimation of the accuracy of the calf respiratory scoring chart and ultrasonography for the diagnosis of bovine respiratory disease in pre-weaned dairy calves. Prev. Vet. Med. 119(3):227-231.

Cramer, M., T. Ollivett, and A. Stanton. 2016. Associations of behavior-based measurements and clinical disease in preweaned, group-housed dairy calves. J. Dairy Sci. 99(9):7434-7443.

Dohoo, I., W. Martin, and H. Stryhn. 2003. Veterinary Epidemiologic Research. AVC Inc., University of Prince Edward Island, 550 University Avenue, Charlottetown, Prince Edward Island, CA.

Færevik, G., M. B. Jensen, and K. E. Bøe. 2010. The effect of group composition and age on social behavior and competition in groups of weaned dairy calves. J. Dairy Sci. 93(9):4274-4279.

Hart, B. L. 1988. Biological basis of the behavior of sick animals. Neurosci. Biobehav. Rev. 12(2):123-137.

Huzzey, J., D. Veira, D. Weary, and M. Von Keyserlingk. 2007. Prepartum behavior and dry matter intake identify dairy cows at risk for metritis. J. Dairy Sci. 90(7):3220-3233.

Jensen, M. B. 2006. Computer- controlled milk feeding of group-housed calves: The effect of milk allowance and weaning type. J. Dairy Sci. 89(1):201-206.

Johnston, D., D. A. Kenny, M. McGee, S. Waters, A. Kelly, and B. Earley. 2016. Electronic feeding behavioural data as indicators of health status in dairy calves. Irish Journal of Agricultural and Food Research 55(2):159-168.

Knauer, W. A., S. M. Godden, A. Dietrich, and R. E. James. 2017. The association between daily average feeding behaviors and morbidity in automatically fed group-housed preweaned dairy calves. J. Dairy Sci. 100(7):5642-5652.

Lago, A., S. M. McGuirk, T. B. Bennett, N. B. Cook, and K. V. Nordlund. 2006. Calf respiratory disease and pen microenvironments in naturally ventilated calf barns in winter. J. Dairy Sci. 89:4014-4025.

McGuirk, S. M. 2008. Disease management of dairy calves and heifers. Vet. Clin. of North Am. Food Anim. Pract. 24(1):139-153.

Ollivett, T. 2014. Understanding the diagnosis and risk factors for respiratory disease in dairy calves. in Department of Population Medicine. Vol. PhD. University of Guelph, Guelph, Ontario, Canada.

Ollivett, T., J. Caswell, D. Nydam, T. Duffield, K. Leslie, J. Hewson, and D. Kelton. 2015. Thoracic Ultrasonography and Bronchoalveolar Lavage Fluid Analysis in Holstein Calves with Subclinical Lung Lesions. J. Vet. Intern. Med. 29(6):1728-1734.

Ollivett, T. L. and S. Buczinski. 2016. On-Farm Use of Ultrasonography for Bovine Respiratory Disease. Vet. Clin. North Am. Food Anim. Pract. 32(1):19-35.

Sivula, N. J., T. R. Ames, W. E. Marsh, and R. E. Werdin. 1996. Descriptive epidemiology of morbidity and mortality in Minnesota dairy heifer calves. Prev. Vet. Med. 27:155-171.

Svensson, C. and M. B. Jensen. 2007. Short communication: Identification of diseased calves by use of data from automatic milk feeders. J. Dairy Sci. 90(2):994-997.

USDA. 2010. Heifer calf health and management practices on U.S. dairy operations, 2007. USDA: APHIS: VS,CEAH, Fort Collins, CO.

3.9 Tables

Table 3.1. Proportion (% (n)) of calves in each category of sex, breed, supplemented milk replacer type, diarrhea, and fever, by BRD status[1]. Observations of diarrhea and fever were limited to the 4 d of feeding behavior observations. Calves (n = 133) were enrolled in the study at 21 ± 6 (mean ± SD) d of age and underwent twice weekly health exams.

	BRD Status		
Variable	CBRD (n = 39)	SBRD (n = 81)	NOBRD (n = 13)
Sex			
Male	85% (33)	73% (59)	77% (10)
Female	15% (6)	27% (22)	23% (3)
Breed			
Holstein	77% (30)	83% (67)	69% (9)
Jersey	23% (9)	17% (14)	31% (4)
Supplemented Milk Replacer Type[2]			
Medicated	95% (37)	73% (59)	54% (7)
Celmanax	5% (2)	27% (22)	46% (6)
Diarrhea[3]			
With	28% (11)	15% (2)	10% (8)
Without	72% (28)	85% (11)	90% (73)
Fever[4]			
With	51% (20)	30% (24)	0 (0)
Without	49% (19)	70% (57)	100% (13)

1- Bovine respiratory disease status; Researchers performed twice weekly health exams, which included a clinical respiratory score (CRS; - or +; calculated with nasal, eye, ear, cough, and rectal temperature scores; McGuirk, 2008) and a lung ultrasound (Ollivett et al., 2016). BRD status for each calf was defined as SRBD (subclinical BRD; calves with lung consolidation ≥ 1cm^2 and CRS-;) or CBRD (clinical BRD; CRS+ with or without lung consolidation), based on the first BRD event. Calves with NOBRD never had lung consolidation ≥ 1cm^2 or CRS+.

2- The farm added milk replacer (32 g milk replacer per 1 L whole milk) in the calf feeders to supplement the whole milk. From February to April, calves were supplemented with medicated 22:20 milk replacer that contained 1600 g/ton neomycin sulfate and 1600 g/ton oxytetracycline (Medicated; Renaissance Nutrition Inc., Roaring Spring, PA, USA). From May through August, the farm supplemented calves with a 22:20 milk replacer (Renaissance Nutrition Inc., Roaring Spring, PA, USA) that contained Celmanax (Arm & Hammer Animal Nutrition).

3- Defined as fecal score ≥ 2: 0 = normal fecal consistency; 1 = semi-formed, pasty; 2 = loose but stays on top of bedding; 3 = watery, sifts though bedding; Mcguirk, 2008)

4- Rectal temperature ≥ 39°C

Table 3.2. Raw values for feeding behaviors, by BRD status[1] for the 3 d prior and day of BRD diagnosis. Calves (n = 133) were enrolled in the study at 21 ± 6 (mean ± SD) d of age and underwent twice weekly health exams.

	BRD Status		
Feeding behavior	CBRD (n = 39)	SBRD (n = 81)	NOBRD (n = 13)
Average daily drinking speed (mL/min; mean ± SD)	720 ± 233	823 ± 205	801 ± 267
Total daily milk intake (L/d; mean ± SD)	9.3 ± 2.9	10.9 ± 3.8	9.2 ± 3.6
Average meal size (L/meal; mean ± SD)	1.6 ± 0.8	1.8 ± 0.8	1.4 ± 0.8
Number of rewarded visits (no./d; median; 1st quartile, 3rd quartile)	6 (4, 9)	7 (5, 9)	7 (5, 11)
Number of unrewarded visits (no./d; median; 1st quartile, 3rd quartile)	0 (0, 3)	0 (0, 1)	0 (0, 1)

Bovine respiratory disease status; Researchers performed twice weekly health exams, which included a clinical respiratory score (CRS; - or +; calculated with nasal, eye, ear, cough, and rectal temperature scores; McGuirk, 2008) and a lung ultrasound (Ollivett et al., 2016). BRD status for each calf was defined as SRBD (subclinical BRD; calves with lung consolidation ≥ 1cm^2 and CRS-;) or CBRD (clinical BRD; CRS+ with or without lung consolidation), based on the first BRD event. Calves with NOBRD never had lung consolidation ≥ 1cm^2 or CRS+.

Table 3.3. Multivariable linear regression model for drinking speed (mL/min) collected from an automated milk feeder in 133 preweaned dairy calves. Calves were enrolled in the study at 21 ± 6 (mean ± SD) d of age and underwent twice weekly health exams. This model contains BRD status[1] as the predictor of interest, included calf as a random effect, and day relative to BRD diagnosis as a repeated measure. Drinking speed was analyzed for 3 d prior to and the d of BRD diagnosis[2]. Estimates for drinking speed and reported *P* – values are from the solutions for fixed effects.

Variable	Drinking speed estimate (mL/min)	Standard Error	*P* - value
Intercept	743.8	38.1	<0.0001
BRD Status			
CBRD	-103.9	35.5	0.004
SBRD	-4.0	54.2	0.94
NOBRD	Referent		
Sex			
Female	-69.0	38.8	0.08
Male	Referent		
Breed			
Holstein	117.3	40.7	0.004
Jersey	Referent		

1- Bovine respiratory disease status; Researchers performed twice weekly health exams, which included a clinical respiratory score (CRS; - or +; calculated with nasal, eye, ear, cough, and rectal temperature scores; McGuirk, 2008) and a lung ultrasound score (0 to 5, based on severity of lung consolidation; Ollivett et al., 2016). BRD status for each calf was defined as SRBD (subclinical BRD; calves with lung consolidation ≥ 1cm^2 and CRS-;) or CBRD (clinical BRD; CRS+ with or without lung consolidation), based on the first BRD event. Calves with NOBRD never had lung consolidation ≥ 1cm^2 or CRS+.

2- Feeding behavior data were collected automatically during the 3 d before and the day of diagnosis (d 0; 4 d total) for SBRD and CBRD calves; for NOBRD calves, the mean age of all calves with SBRD and CBRD (31±8 d old) was used for day 0.

Table 3.4. Multivariable linear regression model for milk intake (L/d), collected from an automated milk feeder in 133 preweaned dairy calves. Calves were enrolled in the study at 21 ± 6 (mean ± SD) d of age and underwent twice weekly health exams. This model contains BRD status[1] as the predictor of interest, included calf as a random effect, and day relative to BRD diagnosis as a repeated measure. Drinking speed was analyzed for 3 d prior to and the d of BRD diagnosis[2]. Estimates for milk intake and reported *P* – values are from the solutions for fixed effects.

Variable	Drinking speed estimate (mL/min)	Standard Error	*P* - value
Intercept	7.4	0.8	<0.0001
BRD Status			
CBRD	0.9	0.9	0.32
SBRD	1.9	0.8	0.02
NOBRD	Referent		
Breed			
Holstein	1.2	0.6	0.03
Jersey	Referent		
Supplemented milk replacer type			
Celmanax	1.9	0.6	0.0009
Medicated	Referent		

1- Bovine respiratory disease status; Researchers performed twice weekly health exams, which included a clinical respiratory score (CRS; - or +; calculated with nasal, eye, ear, cough, and rectal temperature scores; McGuirk, 2008) and a lung ultrasound (Ollivett et al., 2016). BRD status for each calf was defined as SRBD (subclinical BRD; calves with lung consolidation ≥ 1cm^2 and CRS-;) or CBRD (clinical BRD; CRS+ with or without lung consolidation), based on the first BRD event. Calves with NOBRD never had lung consolidation ≥ 1cm^2 or CRS+.

2- Feeding behavior data were collected automatically during the 3 d before and the day of diagnosis (d 0; 4 d total) for SBRD and CBRD calves; for NOBRD calves, d 0 was the mean age of all calves with SBRD and CBRD (31±8 d old).

4 CHAPTER 4: BEHAVIORAL ATTITUDE SCORES ASSOCIATED WITH BOVINE RESPIRATORY DISEASE IDENTIFIED USING CALF LUNG ULTRASOUND AND CLINICAL RESPIRATORY SCORING

M. C. Cramer*, K. L. Proudfoot†, and T. L. Ollivett‡[1]

*Department of Dairy Science, University of Wisconsin, Madison, 53706

†Department of Veterinary Preventive Medicine, The Ohio State University, Columbus 43210

‡Department of Medical Sciences, School of Veterinary Medicine, University of Wisconsin, Madison 53706

4.1 ABSTRACT

Behavioral research to date has focused on using clinical signs to define bovine respiratory disease (**BRD**). However, BRD can occur without clinical signs and little is known about the behavior of calves with subclinical BRD. The objectives of this cohort study were to determine if calves diagnosed with three categories of BRD exhibit differences in behavioral attitude scores on the day of diagnosis. Preweaned dairy calves (n = 280; 21 ± 6 d) were enrolled at entry into a group-housed automated milk feeder barn. Twice weekly health exams included a clinical respiratory score (CRS; - or +), a lung ultrasound to identify consolidation, and an attitude score (0 = normal: bright, alert responsive; or 1 = depressed: dull but responds to stimulation or depressed, slow to stand or reluctant to lie down). BRD status for each calf was defined as SBRD ('subclinical BRD' calves with lung consolidation ≥ 1cm^2 and CRS-; n = 164) or CBRD ('clinical BRD' CRS+ with or without lung consolidation; n = 79), based on their first BRD event. Calves with NOBRD (n = 37) never had lung consolidation ≥ 1cm^2 or CRS+. For calves with SBRD and CBRD, attitude scores on the day of BRD diagnosis were used in the analysis. We determined the mean age of all calves with SBRD and CBRD (31 ± 8 d old), and for the NOBRD calves selected the attitude score from the day that most closely corresponded with this mean age. A mixed logistic model (PROC GLIMMIX) was used to determine if BRD status was associated with attitude score (0 or 1; outcome). Depressed attitude scores were identified in 23% of calves with CBRD, 6% with SBRD, and 0% with NOBRD. Calves with CBRD were 5.2 (95% CI: 1.1 – 23.7) times and 4.5 (95% CI: 2.0 – 10.4) times more likely to have a depressed attitude score compared to calves with NOBRD and SBRD, respectively. There was no difference in the odds of having a depressed attitude score between calves with SBRD and NOBRD. The sensitivity and specificity of the attitude score to identify calves with CBRD were 23% and 95%, respectively. Attitude score did not differentiate between calves

with SBRD and calves with NOBRD, but did differentiate between calves with CBRD and calves with NOBRD. Because the sensitivity for the attitude score was not ideal and it failed to detect calves with SBRD, producers should be cautious using this attitude score as the primary means of detecting calves affected by BRD.

Key words: automated calf feeder, calf lung ultrasound, pneumonia, welfare

4.2 INTRODUCTION

Bovine respiratory disease (**BRD**) is the second most common cause of morbidity (affects 12% of dairy calves) and accounts for almost one-third of preweaned calf mortality in the United States, according to producer-reported data (USDA, 2010). Early disease detection, and therefore early intervention, likely ameliorates the negative consequences of BRD and improves calf welfare. However, producers and veterinarians struggle to identify all calves with BRD (Sivula et al., 1996, Buczinski et al., 2015, Cramer et al., 2016). Detecting sick calves in group housing is additionally challenging due to the difficulty of observing individual animals and our limited knowledge of how sick calves behave in a social environment.

Sickness behavior, which refers to behavioral changes that accompany illness (Hart, 1988), have recently been linked with changes in automated feeding behaviors in dairy calves (Svensson and Jensen, 2007, Borderas et al., 2009, Knauer et al., 2017). Behavioral changes that can quickly be observed by calf caretakers have also been assessed for their usefulness to detect BRD (Cramer et al., 2016).

Research using calf behavior to accurately detect BRD is lacking, and little is known about the behavior of calves with subclinical BRD. Previous studies have used clinical detection tools to identify calves with BRD which included visual observations and lung auscultation (Svensson and Jensen, 2007, Borderas et al., 2009), or the Wisconsin Calf Respiratory Scoring chart (McGuirk, 2008, Cramer et al., 2016, Knauer et al., 2017) to define BRD. However, lung auscultation and BRD scoring systems that rely solely on clinical signs lack sensitivity (Buczinski et al., 2014). The limitations of clinical BRD detection methods preclude our ability to accurately identify all calves with BRD, including subclinical BRD (lung consolidation, but no visible signs of disease), which can range from 23 to 67% (Ollivett and Buczinski, 2016).

Calf lung ultrasound can accurately detect lung consolidation, regardless of clinical status (Ollivett and Buczinski, 2016). The sensitivity and specificity of ultrasound are high (94% and 100%, respectively) for detecting subclinical lung consolidation (Ollivett et al., 2015). The advantages of calf lung ultrasound allow us to accurately detect BRD. We can then determine if behavioral changes differ between types of BRD. Lung ultrasound can be combined with a clinical scoring system to ensure accurate identification of BRD.

Understanding how behavior differs among calves with clinical BRD, subclinical BRD and unaffected calves can provide insight into how BRD affects animal welfare and inform management strategies. Additionally, changes in automated feeding behaviors have been associated with BRD (e.g., Knauer et al., 2017). However, these systems require more validation and are not available on all dairies. Thus, there is a need for a BRD detection tool using observations that can be performed quickly by calf caretakers. The Wisconsin Calf Health Scoring App includes a four-level attitude score for use when performing health examinations on calves. This attitude score might offer an additional way to find sick calves, be more financially feasible on more dairies, and require less calf handling. Therefore, our objectives of this cohort study were to determine if calves with BRD, diagnosed by lung ultrasound and clinical scoring, exhibit differences in behavioral attitude scores on the day of disease.

4.3 MATERIALS AND METHODS

Data collection for this cohort study took place in Ohio, USA between February and August 2016 on a dairy cattle facility that raised heifer and bull calves and freshened only nulliparous heifers. The Institutional Animal Care and Use Committee at the University of Wisconsin - Madison (A005049 - A03) approved this study.

Animal housing, management, and feeding before study enrollment

Calves were separated from their dam within 30 min to 1 h of birth and received 4L of pasteurized maternal colostrum within 3 h of birth or colostrum replacer when adequate quality colostrum (BRIX ≥ 22%) was unavailable. Farm staff recorded birth weight and calving ease score for all calves. The calving ease score ranged from 1 - 5 (1 = no assistance, 2 = minor assistance, 3 = hard pull, 4 = mechanical assistance and hard pull, 5 = surgical delivery). Calves were housed in straw bedded individual pens 1 of 2 identical barns for approximately 3 wk before entering an automated calf feeder barn. Calves were fed 5L whole milk or milk replacer twice daily by bucket while housed individually.

During the pre-enrollment phase of the study (when calves were less than approximately 21 d of age), calves were monitored daily and all treatments were recorded by farm staff on paper treatment records. Farm staff defined a diarrhea event as extremely watery feces during at least 2 consecutive feedings. Farm staff defined a respiratory event as a calf with labored breathing, cough, and droopy ears.

Calf enrollment and housing

Enrollment for this study occurred from February to July 2016. Calves were enrolled into the study upon entry to an automated calf feeder barn at approximately 21 ± 6 d of age. Calves were included in the study if they entered the automated calf feeder barn by 30 d of age. The automated calf feeder barn had 2 identical sides with 4 group pens on each side for a total of 8 pens; each pen measured 3.4m x 18.3m. Pens were bedded with straw from February to March and a combination of shavings and sand from April through August. The automated calf feeder barn was naturally ventilated with curtain sidewalls and supplemental positive pressure tube ventilation. Calves had nose-to-nose contact between pens.

Four automated calf feeders (Lely Calm calf feeder, Lely North America, Pella, IA, USA) served the 8 pens with 1 nipple provided per pen. Calves were allowed *ad libitum* milk access until 40 d of age, from 41 to 46 d of age calves were stepped up from 8 to 9.7 L per d, from 47 to 56 d of age calves were stepped down from 9.7 to 9 L per d, from 57 to 70 d of age calves were gradually weaned from 9 to 2 L per d, and weaned completely at 71 d of age. The age of the calf was entered into the automated feeding system to ensure the feeding program was adjusted for calf age. Whole milk from early lactation cows, purified using ultraviolet light (UVPure calf milk purifier, GEA Group Aktiengesellschaft, Düsseldorf, Germany), was the predominate source of liquid feed in the automated calf feeder barn. The farm chose to supplement this whole milk with milk replacer (32 g milk replacer per 1 L whole milk). More specifically, from February to April, calves were supplemented with medicated 22:20 milk replacer that contained 1600 g/ton neomycin sulfate and 1600 g/ton oxytetracycline (Renaissance Nutrition Inc., Roaring Spring, PA, USA). From May through August, the farm supplemented calves with a 22:20 milk replacer (Renaissance Nutrition Inc., Roaring Spring, PA, USA) that contained Celmanax (Arm & Hammer Animal Nutrition).

Health and attitude score data collection

Research staff performed health examinations on enrolled calves twice weekly until 50 d of age. Health examinations were recorded using the Wisconsin Calf Health Scorer Application (https://www.vetmed.wisc.edu/dms/fapm/apps/chs.htm) and included a clinical respiratory score (**CRS**; Lago et al., 2006, McGuirk, 2008), a 6 level lung ultrasound score **(**Ollivett and Buczinski, 2016), and fecal (0 = normal fecal consistency; 1 = semi-formed, pasty; 2 = loose but stays on top of bedding; 3 = watery, sifts though bedding), navel, and joint scores (McGuirk, 2008). Briefly, the CRS assigned 0 (normal) to 3 (severely abnormal) points for each of the

following categories: nasal discharge, eye discharge, ear position, cough, and rectal temperature. Calves were considered positive for clinical respiratory disease (CRS+) when 2 categories with a score of 2 or greater were observed. Diarrhea status was defined as fecal score ≥ 2. The application also included an attitude score (0 = bright, alert responsive; 1 = dull but responds to stimulation; 2 = depressed, slow to stand or reluctant to lie down; 3 = unresponsive to stimulation). One researcher assigned an attitude score for each calf before health examinations began. If a calf was lying down at the beginning of the health examination, it was stimulated to rise using the following actions, in order: vocal cue, light touch on the back, or vigorously running hands up and down the spine. If a calf was standing at the beginning of the health examination, stimulation included a vocal cue or a light touch on the hindquarters to guide the calf to the corner of the pen, where scoring took place. Three research staff members performed the CRS; one experienced researcher trained two research staff members on how to properly score calves using the CRS prior to the study. Inter-observer agreement for CRS status (CRS+ versus CRS-) was determined prior to the study (kappa = 0.6) and halfway through the study (kappa = 0.7). One researcher performed all ultrasound exams.

For the ultrasound score, a portable linear rectal ultrasound set at a depth of 9 cm, frequency of 6.2 MHz, and gain of 23 dB (Near 13 dB; Far 36 dB; Ibex Pro, E.I. Medical, Loveland, CO, USA) was used. Approximately 150mL 70% isopropyl alcohol was applied to the hair as a transducing agent. The left and right lungs were scanned in a systematic manner using a technique previously described in dairy calves (Ollivett et al., 2015, Ollivett and Buczinski, 2016). During the ultrasound exam, 5 lung lobes (right middle lung lobe, caudal and cranial aspects of the right cranial lung lobe, and the caudal and cranial aspects of the left cranial lung lobe) were examined and considered separate lung lobes for the scoring system (Ollivett

and Buczinski., 2016). The ultrasound scoring system ranged from 0 to 5 (0 = normal or ≤ 1cm^2 consolidation; 1 = diffuse comet tails; 2 = lobular pneumonia: consolidation ≥ 1 cm^2; 3 = lobar pneumonia, 1 entire lung lobe consolidated; 4 = lobar pneumonia, 2 entire lung lobes consolidated; 5 = lobar pneumonia, ≥ 3 entire lung lobes consolidated). Lung characteristics such as necrosis, abscessation, or pleural effusion were also noted.

BRD definitions

Three categories of BRD status were defined: clinical BRD (**CBRD**): CRS+ at their first BRD event, regardless of ultrasound score, subclinical BRD (**SBRD**): lung consolidation ≥ 1cm^2 and CRS– at their first BRD event, or without BRD (**NOBRD**): never CRS+ and never had lung consolidation ≥ 1cm^2 throughout the study.

All CRS+ calves were treated with an antibiotic according to a standardized treatment protocol developed in cooperation with the farm management and farm veterinarians. Calves identified with SBRD at their first BRD event were not treated with an antibiotic; these calves may have later received an antibiotic if they were subsequently identified as CRS+. Research staff administered treatments for the first 2 CRS+ events to ensure consistency and accuracy of treatment records. Ceftiofur crystalline free acid (6.6 mg/ kg subcutaneous at the base of the ear once; Excede; Zoetis Services LLC., Parsippany, NJ, USA) and gamithromycin (6 mg/ kg subcutaneous once; Zactran; ©2017 Merial, Inc., Duluth, GA, USA) were the first and second line treatments, respectively. Calves were not re-treated with an antibiotic within 48 h of their first antibiotic. After 48 h, they received a second antibiotic if they were CRS+ again. For any subsequent CRS+ events, the farm staff was responsible for administering treatments as defined by the farm protocol. Flunixin meglamine (1.1 mg/ kg once intravenous; Vetameg; Aspen

Veterinary Resources, LTD, Greeley, CO, USA) was administered to calves with a rectal temperature ≥ 39.4°C and when the calf was depressed, slow to stand, or reluctant to lie down.

Statistical analysis

All data were stored, cleaned, and analyzed using Microsoft Excel (Microsoft, Redmond, WA) and SAS (version 9.3; SAS Institute Inc., Cary, NC). Data cleaning consisted of checking the data for entry errors and compiling the data for analyses. Attitude scores collected on the day of diagnosis for CBRD and SBRD calves were used for the analyses. We determined the mean age of all calves with SBRD and CBRD (31 ± 8 d old), and for the NOBRD calves selected the attitude score from a health examination day that most closely corresponded with this mean age. A one-way ANOVA was used to ensure that age at the attitude score used for analysis was not different between BRD categories. Calves that entered the automated calf feeder barn at ≥ 30 d of age were not included in final analyses. Calves with NOBRD had to remain in the automated feeder barn until 50 d of age in order to be included in the final analysis; this was to ensure that all calves had an equal opportunity to develop disease.

The experimental unit was the calf. The outcome of interest was the attitude score on the day of BRD diagnosis, or the attitude score on the age-equivalent day for NOBRD calves. Our objective was to determine if BRD status (CBRD, SBRD, and NOBRD) influence attitude score. As such, the outcome was attitude score and the predictor of interest was BRD status. No calves were identified with an attitude score of 3 at their first BRD event and only 5 calves (CBRD) had an attitude score of 2 at their first BRD event. As this would result in inadequate power to determine differences between the BRD categories within an attitude score of 2, we chose to collapse the attitude score into a dichotomous variable (‘normal attitude’ = 0 and ‘depressed

attitude' = 1). A Kruskal Wallis test was used to ensure there was no difference in the proportion of calves with each BRD category in attitude scores of 1 versus 2 prior to collapsing, ($P = 0.87$).

Associations between explanatory variables and attitude score were evaluated using univariable analyses (PROC GLIMMIX in SAS). A variable was considered a potential confounder if it met criteria as described by Dohoo et al. (2003); significant association at the univariable level was defined as alpha < 0.20 level. Backwards stepwise elimination was used to select variables for the final model to include only variables that were significant at alpha ≤ 0.05 level. Prior to the final elimination of a variable, a change in estimate criterion of ≥ 30% for the predictor of interest was used to detect confounding. All potential interactions with attitude score and the remaining variables in the model were assessed and interactions with $P > 0.05$ were removed.

One mixed logistic model (PROC GLIMMIX) was used to determine if BRD status was associated with attitude score. Explanatory variables included dystocia (dichotomized into: no assistance or easy pull = 0; hard pull or surgical delivery = 1), season of birth (calves born on or before February 29th, 2016= 'winter'; calves born between March 1st and April 30th, 2016= 'spring'; calves born on or after May 1st, 2016= 'summer'), and diarrhea at the time of BRD diagnosis (for CBRD and SBRD calves) or at the time of attitude score used for NOBRD calves (dichotomized into: fecal score ≤ 1 = 0; fecal score ≥ 2 = 1).

The initial multivariable model included BRD status, dystocia, season, and diarrhea as fixed effects ($P < 0.20$ at the univariable level). The final model only included BRD status. Predicted probabilities were assessed using the LSMEANS statement and differences between BRD statuses were assessed using differences in LSMEANS. Type 3 tests of fixed effects were used to determine significance at the alpha ≤ 0.05 level. Measures of central tendency for raw

data are presented as mean ± SD or median (1st quartile, 3rd quartile). Estimates from multivariable models are presented as mean ± SE.

A BRD category that was associated with the attitude score was further analyzed to determine the test characteristics. Sensitivity and specificity of the attitude score were calculated as described by Dohoo et al. (2003). The health examinations (BRD status) were the 'gold standard'. Attitude score was treated as the exposure. Sensitivity was calculated as: (number of calves with BRD who had a depressed attitude)/(total number of calves with BRD) x 100. Specificity was calculated as: (number of calves without BRD who had a normal attitude)/(total number of calves without BRD) x 100.

4.4 RESULTS

Of the 835 calves that were born on the facility during the study period, farm records indicated that 259 calves were removed at less than 30 d of age and therefore prior to the age criteria for study enrollment (n = 211 were sold or sent off-site; n = 48 died). The farm elected not to send 268 calves through the automated calf feeder barn due to a lack of space in the barn. A total of 308 calves entered the automated calf feeder barn and were examined for study eligibility. However, 9% (28/308) of calves did not meet the enrollment age criteria for study eligibility, as they entered automated calf feeder barn at 40 d of age. Therefore, final analysis included 280 observations representing 280 calves.

Calves were 21 ± 6 d (mean ± SD) at entry to the automated calf feeder barn and were housed in groups of 13 ± 3. Calf descriptive information and the proportion of calves with diarrhea and fever at the time of BRD diagnosis are presented in Table 4.1. Twenty-eight percent (79/280) and 59% (164/280), of calves were identified with CBRD and SBRD at their first BRD event, respectively. Thirteen percent (37/280) of calves were never identified with CBRD or SBRD and were therefore categorized as NOBRD. The age (mean ± SD) of calves

with NOBRD (33 ± 5 d), calves with CBRD (27 ± 9 d), and calves with SBRD (30 ± 8 d) on the day of the health examination from which the attitude score was collected was not different between BRD groups ($P = 0.73$). The proportion of calves with normal and depressed attitude scores are presented in Table 4.2.

The multivariable model showed an effect of BRD status on the probability of having a depressed attitude score ($P = 0.0008$). Calves with CBRD were 5.2 (95% CI: 1.1 – 23.7) times and 4.5 (95% CI: 2.0 – 10.4) times more likely to have a depressed attitude score compared to calves with NOBRD ($P = 0.0350$) and calves with SBRD ($P = 0.0004$), respectively. There was no difference in the odds of having a depressed attitude score between calves with SBRD and NOBRD ($P = 0.87$). The mean probability of having a depressed attitude, based on BRD status, is depicted in Figure 4.1.

The sensitivity and specificity of the attitude score to identify calves with CBRD were 23% and 95%, respectively (Table 4.3).

4.5 DISCUSSION

To our knowledge, this cohort study was the first to investigate if a behavioral attitude score was associated with BRD, using both calf lung ultrasound and clinical respiratory scoring for BRD diagnosis. Calves with clinical BRD were more likely to have a depressed attitude score compared to both calves with subclinical BRD and without BRD, suggesting that clinical BRD affects calf behavior. Calves with subclinical BRD did not differ in attitude score compared to unaffected herdmates. Although calves with clinical BRD were more likely to have depressed attitude scores, the attitude score had a poor sensitivity; the attitude score would only identify 23% of calves with CBRD if it was the only method used for detection. Therefore, we suggest caution when using this attitude score as the sole means for clinical BRD detection.

Furthermore, subclinical BRD was not associated with the attitude score, meaning the attitude score would not be useful to detect calves with SBRD.

Calves with clinical BRD were more likely to have a depressed attitude score compared to both calves with subclinical BRD and calves with no BRD. One potential explanation for this finding is the greater proportion of calves with fever in the clinical BRD group compared to calves with subclinical and calves with no BRD. Sickness behavior is a term used to describe the behavioral changes that accompany illness (Hart, 1988). It is hypothesized to conserve energy for the febrile response, which serves to increase survival when the animal is infected with a pathogen (Hart, 1988, Kluger, 1991, Johnson, 2002). Sickness behavior is mediated by cytokines, which are released during the host immune response to infection (Dantzer and Kelley, 2007). Previous studies in calves observed less exploratory behavior (Cramer and Stanton, 2015), rumination, hay eating, and self–grooming (Borderas et al., 2008) in response to disease or a lipopolysaccharide challenge.

The probability of a depressed attitude score was not different between calves with subclinical BRD and calves with no BRD. Our findings are supported by previous work in which calves with subclinical BRD had similar drinking speeds and number of visits to the feeder compared to calves with no BRD (Chapter 3). A smaller proportion of calves with subclinical BRD had fever compared to calves with clinical BRD or calves with no BRD. Because sickness behavior serves to conserve energy for the febrile response (Hart, 1988), it is likely that calves who did not have a fever did not express sickness behaviors such as depression and lethargy.

While our findings suggest subclinical BRD does not affect the calf from a behavior perspective, these limited findings are not sufficient to determine if subclinical BRD affects calf

welfare. It is possible that calves with subclinical BRD may experience pain or discomfort even if they do not show outward signs of disease. For example, substance P, a neuropeptide involved with the nervous system's response to painful stimuli, was found to be higher in calves inoculated with *M. haemolytica* compared to control calves (Theurer et al., 2013); all inoculated calves had lung consolidation observed at necropsy. In addition, humans report that pneumonia is a painful condition (Melbye et al., 1992). Producers should also be aware of calves with subclinical BRD, as they may transition to clinical BRD (Binversie, 2017). Thus, intervening when the calf has subclinical BRD may prevent a clinical BRD event.

The differences in attitude scores between calves with clinical BRD and calves with subclinical or no BRD may help inform the management and housing of ill calves. Millman (2007) suggested that compromised dairy cows have different behavioral priorities and needs compared to healthy cows. However, most pens on dairy farms are designed for healthy cattle and may exacerbate the challenges sick cattle face when competing for resources with their healthy pen mates (Millman, 2007). Eighty-two percent of producers surveyed in a study in Iowa reported they had the ability to house a sick calf away from her group (Fogsgaard et al., 2016); however, it is anecdotally recognized that sick calves in group housing are rarely moved to a hospital pen. It is possible that sick calves might be less successful when competing for resources such as feed, water, or lying space compared to healthy calves. Studies using adult dairy cattle have found that cows with lower success in competitive interactions at the feed bunk had greater nonesterified fatty acid and fecal cortisol metabolites compared to cows with greater success in competitive interactions (Huzzey et al., 2012). This suggests that cows with lower success in competitive interactions may be more likely to develop stress-related disease compared to those that are more successful at the feedbunk. Thus, leaving sick calves, who

might be less able to compete for resources, in a competitive environment could put them at risk for physiological stress and subsequent illness. Furthermore, Bach (2011) found that the number of BRD cases in newly weaned calves increased as the proportion of calves with a history of BRD in each pen increased, suggesting that leaving calves with BRD in their home pen puts healthy calves at risk for developing BRD. Therefore, it is possible that leaving sick calves in a pen with healthy calves puts sick at greater risk for stress or other diseases as well as increases the odds of healthy calves getting BRD. More research is warranted in this area.

The sensitivity of the attitude score to detect calves with clinical BRD was lower than ideal. For a screening tool, it would be best to have a sensitivity of 100% so that all truly sick calves are identified. However, most current BRD detection methods do not have a 100% sensitivity (Buczinski et al., 2014). Therefore, any new screening tool proposed should at least have a higher sensitivity than currently available methods, such as the Wisconsin clinical respiratory score (sensitivity = 62%), and calf lung ultrasound (sensitivity = 80%; Buczinski et al., 2014). A previously proposed behavior–based screening tool had a sensitivity of 48% (Cramer et al., 2016). We do not recommend the attitude score in the present study as the sole means of disease detection because other available methods of BRD detection have higher sensitivity, and would therefore be able to identify a greater proportion of calves that are truly sick. Bayesian analysis might be more useful to adjust for the pitfalls of the clinical respiratory score and lung ultrasound (Buczinski et al., 2015).

The sensitivity of the attitude score might have been low due to our use of thoracic ultrasound and the Wisconsin clinical respiratory score, which are designed for early detection of disease. During early disease, a depressed attitude might not occur and may have leading to more false negative calves. Additionally, the attitude score focused on a depressed attitude,

whereas other behaviors such as isolation from the group (e.g., Cramer et al., 2016) were not included in the present score. Including other behaviors in the attitude score may have improved our ability to correctly classify calves. Differences in BRD definitions and reference methods should be carefully considered when comparing diagnostic characteristics between studies. Despite lower sensitivity of our attitude score compared to other screening tools, the attitude score may be a beneficial addition to an existing disease detection program or to implement if no disease detection program is in place.

When interpreting these results, one should consider the potential biases of our study design. We based BRD status on a calf's first BRD event after study enrollment. However, calves were enrolled after 21 d of age; clinical BRD (Cramer and Stanton, 2015) and lung consolidation (Binversie, 2017) have been reported in calves younger than 3 wk and at 7 d of age, respectively. Therefore, it is possible that calves we identified as normal were actually recovered from a BRD event prior to study enrollment. A previous study found an effect of age on the expression of exploratory behavior (Cramer and Stanton, 2015). It is possible that behavior in younger calves is more severely affected compared to older calves. Therefore, we may have introduced selection bias; the exclusion of calves less than 21 d of age may have prevented our ability to find a difference between calves with subclinical BRD and unaffected herdmates. Furthermore, all BRD statuses were based on the first case of BRD identified. Consequently, we did not look at attitude changes based on duration of BRD. It is possible calves with subclinical BRD may have attitude changes later in the course of disease. The researcher that assigned the attitude score also performed some of the clinical scores and all of the ultrasound exams. This methodology could have biased our results in two ways. First, the attitude score could have influences the clinical or ultrasound score assigned by researcher,

potentially increasing the number of calves classified with BRD. Second, although the attitude score was performed prior to the health examination, it is possible that clinical signs (e.g. eye or nasal discharge) were noticeable prior to assigning the attitude score, which may have biased the attitude score.

Our results are most applicable to calves aged 21 to 40 d of age in group housing fed large quantities of milk. We encourage similar studies to explore changes in calf behavior among calves with subclinical BRD, clinical BRD, and unaffected calves less than 21 d of age. More research is needed to determine how different BRD categories affect calf welfare, how behavior changes in relationship to disease duration and severity, and how to improve detection of BRD on dairy farms.

4.6 CONCLUSION

To our knowledge, this was the first study to investigate differences in an attitude score between different categories of BRD, diagnosed using calf lung ultrasound and clinical respiratory scoring. Our attitude score did not differentiate between calves with subclinical BRD and calves with no BRD, suggesting subclinical BRD does not affect the probability of a calf having a depressed attitude. We observed an increased likelihood for depressed attitude scores in calves with clinical BRD, the sensitivity of the attitude score was less than desirable. Therefore, we do not recommend using this attitude score as the sole means of BRD detection. Veterinarians and producers should consider implementing calf lung ultrasound and clinical respiratory scoring tools in calf disease detection programs.

4.7 ACKNOWLEDGEMENTS

This material is based upon work supported by funding from STGenetics (Navasota, Texas, United States, 77868). Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the view of

STGenetics. The authors wish to thank the staff and owners of the calf facility for allowing us to perform research and Ella Jackson and Hannah Donley for their assistance with data collection.

4.8 REFERENCES

Bach, A., C. Tejero, and J. Ahedo. 2011. Effects of group composition on the incidence of respiratory afflictions in group-housed calves after weaning. J. Dairy Sci. 94(4):2001-2006.

Binversie, E. 2017. Using visual observations and thoracic ultrasonography to evaluate treatment success in preweaned dairy calves affected by respiratory disease. in Dairy Science. Vol. MSc. University of Wisconsin- Madison, Madison, WI.

Borderas, T. F., A. M. d. Passille, and J. Rushen. 2008. Behavior of dairy calves after a low dose of bacterial endotoxin. J. Anim. Sci. 86(11).

Borderas, T. F., J. Rushen, M. A. G. von Keyserlingk, and A. M. B. de Passillé. 2009. Automated measurement of changes in feeding behavior of milk-fed calves associated with illness. J. Dairy Sci. 92:4549-4554.

Buczinski, S., G. Forté, D. Francoz, and A. M. Bélanger. 2014. Comparison of thoracic auscultation, clinical score, and ultrasonography as indicators of bovine respiratory disease in preweaned dairy calves. J. Vet. Intern. Med. 28(1):234-242.

Buczinski, S., T. L. Ollivett, and N. Dendukuri. 2015. Bayesian estimation of the accuracy of the calf respiratory scoring chart and ultrasonography for the diagnosis of bovine respiratory disease in pre-weaned dairy calves. Prev. Vet. Med. 119(3):227-231.

Cramer, M., T. Ollivett, and A. Stanton. 2016. Associations of behavior-based measurements and clinical disease in preweaned, group-housed dairy calves. J. Dairy Sci. 99(9):7434-7443.

Cramer, M. and A. Stanton. 2015. Associations between health status and the probability of approaching a novel object or stationary human in preweaned group-housed dairy calves. J. Dairy Sci. 98(10):7298-7308.

Dantzer, R. and K. W. Kelley. 2007. Twenty years of research on cytokine-induced sickness behavior. Brain, Behav., Immun. 21(2):153-160.

Dohoo, I., W. Martin, and H. Stryhn. 2003. Veterinary Epidemiologic Research. AVC Inc., University of Prince Edward Island, 550 University Avenue, Charlottetown, Prince Edward Island, CA.

Fogsgaard, K. K., M. S. Herskin, P. J. Gorden, L. L. Timms, J. K. Shearer, and S. T. Millman. 2016. Management and design of hospital pens relative to behavior of the compromised dairy cow: A questionnaire survey of Iowa dairy farms. Appl. Anim. Behav. Sci. 175:50-55.

Hart, B. L. 1988. Biological basis of the behavior of sick animals. Neurosci. Biobehav. Rev. 12(2):123-137.

Huzzey, J., R. Grant, and T. Overton. 2012. Relationship between competitive success during displacements at an overstocked feed bunk and measures of physiology and behavior in Holstein dairy cattle. J. Dairy Sci. 95(8):4434-4441.

Johnson, R. W. 2002. The concept of sickness behavior: a brief chronological account of four key discoveries. Vet. Immunol. Immunopathol. 87:443-450.

Kluger, M. J. 1991. Fever: role of pyrogens and cryogens. Physiol Rev. 71(1):93-117.

Knauer, W. A., S. M. Godden, A. Dietrich, and R. E. James. 2017. The association between daily average feeding behaviors and morbidity in automatically fed group-housed preweaned dairy calves. J. Dairy Sci. 100(7):5642-5652.

McGuirk, S. M. 2008. Disease management of dairy calves and heifers. Vet. Clin. of North Am. Food Anim. Pract. 24(1):139-153.

Melbye, H., B. Straume, U. Aasebøs, and K. Dale. 1992. Diagnosis of Pneumonia in Adults in General Practice Relative Importance of Typical Symptoms and Abnormal Chest Signs Evaluated Against a Radiographic Reference Standard. Scand. J. Prim. Health Care 10(3):226-233.

Millman, S. T. 2007. Sickness behaviour and its relevance to animal welfare assessment at the group level. Anim. Welfare 16(2):123-125.

Ollivett, T., J. Caswell, D. Nydam, T. Duffield, K. Leslie, J. Hewson, and D. Kelton. 2015. Thoracic Ultrasonography and Bronchoalveolar Lavage Fluid Analysis in Holstein Calves with Subclinical Lung Lesions. J. Vet. Intern. Med. 29(6):1728-1734.

Ollivett, T. L. and S. Buczinski. 2016. On-Farm Use of Ultrasonography for Bovine Respiratory Disease. Vet. Clin. North Am. Food Anim. Pract. 32(1):19-35.

Sivula, N. J., T. R. Ames, W. E. Marsh, and R. E. Werdin. 1996. Descriptive epidemiology of morbidity and mortality in Minnesota dairy heifer calves. Prev. Vet. Med. 27:155-171.

Svensson, C. and M. B. Jensen. 2007. Short communication: Identification of diseased calves by use of data from automatic milk feeders. J. Dairy Sci. 90(2):994-997.

Theurer, M., D. Anderson, B. White, M. Miesner, D. Mosier, J. Coetzee, J. Lakritz, and D. Amrine. 2013. Effect of Mannheimia haemolytica pneumonia on behavior and physiologic responses of calves during high ambient environmental temperatures. J. Anim. Sci. 91(8):3917-3929.

USDA. 2010. Heifer calf health and management practices on U.S. dairy operations, 2007. USDA: APHIS: VS,CEAH, Fort Collins, CO.

4.9 Tables and Figures

Table 4.1. Proportion (% (n)) of calves in each category of dystocia and season of birth. by BRD status[1]. Proportion of calves with diarrhea and fever at the time of BRD diagnosis, by BRD status. Calves were enrolled in the study at 21 ± 6 (mean ± SD) d of age and underwent twice weekly health exams.

	BRD Status		
Variable	CBRD (n = 79)	SBRD (n = 164)	NOBRD (n = 37)
Dystocia[2]			
With	16% (13)	20% (32)	5% (2)
Without	84% (66)	80% (132)	95% (35)
Season of Birth[3]			
Winter	59% (47)	37% (61)	16% (6)
Spring	22% (17)	26% (42)	30% (11)
Summer	19% (15)	37% (61)	54% (20)
Diarrhea[4]			
With	28% (22)	13% (21)	14% (5)
Without	72% (57)	87% (143)	86% (32)
Fever[5]			
With	51% (40)	27% (44)	0% (0)
Without	49% (39)	73% (120)	100% (37)

1- Bovine respiratory disease status; Researchers performed twice weekly health exams, which included a clinical respiratory score (CRS; - or +; calculated with nasal, eye, ear, cough, and rectal temperature scores; McGuirk, 2008) and a lung ultrasound (0 to 5, based on severity of lung consolidation; Ollivett et al., 2016). BRD status for each calf was defined as SRBD (subclinical BRD; calves with any lung consolidation ≥ 1cm^2 and CRS-;) or CBRD (clinical BRD; CRS+ with or without lung consolidation), based on the first BRD event. Calves with NOBRD never had lung consolidation ≥ 1cm^2 or CRS+.

2- With = hard pull or surgery; Without = no assistance or easy pull

3- Calves born on or before February 29th, 2016= winter; calves born between March 1st and April 30th, 2016= spring; calves born on or after May 1st, 2016= summer

4- Diarrhea at the time of BRD diagnosis: defined as fecal score ≥ 2: 0 = normal fecal consistency; 1 = semi-formed, pasty; 2 = loose but stays on top of bedding; 3 = watery, sifts though bedding; Mcguirk, 2008); for calves with NOBRD fecal scores corresponding to the mean age of calves with CBRD and SBRD (31 d) were used.

5- Rectal temperature ≥ 39°C at the time of BRD diagnosis; for calves with NOBRD rectral temperatures from 31 d of age were used

Table 4.2. Proportion (% (n)) of calves with normal or depressed attitude scores at their first BRD event, by BRD status[1]. Calves were enrolled in the study at 21 ± 6 (mean ± SD) d of age and underwent twice weekly health exams.

	BRD Status		
Attitude Score[2]	CBRD (n = 79)	SBRD (n = 164)	NOBRD (n = 37)
Normal	77% (61)	94% (154)	95% (35)
Depressed	23% (18)	6% (10)	5% (2)

1- Bovine respiratory disease status; Researchers performed twice weekly health exams, which included a clinical respiratory score (CRS; - or +; calculated with nasal, eye, ear, cough, and rectal temperature scores; McGuirk, 2008) and a lung ultrasound (Ollivett et al., 2016). BRD status for each calf was defined as SRBD (subclinical BRD; calves with any lung consolidation ≥ 1cm^2 and CRS-;) or CBRD (clinical BRD; CRS+ with or without lung consolidation), based on the first BRD event. Calves with NOBRD never had lung consolidation ≥ 1cm^2 or CRS+.

2- Attitude score obtained from the Wisconsin Calf Health Scoring App; Normal= Bright, alert responsive; Depressed: dull but responds to stimulation; depressed, slow to stand or reluctant to lie down. We determined the mean age of all calves with SBRD and CBRD (31 ± 8 d old) and for the NOBRD calves, selected the attitude score from a health examination day that most closely corresponded with this mean age.

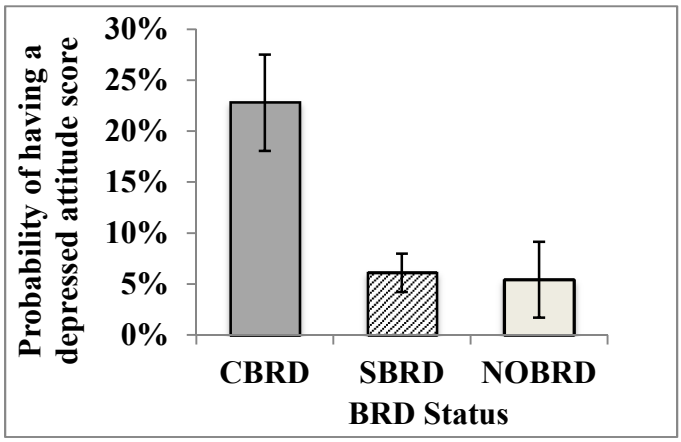
Table 4.3. A 2 x 2 contigency table for attitude scores[1] among calves with CBRD[2] and calves with NOBRD[2].

	Attitude Score	
BRD status	Depressed	Normal
CRBD	18	61
NOBRD	2	35

1- Attitude score obtained from the Wisconsin Calf Health Scoring App; Normal= Bright, alert responsive; Depressed: dull but responds to stimulation; depressed, slow to stand or reluctant to lie down. We determined the mean age of all calves with CBRD (31 ± 8 d old) and for the NOBRD calves, selected the attitude score from a health examination day that most closely corresponded with this mean age.

2- Researchers performed twice weekly health exams, which included a clinical respiratory score (CRS; - or +; calculated with nasal, eye, ear, cough, and rectal temperature scores; McGuirk, 2008) and a lung ultrasound (Ollivett et al., 2016). CBRD (clinical BRD; CRS+ with or without lung consolidation), based on the first BRD event. Calves with NOBRD never had lung consolidation ≥ 1cm^2 or CRS+.

Figure 4.1. The predicted probabilities (LSM ± SEM) of having a depressed attitude score[1], by BRD status[2] (*P* = 0.0008). Proc Glimmix in SAS (version 9.3; SAS Institute Inc., Cary, NC) was used.



1- Attitude score obtained from the Wisconsin Calf Health Scoring App; Normal= Bright, alert responsive; Depressed: dull but responds to stimulation; depressed, slow to stand or reluctant to lie down. We determined the mean age of all calves with SBRD and CBRD (31 ± 8 d old) and for the NOBRD calves, selected the attitude score from a health examination day that most closely corresponded with this mean age.

2- Bovine respiratory disease status; Researchers performed twice weekly health exams, which included a clinical respiratory score (CRS; - or +; calculated with nasal, eye, ear, cough, and rectal temperature scores; McGuirk, 2008) and a lung ultrasound (Ollivett et al., 2016). BRD status for each calf was defined as CBRD (n = 79; clinical BRD; CRS+ with or without lung consolidation) or SRBD (n = 64; subclinical BRD; calves with any lung consolidation ≥ 1cm^2 and CRS-;), based on the first BRD event. Calves with NOBRD (n = 37) never had lung consolidation ≥ 1cm^2 or CRS+.

5 CHAPTER 5: ASSOCIATIONS BETWEEN SEROTONIN AND BOVINE RESPIRATORY DISEASE, DIAGNOSED USING THORACIC ULTRASOUND, IN PREWEANED DAIRY CALVES- AN OBSERVATIONAL STUDY.

M. C. Cramer*, L. L. Hernandez*, S. R. Weaver*, and T. L. Ollivett†[1]

*Department of Dairy Science, University of Wisconsin, Madison, 53706

† Department of Medical Sciences, School of Veterinary Medicine, University of Wisconsin, Madison 53706

5.1 ABSTRACT

The objective of trial 1, a cohort study, was to determine if serum serotonin concentrations in 3 - 5 d old dairy calves were associated with the development of three categories of bovine respiratory disease (BRD) later in the preweaning period. Thirty-four calves from trial 1 were selected for enrollment into a case control study (trial 2). The objective of trial 2 was to describe differences in serotonin concentrations at the time of diagnosis between calves with and without BRD. For trial 1, blood samples were collected at 3 - 5 d of age (n = 194) to assess serum serotonin concentrations. Calves were then monitored for BRD using twice weekly health exams, which included a clinical respiratory score (**CRS**) and a lung ultrasound (score based on amount of lung consolidated) from 21 ± 6 d until 50 d of age. The first BRD event for each calf was defined by three BRD levels: clinical BRD (CBRD; CRS +), subclinical BRD (**SBRD**; CRS –, lung consolidation ≥ 1cm^2), or no BRD (**NOBRD**; never CRS +, never lung consolidation ≥ 1cm^2). For trial 2, blood samples to assess serum serotonin concentrations were taken from BRD + calves (n = 17) and from a matched pen-mate without BRD (n = 17). A multinomial logistic regression model was fit to determine if serotonin at 3 - 5 d of age was associated with BRD (outcome; CBRD, SBRD, or NOBRD; trial 1). A mixed linear regression model was fit to determine if serum serotonin concentrations (outcome) at the time of diagnosis were different between calves with and without BRD (trial 2). In trial 1, for every 1-unit increase in serum serotonin at 3 - 5 d of age, the odds of a calf developing SBRD versus NOBRD increased by 1.00 (95%CI: 1.00 - 1.001); the odds of a calf becoming SBRD versus CBRD tended to increase by 1.00 (95%CI: 1.00 - 1.001). There was no difference in the odds of developing CBRD compared to NOBRD. For trial 2, the serotonin concentration of calves with BRD was 779 ng/mL lower than calves without BRD (2707 ± 201 vs. 3487 ± 201, respectively). Serotonin at 3 - 5 d was associated with developing BRD later in the preweaning period and

serotonin concentrations differed between calves with BRD and matched unaffected controls. The relationship between serotonin and BRD should be further studied to determine the practicality of using serotonin in a farm setting and if serotonergic interventions can prevent BRD.

5.2 INTRODUCTION

Bovine respiratory disease (**BRD**) affects 12% of preweaned calves in the United States and accounts for 22% of all preweaned calf deaths, according to producer-reported data (USDA, 2010). Despite years of research regarding the risk factors for BRD, this disease continues to be problematic in dairy calves, leading to calf welfare concerns (Mellor and Stafford, 2004) and economic concerns for dairy producers (van der Fels-Klerz et al., 2001). The negative consequences of BRD drive the need to explore biomarkers that might indicate or influence a calf's susceptibility to BRD. Previous studies observed differences in concentrations of biomarkers such as haptoglobin (Godson et al., 1996) and serum amyloid-A (Kabu et al., 2016) between calves with and without respiratory disease at the time of diagnosis. However, a novel biomarker that is a both predictive of BRD later in life and can also help explain the immune response at the time of BRD might be useful to target for the management of BRD.

Serotonin, or 5- hydroxytryptamine is one such biomarker that might be associated with BRD in young dairy calves. Serotonin is a monoamine derived from L-tryptophan (Wang et al., 2002). Outside the brain (non-neuronal), serotonin is involved with pulmonary systems in several different species (Berger et al., 2009) and helps regulate breathing through effects on the brainstem (Berger et al., 2009). Additionally, plasma serotonin concentrations are elevated during hypoxia in humans with pulmonary artery hypertension (Kereveur et al., 2000). The relationship between serotonin and pulmonary function make serotonin an attractive biomarker to investigate in order to better understand the underlying mechanism of BRD in dairy calves.

In addition to serotonin's role in pulmonary function, serotonin has important roles in the immune system (reviewed by Ahern, 2011), which make it an attractive biomarker to evaluate as a risk factor for and detection of BRD. Mast cells express the serotonin reuptake transporter and are able to accumulate serotonin; this response is regulated by activation of complement, platelet activating factor, or IgE complexes (Matsuda et al., 1997, Gordon and Barnes, 2003). Dendritic cells take up serotonin from their microenvironment and from activated T-cells (O'Connell et al., 2006). Furthermore, dendritic cells modulate T-cell proliferation and differentiation through the uptake of serotonin at inflammatory sites in the body and shuttle serotonin to naïve T-cells (O'Connell et al., 2006). B-lymphocyte expression of the serotonin reuptake transporter is increased upon B- lymphocyte activation (Meredith et al., 2005). Additionally, serotonin stimulates phagocytosis by neutrophils (Herr et al., 2017), which are present during lung consolidation (Ollivett et al., 2015). Because serotonin plays a role in the modulation of the immune system (reviewed by Ahern, 2011), it is possible that serotonin is involved in the immune response during BRD.

Manipulation of the serotonergic axis might be useful in reducing the risk of BRD in dairy calves. In dairy calves, supplementation of colostrum and milk with the precursor to serotonin (5-hydroxy-L-tryptophan) increased blood mRNA abundance of factors involved with the innate and adaptive immune response (Hernández-Castellano et al., 2018). Furthermore, serotonin may be useful in the development of antimicrobial alternatives for BRD treatment.

Accurately measuring BRD in dairy calves is imperative to fully understand the relationship between BRD and serotonin. However, BRD in dairy calves can be difficult to accurately assess due to the prevalence of subclinical BRD (involves lung consolidation but no outward signs of disease), which can range from 23% to 67% (Ollivett and Buczinski, 2016).

Thoracic ultrasonography can accurately diagnose lung consolidation associated with BRD, including subclinical BRD (Buczinski et al., 2013, Ollivett, 2014). Estimates for the sensitivity and specificity of thoracic ultrasonography are 79.4% (95% CI: 66.4 - 90.9) and 93.9% (95%CI: 88.0 - 97.6), respectively. Therefore, utilizing thoracic ultrasonography in a study investigating BRD ensures accurate detection of BRD.

Improving our understanding of the relationship between serotonin and BRD, particularly when BRD is accurately measured using thoracic ultrasonography, might lead to management interventions that can improve calf health. Therefore, the first objective of this study was to determine if serum serotonin concentrations in 3 - 5 d old dairy calves were predictive of the development of three categories of BRD. The second objective was to describe differences in serotonin concentrations at the time of diagnosis between calves with and without BRD.

5.3 MATERIALS AND METHODS

Data collection for the two trials took place between February and August 2016 on a dairy facility in Ohio, USA. The Institutional Animal Care and Use Committee at the University of Wisconsin-Madison (A005049 - A03) approved this study.

Trial 1

Enrollment. Enrollment for trial 1 occurred from mid-February to July 2016. Calves were enrolled at 3 – 5 d when a blood sample was collected. Calves were housed individually during this time and farm staff monitored calves daily and recorded all treatments. Research staff monitored calves regularly for disease incidence after movement to a group housing barn, which contained automatic calf milk feeders. Calves were observed for disease incidence until 50 d of age, when follow up ended.

Animal management during sampling period. All calves were born from nulliparous dams as determined by the business model of this particular dairy. Calves were separated from

the dam within 30 minutes of birth and received 4L of pasteurized maternal colostrum within 3 hours of birth or colostrum replacer when adequate quality colostrum (BRIX ≥ 22%) was unavailable. Calves were housed individually in barns that contained straw bedded pens until 21 ± 6 d of age. Calves were fed 5L whole milk or milk replacer twice per d by bucket while housed individually.

Research staff collected jugular venous blood samples using a 20 g x 1 inch vacutainer needle and glass tube without anticoagulant (BD Vacutainer Precision Glide, Becton, Dickinson, and Co., Franklin Lakes, NJ) from all 3 – 5 d old calves between 1300 and 1600 h. Blood tubes were stored on ice for up to 2 hours and then centrifuged at 3000 rpm for 20 minutes at 20°C to separate the serum portion. Serum samples were stored in aliquots using 2mL cryovial tubes (Fisher Scientific, Fitchburg, WI, USA). Aliquots were stored at -18°C during data collection, and then transferred and stored at -80°C until lab assays were performed.

Serotonin lab analyses. Serum serotonin concentrations (ng/mL) were analyzed using a Serotonin ELISA kit following the manufacturer's instructions (IM1749, Immunotech, Beckman Coulter, Marseille Cedex, 9, France). All samples were diluted 1:100 (Weaver et al., 2016a) in order to fit within the assay's standard curve range. A quality control of pooled adult cow serum was analyzed on each plate to assess inter-assay coefficient of variation (26%). The intra-assay coeffcient of variation was 4%.

Health observation period. After calves entered the group-housing barn, research staff observed calves twice weekly until 50d of age for BRD. Health examinations were recorded using the Wisconsin Calf Health Scorer App (https://www.vetmed.wisc.edu/dms/fapm/apps/chs.htm) and included a CRS (Lago et al., 2006, McGuirk, 2008) and a 6 level ultrasound score (Ollivett and Buczinski, 2016). Briefly, the CRS

assigned 0 (normal) to 3 (severely abnormal) points for each of the following categories: nasal discharge, eye discharge, ear position, cough, and rectal temperature. Calves were considered positive for clinical respiratory disease (CRS+) when 2 categories with a score of 2 or greater were observed.

Ultrasound exams were performed using a portable linear rectal ultrasound set at a depth of 9 cm, frequency of 6.2 MHz, and gain of 23 dB (Near 13 dB; Far 36 dB; Ibex Pro, E.I. Medical, Loveland, CO, USA) was used. Approximately 150mL 70% isopropyl alcohol was applied to the hair as a transducing agent. One researcher performed all ultrasound exams. The left and right lungs were scanned in a systematic manner using a technique previously described in dairy calves (Ollivett et al., 2015, Ollivett and Buczinski, 2016). Briefly, the ultrasound exam began on the right side of the calf dorsally at the level of the scapula in the right 6^{th} intercostal space (**ICS**) and moved cranially to the right 1^{st} ICS. Within each ICS, the probe was held parallel to the ribs and scanned ventrally until specific ultrasonographic anatomical landmarks were identified prior to moving cranially into the next ICS as described previously (Ollivett and Buczinski, 2016). Normal lung was characterized by observation of a hyperechoic line with reverberation artifact, indicative of the normal pleural interface (Blond and Buczinski, 2009). Consolidated lung appeared hypoechoic and lacked both the hyperechoic line of the pleural surface and reverberation artifact. For the ultrasound score, 5 lung lobes in total (Right side: middle lung lobe, caudal and cranial aspect of the cranial lung lobe; Left Side: caudal and cranial aspects of the cranial lung lobe) were examined and considered separate lung lobes for the scoring system (Ollivett and Buczinski, 2016). The ultrasound score ranged from 0 to 5 (0 = normal or $\leq 1cm^2$ consolidation; 1 = diffuse comet tails; 2 = lobular pneumonia: consolidation $\geq$

1 cm^2; 3 = lobar pneumonia, 1 entire lung lobe consolidated; 4 = lobar pneumonia, 2 entire lung lobes consolidated; 5 = lobar pneumonia, ≥ 3 entire lung lobes consolidated).

BRD definitions. Three categories of BRD status were defined based on the first BRD event: clinical BRD (CRS+ as their first BRD event, regardless of ultrasound score; **CBRD**), subclinical BRD (lung consolidation ≥ 1cm^2 and CRS– as their first BRD; **SBRD**), or no BRD (never CRS+ and never had lung consolidation ≥ 1cm^2; **NOBRD**).

All CRS+ calves were treated with an antibiotic according to a standardized treatment protocol developed in cooperation with the farm management and farm veterinarians. Calves with SBRD were only treated with an antibiotic if they later became CRS+ at a later date. Research staff administered treatments for the first 2 CRS+ events to ensure consistency and accuracy of treatment records. The first respiratory antibiotic on the protocol was ceftiofur crystalline free acid (6.6 mg/ kg subcutaneous at the base of the ear once; Excede; Zoetis Services LLC., Parsippany, NJ, USA) and the second line treatments was gamithromycin (6 mg/ kg subcutaneous once; Zactran; ©2017 Merial, Inc., Duluth, GA, USA). For calves that continued to be CRS +, the next line of treatement was administered no sooner than 48 hours after the first treatment. Flunixin meglamine (1.1 mg/ kg once intravenous; Vetameg; Aspen Veterinary Resources, LTD, Greeley, CO, USA) was administered to calves with a rectal temperature ≥ 39.4°C and when the calf was depressed, slow to stand, or reluctant to lie down. For any subsequent CRS+ events, the farm staff was responsible for administering treatments as defined by the farm protocol.

Animal management in group housing. The automated calf feeder barn had 2 identical halves with 4 pens on each side for a total of 8 pens; each pen measured 3.4m x 18.3m. Pens were bedded with straw from February to March and a combination of shavings and sand from

April through August. The automated calf feeder barn was naturally ventilated with curtain sidewalls and supplemental positive pressure tube ventilation. Calves had nose-to-nose contact between pens.

Four automated calf feeders (Lely Calm calf feeder, Lely North America, Pella, IA, USA) served the 8 pens with 1 nipple provided per pen. Calves were allowed *ad libitum* milk access until 40 d of age, from 41 - 46 d of age calves were stepped up from 8 to 9.7 L per d and from 47 - 56 d of age calves were stepped down from 9.7 to 9 L per d. Feeder correction days were used to adjust for calf age. Whole milk from early lactation cows, purified using ultraviolet light (UVPure calf milk purifier, GEA Group Aktiengesellschaft, Düsseldorf, Germany), was the predominate source of liquid feed in the ACFB. The farm chose to supplement this whole milk with milk replacer (32 g milk replacer per 1 L whole milk). More specifically, from February to April, calves were supplemented with medicated 22:20 milk replacer that contained 1600 g/ton neomycin sulfate and 1600 g/ton oxytetracycline (Renaissance Nutrition Inc., Roaring Spring, PA, USA). From May through August, the farm supplemented calves with a 22:20 milk replacer (Renaissance Nutrition Inc., Roaring Spring, PA, USA) that contained Celmanax (Arm & Hammer Animal Nutrition; Ewing, NJ).

Trial 2

Trial 2 was a case control study and enrollment occurred at the time of blood sampling for BRD diagnosis. The study base population from which case and control blood samples were selected trial 2 included calves who were: previously sampled at 3 – 5 d, had recorded birth weights, and entered the automated feeder barn by 30 d of age. After entry to the automated calf feeder barn, all calves underwent twice weekly health examinations as in trial 1, until 50 d of age. Research staff collected blood samples in March, April, and June 2016 from calves

undergoing twice weekly health examinations. One blood sample was collected between 0800 and 1200 h from each calves on the day of their first BRD event (CBRD or SBRD). Calves with BRD were pair - matched 1:1 with a calf without BRD in the same pen. Samples from calves without BRD were taken at the same day and time as their BRD pair. Calves without BRD were haphazardly selected as controls from a list of eligible calves in each pen. Calves were eligible to be controls if they were not identified with SBRD or CBRD up until the point of sampling. Blood collection occurred only when calves without BRD were available in the same pens as calves with BRD. Blood was processed, serum was stored, and samples were analyzed for serotonin as in trial 1.

Statistical analysis

Calves with missing birth weights as well as those that did not enter the automated calf feeder barn by 30 d of age were excluded from the final analyses for trial 1. For both trials, data were stored, cleaned, and analyzed using Microsoft Excel (Microsoft, Redmond, WA) and SAS (version 9.4; SAS Institute Inc., Cary, NC). Sample size calculations to determine a difference in serum serotonin concenttration were performed *a priori*; we determined the necessary sample size to be 11 calves per group, using an alpha of 0.8 and a mean ± SD serotonin difference of 198 ± 156 ng/mL between cows that did or did not have hypocalcemia (Laporta et al., 2013). We increased our sample size by ~ 6% in a conservative effort to detect a difference between groups given the paucity of published data on serotonin levels in calves.

The individual calf was considered the experimental unit was for all analyses. Continuous data were visually assessed to detect outliers. Explanatory variables included sex (male = 0; female = 1), breed (Holstein = 0; Jersey = 1), season of birth (calves born on or before February 29th, 2016= winter; calves born between March 1st and April 30th, 2016= spring;

calves born on or after May 1st, 2016= summer), and month of serotonin sample (March, April, or June).

Data were checked for outliers using graphical assessment of data and screened for normality using PROC UNIVARIATE. A Wilcoxon-Mann Whitney test was used to determine if median age at the time of sample collection was different between calves with and without BRD in trial 2. Associations between explanatory variables and the outcome of interest were evaluated at the univariable level using either a linear regression (PROC MIXED) or a logistic regression (PROC LOGISTIC), depending on the nature of the outcome variables. A variable was considered a potential confounder at the univariable level if it met criteria as described by Dohoo et al. (2003); significant association at the univariable level was defined as $\alpha < 0.20$ level. Backwards stepwise elimination was used to select variables for the final model to include only variables that were significant at $\alpha \leq 0.05$ level. Prior to the final elimination of a variable, a change in estimate criterion of $\geq 30\%$ for the predictor of interest was used to detect confounding.

All potential interactions with the predictor of interest and the remaining variables in the model were assessed and interactions with $P > 0.05$ were removed. Type 3 tests of fixed effects were used to determine significance at the $\alpha \leq 0.05$ level. Residuals were plotted to check linear model assumptions. Measures of central tendency for raw data are presented as mean ± SD for normally distributed data or median (1st quartile, 3rd quartile) for non-parametric data. Estimates from multivariable models are presented as least squares means ± SE.

For trial 1, a multinomial logistic model (PROC LOGISTIC in SAS) was used to assess if serotonin at 3 - 5 d of age (predictor of interest) was associated with BRD status (CBRD, SBRD, or NOBRD; outcome of interest). The link function was used to specify the multinomial

distribution of the outcome. The full and reduced multinomial logistic models included BRD status, sex, and season of birth. The odds of developing a given BRD status were calculated by taking the log inverse of the intercept for a particular BRD status.

For trial 2, a mixed linear regression model (PROC MIXED) was used to assess if BRD (with BRD or without BRD; predictor of interest) was associated with serum serotonin concentrations at the time of BRD diagnosis (outcome of interest). Pair (1 – 17; each pair included a calf with and without BRD matched within pen) and pair by BRD status were included as random effects in the model. The full model controlled for sex, breed and month of serotonin sample. Only BRD and month of serotonin sample were retained in the final model.

5.4 RESULTS

Trial 1

Of the 488 calves that were born on the facility during the enrollment period, 420 calves were blood sampled (n = 68 were outside the 3 – 5 d age range at the time of blood samples and therefore not sampled). Of the 420 calves with blood samples at 3 – 5 days of age, 188 were lost to follow - up because they died (n = 31), were sold (n = 15) prior to entry into the group housing barn, or the farm elected not to send some calves to the automated calf feeder barn due to inadequate barn space (n = 142). A total of 232 calves entered the automated calf feeder barn. In addition, calves that entered the automated calf feeder barn, but lacked a recorded birth weight (n = 10) or who entered the barn after 30 d of age (n = 28) were excluded from analysis. Therefore, 194 calves were were carried forward for final analysis for trial 1.

Calves were 21 ± 6 (mean ± SD) d at entry to the automated calf feeder barn. Calves were 30 ± 10 d and 31 ± 8 d of age at diagnosis with CBRD and SBRD, respectively ($P = 0.89$). Calf descriptive information is presented in Table 5.1. Twenty-two percent (43/194) and 58% (112/194) of calves were identified with CBRD and SBRD at their first BRD event, respectively.

Twenty percent (39/194) of calves were never identified with CBRD or SBRD and therefore were in the NOBRD group. Raw (mean ± SD) serum serotonin concentrations at 3 - 5 d of age were 2288 ± 959 ng/mL for calves with CBRD, 2691 ± 939 ng/mL for calves with SBRD, and 2207 ± 787 ng/mL for calves with NOBRD.

For 3 – 5 d old calves, the multinomial logistic regression model indicated that for every 1 unit increase in serum serotonin at 3 - 5 d of age, the odds of a calf becoming SBRD versus NOBRD later in the preweaning period increase by 1.00 (95%CI: 1.00-1.001; $P = 0.02$; Table 5.2); the odds of a calf becoming SBRD versus CBRD tended to increase by 1.00 (95% CI: 1.00-1.001; $P = 0.06$; Table 5.2). The odds of developing CBRD compared to NOBRD were not different ($P = 0.55$). There was no interaction between serotonin concentration at 3 - 5 d of age and sex ($P = 0.40$) or season of birth ($P = 0.59$). In this population, the odds of a calf developing CBRD versus NOBRD were 0.7 and the odds of a calf developing SBRD versus NOBRD were 0.8. The odds of developing SBRD versus CBRD were 1.1.

As an example, the difference in raw mean serotonin in our study between calves with SBRD and calves with NOBRD was approximately 480 ng/mL. A 480-unit change in serotonin equates to a 480-time increase in the odds of developing SBRD versus NOBRD; the odds of a calf developing SBRD versus NOBRD would 384 (0.8 x 480).

Results (Trial 2)

A total of 308 calves entered the automated calf feeder barn. However, 12% (38/308) of calves were excluded from analysis because they were missing birth weights (n = 10) or did not meet the study eligibility enrollment age criteria (n = 28; entered the automated feeder barn at 40 d of age). The base population from which cases and controls were selected included a total of 270 calves. Seventeen BRD cases were pair-matched with 17 controls.

Calf descriptive information is presented in Table 5.3. The median (1st quartile, 3rd quartile) age at sampling was 40 (36, 44) d for calves with BRD and 40 (33, 47) for calves without BRD ($P = 0.25$). Of the 17 calves without BRD at the time of sampling, 59% (10/17) remained without BRD until 50 d of age, 35% (6/17) were later identified with SBRD, and 6% (1/17) were later identified with CBRD. Raw mean serum serotonin concentration at the time of BRD diagnosis was 2538 ± 1319 ng/mL for calves with BRD and 3317 ± 698 ng/mL for calves without BRD. The multivariable linear regression analysis revealed that calves with BRD had 779 ng/mL less serotonin compared to calves without BRD (2707 ± 201 vs. 3487 ± 201, respectively; Table 5.4; $P = 0.0092$). Season of sample collection was also associated with BRD ($P = 0.0002$). There was no interaction between BRD and season of sample collection ($P = 0.21$).

5.5 DISCUSSION

To our knowledge, this study was the first to investigate the association between serotonin and BRD in dairy calves using both lung ultrasonography and clinical respiratory scoring to identify calves affected by BRD. In trial 1, higher serum serotonin at 3 - 5 d of age was associated with increased odds of a calf developing SBRD versus NOBRD and tended to increase the odds of a calf developing SBRD versus CBRD. The association between serotonin at 3 – 5 d of age and development of BRD later in life suggests that serotonin warrants further investigation to determine its usefulness as a potential biomarker in young calves for BRD prevention. In trial 2, serum serotonin was lower in calves at the time of BRD diagnosis compared to unaffected controls, suggesting that changes in the serotonergic axis might occur during or leading up to BRD.

We observed that lower serum serotonin at 3 - 5 d of age was protective against the

development of subclinical BRD in trial 1. These results were somewhat unexpected, considering that Hernández-Castellano et al. (2018) found higher levels of serotonin to be associated with increased innate and adaptive immune factors. However, the clinical implications of the increased immune factors were not elucidated in Hernández-Castellano et al. (2018). The previous study (Hernández-Castellano et al., 2018) supplemented newborn calves with the precursor to serotonin (5-hydroxy-L-tryptophan). However, supplementation with the precursor to serotonin does not precisely replicate the role of endogenous serotonin in response to a challenge on the immune system (Young et al., 1993), as would occur with BRD infection. Furthermore, Hernández-Castellano et al. (2018) did not include health information, so the health status of controls is unknown. As such, there are difficulties in comparing the present study to Hernández-Castellano et al. (2018).

It is interesting that lower serum serotonin at 3 - 5 days of age was protective against the development of subclinical BRD, but not clinical BRD. This finding is of particular importance because subclinical BRD might be less likely to be detected, due to the absence of visible signs of disease. Therefore, lower serum serotonin concentrations early in life may indicate which calves should be monitored more frequently using lung ultrasound. It might also be particularly useful to investigate the manipulation of serotonin in colostrum (e.g., Hernández-Castellano et al. 2018). It is possible that serotonin concentration in colostrum could be driving serum serotonin concentration in the calf and the absorption of antibodies. Future studies could collect colostrum and blood samples from calves simultaneously to determine how maternal transfer of serotonin through colostrum impacts calf immunity and health. Future work could also investigate how calf immunity and health are affected by the maternal serotonin pool through collection of both dam and calf blood samples. Manipulation of the maternal serotonergic axis during pregnancy

and lactation has previously been shown to worsen hyperoxia-induced lung damage in neonatal rats (Porzionato et al., 2012) and increase the risk of pulmonary disease in children (Ter Horst et al., 2013). We encourage more research into the relationship between serotonin and BRD later in life, in order to develop new tools for manipulating serotonin *in utero* or in colostrum to affect calf health.

We observed lower serotonin concentrations at the time of diagnosis in calves with BRD compared to calves without BRD. Serotonin has been implicated in the immune response, which is involved with BRD. Differential cell counts from bronchoalveolar lavage fluid are inflammatory in nature, showing neutrophil and macrophage proportions in calves with subclinical BRD compared to unaffected herdmates (Ollivett et al., 2015) and greater macrophage and lymphocyte proportions in calves with clinical BRD compared to calves without clinical BRD (Allen et al., 1992). In addition, B-lymphocyte expression of the serotonin reuptake transporter is increased upon B-lymphocyte activation (Meredith et al., 2005), and macrophages contain serotonin receptors (Nakamura et al., 2008, Mikulski et al., 2010). Based solely on the role of serotonin in the immune system, we would expect serotonin to be higher in calves with BRD making our results surprising. However, serotonin is derived from tryptophan (Wang et al., 2002), which can be converted to kynurenine through the indoleamine 2,3-dioxygenase (**IDO**) pathway or to serotonin via the tryptophan hydroxylase pathway (Gál and Sherman, 1980). Tryptophan is required for the growth of microorganisms and IDO is thought to inhibit microbial growth by converting tryptophan to kynurenine (Meier et al., 2017). Tryptophan, IDO, and kynurenine were implicated in community-acquired pneumonia in humans (Meier et al., 2017); researchers observed increased activation of IDO, leading to a shift in metabolism from tryptophan to kynurenine in patients with pneumonia. It is possible that we

observed lower serotonin in calves with BRD because tryptophan was shunted away from the serotonin pathway and towards the formation of kynurenine. Future studies should consider measuring tryptophan, kynurenine, and serotonin simultaneously to elucidate this relationship.

The difference in serotonin concentrations between calves with and without BRD may be useful to target for therapeutic purposes. Exploring antimicrobial alternatives that effectively treat BRD, through manipulation of the serotonergic system, may help the dairy industry improve judicious use of antibiotics, particularly those classified as "highest priority critically important" by the World Health Organization (World Health Organization Critically Important Antimicrobials for Human Medicine—5th Rev). However, our results should be interpreted cautiously, as this study population represents a small group of calves at one time during a specific disease. We suggest studies that examine serotonin concentrations in calves over time and throughout the course of various diseases. Serotonin is involved with multiple different systems (Berger et al., 2009), hence it is imperative that the relationship between serotonin and BRD is carefully studied prior to the development of pharmacological agents.

Our data suggest that the relationship between serotonin and BRD may change depending on calf age. Lower serum serotonin at 3 - 5 d of age had a protective effect against the development of subclinical BRD. We found the opposite pattern in calves at the time of diagnosis whereby calves with BRD had lower serotonin concentrations compared to calves without BRD. A previous study found calves had higher serotonin concentrations overall compared to adults (Laporta et al., 2014). Because the gut produces the majority of circulating serotonin in the body (Kim and Khan, 2014), Laporta et al. (2014) hypothesized calves had higher serotonin concentrations compared to cows because calves are similar to monogastrics at birth (Swanson and Harris, 1958). In addition to the transition from a monogastric to a ruminant,

calves experience rapid growth, changes in diet, and sometimes, changes in housing and social interaction during the preweaned period. Our trials were not designed to address how serotonin concentrations change throughout a calf's life and in response to environmental changes, but we support future research in this area.

The mean serum serotonin concentration at 3 - 5 d for calves across all BRD categories in our study was approximately 1300 ng/mL less than the serum serotonin values reported for both controls (calves not supplemented with 5-hydryoxy-tryptophan) in Hernández-Castellano et al. (2018) and controls (calves born to dams not supplemented with 5-hydryoxy-tryptophan; sampled at 4 h, 12 h, and 3 weeks after birth) in Laporta et al. (2014). Serotonin can differ due to breed of cattle (Weaver et al., 2016b), season (Badcock et al., 1987), and age (Badcock et al., 1987). Additionally, the preparation of serotonin samples can affect the measured concentration. The extent to which platelets are allowed to activate (ie: the amount of time that the sample sits before processing) can affect the concentration (Brand and Anderson, 2011), as can the type of assay (Vatassery et al., 1981, Beck et al., 1993). Serotonin in calves should be studied when breed, season, age, sampling technique, and other factors are taken into account.

Certain limitations are present in our study, which should be considered carefully. We based BRD status on a calf's first BRD event after study enrollment. However, we did not enroll calves until 21 d of age. Clinical BRD (Cramer and Stanton, 2015) and lung consolidation (Binversie, 2017) have been reported in calves younger than 3 weeks and at 7 d of age, respectively. Therefore, it is possible that our unaffected controls were actually recovered cases from a previous BRD event. Serotonin concentrations at 3 - 5 days may have affected BRD events in calves <21 d of age more prominently compared to BRD events >21 d of age. We may have introduced selection bias as only the calves that survived until 21 d of age were eligible for

study enrollment. The exclusion of calves that died prior to 21 d of age may have biased our results towards the null, as calves that died were likely most affected by disease. We also recognize that haphazardly selecting calves without BRD as pen-mate matches was not ideal and may have introduced selection bias, which might have been different than a random sample.

More research is warranted to investigate the relationship of serotonin in preweaned calves and health events to determine how serotonin changes in response to illness over time and if serotonin is a good predictor of mortality. Furthermore, a better understanding of how serotonin changes with calf age, time of day, and environmental factors are necessary before serotonin can be used on-farm to identify calves at risk for BRD. We suggest large cohort studies to investigate the impact of serotonin on actual health outcomes, as well as immunity factors, so the mechanism between serotonin and illness can be understood.

5.6 CONCLUSION

To our knowledge, our study is the first to investigate the association between serotonin and BRD as diagnosed using lung ultrasound and clinical respiratory scoring in preweaned dairy calves. We found lower concentrations of serum serotonin concentrations at 3 - 5 d of age were protective against the development of subclinical BRD in dairy calves. At the time of diagnosis with BRD, calves with BRD had lower serum serotonin concentrations compared to calves without BRD. Our results demonstrate that serotonin may potentially be a useful factor to target in order to prevent BRD or treat BRD in dairy calves. However, serotonin is involved in many different body systems and is not well understood in calves. Therefore, extensive research is needed before prevention or therapeutic interventions in the serotonergic system are used in calves.

5.7 ACKNOWLEDGEMENTS

This material is based upon work supported by funding from STGenetics (Navasota, Texas, United States, 77868). Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the view of STGenetics. The authors wish to thank the staff and owners of the calf facility for allowing us to perform research and Ella Jackson and Hannah Donley for their assistance with data collection.

5.8 REFERENCES

Ahern, G. P. 2011. 5-HT and the immune system. Curr. Opin. Pharm. 11(1):29-33.

Allen, J. W., L. Viel, K. G. Bateman, S. Rosendal, and P. E. Shewen. 1992. Cytological findings in bronchoalveolar lavage fluid from feedlot calves: associations with pulmonary microbial flora. Can. J. Vet. Res. 56(2):122-126.

Badcock, N. R., J. G. Spence, and L. M. Stern. 1987. Blood Serotonin Levels in Adults, Autistic and Non-Autistic Children—With a Comparison of Different Methodologies. Ann. Clin. Biochem. 24(6):625-634.

Beck, O., N. H. Wallén, A. Broijersén, P. T. Larsson, and P. Hjemdahl. 1993. On the accurate determination of serotonin in human plasma. Biochem. Biophys. Res. Commun. 196(1):260-266.

Berger, M., J. A. Gray, and B. L. Roth. 2009. The expanded biology of serotonin. Annu. Rev. Med. 60:355-366.

Brand, T. and G. M. Anderson. 2011. The measurement of platelet-poor plasma serotonin: a systematic review of prior reports and recommendations for improved analysis. Clin. Chem. 57(10):1376-1386.

Buczinski, S., G. Forté, and A. Bélanger. 2013. Short communication: Ultrasonographic assessment of the thorax as a fast technique to assess pulmonary lesions in dairy calves with bovine respiratory disease. J. Dairy Sci. 96(7):4523-4528.

Dohoo, I., W. Martin, and H. Stryhn. 2003. Veterinary Epidemiologic Research. AVC Inc., University of Prince Edward Island, 550 University Avenue, Charlottetown, Prince Edward Island, CA.

Gál, E. M. and A. D. Sherman. 1980. l-Kynurenine Its synthesis and possible regulatory function in brain. Neurochem. Res. 5(3):223-239.

Godson, D. L., M. Campos, S. K. Attah-Poku, M. J. Redmond, D. M. Cordeiro, M. S. Sethi, R. J. Harland, and L. A. Babiuk. 1996. Serum haptoglobin as an indicator of the acute phase response in bovine respiratory disease. Vet. Immunol. Immunopathol. 51(3):277-292.

Gordon, J. and N. M. Barnes. 2003. Lymphocytes transport serotonin and dopamine: agony or ecstasy? Trends Immunol. 24(8):438-443.

Hernández-Castellano, L. E., R. Özçelik, L. L. Hernandez, and R. M. Bruckmaier. 2018. Supplementation of colostrum and milk with 5-hydroxy-l-tryptophan affects immune factors but not growth performance in newborn calves. J. Dairy Sci. 101(1):794-800.

Herr, N., C. Bode, and D. Duerschmied. 2017. The effects of serotonin in immune cells. Frontiers in cardiovascular medicine 4:48.

Kabu, M., B. Elitok, and I. Kucukkurt. 2016. Detection of serum amyloid-A concentration in the calf clinically diagnosed with pneumonia, enteritis and pneumoenteritis. Ciencia Rural 46(2):293-299.

Kereveur, A., J. Callebert, M. Humbert, P. Herve, G. Simonneau, J.-M. Launay, and L. Drouet. 2000. High plasma serotonin levels in primary pulmonary hypertension: effect of long-

term epoprostenol (prostacyclin) therapy. Atertio. Thromb. Vasc. Biol. 20(10):2233-2239.

Kim, J. J. and W. I. Khan. 2014. 5-HT7 receptor signaling: improved therapeutic strategy in gut disorders. Front. Behav. Neurosci. 8(396).

Laporta, J., J. J. Gross, T. Crenshaw, R. Bruckmaier, and L. Hernandez. 2014. Timing of first milking affects serotonin (5-HT) concentrations. J. Dairy Sci. 97(5):2944-2948.

Laporta, J., S. Moore, M. Peters, T. Peters, and L. Hernandez. 2013. Circulating serotonin (5-HT) concentrations on day 1 of lactation as a potential predictor of transition-related disorders. J. Dairy Sci. 96(8):5146-5150.

Matsuda, H., H. Ushio, G. P. Geba, and P. W. Askenase. 1997. Human platelets can initiate T cell-dependent contact sensitivity through local serotonin release mediated by IgE antibodies. The Journal of Immunology 158(6):2891-2897.

Meier, M. A., M. Ottiger, A. Vögeli, C. Steuer, L. Bernasconi, R. Thomann, M. Christ-Crain, C. Henzen, C. Hoess, and W. Zimmerli. 2017. Activation of the tryptophan/serotonin pathway is associated with severity and predicts outcomes in pneumonia: results of a long-term cohort study. Clinical Chemistry and Laboratory Medicine (CCLM) 55(7):1060-1069.

Mellor, D. J. and K. J. Stafford. 2004. Animal welfare implications of neonatal mortality and morbidity in farm animals. Vet. J. 168(2):118-133.

Meredith, E. J., M. J. Holder, A. Chamba, A. Challa, A. Drake-Lee, C. M. Bunce, M. T. Drayson, G. Pilkington, R. D. Blakely, and M. J. Dyer. 2005. The serotonin transporter (SLC6A4) is present in B-cell clones of diverse malignant origin: probing a potential anti-tumor target for psychotropics. The FASEB journal 19(9):1187-1189.

Mikulski, Z., Z. Zasłona, L. Cakarova, P. Hartmann, J. Wilhelm, L. H. Tecott, J. Lohmeyer, and W. Kummer. 2010. Serotonin activates murine alveolar macrophages through 5-HT2C receptors. American Journal of Physiology-Lung Cellular and Molecular Physiology 299(2):L272-L280.

Nakamura, K., T. Sato, A. Ohashi, H. Tsurui, and H. Hasegawa. 2008. Role of a serotonin precursor in development of gut microvilli. The American journal of pathology 172(2):333-344.

O'Connell, P. J., X. Wang, M. Leon-Ponte, C. Griffiths, S. C. Pingle, and G. P. Ahern. 2006. A novel form of immune signaling revealed by transmission of the inflammatory mediator serotonin between dendritic cells and T cells. Blood 107(3):1010-1017.

Ollivett, T. 2014. Understanding the diagnosis and risk factors for respiratory disease in dairy calves. in Department of Population Medicine. Vol. PhD. University of Guelph, Guelph, Ontario, Canada.

Ollivett, T., J. Caswell, D. Nydam, T. Duffield, K. Leslie, J. Hewson, and D. Kelton. 2015. Thoracic Ultrasonography and Bronchoalveolar Lavage Fluid Analysis in Holstein Calves with Subclinical Lung Lesions. J. Vet. Intern. Med. 29(6):1728-1734.

Ollivett, T. L. and S. Buczinski. 2016. On-Farm Use of Ultrasonography for Bovine Respiratory Disease. Vet. Clin. North Am. Food Anim. Pract. 32(1):19-35.

Porzionato, A., P. Zaramella, V. Macchi, D. Grisafi, R. Salmaso, M. Baraldi, E. Fornaro, E. Tassone, V. Masola, and M. Onisto. 2012. Fluoxetine may worsen hyperoxia-induced lung damage in neonatal rats. Histol. Histopathol. 27(10):1599.

Swanson, E. W. and J. D. Harris, Jr. 1958. Development of Rumination in the Young Calf. J. Dairy Sci. 41(12):1768-1776.

Ter Horst, P., H. Bos, L. De Jong-Van De Berg, and B. Wilffert. 2013. In utero exposure to antidepressants and the use of drugs for pulmonary diseases in children. Eur. J. Clin. Pharmacol. 69(3):541-547.

USDA. 2010. Heifer calf health and management practices on U.S. dairy operations, 2007. USDA: APHIS: VS,CEAH, Fort Collins, CO.

van der Fels-Klerz, H. J., J. T. Sorensen, A. W. Jalvingh, and R. B. M. Huirne. 2001. An economic model to calculate farm-specific losses due to bovine respiratory disease in dairy heifers. Prev. Vet. Med. 51(1-2):75-94.

Vatassery, G. T., M. A. Sheridan, and A. M. Krezowski. 1981. Spectrophotofluorometry of serotonin in blood platelets. Clin. Chem. 27(2):328-330.

Wang, L., H. Erlandsen, J. Haavik, P. M. Knappskog, and R. C. Stevens. 2002. Three-Dimensional Structure of Human Tryptophan Hydroxylase and Its Implications for the Biosynthesis of the Neurotransmitters Serotonin and Melatonin. Biochemistry 41(42):12569-12574.

Weaver, S. R., J. Laporta, S. A. Moore, and L. L. Hernandez. 2016a. Serotonin and calcium homeostasis during the transition period. Domest. Anim. Endocrinol. 56:S147-S154.

Weaver, S. R., A. P. Prichard, E. L. Endres, S. A. Newhouse, T. L. Peters, P. M. Crump, M. S. Akins, T. D. Crenshaw, R. M. Bruckmaier, and L. L. Hernandez. 2016b. Elevation of circulating serotonin improves calcium dynamics in the peripartum dairy cow. J. Endocrinol. 230(1):105-123.

World Health Organization Critically Important Antimicrobials for Human Medicine—5th Rev. World Health Organization, 2017.

Young, M., J. Kut, M. Coogan, M. Wright, M. Young, and J. Matthews. 1993. Stimulation of splenic T-lymphocyte function by endogenous serotonin and by low-dose exogenous serotonin. Immunology 80(3):395.

5.9 Tables

Table 5.1. Proportion of preweaned dairy calves (n = 194) in each category of sex, breed, and season of birth, by BRD status[1] for trial 1[2]. Calves were enrolled in the study at 21 ± 6 (mean ± SD) d of age and underwent twice weekly health exams.

	BRD Status		
Variable (n)	CBRD (n = 43)	SBRD (n = 112)	NOBRD (n = 39)
Sex			
Male	27% (38)	50% (72)	23% (33)
Female	10% (5)	15% (40)	12% (6)
Breed			
Holstein	21% (31)	61% (89)	18% (26)
Jersey	25% (12)	48% (23)	27% (13)
Season of Birth[2]			
Winter	39% (7)	28% (5)	33% (6)
Spring	26% (21)	58% (47)	16% (13)
Summer	16% (15)	63% (60)	21% (20)

1- Bovine respiratory disease status; Health exams included a clinical respiratory score (CRS; - or +; calculated with nasal, eye, ear, cough, and rectal temperature scores; McGuirk, 2008) and a lung ultrasound (Ollivett et al., 2016). BRD status for each calf was defined as SRBD (subclinical BRD; calves with any lung consolidation ≥ 1cm^2 and CRS-;) or CBRD (clinical BRD; CRS+ with or without lung consolidation), based on the first BRD event. Calves with NOBRD never had lung consolidation ≥ 1cm^2 or CRS+.

2- Trial 1 examined the effect of serum serotonin concentrations at 3 - 5 d of age on BRD status between 21-50 d of age.

Table 5.2. Multinomial logistic regression model for the probability of BRD status[1] in 194 preweaned dairy calves. The predictor of interest was serum serotonin concentration at 3 - 5 d of age. Calves were enrolled in the study at 21 ± 6 (mean ± SD) d of age and underwent twice weekly health exams, which included a clinical respiratory score and a lung ultrasound. Estimates and reported *P* – values are from the analysis of maximum likelihood estimates.

Variable	Likelihood Estimate	Standard Error	Odds Ratio	95% CI for Odds Ratio	*P* - value
Reference Category: NOBRD					
Intercept					
CBRD	-0.35	0.66	1.000	1.000 - 1.001	0.60
SBRD	-0.23	0.58	1.001	1.000 - 1.001	0.70
Serotonin concentration at 3 - 5 d of age					
CBRD	0.00016	0.00026	-	-	0.55
SBRD	0.00055	0.00027	-	-	0.02
Sex					
Female (relative to Male); CBRD	-0.16	0.33	0.721	0.198 - 2.627	0.62
Female (relative to Male); SBRD	0.51	0.25	2.756	1.037 - 7.326	0.04
Season of Birth					
Winter (relative to Summer); CBRD	0.06	0.06	1.645	0.436 – 6.204	0.88
Winter (relative to Summer); SBRD	-0.57	-0.57	0.495	0.128 - 1.911	0.20
Spring (relative to Summer); CBRD	0.67	0.37	2.231	0.845 - 5.888	0.25
Spring (relative to Summer); SBRD	0.44	0.44	1.361	0.596 - 3.109	0.16
Reference Category: CBRD					
Intercept					
NOBRD	0.35	0.66	1.000	0.999 – 1.000	0.60
SBRD	0.12	0.60	1.000	1.000 – 1.001	0.83
Serotonin concentration at 3 - 5 d of age					
NOBRD	-0.00016	0.00026	-	-	0.55
SBRD	0.00040	0.00021	-	-	0.06
Other model variables not shown					

1- Bovine respiratory disease status; Health exams included a clinical respiratory score (CRS; McGuirk, 2008) and a lung ultrasound (Ollivett et al., 2016). BRD status for each calf was defined as SRBD (subclinical BRD; calves with any lung consolidation ≥ 1cm^2 and CRS-;) or CBRD (clinical BRD; CRS+ with or without lung consolidation), based on the first BRD event. Calves with NOBRD never had lung consolidation ≥ 1cm^2 or CRS+.

Table 5.3. Proportion of preweaned dairy calves (n = 34) in each category of sex, breed, and sample month, by BRD status[1] for trial 2[2]. The median (1st quartile, 3rd quartile) age at BRD was 40 (36, 44) d for calves with BRD and 40 (33, 47) for calves without BRD. Calves underwent twice weekly health exams.

	BRD Status	
Variable (n)	With BRD (n = 17)	Without BRD (n = 17)
Sex		
Male	52% (14)	48% (13)
Female	43% (3)	57% (4)
Breed		
Holstein	50% (11)	50% (11)
Jersey	50% (6)	50% (50)
Sample month[3]		
March	50% (4)	50% (4)
April	50% (8)	50% (8)
June	50% (5)	50% (5)

1- Bovine respiratory disease status; Health exams included a clinical respiratory score (CRS; - or +; calculated with nasal, eye, ear, cough, and rectal temperature scores; McGuirk, 2008) and a lung ultrasound (Ollivett et al., 2016). BRD status for each calf was defined as SRBD (subclinical BRD; calves with any lung consolidation $\geq 1cm^2$ and CRS-;) or CBRD (clinical BRD; CRS+ with or without lung consolidation), based on the first BRD event. Calves with NOBRD never had lung consolidation $\geq 1cm^2$ or CRS+.

2- Trial 2 examined differences in serotonin levels at the time of BRD diagnosis. Blood samples were take from a calf with BRD and from a pen mate without BRD.

Table 5.4. Multivariable linear regression model for serum serotonin concentration (ng/mL) at the time of diagnosis with or without BRD[1] in 34 preweaned dairy calves. At the time of diagnosis for sick calves, a blood sample was taken from a pen-mate without BRD. Calves were enrolled in the study at 21 ± 6 (mean ± SD) d of age and underwent twice weekly health exams. This model contains BRD as the predictor of interest. Estimates and reported *P* – values are from solutions fixed effects.

Variable	Serum serotonin concentration estimate (ng/mL)	Standard Error	*P* - value
Intercept	4122.22	292.17	<0.0001
BRD			
With BRD	-779.38	278.57	0.0092
Without BRD	Referent		
Sample season			
March	-393.33	385.25	0.3160
April	-1513.57	327.40	<0.0001
June	Referent		

1- Bovine respiratory disease status; Health exams included a clinical respiratory score (CRS; - or +; calculated with nasal, eye, ear, cough, and rectal temperature scores; McGuirk, 2008) and a lung ultrasound (Ollivett et al., 2016). BRD status for each calf was defined as SRBD (subclinical BRD; calves with any lung consolidation ≥ 1cm^2 and CRS-;) or CBRD (clinical BRD; CRS+ with or without lung consolidation), based on the first BRD event. Calves with NOBRD never had lung consolidation ≥ 1cm^2 or CRS+.

6 CONCLUSIONS AND FUTURE RESEARCH

This dissertation used accurate BRD diagnostic methods to investigate growth, potential on-farm disease detection tools, and serotonin in calves (Table 6.1). Our results will provide more information to veterinarians using lung ultrasound to measure calf growth. Additionally, our findings will inform how BRD detection tools, such as automated milk feeders and attitude scores, are used on farm. Our results regarding serotonin and BRD provide evidence that this relationship should be investigated further to determine if serotonergic interventions can prevent or treat BRD. Finally, we used accurate BRD diagnostic methods to ensure the correct classification of calves with BRD and unaffected herdmates; this can serve as a model for other researchers.

Chapter 2 identified a cut point on a 6-level lung ultrasound score at which calf growth was impacted. In addition, we also found that the clinical status of the calf independently affected calf growth. Both the lung status and the clinical status explained some variation in calf growth, suggesting both diagnostic tools should be used in calf programs. Our simplified lung ultrasound score is most useful for veterinarians interested in identifying calves at risk for poor growth or lung consolidation prevalence. However, the simplified score is not useful to measure lung consolidation severity or changes in the amount of lung consolidation over time. We focused on the first BRD event, which occurred between 30 and 50 days of age for this population of calves. However, future research is warranted to understand how the duration of lung consolidation and BRD in younger calves affects growth.

Chapter 3 was the first study to our knowledge to investigate if feeding behavior (milk intake, drinking speed, number of feeder visits, and average meal size) in calves with subclinical BRD differed from calves with clinical or no BRD. Calves with subclinical BRD did not differ

from calves without BRD in any behavior except intake, and subclinical BRD calves drank more than normal calves. Feeding behavior did not adequately differentiate between calves with subclinical BRD, clinical BRD and unaffected herdmates. Therefore, producers should use caution when using feeding behavior as the primary means for BRD detection. Chapter 4 had similar findings whereby a behavioral attitude score did not differ between calves with subclinical BRD and calves with no BRD. In contrast, calves with clinical BRD were more likely to have a depressed attitude compared to the other BRD categories. However, the attitude score had poor sensitivity and should not be used as the primary means of BRD detection.

Future research examining behavioral effects of BRD is warranted. Particularly, changes in behavior associated with BRD in calves less than 30 days of age. Younger calves might be more affected by BRD and it would be useful to understand the impact of BRD during this age. Additionally, we can use behavior to inform the management and housing of sick calves. It is possible that calves with clinical BRD might be less likely to compete for resources in a social setting. Thus, leaving sick calves in a pen with unaffected herd mates could put sick calves at risk for physiological stress and subsequent worsening illness.

Chapter 5 examined the relationship between serotonin and BRD. We found that a 1-unit increase in serotonin at 3-5 d of age was associated with an increased odds of developing subclinical BRD later in life. Additionally, we observed lower serotonin in calves at the time of BRD diagnosis compared to unaffected herd mates, suggesting that changes in the serotonergic axis might occur during BRD. This relationship should be investigated further to determine if serotonin is a potential biomarker to target for BRD prevention and treatment at the time of illness.

We encourage researchers to utilize lung ultrasound and clinical respiratory scoring in studies to ensure the correct classification of calves. More broadly, it would be useful to know the underlying mechanism behind why some calves show clinical signs and others do not. Understanding this would allow us to better explain differences in behavior and could inform treatment decisions. Additionally, studies that examine pain in calves with BRD are warranted. This information would inform how we treat sick calves and if pain mitigation is necessary.

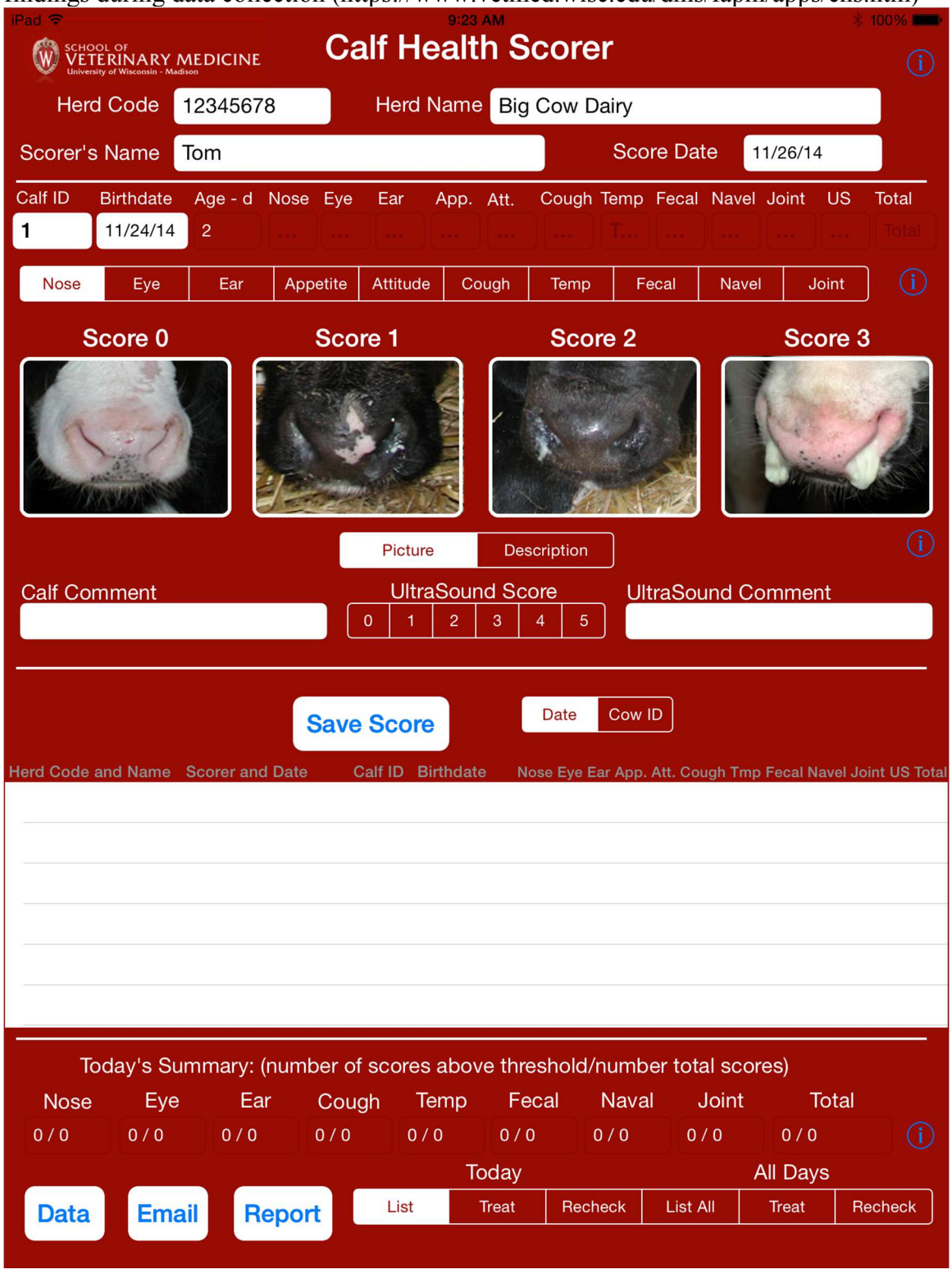
6.1 Tables

Table 6.1. Summary of findings.

	Outcomes								
Predictors	ADG	Predictors	Drinking speed	Milk Intake	Average meal size	Number of visits	Behavioral attitude	Serum serotonin concentration at 3-5 d of age	Serum serotonin concentration at BRD diagnosis
Lung consolidation	Decreased	Subclinical BRD	No difference	Increased	No effect of BRD	No effect of BRD	No difference	1 unit increase = 1 time increase in odds	Lower
Clinical BRD	Decreased	Clinical BRD	Decreased	No difference	No effect of BRD	No effect of BRD	More likely to be depressed	No difference	
		No BRD	Referent	Referent	No effect of BRD	No effect of BRD	Referent	Referent	Referent

7 APPENDIX

Appendix 1: The Wisconsin Calf Health Scoring App was used to enter health examination findings during data collection (https://www.vetmed.wisc.edu/dms/fapm/apps/chs.htm)



Appendix 2. Example of the calf treatment records used during data collection. Treatment records were entered and store on an iPad. Recorded information included calf ID, date, condition, person who diagnosed the condition, drug (including dosage, route, and withdraw information), the amount of antibiotic given, and the amount of flunixin administered, if needed.

ID	date1	condition1	drug1	ccSQdrug1	ccIVflunix1	date2	condition2	drug2	ccSQdrug2
123	2/19/16	Resp_Catie HS	Excede_(1.5cc/100lb SQ in ear once) 13d meat	3					
456	2/16/16	Resp_Catie HS	Zactran_(2cc/110lb SQ once) 35d meat	4	1.5				
789	2/25/16	Resp_Catie HS	Excede_(1.5cc/100lb SQ in ear once) 13d meat	3		3/21/16	Resp_Catie HS	Zactran_(2cc/110 lb SQ once) 35d meat	4.6
012	2/11/16	Resp_Catie HS	Excede_(1.5cc/100lb SQ in ear once) 13d meat	3		2/18/16	Resp_Catie HS	Zactran_(2cc/110 lb SQ once) 35d meat	4

Appendix 3. Serotonin Enzyme Immunoassay (EIA) Protocol for Blood Serum Samples. Beckman Coulter, Inc. (summarized by Jimena Laporta)

Thaw frozen samples (~ 30 min – 1 hr). Milk typically thaws more easily in a warm water bath.

Can run 41 samples and 1 pool (QC) per plate in duplicate

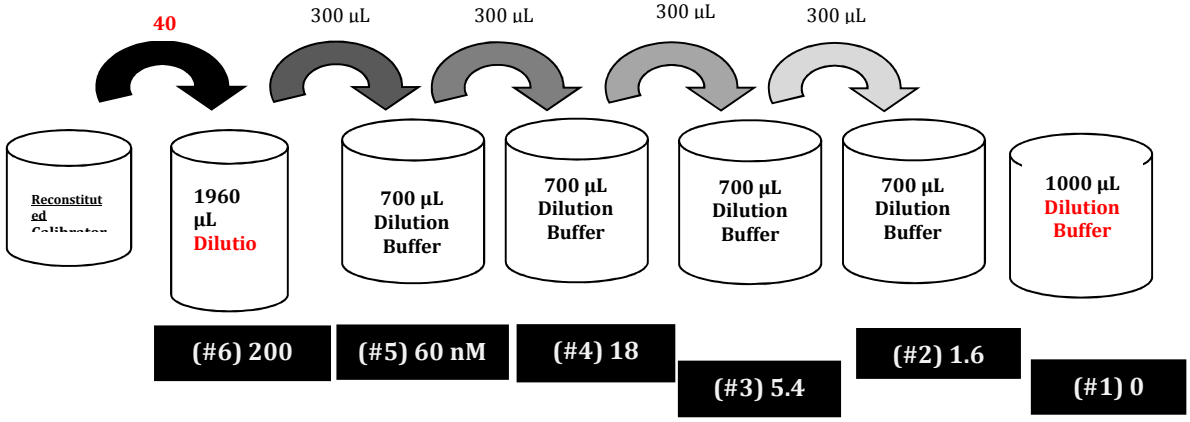
Set out assay kit to equilibrate to room temperature (30 min before you start)

Steps

1. Reconstitute the lyophilized **CALIBRATOR with 1 mL of distilled water** (Wait ≥30 min before dispensing; mix gently, *DO NOT VORTEX*)

2. **DILUTE SERUM SAMPLES (1:100)** in plastic labeled tubes (1-41) by adding:
   a. **4 µL** of sample **(REVERSE PIPET PLEASE!!!)**
   b. **396 µL of DILUTION BUFFER**
      *don't forget the pool
      *for **MILK**, dilute **1:5** → 80 **µL of sample into 320 µL of dilution buffer**
      *colostrum can be very sticky, use caution to assure that you are pipetting uniform volumes

3. Prepare **STANDARD CURVE** starting with reconstituted **CALIBRATOR** solution in plastic tubes: (SC #6 will be a 10ml conical tube, the rest can be 1.5ml Eppendorf. First add 1960 ul to SC#6, then 700ul to SC# 5, 4, 3, 2 and 100ul to SC#1. Next add 40uL of calibrator to SC#6, vortex really well. Then add 300ul of SC#6 to #5 and so on…*SC#1 will be Dilution buffer only)



4. To containing **ACYLATING REAGENT TUBE** (label them 1-41) add:
   a. **100 µL of standard curve or diluted sample**
   b. **50 µL** of **ACYLATION BUFFER**

5. Recap and immediately vortex until dissolution

6. Incubate at room temperature for **30 min in the DARK (drawer)**

    a. Reconstitute the lyophilized **CONJUGATE** with **25 mL of distilled** water. Wait 5-10 min before homogenizing

7. To coated wells (**PLATE**), add:
    a. 20 µL of standard curve or sample
    b. 200 µL of CONJUGATE
8. Incubate at room temperature for **3 hr in the DARK on PLATE SHAKER**

9. Dilute 50 mL of **WASH SOLUTION** with 950 mL of distilled water
    Reconstitute the **SUBSTRATE** with 25 mL of distilled water (20 min before the 3 hrs) Wait 5-10 min before homogenizing

10. Dump out contents of the plate. Add 300 µL of **WASH SOLUTION** and dump it, do it 3 times
11. Add **200 µL of SUBSTRATE** to all wells
12. Incubate at room temperature for **15-20 min in the DARK on PLATE SHAKER**
13. Add 50 µL of **STOP SOLUTION**

14. Read absorbance at **405 nm**
    IMPORTANT: ***Copy and paste an old plate *** change the number of plate and date
    ***keep track of the plate number – PRESS SAVE AFTER YOU READ IT!!!!**