Transmission dynamics, characterization, diagnosis, and control of Bovine Respiratory Disease

by

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B.S., University of Florida, 2008
D.V.M., University of Florida, 2013

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Diagnostic Medicine and Pathobiology
College of Veterinary Medicine

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Abstract

The diagnosis and prevention of bovine respiratory disease (BRD) is challenging due to the multifactorial nature of the disease. *Mannheimia haemolytica*, *Pasteurella multocida*, and *Histophilus somni* are gram negative bacteria that are commonly considered three of the most important bacterial agents involved in the etiology of BRD; all three are also commensals in the upper respiratory tracts of healthy cattle. However, despite decades of study, questions still remain regarding the transmission dynamics and characterization of these bacteria, the ability of diagnostic sampling methods to accurately portray the causative bacteria, and ways to mitigate the effects of risk factors for BRD such as long-distance transportation.

Through our research, we have demonstrated how variable the culture results of a single nasopharyngeal swab can be and the challenges of using an individual culture to truly represent animal *M. haemolytica* status. Additionally, comparison of the diagnostic performance of two antemortem sampling methods, nasopharyngeal swabs (NPS) and bronchoalveolar lavages (BAL), revealed high agreement, high negative predictive values of NPS for the presence of *M. haemolytica*, *P. multocida*, and *H. somni* in the lungs, and the potential for different susceptibility profiles from paired NPS and BAL samples. As agreement and predictive values can vary with disease prevalence, interpretation of diagnostic test results should be done carefully and with due consideration of the sample population in which the test is being applied.

An investigation into cattle behavior following a relatively innocuous handling procedure indicated that some behaviors are altered after handling and restraint in a squeeze chute. Additionally, we demonstrated that cattle with different temperament scores may have

different activity levels and spend different amounts of time within 1 m of the hay bunk, grain bunk, waterer, and shed after handling. Consequently, there is also the potential to improve upon disease detection algorithms by incorporating behavioral changes that may occur after handling events and the need for careful trial design when behavioral parameters are a trial outcome. Additionally, this study indicated that some behaviors may vary for calves that react differently when handled, which also has potential implications when behavior is considered a variable of interest.

Examination of the potential for 1 mg/kg oral meloxicam administered pre-transport to mitigate the effects of long-distance transportation revealed that meloxicam did not have a statistically significant effect on the maintenance of leukocyte function or the reduction of inflammation during or after long-distance transportation in healthy steers. Finally, a report on two separate experiments regarding the effects of 1 mg/kg oral meloxicam administered pre-transport on the movement, feeding, and drinking behaviors and performance of transported and non-transported calves demonstrated that there was a significant day effect on behaviors in transported calves but meloxicam did not affect behavior or performance. However, meloxicam did modify the effect of day on daily distance traveled in non-transported calves but there were no significant within-day comparisons between non-transported meloxicam and non-transported control calves. These results do not provide evidence for the benefit of administering a single dose of 1 mg/kg oral meloxicam prior to long-distance transportation.

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Dedication

To my strong tower, to my family, and to my friends who might as well be family. Your love and support over the years has been invaluable and I am so blessed to have you all in my life.

Preface

The multifactorial nature of the epidemiology of bovine respiratory disease (BRD) is reflected in this compilation of work. Chapter 1 begins with a review of antemortem sampling methods for establishing the presence of BRD pathogens including aspiration of nasal mucus, anterior nasal swabs, nasopharyngeal swabs, tonsillar washes, transtracheal sampling, and bronchoalveolar lavage sampling. Investigations into our ability to characterize one of the most commonly implicated BRD pathogens, *Mannheimia haemolytica*, are explored in Chapter 2. A comparison of the diagnostic agreement and predictive values of nasopharyngeal swab and bronchoalveolar lavage culture results is found in Chapter 3. Chapter 4 details a study on the activity, eating, and drinking behavior of clinically healthy calves following a relatively innocuous handling event similar to what cattle may experience when examined for evidence of BRD. Finally, Chapters 5 and 6 include investigations into the effects of long-distance transportation, a common stressor implicated in increasing BRD risk in feedlot cattle, on various markers of stress, inflammation, and leukocyte function as well as several behavioral parameters.

Chapter 1 - Review of Sampling Procedures for Bovine Respiratory Disease Pathogens

Introduction

Mannheimia haemolytica, *Pasteurella multocida*, and *Histophilus somni* are commonly considered three of the most important bacterial agents involved in the multifactorial disease process known as bovine respiratory disease (BRD).[1] These gram negative bacteria are considered to be commensals in the upper respiratory tracts of healthy cattle[1] but can become pathogens of the lower respiratory tract following stress, lowered immune function, or exposure to viruses.[1-3] Although BRD has been the subject of decades of study, relatively little research has been aimed at defining the most appropriate methods for obtaining biological samples for subsequent culture and additional diagnostics. The diagnosis of BRD is challenging for many reasons including our inability to correctly classify animals as sick or not sick before necropsy.[4,5] Likewise, diagnosing the causative agent of a particular case of BRD is also challenging given the imperfect ability of our diagnostic methods.[6] Ultimately, there are many factors which play a role in whether or not a particular test will be positive for a specific agent or not. The focus of this chapter is to review the available literature comparing the utility and diagnostic performance of some of the most commonly applied methods for obtaining respiratory tract samples for culture purposes. Additionally, several manuscripts that did not compare sampling methods but which reported results from paired respiratory tract samples and provided enough data for this author (SC) to calculate either their positive and negative predictive values or the agreement between methods will also be discussed.

Sampling methods

Various methods exist for obtaining an antemortem culture from cattle and they require varying amounts of skill, restraint, and/or sedation. One of the most common and simplest methods is to aspirate[7] a bit of mucus from the anterior nasal cavity or swab the same area with a guarded[8] or unguarded nasal swab (NS).[9] Another method employed in several papers is the tonsillar wash (TW) which involves inserting and immediately aspirating a wash solution from the tonsillar sinuses.[7,10] More recently, nasopharyngeal swabs (NPS), which are either single or double-guarded swabs that are inserted deeper into the upper respiratory tract to obtain a sample of the nasopharyngeal region have been employed (Figure 3.1).[6,11-14] Antemortem lower respiratory tract samples can also be obtained via several methods including transtracheal washes[11,15] or swabs[16] (TTS) and bronchoalveolar lavages (BAL) which can be performed either blinded[17-21] or via endoscopic guidance.[12]

Some details that must be considered when evaluating which sampling method is best include animal welfare considerations, degree of invasiveness, potential for cross contamination both within and between cattle, required equipment and ease of equipment sterilization, and the required skill level of the sample taker. Certain methods also become relatively impractical if frequent serial sampling is needed. Some researchers, including the author of this dissertation (SC), have found guarded NPS to be a simple method for obtaining an upper respiratory tract sample that requires relatively little in terms of equipment or restraint, and can be done quickly and frequently in large volumes.[13] While it is true that some may consider the NPS more invasive than an anterior nasal swab, the author (SC) has observed that cattle are most reactive when swabs are guided within the first few inches of the nasal cavity but many tolerate the

advancement of a NPS past that point very well. Another consideration when comparing upper respiratory sampling sites is the possibility that samples taken deeper in the upper respiratory tract may be preferred due to less environmental contamination (Personal communication, B. Lubbers Sept. 2016).

When considering antemortem lower respiratory tract sampling techniques, BALs are often considered to be less invasive, quicker, and easier to obtain than TTS.[18] Proponents of TTS are drawn to the fact that there is no contamination of the sample from the upper respiratory tract[15,21] but Grey *et al.* demonstrated in 1971 that variable concentrations of *M. haemolytica* could be found in the tracheal air of individual clinically normal calves that also had *M. haemolytica* recovered from nasal swabs taken on the same day.[22] Therefore, the possibility of tracheal contamination exists, at least for *M. haemolytica*, simply via normal respiration. Undoubtedly the potential for cross contamination between the nasal cavities and the lungs exists with unguarded BAL techniques, yet the use of a guard[12] as described by Allen *et al.* has not been widely reported. There are many variations on the BAL procedure including endoscopic[12] and blind techniques[17,18,21]; some involve sedation while others use varying methods of restraint depending on the size and age of the animal being sampled. The author (SC) has found that, like NPS, most animals tolerate blind BALs well once the tubing has passed the first few inches of the nasal cavity and that an experienced person can perform a blind BAL in a few minutes. For more discussion on blind vs. endoscopic BALs, see Chapter 3 of this dissertation.

Evaluating the performance of sampling methods

Although a detailed review of predictive values and kappa values are beyond the scope of this literature review, a brief introduction regarding their calculation, use, and limitations is warranted given their importance in the subsequent literature cited. Predictive values are routinely misunderstood and often easily confused with the diagnostic sensitivity and specificity of a test. However, they provide important information about the probability of disease given a positive or negative test result while taking into account both the sensitivity and specificity of the test used and the true prevalence of the disease in the sampled population. Therefore, predictive values can change when the same tests are used within different populations with different prevalences (e.g., in healthy cattle vs. clinically ill cattle). A positive predictive value (PPV) is interpreted as "the probability that given a positive test, the animal actually has the disease" while a negative predictive value (NPV) is interpreted as "the probability that given a negative test, the animal does not have the disease".[23] Predictive values were calculated by the author of this review (SC) as indicated in Dohoo *et al.*[23]

It is important when comparing the results of two diagnostic tests to consider that there will always be some agreement between the two tests simply due to chance alone.[23] The kappa statistic is a method for evaluating whether two tests agree with each other beyond what agreement could be expected due to chance but it is influenced by both the prevalence of the disease within the group of animals examined and the presence of bias.[23,24] The kappa values calculated in this dissertation were calculated as described elsewhere[23] and Exact McNemar's *P*-values were obtained for each kappa statistic using Stata (Version 12.1, StataCorp LP, College Station, Texas). A significant Exact McNemar's *P*-value indicates that there is evidence of

bias[23,25] between the two tests and therefore the kappa value is biased. The scale used to interpret the kappa values herein is the Landis and Koch scale[26] where ≤ 0 indicates poor agreement, 0.01 to 0.2 indicates slight agreement, 0.21 to 0.4 indicates fair agreement, 0.41 to 0.6 indicates moderate agreement, 0.61 to 0.8 indicates substantial agreement, and 0.81 to 1.0 indicates almost perfect agreement. More detailed information on the kappa statistic and its limitations can be found in the published literature.[23,24,27-30]

Organisms evaluated

Of the three bacteria previously mentioned, the focus of the vast majority of the literature examined for this review revolves around the isolation of *M. haemolytica* which has been found in the lungs of cattle without lung lesions[31], the oral cavity[22], and in all areas of the nasal cavity[32] including the tonsils[7,31], nasopharyngeal lymph nodes[31], and nasopharynx.[12] Various methods for differentiating between *M. haemolytica* isolates have been explored in the literature including serotyping[14,33], antimicrobial susceptibility profiling[34], plasmid identification[34], PCR[14], pulsed-field gel electrophoresis (PFGE)[6,14,35], as well as other advanced genomics methods.[18,36,37] Although some manuscripts discussed herein also explore the presence of *P. multocida* and *H. somni*, there is considerably less literature covering diagnostic performance in those organisms when compared to *M. haemolytica*.

Comparison of antemortem upper respiratory tract methods

Within the published literature, several challenge studies employing unique *M. haemolytica* strains provided culture results from two separate antemortem upper respiratory tract sampling methods. Data reported by Frank & Briggs showed that, after infecting the tonsils

of calves with *M. haemolytica*, there were differences in their ability to detect *M. haemolytica* between nasal mucus samples and tonsillar wash samples taken bi-weekly over the course of 25 days.[7] The authors of that study did not formally evaluate PPV, NPV or kappa values but did provide sufficient data in the text and in Figure 1 of the manuscript for this author (SC) to do so (Table 1.1). Although the sample size was small, the results of that study illustrate that *M. haemolytica* can be found in the tonsils even if it is not found in the nasal mucus. Subsequent work done by Frank *et al.* in 1994 also indicated variability in recovery of *M. haemolytica* from the nasal mucus and tonsils of non *M. haemolytica*-vaccinated calves over time; calculations of agreement, PPV, and NPV were performed from the data provided by the authors (Table 1.1). Another study reported by Briggs *et al.* which examined 10 non-challenged calves placed in a pen with 4 calves who were challenged with a unique strain of *M. haemolytica*[34] also provided enough data for comparison of nasal mucus and tonsillar wash samples (Table 1.1). Again, variability in recovery of *M. haemolytica* from the two sample locations was apparent.

Comparison of antemortem upper and lower respiratory tract methods

Challenge studies employing both upper and lower respiratory tract samples were also found in the literature. One study examined the isolation of *M. haemolytica* from nasal mucus samples and BALs following challenge with *M. haemolytica* and vaccination in half the calves (n = 6 vaccinated, n = 6 controls).[38] Variability in the recovery of the organism at given time points was reported between the vaccinated and control animals yet sufficient data was not reported to calculate agreement or predictive values. In another report, even after aerosol challenge with a nalidixic acid resistant *M. haemolytica* isolate, there was variability in the recovery of the organism both within and between calves from tracheal and nasopharyngeal swabs taken

repeatedly during the first 23 hours after challenge.[39] However, as the study comprised only 4 calves in each treatment group, formal calculation of agreement or predictive values was not performed.

As previously noted, Grey *et al.* examined the tracheal air of calves and compared the presence and quantity of *M. haemolytica* found in the tracheal air to the presence and quantity of *M. haemolytica* in the anterior nasal passages.[22] Although the authors did not report any direct correlation in bacterial numbers between the two sampling sites, they did not obtain *M. haemolytica* from the tracheal air of calves if there were none in the nasal swabs.[22] However, 86% (6/7) of the nasal and tracheal air negative calves had *M. haemolytica* present in the oral swabs that were taken daily.[22] Data from Confer *et al.*[40] was used to calculate kappa values for anterior nasal and tracheal cultures obtained via the oropharynx from clinically healthy, low-risk calves and revealed slight to poor agreement for isolating *M. haemolytica* (Table 1.2). Calculations from the same data indicated that the PPV of a nasal swab compared to tracheal cultures was between 10% and 29%, whereas the NPV was higher at 88% to 95% (Table 1.2).

One study that specifically set out to compare an upper and lower respiratory tract sampling method for three major BRD pathogens was performed by Allen *et al.* in 1991. The authors found moderate agreement between NPS and guarded endoscopic BAL samples in terms of *M. haemolytica* (kappa = 0.47), *P. multocida* (kappa = 0.61), and *H. somni* (kappa = 0.55) when all the calves (cases and controls) were considered together but also noted that there was considerable variability in where the true estimate of agreement should lie.[12] Therefore, the authors concluded that NPS was not able to accurately predict BAL results in individual calves

and would be more appropriately used on the group level.[12] Further calculations by this author (SC) of the PPV, NPV, and kappa values from data presented in the 1991 study also indicated variability in the performance of the two sampling methods when data for cases and clinically healthy controls were considered separately (Table 1.3). Then, in another report concerning the same group of calves, Allen *et al.* also found that there was variability in both BAL and NPS culture results that were repeated over time.[41]

Another study by DeRosa *et al.* examined paired anterior guarded nasal swabs and transtracheal swabs taken from calves with clinical BRD. Their work demonstrated that it is possible to obtain different organisms from the two methods 31.6% of the time.[16] Additionally, when a single *M. haemolytica* or *P. multocida* isolate from each paired positive sample was tested, there were instances where the minimum inhibitory concentrations were not within one twofold dilution of each other, though the authors indicated that from a therapeutic perspective, they were similar.[16] However, when the genetic identity of all paired *M. haemolytica* and *P. multocida* isolates were compared via ribotyping, the ribotypes were not identical between the two methods in 30% of the positive paired samples.[16] Further calculation of the kappa values for *M. haemolytica*, *P. multocida*, and *H. somni* from the paired samples taken in this study showed fair to poor agreement within organism for all three organisms (Table 1.2). Additional comparisons of calculated predictive values also reveal that although the PPV of nasal swabs for *M. haemolytica* were almost perfect, the PPV was considerably lower for *P. multocida* and *H. somni* (Table 1.2). Therefore, considerable variability exists in the ability of a guarded anterior nasal swab to predict whether *M. haemolytica*, *P. multocida*, or *H. somni* are in the lower respiratory tract.

In 2013 Timsit *et al.* reported only moderate agreement (kappa = 0.50) between repeated nasopharyngeal swabs and transtracheal aspirations for *M. haemolytica* and demonstrated that both clinically healthy and clinically ill animals could have *M. haemolytica* in the upper respiratory tract but not the lungs and vice versa. They also noted that different *M. haemolytica* PFGE types could be obtained from 33% of the positive matched paired samples.[11] The authors hypothesized that this difference may be explained by previous evidence provided by Briggs *et al*. that indicated that different *M. haemolytica* can be present in different parts of the upper respiratory tract within the same calf[34] or that the sampling methods of only choosing one isolate per sample may have resulted in bias. However, Timsit *et al*. examined multiple isolates (8-10 colonies per sample) from a small subset of eight transtracheal aspirations and did not identify any PFGE diversity within a single transtracheal aspiration sample.[11]

Overall, these reports point to considerable variability in the agreement of antemortem upper and lower respiratory tract sampling methods. They also indicate that the ability of antemortem upper respiratory tract methods to predict what is in the lungs is suboptimal and can be quite poor depending on the population of cattle examined. Additionally the comparison of bacterial isolates from paired samples indicate that it is possible to obtain organisms that are genetically different and that have different antimicrobial susceptibilities in the two locations. Therefore, even if an antemortem upper respiratory tract sample indicates the presence of a particular bacteria, the bacterial population in the lungs may be different.

Comparison of antemortem upper respiratory tract and postmortem lower respiratory tract sampling collection methods

Barbour *et al.* compared anterior nasal swabs and post-mortem lung cultures of clinically ill and clinically healthy 3 month old Holstein calves and found *M. haemolytica* in the lungs of all sick calves that were necropsied (n = 5) but in only 16 of the nasal swab samples from sick calves (n = 34).[42] Among the clinically healthy calves, no *M. haemolytica* was found in the lungs of the calves that were necropsied (n = 5) but it was found in 14 of the nasal swabs taken from clinically healthy calves (n = 40).[42] However, information about direct comparisons of paired samples taken from individual calves was not available in this manuscript, and therefore inferences about the predictive values or agreement between the isolation of *M. haemolytica* from the anterior nasal passages before death and the lungs after death was not possible.

In 2007, Godinho *et al.* compared nasopharyngeal swabs with postmortem lung lavages, lung swabs, and lung tissue sections for the presence of *M. haemolytica* in 20 calves with clinical BRD. The authors reported that minimum inhibitory concentrations for tulathromycin of isolates from paired positive samples were within a twofold dilution and therefore considered equivalent.[13] Additionally, when the authors compared the randomly amplified polymorphic DNA (RAPD) and denaturing gradient gel electrophoresis (DGGE) of 9 positive paired NPS and lung lavage samples, they found that 8/9 were identical via RAPD and all were identical via DGGE.[13] However, PPVs of NPS for lung lavages, lung tissue, and lung swabs ranged from 50% to 100% while the NPVs ranged from 70 to 100% respectively as calculated by the author of this dissertation (SC) (Table 1.4). Therefore, it seems that the comparison of an antemortem upper respiratory tract sample and some postmortem lower respiratory tract sampling methods in

clinically ill calves also point to the potential for disagreement regarding presence of the organisms. However, limited data on the multiple methods evaluated and the potential for different results within populations of animals with different prevalences makes it difficult to make firm conclusions regarding the utility of antemortem upper respiratory tract samples when compared to postmortem lower respiratory tract samples.

Conclusions

Over the years, studies have utilized different sampling methods to obtain samples for subsequent testing. Study design, sample population demographics, sample sizes, and data reported are variable among the literature examined herein but the common theme is that upper respiratory tract samples are not perfectly predictive of what is found in the lungs; indeed, their predictive values and agreement vary between BRD cases and clinically healthy animals and between pathogens. It is clear that the ecology of the three main bacteria involved in BRD is complex and that additional work needs to be done to better characterize the relationship of these organisms in the upper and lower respiratory tracts. Without further knowledge of how these sampling methods compare, the usefulness and interpretation of the diagnostics performed on the samples is questionable.

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Figure 1.1 – Schematic representation of the sampling location of a nasopharyngeal swab (NPS) in cattle. Image created by Mal Hoover.

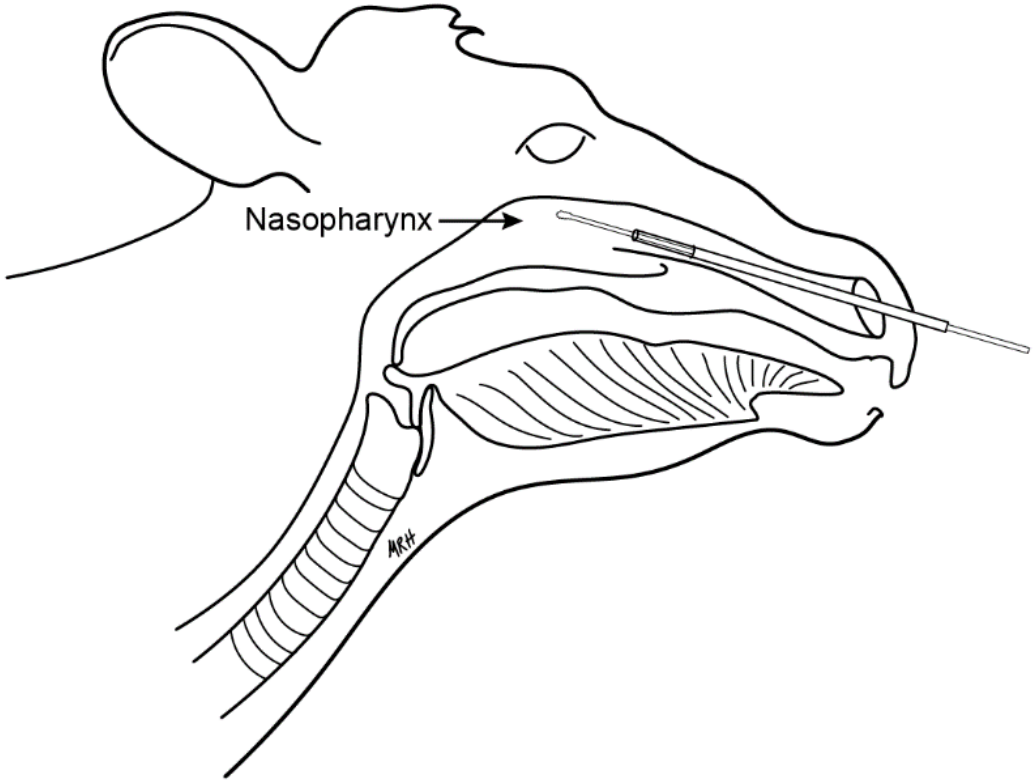

Table 1.1 – Summary of positive and negative predictive values (PPV and NPV, respectively) and kappa statistics of paired upper respiratory tract samples for *Mannheimia haemolytica* as calculated by the author of this dissertation (SC) from data obtained from 3 challenge studies. Calves in the Frank & Briggs study were challenged in the tonsils with *M. haemolytica* on day 0. Cells containing "n/a" indicate that the predictive value was calculated as 0/0 which is undefined.

Organism	Animal status	Study day	Sample size	Sample Type				Kappa	Exact McNemar's *P*-value[a]	Reference
				Nasal mucus		Tonsillar wash				
				PPV (%)	NPV (%)	PPV (%)	NPV (%)			
M. haemolytica	Challenged	Day 4	10	100.0	0.0	20.0	n/a[b]	0.00	0.01	Figure 1 and text Frank & Briggs, 1992
		Day 7	10	100.0	0.0	10.0	n/a[b]	0.00	< 0.01	
		All other days[b]	10	n/a[b]	0.0	0.0	n/a[b]	0.00	< 0.01	
M. haemolytica; all serotypes	Non-vaccinated for *M. haemolytica*	Day 1	44	59.1	13.6	40.6	25.0	-0.27	0.09	Table 4 Frank *et al.*, 1994
		Day 8	44	68.2	54.5	60.0	63.2	0.23	0.63	
		Day 29	44	85.7	50.0	44.4	88.2	0.29	< 0.01	
M. haemolytica	Non-inoculated calves	Day 7	10	14.3	100.0	100.0	33.3	0.09	0.03	Tables 4 and 5 Briggs *et. al.*, 1998
		Day 14	10	28.6	66.7	66.7	28.6	-0.03	0.22	
		Day 24	10	0.0	66.7	0.0	85.7	-0.18	0.63	

[a] A significant *P*-value indicates there is evidence of bias between the sampling methods and therefore the kappa value is biased.
[b] Results between nasal mucus samples and tonsillar washes were identical on the remaining 5 collection days.

Table 1.2 – Positive and negative predictive values (PPV and NPV, respectively) and kappa values for nasal swabs compared to tracheal and transtracheal swabs. In the first study, culture results for *Mannheimia haemolytica* were evaluated in clinically healthy calves. In the second study, culture results for *M. haemolytica*, *Pasteurella multocida*, and *Histophilus somni* were evaluated in bovine respiratory disease (BRD) cases at the time of BRD diagnosis.

Organism	Animal status	Study day	Sample size	Sample Type				Kappa	Exact McNemar's *P*-value[a]	Reference
				Nasal swab		Tracheal swab				
				PPV (%)	NPV (%)	PPV (%)	NPV (%)			
M. haemolytica	All calves; clinically healthy	Day 0	164	10.0	95.1	22.2	88.4	0.07	0.04	Table 2 Confer *et. al.*, 1983
	Group B; clinically healthy	Before slaughter	55	28.6	87.8	44.4	78.3	0.19	0.30	
				Nasal swab		Transtracheal swab				
				PPV (%)	NPV (%)	PPV (%)	NPV (%)			
M. haemolytica	BRD Cases	BRD diagnosis	40	85.2	38.5	74.2	55.6	0.26	0.39	Table 1 DeRosa *et al.*, 2000
P. multocida			40	37.5	90.6	50.0	85.3	0.31	0.73	
H. somni			40	0.0	97.4	0.0	97.4	-0.03	1.00	

[a] A significant *P*-value indicates there is evidence of bias between the sampling methods and therefore the kappa value is biased.

Table 1.3 – Positive and negative predictive values (PPV and NPV, respectively) and kappa values for nasopharyngeal swab (NPS) and bronchoalveolar lavage (BAL) samples obtained from bovine respiratory disease (BRD) cases, clinically healthy controls, and all calves. In this study, paired samples were obtained from cases and matched controls on the day of BRD diagnosis; three organisms, *Mannheimia haemolytica*, *Pasteurella multocida*, and *Histophilus somni* were cultured from the samples. Kappa values from all calves were reported by the original author but the remaining values were calculated by the author of this manuscript (SC) from data provided in the original manuscript.

Organism	Animal status	Study day	Sample size	Sample Type				Kappa	Exact McNemar's *P*-value[a]	Reference
				NPS		BAL				
				PPV (%)	NPV (%)	PPV (%)	NPV (%)			
M. haemolytica	BRD Cases	BRD diagnosis	59	66.7	96.0	75.0	94.1	0.66	1.00	Allen *et al.*, 1991
	Controls		60	31.6	97.6	85.7	75.5	0.35	< 0.01	
	All calves		119	42.9	96.7	80.0	84.6	0.47	< 0.01	
P. multocida	BRD Cases		59	85.4	72.2	87.5	68.4	0.57	1.00	
	Controls		60	75.0	84.4	80.8	79.4	0.60	0.77	
	All calves		119	81.2	80.0	84.8	75.5	0.61	0.68	
H. somni	BRD Cases		59	42.9	92.3	42.9	92.3	0.35	1.00	
	Controls		60	75.0	100.0	100.0	98.2	0.85	1.00	
	All calves		119	54.5	96.3	60.0	95.4	0.53	1.00	

[a] A significant *P*-value indicates there is evidence of bias between the sampling methods and therefore the kappa value is biased.

Table 1.4 – Positive and negative predictive values (PPV and NPV, respectively) and kappa values for antemortem nasopharyngeal swabs (NPS) and several postmortem lower respiratory tract sampling methods for *Mannheimia haemolytica*. Calves in this study were all clinically ill with bovine respiratory disease (BRD) and were sampled at BRD diagnosis. Values in this table were calculated by the author of this manuscript (SC) from the data provided in Table 1 of the original manuscript.

Organism	Animal status	Study day	Sample size	Sample Type				Kappa	Exact McNemar's *P*-value[a]	Reference
				NPS		Postmortem lung lavage				
				PPV (%)	NPV (%)	PPV (%)	NPV (%)			
M. haemolytica	BRD cases	BRD diagnosis	20	100.0	100.0	100.0	100.0	1.00	1.00	
				NPS		Postmortem lung tissue				
				PPV (%)	NPV (%)	PPV (%)	NPV (%)			
M. haemolytica	BRD cases	BRD diagnosis	20	50.0	100.0	100.0	25.0	0.21	< 0.01	Table 1 Godinho *et al.*, 2007
				NPS		Postmortem lung swab				
				PPV (%)	NPV (%)	PPV (%)	NPV (%)			
M. haemolytica	BRD cases	BRD diagnosis	20	50.0	70.0	90.0	20.0	0.10	0.04	

[a] A significant *P*-value indicates there is evidence of bias between the sampling methods and therefore the kappa value is biased.

Chapter 2 - Characterization of *Mannheimia haemolytica* in beef calves via nasopharyngeal culture and pulsed-field gel electrophoresis

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Abstract

Mannheimia haemolytica is a major bacterial component of bovine respiratory disease (BRD); unfortunately, very little is known about *M. haemolytica* transmission dynamics among cattle. Identifying potential variation in *M. haemolytica* populations over time and induction of nasopharyngeal colonization and subsequent shedding are 2 areas where knowledge is lacking. In our study, 2 separate loads of 20 mixed-origin, male calves were purchased through an order buyer on different dates. Deep nasopharyngeal cultures (NPC) were performed on all calves on arrival and, if *M. haemolytica*–negative, a second screening culture was obtained. Calves that were negative on 2 initial NPCs (NEG; $n = 4$) were subsequently challenged with a previously isolated field strain of *M. haemolytica* in both the upper and lower respiratory tract, individually

housed, and then monitored for *M. haemolytica* shedding via NPCs at 0.5, 1, 3, 5, 7, and 9 days postchallenge. Naturally *M. haemolytica*–positive calves (2 per load) were kept for additional daily cultures (POS; $n = 4$). Individual calf *M. haemolytica* status for both the POS and NEG groups was inconsistent between study days. Additionally, pulsed-field gel electrophoresis performed on isolates from the positive cultures showed that the NEG calves did not shed the *M. haemolytica* challenge strain, but rather 2 distinct clusters of *M. haemolytica* were shared among POS and NEG calves regardless of their initial status. Although sample sizes were small, these findings illustrate how variable the results of a single nasopharyngeal swab can be and the challenges of using an individual culture to truly represent animal *M. haemolytica* status.

Introduction

Bovine respiratory disease (BRD) is widely considered the most common and costly postweaning beef cattle disease.[17,18,25] Although BRD is truly a multifactorial disease with bacterial, viral, environmental, and host factors involved, *Mannheimia haemolytica* is the most frequently isolated bacterial pathogen from BRD cases.[16,17,24] *Mannheimia haemolytica* has many virulence factors that have been identified, and *M. haemolytica* may transition from being a commensal organism within the nasal passages into a pulmonary pathogen.[16] Unfortunately, it is unclear how *M. haemolytica* makes this transition, and whether a specific *M. haemolytica* isolate is likely to transfer from calf to calf within a population. A variety of methods have been used to explore *M. haemolytica* population dynamics over the years including serotyping,[1] plasmid typing,[7] and selective culture[15] with pulsed-field gel electrophoresis (PFGE) gaining popularity because of its high discriminatory power and medium-to-high repeatability.[18,24]

Because definitive antemortem diagnosis of BRD based on clinical signs can be difficult,[4] additional diagnostic aids such as nasal cultures, transtracheal cultures, transtracheal washes, and bronchoalveolar lavages have been used in both research and clinical settings.[2,6,9,10] Although several studies[13,15,19] have attempted to use nasopharyngeal culture (NPC) as a way to describe the population of *M. haemolytica* within individual calves and how it moves within a group of calves, it remains an imperfect test. Other studies have attempted to correlate NPC results with cultures obtained elsewhere in the respiratory tract with mixed results.[2,8,10,15,24] Although inoculation of calves with *M. haemolytica* can induce BRD,[7,14] a repeatable model for *M. haemolytica* inoculation that results in subsequent shedding has not been established.

The objectives of our pilot study were to describe the arrival *M. haemolytica* status in a group of calves via NPC, identify negative animals (NEG), and evaluate the ability of an artificially inoculated *M. haemolytica* strain to colonize and shed from those negative calves. Additionally, this pilot study sought to identify healthy, naturally *M. haemolytica*– positive calves (POS) on arrival and evaluate the shedding patterns of *M. haemolytica* in those calves via NPC.

Materials and Methods

Calf selection and management

Two loads of 20 male calves were procured through an order buyer from local livestock auction markets and transported to the Kansas State University Large Animal Research Center (Manhattan, Kansas; total $n = 40$). Within a few hours of arrival, all calves were weighed and individually identified with ear tags. Additionally, the nasopharynx of each calf was cultured by passing a single guarded calcium alginate swab[a] through the right external nares. The guarded swab was then guided into the

caudal nasal passage where the inner swab was passed through the guard, rotated against the nasal mucosa, and retracted back into the guard prior to removal from the nasal passages. The swab was placed immediately into sterile transport media[b] and either temporarily refrigerated or submitted directly to the Kansas State Veterinary Diagnostic Laboratory (Manhattan, KS) for aerobic culture. None of the calves received antimicrobial treatment for the control of BRD, vaccinations, or implants at any time during the study, and any animal that required antimicrobial treatment for clinical disease was removed from the study. Based on the results of the initial screening culture(s), calves were segregated into culture-positive (POS) and culture-negative (NEG) groups (Figure 2.1).

During the prechallenge period, all calves were housed in open-air, dirt-floor group housing pens with a total area of 297 m^2 per pen and provided with an adequate amount of bunk space, access to water, and shelter. In order to prevent any possible nose-to-nose contact, calves were individually housed immediately following challenge in either identical open-air, dirt-floor pens or in identical 13.4 m^2 stalls. Within the 2 types of housing, the challenged calves were managed similarly. Throughout the trial, calves were fed a diet consisting of a grain mix with grass hay and water made available ad libitum. Calf management and sampling procedures described were part of a protocol approved by the Kansas State University Institutional Animal Care and Use Committee.

Preparation of challenge media

A *M. haemolytica* isolate obtained from a field case of BRD and used in a prior challenge study[5] was grown on a 5% ovine blood agar plate in a CO_2 incubator at 37℃ for 18–22 hr. Isolated colonies were inoculated into brain–heart infusion broth and incubated for 16–18 hr at 37℃ on a rotary shaker. The bacteria were then pelleted via centrifugation at 15,000 × *g* for 20 min, washed

3 times in sterile phosphate buffered saline (PBS), and re-suspended in sterile PBS to an optical density (OD) of 1.8–2.0 at 600 nm. This OD corresponds to a bacterial concentration of 3×10^8 colony forming units (CFU)/mL as determined by a standard curve of *M. haemolytica* CFUs versus OD at 600 nm.

Challenge and sampling of negative calves

Four NEG calves were restrained in a chute with their heads elevated and stabilized using a rope halter. A 5.9-mm endo-scope with 2-mm biopsy channel[c] was introduced into the right nasal passage of each calf and passed through the nasopharyngeal region and laryngeal folds into the trachea and advanced to the right cranial tracheobronchus. A 140-cm polyurethane catheter was then passed through the endoscopic biopsy channel, and 10 mL of *M. haemolytica* inoculum (3×10^8 CFU/mL in PBS) followed by 60 mL of sterile PBS was administered. Following the endoscopic challenge, calves were also inoculated with 2 mL of *M. haemolytica* (3×10^8 CFU/mL in PBS) deposited into the right nasal passages via a syringe.

Using the deep nasopharyngeal sample collection technique described above, samples for culture were collected at 0.5, 1, 3, 5, 7, and 9 days postchallenge from the right nasopharyngeal area. Rectal temperatures were also recorded at the time of each NPC. Potential shedding of the challenge organism was determined by comparing the PFGE pattern of a single *M. haemolytica* isolate from each positive culture plate with the known challenge strain.

Challenged calves were euthanized via a penetrating captive bolt[d] according to the American Veterinary Medical Association Guidelines (https://www.avma.org/KB/Policies/

Documents/euthanasia.pdf), and a full autopsy was performed 10 days postchallenge. During autopsy, the lungs were removed intact and then weighed; lung lesions were scored by an experienced veterinarian using a standardized system[12] similar to previous work.[4,5,23]

Monitoring of naturally *M. haemolytica*-positive calves

Four naturally POS calves (2 from each load) were retained after initial screening for further monitoring of NPC status. Prior to the start of daily cultures, all 4 calves were confirmed culture positive for *M. haemolytica*, and up to 10 isolates were selected and frozen per calf for later PFGE comparison. If <10 distinct colonies were present on the plate, all *M. haemolytica* isolates were selected from the plate and frozen for later PFGE comparison. Once the second confirmatory culture results were obtained, NPCs were performed daily for 3 days as the calves continued to be monitored for clinical signs of illness. Single isolates were retained from each positive daily culture and examined by PFGE.

Clinical illness scores

All calves were observed by the same veterinarian (SF Capik) twice daily throughout the study (morning and evening) for any signs of illness and assigned a clinical illness score (CIS) based on the following criteria: 0 = normal calf, 1 = mild signs of depression, 2 = moderate depression, 3 = severe depression, and 4 = severe prostration and/or recumbence.[21] Following arrival and prior to challenge, any calf with a CIS of >0 was evaluated by a veterinarian and appropriate treatment applied. If antimicrobial treatment was deemed necessary at any time after arrival, the animal was treated and excluded from the study. Any calf given a clinical illness score of 4 at any point during the trial was humanely euthanized and an autopsy performed.

Microbiology culture methods

Nasopharyngeal swabs were rolled directly onto trypticase soy agar plus 5% sheep blood (SBA) and chocolate agar plates. Using a sterile loop, multiple passes (in a single continuous streak) were made through the inoculated area of the agar plates. A second sterile loop was used, in the same manner, from the second to third quadrants of the agar plate so that isolated colonies would be present. The SBA and chocolate plates were incubated at 37°C in 5% CO_2 for 18–24 hr. Following incubation, plates were examined, and colonies suspected to be *M. haemolytica* were either re-streaked for isolation or identified directly (depending on degree of colony isolation and phase of project). For the second confirmatory culture of POS calves in which up to 10 isolates were selected for PFGE, isolates were chosen preferentially from colonies that were either 1) phenotypically different or 2) distinct from background growth. All bacterial identification was performed using matrix-assisted laser desorption ionization[e] according to the manufacturer's instructions. Isolates, in pure culture, were stored in *Brucella* broth plus 10% glycerol at –80ºC for further PFGE analysis.

Pulsed-field gel electrophoresis

Actively growing, pure bacterial cultures on blood agar plates were collected using sterile cotton swabs and resuspended in a buffered resuspension solution (100 mM Tris–HCl, pH 8.0, and 100 mM ethylenediamine tetra-acetic acid [EDTA]). Cell concentration was adjusted to 0.72 units,[f] and 200 µL of the cell suspension was mixed with 200 µL of 1.2% agarose in water and proteinase K at 0.5 mg/mL[g] and dispensed into plug molds.[h] Bacteria-containing agarose plugs were lysed in the presence of 50 mM Tris–HCl (pH 8.0), 50 mM EDTA, 1% sarkosyl, and 0.32 of mg/mL proteinase K at 54°C with constant agitation. Plugs were successively washed with distilled water and 10 mM Tris–HCl (pH 8.0) and 1

mM EDTA at 50°C with constant agitation. Agarose slices were digested with *Sma*I restriction enzyme[i] for 2 hr according to manufacturer's instructions, and electrophoresis was performed[j] on a 1% agarose gel[k] with 0.5× Tris–borate–EDTA buffer (50 mM Tris–HCl, pH 8.4, 45 mM boric acid, and 0.5 mM EDTA) at 14°C. *Salmonella enterica* subsp. *enterica* serovar Braenderup ATCC BAA-664 was used as a molecular weight marker strain, and plug DNA from that organism was digested with *Xba*I.[l] The molecular weight marker DNA was interspersed in lanes at regular intervals across each agarose gel in order to normalize banding patterns both within and between agarose gels. Electrophoresis conditions were Block 1: Initial Switch Time 2.0 sec, Final Switch Time 5.0 sec, Run Time 12 hr; Block 2: Initial Switch Time 2.0 sec, Final Switch Time 5.0 sec, Run Time 12 hr; for both blocks: colts/cm: 5.6, V, Included Angle: 120°, 24 hr total. Agarose gels were stained with 1 μg/μL of ethidium bromide, destained, and an image obtained.[m] Clustering analysis was performed[n] using the Dice coefficient and the unweighted pair group method with arithmetic mean clustering method with settings for optimization: 1.0%, band tolerance: 1.5%. A 90% similarity threshold cutoff value was used when comparing the challenge strain and the cultured isolates.[26]

Results

The average weights of load 1 (arrived on 06/10/2013) and load 2 (arrived on 06/14/2013) were 169.3 kg and 165 kg, respectively. Between the 2 loads, a total of 32 bulls and 8 steers were enrolled in the study (Figure 2.1). After the initial screening culture, only 26 culture-negative calves remained eligible for the second screening culture. Following the second screening culture, 4 calves remained culture-negative (2 from each load) and were retained for challenge (NEG). Additionally, 4 culture-positive calves (2 from each load) were kept for additional monitoring (POS).

NEG calves

Following an observation period of 7–10 days in which the NEG calves (n = 4) were determined to be healthy, challenge was performed on trial day 0. Throughout the 10-day postchallenge period, only 1 calf (no. 75) received a clinical illness score of 1 on trial days 3, 4, 7, 8, and 9 after showing signs of mild depression and heat stress. All other calves appeared clinically normal throughout the trial, and no signs of respiratory disease were noted. Although rectal temperatures did not directly correlate with CIS or culture results, it was noted that calf 75 had a consistently elevated rectal temperature (>40°C) throughout the trial. Two other calves (23 and 54) had elevated rectal temperatures (>40°C) on trial day 0.5, and calf 23 also had an elevated rectal temperature (>40°C) on trial day 3.

Postchallenge NPCs revealed consistent results for 3 of the 4 negative calves but inconsistent shedding and day-to-day variability in 1 calf (Table 2.1). The use of PFGE analysis on a single isolate taken from each *M. haemolytica*–positive culture plate showed 2 distinct clusters of *M. haemolytica* (cluster A and cluster B) using a 90% similarity threshold cutoff value.[26] Comparison of cluster A and cluster B to the challenge strain using the same 90% similarity threshold cutoff value revealed that the challenge strain was unrelated to all *M. haemolytica* isolates selected from study animals (Figure 2.2). Additionally, within the 3 calves that cultured positive after challenge, calves 54 and 23 shed isolates that fell within cluster B while calf 75 shed isolates that were consistent with cluster A.

All 4 NEG challenged calves had prominent right cranioventral pulmonary lesions. Among the calves, total consolidation ranged from 10.1% to 37.6% with a median lung score of 19%, and lung weight as a percentage of body weight ranged from 0.93% to 1.47% with a median of 1.04%. Pleural adhesions were present in 3 of the 4 calves and 1 calf (no. 75) had reactive tracheobronchial lymph nodes. Despite gross evidence of BRD consistent with previous *M. haemolytica* challenge models, bacterial cultures of fresh lung samples from all 4 calves were negative for *M. haemolytica*.

POS calves

All 4 naturally *M. haemolytica*–positive calves remained clinically healthy throughout the monitoring period. Despite each of the calves having 2 prior positive cultures, the culture results from the 3 days of monitoring were inconsistent (Table 2.2). The PFGE analysis of the multiple isolates taken from the second confirmatory culture (calf 24: 10 isolates; calf 51: 1 isolate; calf 59: 10 isolates; calf 68: 5 isolates), and the single isolates taken from the daily culture fell within the same cluster A and cluster B equivalent to those seen in the NEG calves. Additionally, calf 59 had isolates that were consistent with both cluster A (9/10 isolates) and cluster B (1/10 isolates) on the second confirmatory culture. The isolates obtained from calf 51 alternated between cluster A and cluster B depending on the sampling day.

Discussion

The fluctuations in culture status and cluster seen within all phases of our pilot study raise questions about both the pattern of *M. haemolytica* shedding and the ability of an individual culture swab to give a true representation of the *M. haemolytica* status of a calf. Prior work[20] found *M. haemolytica* present only on the surface of the nasal epithelium and indicated that a negative nasal

swab culture did not necessarily mean that *M. haemolytica* was absent from the nasal cavity. Other work also showed that *M. haemolytica* was isolated irregularly even when both right and left sides of the anterior and posterior nasal meatuses of calves were swabbed multiple times a day for several days in a row.[19] The results of our study seem to support these previous studies, as the culture status of each calf within the NEG and POS groups was not always consistent even when daily samples were taken.

Given the multifactorial nature of BRD, the limitations of available diagnostic tests, and the knowledge gaps that exist regarding the hypothesized conversion of *M. haemolytica* from commensal to pathogenic, the prevalence of *M. haemolytica* in calves on arrival at a facility can be difficult to establish. Our study found considerable differences in the results of the arrival screening cultures performed on the 2 separate loads of calves we received, with load 1 having an apparent prevalence of 60% and load 2 having an apparent prevalence of 10% (Figure 2.1). However, when the second screening culture was performed several days later on previously culture-negative calves, the cumulative apparent prevalence for each load became 88.2% and 89.5%, respectively. The drastic change in apparent prevalence seen in load 2 may reflect the limitations of a single culture and the unknown dynamic nature of *M. haemolytica* shedding. Because these calves were commingled while awaiting the results of the culture, it is also possible that this change in prevalence could signify potential transmission. However, since our study was not designed to identify transmission, PFGE was not performed on the arrival screening cultures and therefore we are unable to provide evidence either for or against transmission of *M. haemolytica* in this study. Regardless of the exact cause, the change in apparent prevalence between screening cultures further emphasizes the potential problem with using a single culture at a single point in time or even 2 cultures interpreted in parallel to give an accurate assessment of *M. haemolytica* status.

There are many possible reasons why a single culture may not be sufficient to accurately represent the culture status of a calf even when samples are obtained and handled properly. First, when compared with the large surface area of a calf's nasal passages, the surface area of a culture swab and the area it can sample are relatively small. Taking into account the potential diverse and dynamic microbial flora of the nasal mucosa of a calf, the potential for competitive inhibition, the unknown natural shedding pattern of *M. haemolytica*, and the variable rate of mucous production, it is not surprising that 1 culture swab cannot necessarily correctly classify a calf as negative.

Analysis by PFGE was instrumental in helping to characterize the clusters of *M. haemolytica* that were isolated, and allowed for the comparison of the similarity of the challenge strain to each selected isolate. Additionally, the PFGE results raised concerns about expecting a single culture to provide a true representation of the *M. haemolytica* population within the nasopharynx. The challenge strain used in this study was taken from a prior clinical case of BRD, had been utilized in another challenge study,[5] and, based on lesions observed during autopsy, was successful in inducing comparable pneumonic lesions. However, none of the isolates saved from the postchallenge NPCs matched the challenge strain, based on a 90% similarity threshold value, even though the challenge strain was placed in both the nasal cavity and the lung. Whether this was a result of the challenge strain not being an efficient colonizer in the presence of the other *M. haemolytica* types isolated from the NEG calves, not being present in the small area from which the swab was taken, or not being the single isolate selected from the colonies on the plate for PFGE analysis remains unclear.

The PFGE results from the POS calves showed that it is possible, when multiple isolates are taken from a single plate, to find more than 1 cluster of *M. haemolytica*. A preliminary report presented as a poster at the 2014 BRD Symposium (Taylor et al., Use of pulsed-field gel electrophoresis for characterization of *Mannheimia haemolytica* isolates from the upper and lower respiratory tracts of cattle, Poster presentation at the Bovine Respiratory Disease Symposium, 2014 July 30–31, Denver, Colorado) also indicated that more than 1 pulse-type of *M. haemolytica* can be found in an animal. Another study[7] found evidence that, based on plasmid profile, 2 or more strains of *M. haemolytica* can be found within a single calf. Current bacteriology practices depend on the assumption that if a single isolate is selected randomly from a plate it is likely to be the most predominant one on the culture plate and therefore the nasopharynx.[22] Although there may indeed be a "dominant strain" of *M. haemolytica* within a given sample, the selection of a single isolate is unlikely to be truly random and instead may suffer from unintentional selection bias due to potential differences in *M. haemolytica* colony morphology. Additionally, given the previously mentioned inherent limitations of NPCs, it may be unrealistic to extrapolate the results from the single isolate level to the entire nasopharynx. In addition to the multiple assumptions routinely made when performing standard bacterial culture techniques,[22] it is important to remember that the goal of routine diagnostic methods is not to identify diversity within a sample, but to separate colonies for further characterization such as antimicrobial susceptibility testing. Therefore, it is possible that routine diagnostic methods may not be optimal for detecting diverse strains of bacteria within the original sample. Furthermore, controversy exists regarding the correlation of the bacteria within the upper respiratory tract with the bacteria in the lung[8] especially at the individual calf level.[2] If paired culture and PFGE are to be used as diagnostic tools for *M. haemolytica* in future studies, it would be extremely beneficial to know how many isolates from each culture need to be subjected to PFGE to

gain a true representation of the *M. haemolytica* strains present within each culture. Several statistical models involving Bayesian inference have been developed to estimate this number.[3,11,22] However, to our knowledge, this approach has not yet been applied to *M. haemolytica*.

Other data collected during this pilot included CIS scores, rectal temperatures, and in the case of the challenged calves, lung scores, gross postmortem lesions, and lung cultures. In all phases, CIS and rectal temperature did not seem correlated with either NPC status or, where applicable, with lung lesions or lung cultures at autopsy. The negative results seen on the postmortem lung cultures could be a result of both the innate limitations of culture and the successful immune clearance of the bacteria by day 10. It is well established that rectal temperature and CIS are relatively insensitive and unspecific methods of diagnosing BRD[27] especially when compared to gross pulmonary examination at autopsy[17] or slaughter. Therefore, the lack of correlation between CIS, rectal temperatures, and autopsy findings seen in our study is not surprising and may be due to several confounding factors including individually housing the calves, high ambient temperatures, and the large amount of normal lung tissue left in each calf. Despite the presence of pulmonary lesions that were comparable to those seen in other challenge studies,[5,17] these calves still had a large amount of normal lung tissue remaining and therefore may not have had appreciably abnormal respiratory character or behavior. Assignment of clinical illness scores may also have been confounded by individually housing the calves and thus changing their behavior and willingness to show clinical signs when under observation. Additionally, given the high ambient temperature during this trial, it was difficult to discern between mild signs of respiratory disease and mild signs of heat stress. These 3 factors, among others, collectively made it difficult to accurately judge clinical illness when compared with a more typical commercial setting.

Even though the sample size within each portion of our study was limited, the inconsistencies seen in culture status and the *M. haemolytica* isolates obtained suggest that caution should be used when interpreting the results of a single NPC and that extrapolation of the results to the entire nasopharynx or to the lower respiratory tract may not be possible. Furthermore, this study highlights the complex role of *M. haemolytica* in both healthy and ill animals and emphasizes the need for future research to further explore the epidemiology of *M. haemolytica* transmission.

Sources and manufacturers

a. JorVet #J-272, Jorgensen Laboratories, Loveland, CO.
b. Remel BactiSwab gel collection and transport system, Amies clear media; Thermo Fisher Scientific, Lenexa, KS.
c. VetVu VFS-2B, Swiss Precision Products Inc., Spencer, MA.
d. CASH dispatch kit, Accles & Shelvoke Ltd., Sutton Coldfield, West Midlands, United Kingdom.
e. Bruker Daltonics Inc., Billerica, MA.
f. Microscan turbidity meter, Dade-Behring, West Sacramento, CA.
g. Sigma-Aldrich, St. Louis, MO.
h. Bio-Rad Laboratories, Hercules, CA
i. Life Technologies, Grand Island, NY.
j. CHEF-Mapper XA system, Bio-Rad Laboratories, Hercules, CA
k. SeaKem-Gold, Lonza Rockland Inc., Rockland, ME.
l. Life Technologies, Grand Island, NY.
m. VersaDoc imager, Bio-Rad Laboratories, Hercules, CA.

n. Bionumerics v7.1, Applied Maths Inc., Sint-Martens-Latem, Belgium.

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Figure 2.1 – Summary of *Mannheimia haemolytica* screening cultures performed on each load of calves and selection of calves for inclusion within the final NEG challenge pilot. NEG = negative for *M. haemolytica* on 2 screening nasopharyngeal cultures interpreted in parallel.

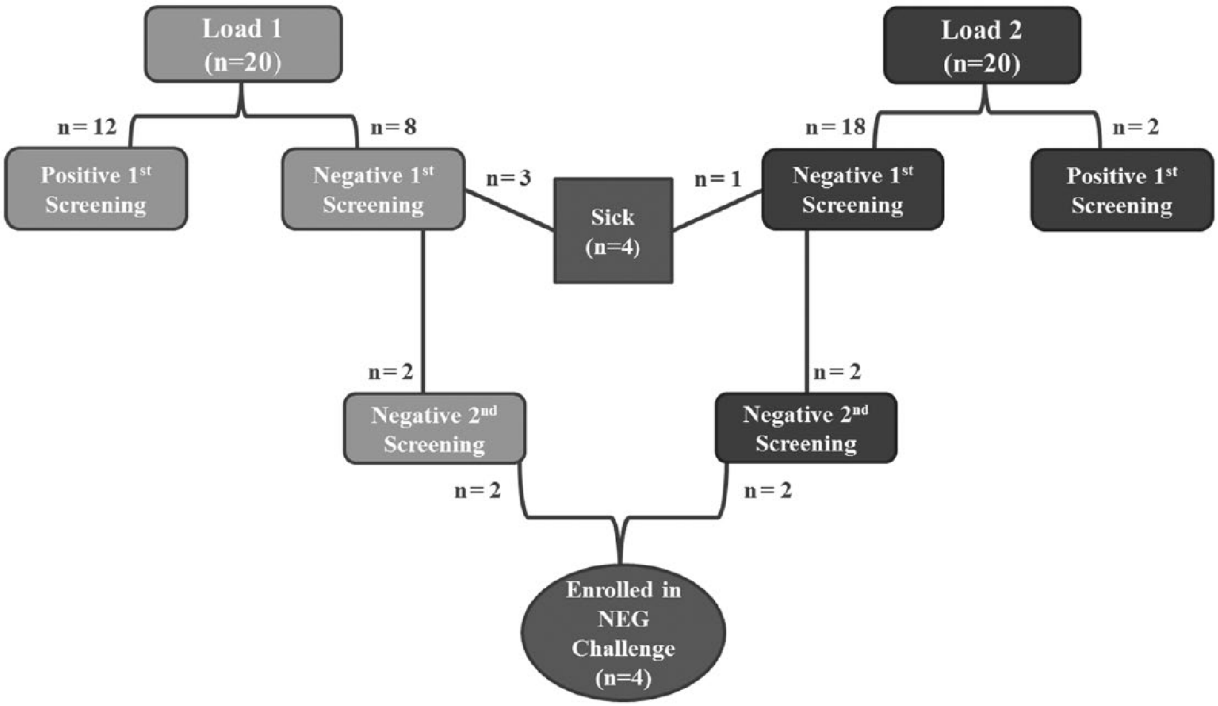

Figure 2.2 –Pulsed-field gel electrophoresis dendrogram and cluster analysis for all *Mannheimia haemolytica*–positive nasopharyngeal cultures following challenge of NEG calves (n = 4). A single isolate was obtained from each *M. haemolytica*–positive culture plate and compared to the Kansas State University (KSU) *M. haemolytica* challenge strain using a 90% similarity threshold cutoff value. Two distinct clusters of *M. haemolytica* were identified as denoted by the A and B. NEG = negative for *M. haemolytica* on 2 screening nasopharyngeal cultures interpreted in parallel.

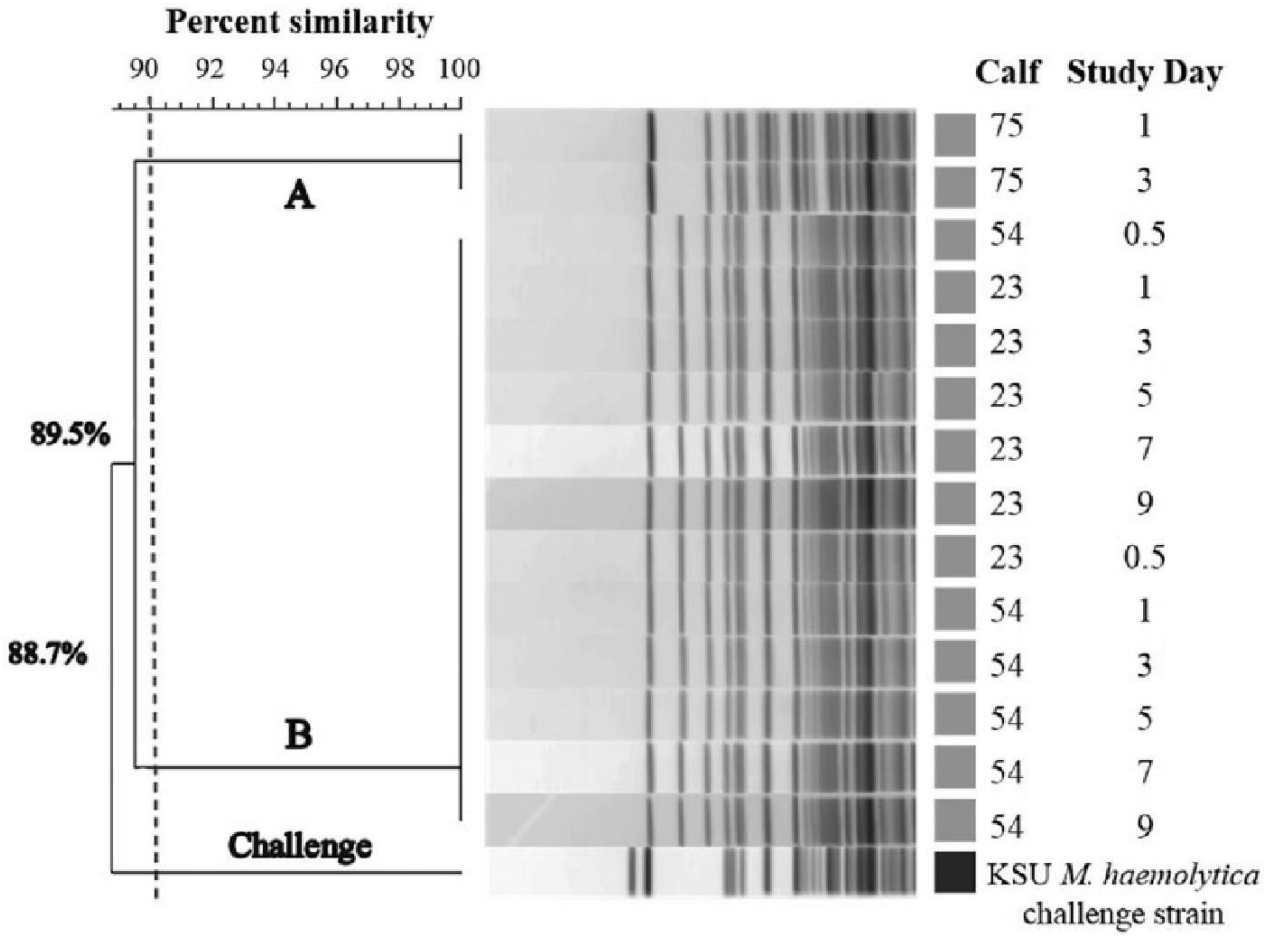

Table 2.1 – Nasopharyngeal culture results over time from NEG calves (n = 4) challenged with *Mannheimia haemolytica* via both intranasal and endoscopic routes at time 0.*

	Nasopharyngeal culture result for *M. haemolytica* on the indicated day						Lung culture result for *M. haemolytica*
Calf no.	Day 0.5	Day 1	Day 3	Day 5	Day 7	Day 9	Lung, Day 10
63	–	–	–	–	–	–	–
23	+	+	+	+	+	+	–
54	+	+	+	+	+	+	–
75	–	+	+	–	–	–	–

* NEG = negative for *Mannheimia haemolytica* on 2 screening nasopharyngeal cultures interpreted in parallel; + = positive; – = negative.

Table 2.2 – Nasopharyngeal culture results for *Mannheimia haemolytica* from confirmatory cultures performed on day –4 (5 days prior to initiation of monitoring period) and subsequent daily samples obtained from naturally *M. haemolytica*–positive calves (n = 4).

Calf no.	Day -4	Day 1	Day 2	Day 3
24	+	–	+	–
51	+	–	+	+
59	+	–	+	+
68	+	–	+	–

Chapter 3 - Comparison of the diagnostic performance of nasopharyngeal swabs and bronchoalveolar lavages in calves diagnosed with bovine respiratory disease

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Abstract

Objective

Examine the culture results, gamithromycin susceptibility, predictive values, and agreement of pooled bilateral nasopharyngeal swabs (NPS) and bronchoalveolar lavages (BAL) for identification of *Mannheimia haemolytica* genotypes, *Pasteurella multocida*, and *Histophilus somni* in calves treated for Bovine Respiratory Disease (BRD).

Animals

28 beef calves with clinical BRD.

Procedures

Bilateral pooled NPSs and blind BALs were obtained from calves pre-treatment and at several points post-treatment with gamithromycin treatment. Up to 12 *M. haemolytica*, 6 *P. multocida*, and 6 *H. somni* colonies were selected from each sample for gamithromycin susceptibility testing; whole-genome sequencing was performed on all *M. haemolytica* isolates. Kappa values and predictive values for organism presence were calculated from the 28 paired samples obtained on day 5 post-treatment.

Results

Prevalence of *M. haemolytica*, *P. multocida*, and *H. somni* was 21%, 21%, and 11% respectively 5 days post-treatment and kappa values for BAL/NPS were 0.71, 0.81, and 0.78 respectively. When BAL results were considered the gold standard, the positive and negative predictive values of NPS were: 67% and 100% for *M. haemolytica*; 75% and 100% for *P. multocida*; and 100% and 96% for *H. somni*, respectively. Gamithromycin susceptibility of isolates varied within the same sample and between paired NPS and BAL samples.

Conclusions and Clinical Relevance

In this study, NPS and BAL showed high agreement and a high negative predictive value of NPS along with the potential for different susceptibility profiles from paired NPS and BAL samples.

Abbreviations:

BAL Bronchoalveolar Lavage

BRD Bovine Respiratory Disease

LRT Lower Respiratory Tract

MIC Minimum inhibitory concentration

NPS Nasopharyngeal Swab

NPV Negative predictive value

PPV Positive predictive value

SE Sensitivity

SP Specificity

URT Upper Respiratory Tract

Introduction

Although extensively studied for many years, BRD is still the most costly disease facing the beef cattle industry[1] and significant knowledge gaps exist regarding the epidemiology of BRD. As a disease with a complex causal-web, BRD has multiple contributing factors including host immunity, stressors, viruses, and bacterial agents such as *Mannheimia haemolytica*, *Pasteurella multocida*, and *Histophilus somni*.[2] These three bacteria are commonly implicated, either together or separately, in BRD outbreaks and diagnostic testing of high-risk or ill animals

to identify these bacteria and their *in vitro* antimicrobial susceptibility can be used to formulate control and therapeutic regimens.

Several studies have explored the use of various antemortem and postmortem methods for obtaining samples for bacterial cultures from the respiratory tracts of calves including: nasal swabs, transtracheal swabs, transtracheal washes, nasopharyngeal swabs (NPS), bronchoalveolar lavages, lung lavages, lung swabs, and lung tissue cultures, for further diagnostic testing.[3-9] Each sampling technique has strengths and limitations based on materials and equipment needed, level of restraint required, degree of invasiveness, time and skill needed to obtain the samples, and area of the respiratory tract sampled during the procedure. When comparing potential sampling locations, obtaining an antemortem URT sample is simpler than an antemortem LRT sample as it requires less materials, time, and skill. However, if an antemortem sample of the LRT is truly desired, performing a blind BAL is more practical, economical, and faster than methods requiring an endoscope or transtracheal wash; this is especially true when multiple calves need to be sampled over a short time-span without cross-contamination. Additionally, BALs are less invasive than transtracheal washes and are well-tolerated without sedation. One advantage to using an endoscope-guided sampling method lies in the ability to obtain a sample from a specific lung lobe, usually the right cranial lung lobe, which is commonly affected in calves experiencing BRD[5] and may yield different diagnostic results than other, less affected lung lobes. However, a significant disadvantage of using an endoscope lies in the need to decontaminate the endoscope between animals versus using a single sterile tube for each BAL collection. One disadvantage of both blind and endoscopic BALs is the potential introduction of organisms from the nasal passages into the lungs. This can be partially mitigated via the use of a guarded endoscope, but a

comparison of guarded vs. unguarded BAL and the impact it has on bacterial flora has not, to the authors' knowledge, been performed. However, regardless of sampling location or method, all sampling techniques are eventually hampered by the limitations associated with culturing the organisms and the potentially complex ecology of the native microbial flora in an individual animal.[10]

This current project focused on the use of bilateral NPS as a means for antemortem sampling of the URT and BAL as a means for antemortem sampling of the LRT. Each can be done relatively easily and quickly in the field using basic restraint via a squeeze chute and halter(s) and are relatively non-invasive compared to other techniques. Additionally, both sample collection methods are relatively well-tolerated by calves and require minimal technical expertise. However, there is some controversy in the literature regarding the agreement of LRT and URT samples and their ability to adequately portray the organisms present.[4-6] Furthermore, to the authors' knowledge, there is no comparison of bacterial culture results from pooled bilateral NPS and BAL samples available in the literature. Therefore, the purpose of this report is to compare the culture results, gamithromycin susceptibility profiles, positive and negative predictive values, and agreement of pooled bilateral NPS vs. BAL for identification of *M. haemolytica* genotypes, *P. multocida*, and *H. somni* in beef calves that have been treated for BRD.

Materials and Methods

Animals

Twenty-eight calves originating from a larger study population of 180 commingled, mixed-breed high-risk steers described elsewhere[3] met the case definition for BRD and were treated according to label directions with gamithromycin. Data from two morbid calves who, because they did not finish their post-treatment interval and could not be classified as a treatment success or failure were not included in the previously mentioned publication[3], are included in this study since day 5 post-treatment samples were obtained from them. Calves were randomly assigned to sampling group 1, 2 or 3 so that approximately equal numbers of calves were assigned to each sampling group. Samples were obtained via NPS and/or BAL at the time of treatment (Day 0), 0.5, 1, and/or 5 days after treatment with gamithromycin according to assigned sampling group as described in Table 3.1 as part of the sample collection required for another study[3] which was approved by the Kansas State University Institutional Animal Care and Use Committee (Protocol # 3338).

NPS method

Nasopharyngeal swabs were obtained from both nostrils of each sick animal via double guarded sterile equine culture swabs[a] that were guided through the nasal passages until encountering resistance at a point approximately equivalent to the distance between the medial canthus of the eye and the end of the muzzle. The entire double guarded swab was then retracted a short distance so that the culture swab could be advanced through both the outer and inner guards and rotated against the nasopharyngeal mucosa; once several rotations were made, the swabs were retracted back into the guards for removal from the nasal cavity. The samples from

the left and right nasopharyngeal areas were pooled into a single vial of liquid Amies culture media and placed on ice prior to culture as described by DeDonder *et al*.[3]

BAL method

Bronchoalveolar lavage fluid was obtained in a manner adapted from a previous publication[8] by restraining non-sedated cattle in a squeeze chute with two rope halters to lift and extend their heads as depicted (Figure 3.1). A BAL-240 tube[b] was then inserted through the nares and guided into the trachea until blindly wedging in a bronchus. Correct placement was verified via the elicitation of the coughing reflex, movement of air in the tube during breathing, and/or the absence of rumen contents, gurgling, or rumen odor coming from the tube. Once wedged in the appropriate location, a total of 240 mL of sterile saline was infused and immediately aspirated in the following manner without inflating the cuff on the tube: first dose of 120 mL, second dose of 60 mL, and third dose of 60 mL. Approximately 135-175 mL of bronchoalveolar lavage fluid was obtained from each calf and was divided equally into 4 conical vials. Each vial was kept on ice until centrifugation within 40 minutes of collection; a cell pellet was chosen at random and then resuspended in liquid Amies for bacterial culture as described previously.[3]

Bacterial culture and determination of gamithromycin susceptibility

Microbiological culture methods and sample handling for both NPS and BAL samples are described in detail elsewhere.[3] For the purposes of calculating kappa and predictive values in this study, the presence of a single *M. haemolytica*, *P. multocida*, or *H. somni* colony on a plate was interpreted as positive for that NPS or BAL sample. Detailed methods regarding the

performance of gamithromycin susceptibility testing via broth microdilution of up to 12 *M. haemolytica* colonies and up to 6 *P. multocida* colonies are also described elsewhere[3] with up to 6 additional *H. somni* colonies per sample tested with slight modification of CLSI guidelines.[11] In brief, *H. somni* cultures were cultivated on Chocolate II agar plates in 5% ± 2% CO_2, at 37°C, for 24 h. Resulting colonies were picked to 5 ml cation-adjusted Mueller-Hinton broth tubes to achieve an optical density equivalent to 0.5 McFarland standard. This suspension was used to inoculate 2X Veterinary Fastidious Medium[c] (15 µL per 1 mL), and the resulting bacterial suspension was dispensed into custom frozen susceptibility plates[d], 50 µL per well, then sealed with sealing film prior to incubation in 5% CO_2 at 37°C for 24 h. Growth was visually inspected and gamithromycin MIC determined as the lowest concentration preventing visible growth. Current CLSI guidelines were used in establishing whether an isolate was susceptible (S), intermediate (I), or resistant (R) to gamithromycin.[11]

Genomic sequencing, bioinformatics, and phylogenetic analysis of *M. haemolytica*

A nucleotide polymorphism-based typing method was developed from the *M. haemolytica* isolates characterized in this study, and additional isolates from other sources; the method is being reported in detail in a separate manuscript.[12] Briefly, single colony isolates of *M. haemolytica* were grown overnight (approx. 16-20 h) in 1 mL of Brain Heart Infusion broth with no shaking in 96 deep well blocks. DNA extractions were performed[e] and DNA samples were quantified via fluorometer[f] according to the manufacturer's instructions. Nextera XT DNA libraries were constructed with original A Indices kits and sequenced.[g] A minimum of 10X sequence reads per genome was obtained for each isolate sequenced. Each library was mapped to a closed circular *M. haemolytica* genome available in GenBank (CP004752),[13] using the Bowtie 2 mapping

algorithm for SNP identification. Bootstrapped Neighbor-Joining phylogenetic trees were constructed from concatenated SNP genotypes of each isolate using an F84 substitution.[h] The trees were generated and graphically displayed using publicly available software.[i,14]

Data analysis

Due to the small number of BRD cases, 2 x 2 tables were created for the results of the paired NPSs and BALs performed 5 days post-treatment (n = 28 calves) for each of the three organisms identified: *M. haemolytica*, *P. multocida*, and *H. somni*. The PPV and NPV of NPS were calculated using the BAL result as the gold standard according to formulas described elsewhere.[15] Additionally, the kappa statistic[15] was calculated as a measure of agreement between the two diagnostic methods for each target organism isolated. A kappa statistic ≤ 0 was indicative of poor agreement, from 0.01 to 0.2 was indicative of slight agreement, from 0.21 to 0.4 was indicative of fair agreement, from 0.41 to 0.6 was indicative of moderate agreement, from 0.61 to 0.8 was indicative of substantial agreement, and from 0.81 to 1.0 was indicative of almost perfect agreement.[16] To evaluate the potential for bias between NPS and BAL, the Exact McNemar significance probability[j] was determined for each kappa statistic with values ≤ 0.05 considered significant evidence of bias. Predictive values and kappa values for each organism were only calculated on day 5 post-treatment due to the small number of calves sampled on each of the other study days but each calf's NPS and BAL microbiological profile was evaluated descriptively over time.

Results

Bacterial culture results

Overall, 10, 15, and 4 paired NPS and BAL cultures were positive for *M. haemolytica*, *P. multocida*, and *H. somni*, respectively over all 4 sampling days. Counts of NPS or BAL samples positive for *M. haemolytica, P. multocida,* and *H. somni* on day 5 post-treatment are found in Table 3.2. A total of 287 separate colonies of *M. haemolytica* were retained for whole-genome sequencing from 22 individual *M. haemolytica*-positive NPS samples and 10 individual *M haemolytica*-positive BAL samples obtained over all 4 sampling days. Individual NPS culture results for each calf on each of the sampling days for all 3 target organisms are located within Table A.1. Similarly, individual BAL culture results for calves within each group are presented in Table A.2. Comparison of results in Tables A.1 and A.2 revealed variation in microbiological profile within the same calf when comparing culture results over time; in several instances calves were negative for an organism prior to treatment only to be culture-positive for that organism at a later time point.

Agreement and predictive values of paired NPS and BAL samples collected on day 5 post-treatment

The PPV and NPV of NPS for *M. haemolytica* when BAL was considered the gold standard were 67% and 100%. Agreement between NPS and BAL for *M. haemolytica* was substantial as indicated by a kappa statistic of 0.73.[15] The Exact McNemar significance probability for *M. haemolytica* was 0.25.

The PPV and NPV of NPS for *P. multocida* when BAL was considered the gold standard were 75% and 100%. Agreement between NPS and BAL for *P. multocida* was almost perfect as indicated by a kappa statistic of 0.81.[15] The Exact McNemar significance probability for *P. multocida* was 0.50.

The PPV and NPV of NPS for *H. somni* when BAL was considered the gold standard were 100% and 96%. Agreement between NPS and BAL for *H. somni* was substantial as indicated by a kappa statistic of 0.78.[15] The Exact McNemar significance probability for *H. somni* was 1.0.

Whole-genome sequencing of *M. haemolytica*

Of the 287 *M. haemolytica* isolates obtained from NPS and BAL samples across all time points, genetic subtype information was not discernable for 11 isolates. The remaining 276 *M. haemolytica* isolates were split into two clearly discernable phylogenetic clades (Figure 3.2) and were each further subdivided into subtypes. Of the 10 paired NPS and BAL samples where both sample types were both positive for *M. haemolytica*, all agreed in terms of subtype (Table A.3). Of the 17 total individual *M. haemolytica* positive NPS samples and the 10 total individual *M. haemolytica* positive BAL samples, the only instance where >1 subtype of *M. haemolytica* was found in a sample at a single point in time was the NPS sample from calf #150 on day 0.

Gamithromycin susceptibility profiles

Due to the small number of positive paired samples for each organism both overall and within time point, no formal statistical measurement of agreement of gamithromycin MIC was

possible. Across all time points, gamithromycin susceptibility results agreed, in terms of S, I, R breakpoints[11] for 70% of the 10 paired NPS and BAL samples that were concurrently positive for *M. haemolytica*. Of the 15 paired samples that were concurrently positive for *P. multocida*, gamithromycin susceptibility results agreed for 87%. All paired samples that were concurrently positive for *H. somni* (n = 4) agreed in terms of gamithromycin susceptibility results. A descriptive breakdown of gamithromycin susceptibility agreement by day and by organism can be found in Table 3.3. Within all individual NPS and all individual BAL samples that were obtained across all time points, mixed gamithromycin susceptibility phenotypes were obtained from a total of 4 of 22 NPS and 1 of 10 BAL samples that were positive for *M. haemolytica*, 3 of 35 NPS and 2 of 17 BAL samples that were positive for *P. multocida*, and 2 of 14 NPS and 0 of 6 BAL samples that were culture positive for *H. somni* (Tables A.1 and A.2).

Discussion

Overall, these results indicate that the agreement between NPS and BAL for *M. haemolytica*, *P. multocida*, and *H. somni* in BRD cases 5 days after treatment with gamithromycin varied from substantial to almost perfect according to the scale reported by Dohoo *et al*.[15] The measurement of agreement through the use of a kappa statistic is helpful in situations where there is no true gold standard or, as in this case, the more common true gold standard for BRD diagnosis via postmortem lung examination and culture is not feasible.[15] Although postmortem lung lavages and nasopharyngeal swabs had perfect observed agreement for *M. haemolytica* according to Table 1 in Godinho *et al*., their methodology for performing the postmortem lung lavages is not equivalent to antemortem BALs as a larger portion of the lungs was sampled postmortem than would be feasible antemortem.[6] A review of other available

literature revealed that Allen *et al*.[5] found lower levels of agreement between a single-sided NPS and endoscopic BAL culture obtained prior to treatment from a group of cases and controls; NPS did not truly predict the organisms isolated from BALs in a repeatable fashion for individual animals. Within this study, the samples obtained from the left and right sides of the nasopharynx were pooled which may have improved the ability to identify organisms within the URT and is one explanation for the improved agreement between NPS and BAL. Additionally, although the kappa values reported here are higher than in previous work[5], it is important to remember that kappa is influenced, in part, by prevalence[17] which may vary according to disease status or treatment status; therefore, agreement of NPS and BAL may vary when sampling clinical vs. non-clinical animals or treated vs. untreated animals. Ultimately, careful consideration of the sample population is important when interpreting results and it is vital to understand that the agreement between NPS and BAL is a dynamic value that is not identical in all populations of cattle.

Across the three organisms studied on day 5 post-treatment, there was only a single instance where a NPS classified an animal as negative for *H. somni* but the organism was found in the BAL fluid; otherwise NPS had perfect negative predictive values for *M. haemolytica* and *P. multocida* when BAL was considered the gold standard. This indicates that if an animal is negative via NPS for an organism after treatment, it was unlikely that the organism would be found in BAL fluid at that same point in time. Interestingly, there were several instances where *M. haemolytica* or *P. multocida* were found within the nasopharynx but were not isolated from the lung which lowered the post-treatment PPV for NPS when BAL was considered the gold standard. This conflicts with Table 1 from Godinho *et al*.[6] where, in a population of untreated

calves, perfect positive and negative predictive values were obtained with single-sided nasopharyngeal swabs compared to postmortem lung lavages for *M. haemolytica*. Unfortunately, Godinho *et al*.[6] did not culture much *P. multocida* and did not have any samples culture positive for *H. somni* and, consequently, were unable to comment on the predictive values of NPS for those two organisms in their population. Since the cultures here were performed 5 days after treatment, it is possible that the organisms were newly acquired from other calves and had not had the chance to proliferate sufficiently in the LRT to be detected by BAL. Alternatively, the organisms may have been present in another portion of the LRT that was not sampled with the BAL technique, or there could be a differential drug effect within the URT and LRT that had an impact on microbial growth. Additionally, given the proposed commensal status of *M. haemolytica*, *P. multocida*, and *H. somni*[18] it is possible that the poor PPV of NPS at 5 days post-treatment is analogous to the presence of these organisms in the upper airways of healthy calves and merely reflects a return to "normal" commensal status. Whatever the cause, these results indicate it is possible that the presence of an organism in the nasopharynx after treatment is not a good indicator of whether or not that organism is also in the lung. However, it is important to remember that predictive values are dependent on prevalence.[15] Given the same diagnostic test, if prevalence is low, NPV will be higher and PPV will be lower; alternatively, if prevalence is high, PPV will be higher and NPV will be lower. The overall number of sick calves was small in this study and, as expected, the overall apparent prevalence of each organism was low on day 5 both within the nasopharynx and the lungs. Therefore, it is unsurprising that NPS had high NPVs and lower PPVs after treatment.

While comparing the results of the NPSs across time is not straightforward due to the unknown effect of gamithromycin administration and, due to the small number of cases, NPS results are difficult to compare beyond simple descriptive results, it is interesting to note that there were several instances where a calf was negative for an organism prior to treatment and yet the organism was found at a later point. Similar results were seen within the pre- and post-treatment BAL culture results of the Group 1 calves; the post-treatment BAL culture results in Groups 2 and 3 also varied within organism over time. This phenomenon could be a result of transmission between calves in the same pen; it could also be that the organism was in fact present in the calf's respiratory tract on day 0 but was not successfully cultured for multiple reasons[10] including competitive inhibition. Additionally, there were numerous animals whose nasopharyngeal status remained the same – either positive or negative – for a given organism both on day 0 and day 5. The consistent positives could be a result of transmission between calves; it is also possible that some calves may require longer than 5 days to clear an organism after treatment due to differences in immune response or that antimicrobial resistance within a given strain of bacteria would preclude the clearance of the infection. The consistent negatives may be reflective of the relatively low sensitivity of culture or indicate a true absence of a given organism in that particular calf. Although the interpretation of these culture results is complicated by the timing of treatment, variation over time is not a new finding. For example, Magwood *et al*. found considerable variability in the isolation of *M. haemolytica* and *P. multocida* between the left and right nasal cavities of calves when they sampled multiple times a day for 5 days.[9] Additionally, in a previous study, inconsistencies were found in the apparent prevalence of *M. haemolytica* in a small group of healthy calves when a single NPS was performed daily for 3 days.[10] These results point to a dynamic relationship between organism

and calf and show that a negative or positive result at a given point in time does not always mean that the organism will be present or absent at a different sampling point. Firm conclusions as to causation would require additional investigation and are outside the scope of this publication.

When comparing agreement of gamithromycin MIC within paired NPS and BAL samples, this study was hampered by the small sample size of clinically ill animals and the small number of paired samples that were both positive for each organism. Therefore, the analysis and discussion of phenotypic gamithromycin susceptibility are limited to a descriptive comparison. Overall, the two sample sources agreed the majority of the time in terms of phenotypic susceptibility classifications when both NPS and BAL were positive for the organism. The susceptibility results for *H. somni* were phenotypically the same in every instance where both NPS and BAL were positive, but as this only occurred 4 times out of the 56 possible paired samples that were taken this seeming high level of agreement may simply be an artifact of the low prevalence of *H. somni* in these calves. For *M. haemolytica* and *P. multocida*, there were several instances where either a BAL or NPS sample showed isolates with different gamithromycin susceptibility profiles but a single susceptibility phenotype was obtained from the isolates in the corresponding paired sample taken at the same time. It would seem that *P. multocida* isolates from paired NPS and BAL samples agreed, in terms of gamithromycin susceptibility phenotype, more frequently than *M. haemolytica* isolates. However, only up to 6 isolates of *P. multocida* were taken from each plate while up to 12 *M. haemolytica* isolates were tested. This means that there were potentially less chances, per sample, of finding a *P. multocida* isolate with a different susceptibility profile. Although a relatively rare occurrence in this population of clinically ill animals, the results indicate that it is possible for more than one

susceptibility phenotype to exist within a genera and species of bacteria isolated from a single sample and that the susceptibility results obtained from the nasopharynx may not always equal the lung and vice versa. However, it is important to remember that in this project multiple isolates of each bacterial species were tested from each plate – something that is not commonly done in routine susceptibility testing – therefore with routine diagnostic testing of a single sample it is unlikely to observe mixed antimicrobial susceptibility results for a bacterial organism.

Although finding more than one genetic subtype of *M. haemolytica* in a single sample of the URT or LRT was a rare occurrence in this study, the overall prevalence of *M. haemolytica* was quite low and therefore the frequency of this occurring when prevalence is higher or in different populations may be different. Additionally, a cap of no more than 12 isolates were sequenced from any given plate regardless of the number of suspect *M. haemolytica* colonies present so it is possible that there were other isolates whose genetic subtypes are not represented in the results. It is also possible, given the limitations of culture methods and the dynamic microbial ecology within a calf's respiratory tract, that additional *M. haemolytica* subtypes were present in the calves but were not successfully cultured. Other possible explanations include competitive inhibition between certain *M. haemolytica* subtypes and other microbes, selective inhibition of certain *M. haemolytica* subtypes by the antimicrobial treatment or the immune system, selection bias of isolates in the diagnostic lab, or the presence of a true genetic subtype monoculture. However, this portion of the study is merely descriptive in nature as sample size and low prevalence precluded statistical analysis of the likelihood of a mixed subtype sample. Therefore, the only firm conclusion that can be taken from this comparison of *M. haemolytica*

subtypes between NPS and BAL is that it is possible to isolate different genetic *M. haemolytica* subtypes from a single NPS sample when up to 12 isolates are examined. If the antimicrobial susceptibility results and subtype results for *M. haemolytica* are compared, it also becomes apparent that, although there was agreement in terms of subtype between the positive paired samples, there were variations in antimicrobial resistance patterns within the same *M. haemolytica* subtype. This highlights the fact that while isolates of the same subtype are genetically similar, they are not genetically identical.

Even with the technology and knowledge available today, the interpretation of diagnostic tests from calves affected with BRD remains difficult. Whether obtained via NPS, BAL, or some other method, each technique is limited in the relatively small area that is being sampled,[10] the potentially complex interactions of other microbial flora that may be present in some animals but not others, and the inherent limitations of current culture methods themselves.[19] Further complicating matters is the fact that our ability to identify clinical vs. subclinical animals is often suboptimal[20] and there may be differences in our ability to isolate target organisms during different stages of BRD. Although it is possible that NPSs and BALs may not be representative of the entire URT and LRT,[5] and disagreements regarding the presence of organisms and their susceptibilities can occur, there remain few alternatives for antemortem sampling in a field setting. Still, when antimicrobial susceptibility testing is performed and results are used to guide treatment and management decisions, it is vital that the limitations of the diagnostic test be carefully considered. While predictive values and kappa values were not calculated or compared at other time points due to the limited number of samples, gaining knowledge of the utility of URT and LRT samples following treatment is still valuable to the industry. Often in clinical and

field settings, diagnostic samples for the purpose of culture and susceptibility testing are not obtained prior to treatment; samples are only taken on calves that either fail to respond to treatment or die and are necropsied. Additionally, mass medication of cattle on arrival is quite common, therefore many clinically ill cattle will already have some level of antimicrobial exposure prior to treatment. Due to the aforementioned differential performance of tests within different populations of animals and the knowledge gaps that remain regarding the bacteria involved in BRD, information regarding test performance and the organisms obtained at different time points relative to treatment could only improve the ability of consumers of diagnostic tests to correctly interpret their results; further research at other time points relative to treatment is needed.

Overall, the high level of agreement and high negative predictive values of NPS were influenced by the relatively low prevalence of the organisms on day 5 after treatment and may differ if the same tests are applied in different populations. Additionally, both NPS and BAL results indicate there is variation in the organisms cultured over time within a given calf and that it is possible to obtain different subtypes of *M. haemolytica* from the nasopharynx. It is also possible for a mixture of gamithromycin susceptibility phenotypes to be present in a single NPS or BAL sample and for paired NPS and BAL samples obtained at the same time to have different susceptibility phenotypes when multiple isolates of *M. haemolytica* and *P. multocida* are tested from each sample. Therefore, consumers of diagnostic test results should consider the expected prevalence of a population and interpret diagnostic tests relying on the results of a single isolate with caution.

Footnotes

[a]VetOne, Boise, Idaho, USA

[b] SurgiVet, Smiths Medical, Dublin, OH, USA

[c] Sensititre-TREK Diagnostics, Thermo Scientific, Waltham, MA

[d] Sensititre-TREK Diagnostics, Thermo Scientific, Waltham, MA

[e] Mo Bio Ultra Clean ®-htp 96 well DNA kits, Carlsbad, CA, USA

[f] Promega Quantus, Madison, WI, USA

[g] Illumina MiSeq DNA sequencer, San Diego, CA, USA

[h] PHYLIP, Felsenstein J. PHYLIP (Phylogeny Inference Package). Version 3.69. Department of Genetics, University of Washington, Seattle: Distributed by the author, 1993.

[i] Dendroscope, Version 3.2.10. Daniel H. Huson and Celine Scornavacca. Dendroscope 3: An interactive tool for rooted phylogenetic trees and networks, Systematic Biology (2012), http://sysbio.oxfordjournals.org/cgi/content/abstract/sys062?ijkey=ZCxPRbYt74aQJhR&keytype=ref, software freely available from www.dendroscope.org.

[j]. STATA, version 12.1, StataCorp LP, College Station, TX

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Figure 3.1 – Photograph illustrating the level of restraint required for performing a BAL in a calf without sedation. Each calf must be caught in either a manual or hydraulic squeeze chute and two halters must be placed to elevate and extend the head. Although a neck extender is pictured here and assists in maintaining the correct head position, it is not required to perform the procedure. With proper restraint and sufficient practice, a BAL fluid sample can be obtained in less than 3 minutes.

Figure 3.2 – Neighbor-Joining trees of *M. haemolytica* clades 1 and 2 based on genome-wide concatenated SNPs from 276 isolates subjected to whole-genome sequencing. All *M haemolytica* isolates were acquired from nasopharyngeal swab or bronchoalveolar lavage fluid samples that were obtained from bovine respiratory disease cases either pre-treatment with gamithromycin according to label directions or on day 0.5, 1, or 5 post-treatment. Genetic subtypes within each clade are denoted by lower case letters. Numbers beside internal nodes of the tree represent bootstrap percentage values from 100 pseudo-alignments. The scale bar represents substitutions per site within trees. The genetic distance between clades 1 and 2 exceeds the genetic distance within the two clades and is not shown.

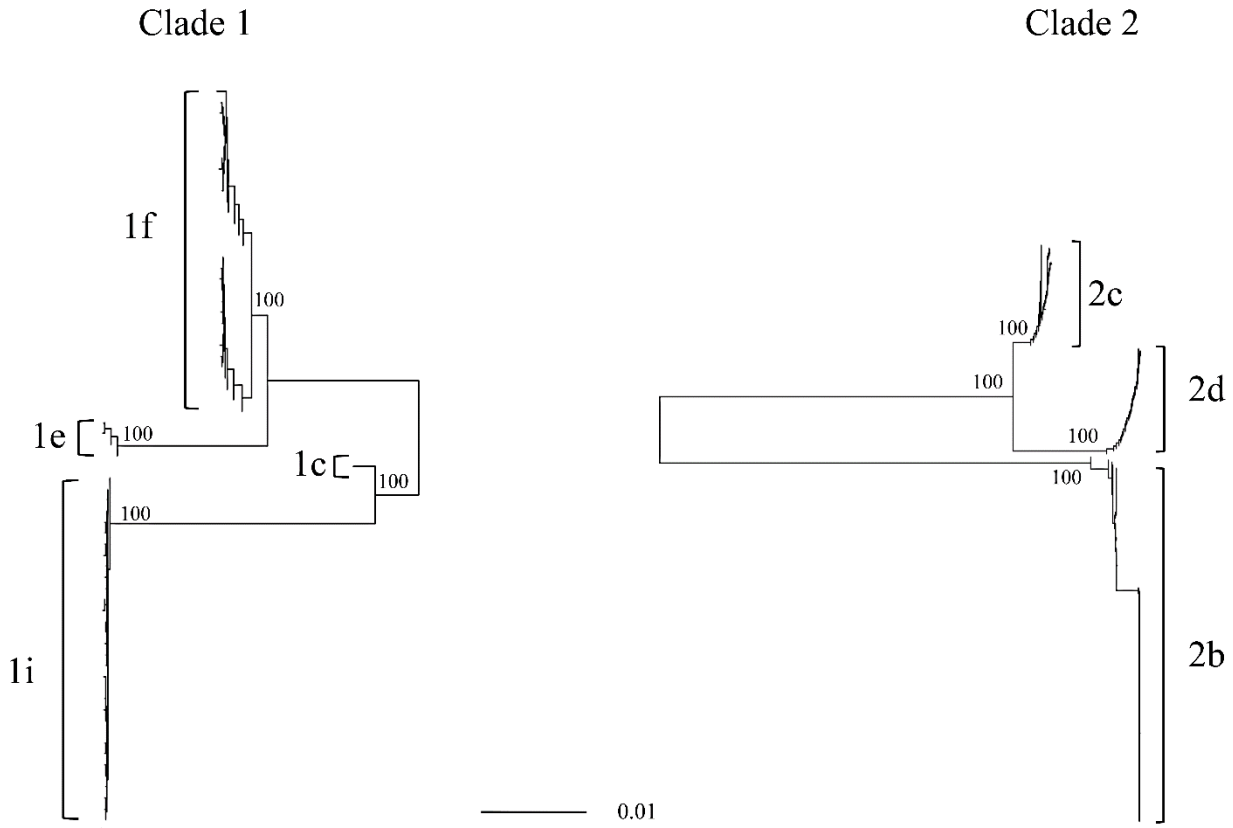

Table 3.1 – Sampling scheme applied to BRD cases (n = 28). Calves were randomly assigned to a sampling group in blocks of 3 at the time they met the criteria for being a BRD case. Day 0 began immediately after meeting case criteria but prior to treatment for BRD.

Total n = 28	Day 0	Day 0.5	Day 1	Day 5
Group 1	NPS	-	-	NPS
n = 9	BAL	-	-	BAL
Group 2	NPS	NPS	-	NPS
n = 10	-	BAL	-	BAL
Group 3	NPS	-	NPS	NPS
n = 9	-	-	BAL	BAL

Table 3.2 – Two by two table depicting the count of NPS or BAL samples that were positive for (a) *Mannheimia haemolytica* on day 5 after treatment with gamithromycin, (b) *Pasteurella multocida* on day 5 after treatment with gamithromycin, and (c) *Histophilus somni* on day 5 after treatment with gamithromycin.

a)

Mannheimia haemolytica				
		NPS		
		Positive	Negative	Total
BAL	Positive	6	0	6
	Negative	3	19	22
	Total	9	19	**28**

b)

Pasteurella multocida				
		NPS		
		Positive	Negative	Total
BAL	Positive	6	0	6
	Negative	2	20	22
	Total	8	20	**28**

c)

Histophilus somni				
		NPS		
		Positive	Negative	Total
BAL	Positive	2	1	3
	Negative	0	25	25
	Total	2	26	**28**

Table 3.3 – Descriptive summary results of gamithromycin susceptibility agreement between paired NPS and BAL cultures that were both positive for *Mannheimia haemolytica*, *Pasteurella multocida*, or *Histophilus somni*. Samples were collected from 28 mixed-breed beef steers pre-treatment (Day 0) and 0.5, 1, and 5 days post-treatment with gamithromycin. Susceptibility agreed when the gamithromycin susceptibility phenotype of the organism was the same for both paired samples and disagreed when the phenotype of the organism differed between the paired samples.

		M. haemolytica	*P. multocida*	*H. somni*
Day 0	Susceptibility agreed	2	2	1
	Susceptibility disagreed	0	1	0
	Both NPS and BAL positive	2	3	1
Day 0.5	Susceptibility agreed	1	5	0
	Susceptibility disagreed	1	0	0
	Both NPS and BAL positive	2	5	0
Day 1	Susceptibility agreed	0	1	1
	Susceptibility disagreed	0	0	0
	Both NPS and BAL positive	0	1	1
Day 5	Susceptibility agreed	4	5	2
	Susceptibility disagreed	2	1	0
	Both NPS and BAL positive	6	6	2

Chapter 4 - Changes in cattle behavior following handling

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Abstract

The objective of this study was to evaluate and quantify behavioral differences over time of cattle with different temperaments after a handling event involving restraint in a squeeze chute. Fifty-one male crossbred beef calves were obtained from local livestock auctions on two different days and fitted with accelerometers and real-time location system transmitters. Once acclimated, cattle were brought to the working facility where each calf was restrained in a chute for approximately 60 seconds while their rectal temperature was taken. A dichotomous chute score, vocalization score, and exit score were recorded chute-side for each calf during handling. Calves were returned to their home pens where daily distance traveled, percent time spent lying, and percent time spent within 1 m of the hay rack, grain bunk, shed, and waterer over the following 3 days were compared to the baseline established the day prior to handling. The trial was repeated one week later with the same group of calves. Significant interactions ($P < 0.05$) were found between trial day and exit score for percent time spent within 1 m of the waterer and

percent time spent within 1 m of the shed, trial day and vocalization score for percent time spent within 1 m of the hay bunk and distance traveled, and trial day and chute score for distance traveled. Regardless of exit score, calves spent a significantly ($P < 0.01$) greater percentage of time near the shed following handling when compared to pre-handling levels but calves with different exit scores showed different patterns of behavior regarding the waterer. Calves that left the chute at a walk spent numerically less time near the waterer on the day of handling and on day 2 but numerically more time near the waterer on day 1 while calves who left the chute faster than a walk showed the inverse behavioral pattern. There was no significant effect of the three temperament scores on percent time spent lying, but there was an effect of trial day with calves spending significantly less time lying ($P < 0.01$) on day 1 when compared to pre-handling. Our results indicate that some behaviors are altered after handling and restraint in a squeeze chute; cattle with different temperament scores may have different activity levels and spend different amounts of time within 1 m of the hay bunk, grain bunk, waterer, and shed after handling.

Introduction

Stress can have negative effects on the health and performance of cattle and is considered an important component in the multifactorial nature of bovine respiratory disease complex (BRDC). Different forms of stress such as fasting, dehydration, exhaustion, restraint, or handling[1] can interact to create changes in behavior that can vary in magnitude and can be modified by other factors including genetics, temperament, past experiences, etc. Although some behaviors, such as lying, tend to follow circadian rhythms[2], some can potentially be modified by weather events, social dynamics within a group of animals[3], and interaction with humans.

Cattle behavior has been studied in multiple ways, and recent technological advances have improved our ability to objectively measure behavior. Animal movement and behavior can be quantified using various methods such as: video monitoring[4], pedometers[4], accelerometers[5], radio-frequency identification (RFID) tags, real-time location systems (RTLS), global positioning systems (GPS)[6,7], or combinations of these methods.[4,8] Accelerometer data gives an accurate, objective measure of lying behavior in cattle[2,8,9] and, combined with RTLS location and activity data, have been used to formulate remote disease detection algorithms for BRDC.[10,11] Both accelerometers and RTLS allow for objective quantification of cattle behavior without the potential influence of human presence during behavioral measurements.

Attempts to categorize cattle temperament and evaluate these categories for potential associations with health and performance outcomes have shown conflicting results regarding agreement and repeatability. Several subjective categorical scales have been used[12-14] to describe the behavior of cattle in working facilities and in pens. Objective measurements of vocalization characteristics such as count, duration, frequency, power density, and intensity as measured by computer software[15], movement as monitored by a movement measuring device[16,17], and exit velocities determined by a system of light beams and reflectors[18,19] have been used to measure aspects of temperament. However, differences in study population, trial design, measurements, scoring systems, and statistical analyses used make results difficult to summarize, interpret, and apply. When combined with the many other potential influential factors such as genetics or prior experiences that result in an animal responding in a “calm” or “not calm” fashion, interpreting the results of behavioral research becomes more complex. Additionally, it is possible that our

current behavioral measurement and scoring systems are not capturing or we are not correctly interpreting enough of these complex factors to adequately classify temperament in cattle. Since the visual observation of cattle behaviors such as depression and anorexia remains a commonly used method of pre-mortem diagnosis of BRDC[20,21], a greater understanding of the influences that temperament may have on these behaviors has important implications for industry and research.

Cattle are handled throughout their lives for routine husbandry and care including applying identification tags, administering vaccinations, anthelmintics, and parasiticides, and diagnosing and treating illness. Additionally, when utilized as research animals, cattle are often handled to obtain physiologic samples and measurements. Because quantification of feeding[22] and drinking behavior and measuring activities such as walking and lying[23] have shown promise as early indicators of illness and are used as outcomes in research projects, knowing the impact, if any, of handling cattle on behavior over time is potentially important to both researchers and producers.

Directly comparing objective behavioral data such as distance traveled and time spent eating, drinking, or lying down could provide additional information about the potential effects of cattle handling on healthy cattle behavior throughout the days following a handling event. The objective of this study was to evaluate and compare behavioral differences of cattle classified based on a chute score (CS), exit score (ES), and vocalization score (VS) prior to and for three days after being handled in a squeeze chute.

Materials and Methods

Calf Selection and Management

For this pilot project, 51 male crossbred beef calves (five bulls; 46 steers; average weight (± SD): 234 kg ± 16.8 kg) were obtained from a local livestock market and arrived at the Kansas State University Large Animal Research Center, in Manhattan, KS, USA, in two separate truck loads delivered three days apart. Bulls and steers were randomly assigned to one of three adjacent dirt-floor, group housing pens (297 m^2) and allowed to acclimate to the housing facility for approximately 6 to 9 days prior to the beginning of behavior monitoring. Each pen included four areas of interest; an automatic ball waterer, hay rack, grain bunk, and open-faced shed. Around each area of interest, a 1 m perimeter (dashed line within Figure 4.1) was created so that each area was mutually exclusive (Figure 4.1). Throughout the trial, all calves were fed a grain mix appropriate for their age and weight and offered free choice grass hay. This study was a part of a protocol (#3417) approved by the Kansas State University Institutional Animal Care and Use Committee.

Cattle in each pen were fed and observed for clinical signs of illness by the same person (SC) at the same time each day to minimize variations in behavior related to those events. Any calf that showed signs of illness was examined and treated appropriately according to a prescribed protocol. Calves that became lame were removed from the study and relocated to a hospital pen; all other calves were returned to their home pens following treatment but their behavioral data was not included in the final analysis.

Behavioral Monitoring

Two days prior to the beginning of the behavioral monitoring period, all calves were fitted with and allowed to acclimate to both real-time location system (RTLS) tags (Ubisense Series 7000 Compact Tag, Ubisense, Inc., Colorado, USA) and accelerometers ((Track a))) Cow™, Animart, Wisconsin, USA). Each RTLS tag was attached to a commercial ear tag button and placed on the dorsal aspect of the right ear. The tag used real-time location monitoring to report the location of each calf within the pen according to *x* and *y* coordinates in relation to locations of interest within each pen such as the waterer, grain bunk, hay rack, and shed as described elsewhere.[24] The percent time spent within 1 m of each location of interest was calculated via software (MySQL, Oracle Corporation, California, USA) using calculations similar to other studies.[23,25] Additionally, by tracking the movement of each calf, the total distance traveled within the pen in meters was calculated per trial day. A three dimensional accelerometer was attached to the distal aspect of the hind limb of each study animal to record the percent time lying; this device communicated with a wireless sensor approximately every 7 minutes. All behavioral measurements were summed into hourly totals and aggregated into daily values. Trial days were composed of the 21 hours immediately prior to working the cattle (day -1: Pre-handling) and the following three days (day 0 to day 2: Post-handling) with each trial day including a total of approximately 21 hours of data. The entire trial was repeated one week later with the same group of calves following the same timeline.

Collection of Weather Data

Daily weather information was gathered for all trial days from a website that collected information from the local airport weather station (Manhattan Regional Airport, Manhattan, KS,

USA).[26] This weather station was located approximately 11 km southwest of the Kansas State Large Animal Research Center. The pattern of weather was compared between the two replicates to determine whether it was reasonable to combine the data from each replicate and to identify any extreme weather events that may have influenced behavior.

Cattle Handling Facilities

The three cattle pens were located approximately 101-125 m from the cattle handling facility along an approximately 4 m wide gravel alley. The cattle handling facility consisted of a large enclosed metal barn with one entry leading to a 135˚ solid sweep (Priefert, Pleasant, Texas, USA) that exited into a straight 4.9 m working alley consisting of metal sheeted alley panels and metal sheeted adjustable alley gates (Priefert, Pleasant, Texas, USA) that created two separate sections prior to the manual squeeze chute (SC11, Priefert, Pleasant, Texas, USA).

Scoring Methods and Handling Protocol

Cattle in each pen were brought up to the cattle handling facility as a group and then returned to their pens after handling as a group in the same pen order for both replicates. All personnel who interacted with the cattle implemented low-stress cattle handling techniques and were supervised by a Beef Quality Assurance certified veterinarian (SC). Efforts were made to ensure cattle were handled in a similar manner during both replicates. During each handling event, every calf was caught and squeezed in the manual squeeze chute, their head was tied to the side with a rope halter and their rectal temperature was taken. Each calf remained restrained in the squeeze chute for approximately 60 seconds prior to being released. The time from handling the first calf in each pen to handling the last calf in each pen was approximately 23.0 ±

1.8 minutes on average ± SD. For each replicate, calves were assigned a chute and vocalization score (CS and VS) while they were in the squeeze chute and an exit score (ES) for the first few strides as they were released from the chute (Table 4.1).

Statistical Analysis

Generalized linear mixed models were fitted using the GLIMMIX procedure (SAS 9.4, SAS Institute, Inc., North Carolina, USA) for all statistical models. Distance traveled in meters, as a normally distributed continuous dependent variable, was modeled via a Gaussian distribution; beta distributions were fitted for models of continuous proportions such as percent time spent lying and percent time spent within 1 m of the shed, hay rack, waterer, or grain bunk. All models used pen as a random intercept to account for the clustering of calves within pens and a random residual term of calf ID nested within replicate to account for repeated measures among calves using covariance structures such as toeplitz (distance traveled); first-order autoregressive (percent time spent lying, percent time spent within 1 m of the waterer, and percent time spent within 1 m of the hay rack); or heterogeneous first-order autoregressive (percent time spent within 1m of the shed and percent time spent within 1 m of the grain). Model selection for each behavioral response began with a main-effects model including fixed effects of trial day (a four level categorical variable), VS (a two level categorical variable), CS (a two level categorical variable), and ES (a two level categorical variable). Two-way interactions between trial day and each of the three types of scores were then tested in a forward fashion using a $P < 0.05$ for inclusion of each interaction. If interactions were not significant, fixed effects of trial day, VS, CS, and ES were kept in each model. A more conservative value of $P < 0.01$ was applied when examining pairwise comparisons between pre-handling and post-handling

trial days in order to reduce the likelihood of Type I errors. Results are presented as model-adjusted mean ± SEM.

Results

Replicate 1 began on 6/12/14 and extended through 6/16/14 whereas replicate 2 began on 6/19/14 and extended through 6/23/14. Weather patterns for the behavioral control day (day -1), the day of handling (day 0), and the two days of observation following handling (days 1 and 2) were similar between replicates in terms of precipitation, maximum humidity, and maximum temperature. Compiled daily weather data for both replicates can be found in Table 4.2.

Randomization resulted in 1 bull being placed in pens 1 and 2 while 3 bulls were placed in pen 3. Overall, two calves became clinically ill with BRDC and were removed from replicate 2 while five calves became lame during replicate 1 and were removed from both replicate 1 and 2. One accelerometer malfunctioned during replicate 1 and was replaced before replicate 2; that calf's accelerometer data are considered only for replicate 2. Two RTLS tags were lost during replicate 1 and were replaced before replicate 2; the RTLS data from those two calves are only present in replicate 2. The total number of calves whose behavior was analyzed within each replicate as well as descriptive statistics for the three subjective temperament scores can be found in Table 4.3.

There was a significant interaction between trial day and ES ($P < 0.05$) for time spent within 1 m of the waterer (Figure 4.2a) and shed (Figure 4.2b). The interaction between trial day and VS was significant ($P < 0.01$) for time spent within 1 m of the hay (Figure 4.2c), however

there were no significant effects of VS, CS, ES, or trial day on the time spent within 1 m of the grain.

Trial day had a significant effect on percent time lying; calves spent significantly less time lying ($P < 0.01$) on day 1 (46.5% ± 1.5%) after handling when compared to the pre-handling day -1 (51.3% ± 1.5%). Percent time spent lying on day 0 (50.1% ± 1.5%) and on day 2 after handling (48.7% ± 1.5%) were numerically lower but not significantly different than percent time lying during the pre-handling day (day -1). There were no significant effects of VS, CS, or ES on the percent time lying.

The effects of VS and CS on distance traveled depended on the day of the trial ($P < 0.05$) (Figure 4.3). Calves with a VS of 0 traveled significantly less distance ($P < 0.001$) on all three days following handling (day 0: 4413.74 m ± 184.2 m; day 1: 4350.02 m ± 184.68 m; day 2: 4349.84 m ± 184.04 m) compared to the pre-handling baseline (day -1: 4702.42 m ± 183.75 m) whereas calves with a VS of 1 did not travel a significantly different distance on any of the days following handling (day 0: 4669.35 m ± 305.6 m; day 1: 4482.25 m ± 305.6 m; day 2: 4405.02 m ± 305.59 m) compared to the baseline (day -1: 4318.05 m ± 305.59 m). None of the pairwise comparisons between pre-handling baseline and post-handling days within CS 0 or 1 were significantly different. There was no significant effect of ES on distance traveled.

Discussion

To the authors' knowledge this is the first study that evaluates and quantifies the potential changes that occur in some behaviors over time specifically after handling healthy cattle with

different temperament scores. Even when low-stress handling methods were used, handling cattle was associated with alterations of some behaviors for a few days post-handling. Additionally, the effect of some temperament scores on the monitored behaviors varied by day after handling.

When considering distance traveled, the effect of CS and VS varied on different days; however, only the pairwise comparisons between pre-handling and post-handling time points in calves that did not vocalize (VS of 0) were significantly different. Given their association with distance traveled, CS or VS could be important scores to measure if distance traveled is considered a proxy for overall activity level. Activity or distance traveled can also be considered a potential surrogate measure of depression – one of the main criteria for BRDC diagnosis. Therefore, any altered levels of activity following a handling event may impact diagnostic accuracy of disease detection algorithms and may also be an important consideration when research trials with intensive sampling use activity as an outcome variable. If the frequency or timing of handling events vary between treatment groups, it may not be appropriate to compare activity directly across treatment groups without adjusting for the influence of handling. In contrast to a study that found flight speed (in m/s) but not chute score was related to activity level in the pen[5], we found no significant differences in distance traveled among calves with different ES. These conflicting results could be due to differences between the studies in measurement or scoring system, analysis of the data, handling systems, study population, or other unknown variables; therefore, further research to test the repeatability of these findings is necessary prior to implementing any changes in current research protocols.

Calves in this study spent approximately 46-51% of their time lying on average which is similar to values reported elsewhere.[2] When compared to the pre-handling baseline, percent time spent lying was numerically lower on both day 0 and day 2 and significantly lower for day 1 post-handling; intuitively, one might expect calves to spend more time resting after a perceived stressful event. It is possible that the relatively short duration of our handling event, the short distance cattle actually had to travel from pen to handling facility, and the relatively innocuous nature of the procedures we employed during handling were not overly taxing to the calves. There is considerable variability in the time required to complete different procedures, the distance cattle must travel to reach handling facilities, and the design of the handling facilities at different operations and research facilities. Other routine practices that are inherently more invasive such as vaccinations, implants, branding, dehorning, castration, etc. may also occur at certain stages of production while research projects also vary in what types of samples are taken and in the frequency of handling events. Although many high-risk feedlot cattle are brought to a chute, restrained briefly, and have their temperature taken at some point in their lives, the handling event evaluated herein is not universally applicable to every operation or research project; it is likely different behavioral responses could be seen with different handling events. It is also possible that other unmeasured confounders such as subclinical illness or prior handling experiences altered the association between handling and lying behavior. In this study, none of the three temperament scores were significantly associated with the percent time spent lying post-handling which agrees with other work indicating that flight speed and chute scores were not significantly correlated with lying behavior.[5] However, given the relatively small sample size of this study, it is possible that a significant statistical association between temperament and lying behavior does exist but was not found. Additionally, it is possible that the association

between these two variables may be more pronounced in other populations with different distributions of breeds, ages, or health statuses. More research is needed to determine if disease detection algorithms that incorporate lying behaviors should be adjusted based on handling events.

Surprisingly, there were no temperament scores significantly associated with the percent of time calves spent near the grain bunk. This is in contrast to previous work which indicated that there was a negative correlation between ADFI and flight speed[5] and work which showed that higher flight speeds were associated with less time spent eating.[27] The inherent difference between time spent within 1 m of the grain bunk which is a proxy measure of time spent actually eating and likely includes time spent not consuming any grain may be the reason behind this difference in findings; the use of a categorical ES compared to a continuous m/s flight speed may also contribute. There was also no effect of trial day on the percent of time calves spent near the grain bunk. As previously mentioned, it is possible that our handling event was not stressful enough to induce a change in this behavior and in other cattle that experience more stressful handling events an effect may actually exist. Time spent near the grain bunk is a relatively rare behavior (1.8-2.4% on average in this study) when the total daily time expenditure of a calf is considered and thus it is also possible that our sample size was insufficient to detect a difference in this infrequent behavior. With larger groups of calves or in a production environment where handling events are more frequent or more stressful, a difference in behavior might be detected. Additionally, only healthy animals were included in this analysis; therefore it is possible that in animals that suffer from BRDC or lameness, the effects their illness already has on eating behavior could be more profoundly impacted by handling.

Time spent near the shed was significantly higher at all time points compared to baseline levels for all calves regardless of ES but the magnitude of the differences were numerically different for calves with different ES (Figure 4.2b). The increase post-handling likely indicates an increase in standing resting behavior as the lying behavior results previously discussed indicated that no temperament score and day interactions were significant and calves actually spent less time lying post-handling. A numerical difference in magnitude and direction of effect was observed when the interaction between trial day and ES was examined for time spent near the water; calves that left the chute at a walk (ES of 0) spent less time near the waterer on the day of handling and on day 2 but more time near the waterer on day 1 while calves with an ES of 1 displayed the opposite behavioral pattern. Like time spent near the grain, time spent near the water is a small portion of a calf's daily time budget and the lack of significant differences of each day post-handling compared to baseline are possibly explained by the large standard errors associated with these estimates and the small sample size in this study. Additionally, the cattle in this study were handled early in the morning to minimize possible heat stress and each pen of cattle spent a very short amount of time in the handling facility away from water. If handled during the heat of the day or for a longer period, it is possible that the behavioral pattern differences seen in cattle with different ES could be more pronounced. The effect of VS depended on trial day for time spent near the hay bunk but no other temperament scores had a significant effect on this behavior. Although the trial day by VS interaction was significant, none of the between-day comparisons within VS were significantly different; the main driver of this significant interaction appears to be the markedly larger difference in percentage of time spent near the hay bunk on day 2 between calves with different VS. It is unclear why vocalizing

calves spent so much more time near the hay on day 2 especially when VS had no significant interaction with day for any of the other locations of interest; however, it is important to note that in this trial, very few calves vocalized during handling (total n = 11; Table 4.3).

By investigating the effects of each score separately, this study could examine whether they were different in their effect on behavior rather than combining them to make an overall temperament categorization. Such a summary of temperament may be appropriate for some outcomes but for others may be an oversimplification of the complex nature of temperament and behavior. Furthermore, only some scores were significantly associated with some behaviors indicating either a lack of power or that the underlying traits or circumstances that drive the different responses we observe during handling do not have the same impacts on behavior. Although further research into current methods of evaluating temperament and their relationship with subsequent behavior is needed to examine the repeatability of our findings, the results of this study imply that some measures of temperament taken during handling may be associated with certain behaviors afterwards but that the effect of a given temperament score is not uniform. If temperament does indeed contribute to the variability in behavior of cattle post-handling, accounting for the temperament of calves could improve disease detection algorithms or research projects that rely on behavioral outcomes.

While cattle with different temperament scores exhibited varying levels of certain behaviors in the pen environment, it is unclear if those variations in behavior may translate into performance differences as our study did not evaluate performance variables. Burrow *et al.*[18] indicated that temperament, as measured by flight speed, could affect some aspects of

performance in some of the cattle they studied but not others. Francisco *et al*.[28] only found a difference in weaning weight between cattle with "adequate" vs. "excitable" temperaments and no statistical difference in the carcass traits or other feedlot performance measurements they examined. Many other attempts have been made to describe the relationship of cattle temperament with different production outcomes[16,27,29] with conflicting results between the various measures of temperament and some performance parameters. The inconsistencies in results likely reflect the variation in study implementation, statistical analyses, and the complex role that factors such as genetics, environment, and nutrition play. Additional research to correlate objective measurements of eating and drinking behavior with temperament is needed to determine whether there is an appreciable impact on performance factors.

A potential limitation of this study was the possibility of variable weather conditions over time during both replicates and its unknown impact on behavior. Although weather patterns were descriptively similar in both replicates, it is unlikely that weather was the sole cause of the differences observed between cattle with different temperament scores nor can it completely explain the differences observed in behavior pre- and post-handling. Nonetheless, the potential impact of weather on behavior cannot be ignored completely and different results may be observed if the same experiment was repeated under different weather conditions.

Another limitation of this study was the short duration of the post-handling monitoring period; as we saw differences in some behaviors for the entire 3-day period, we cannot say if or when behavior returned to baseline. Since research studies have variable sampling timelines, it may be important to know when behavior returns to baseline following a handling or sampling

event. Also, operations may implement post-treatment or post-metaphylaxis non-treatment intervals that exceed 3 days[30] when monitoring cattle behavior for the purposes of disease diagnosis; therefore, in future studies, a longer post-handling monitoring period would be useful. Additionally, as this study involved a relatively low-stress handling event, it is unclear how more stressful handling involving more noxious stimuli such as blood draws or vaccination may affect the duration of behavioral modification following handling.

Conclusions

The potential for some behaviors to be altered for at least 3 days after calves are handled indicates the need for careful trial design when behavioral parameters are a trial outcome; if the frequency or timing of handling events varies between treatment groups, it may not be appropriate to compare all behaviors across treatment groups without appropriately adjusting for those differences. There is also the potential to improve upon disease detection algorithms by incorporating behavioral changes that may occur after handling events. Additionally, this study indicates that some behaviors may vary for calves that react differently when handled which also has potential implications when behavior is used as a research variable. Further research on the impacts of handling on behavior is needed as behavioral measurements become more important in disease detection algorithms and case definitions.

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Figure 4.1 – Diagram showing the four locations of interest (solid boxes) and their 1 m zones of interest (dashed boxes) within each 24.4 m x 12.2 m pen. Real-time location system (RTLS) sensors were located around the periphery of the pens and received data from the RTLS tags placed along the dorsal aspect of each calf's right ear approximately every 5 seconds on average. Time spent within 1 m of the grain bunk, hay rack, shed, and waterer was aggregated on a daily basis for each calf along with the total daily distance traveled (m) within the pen.

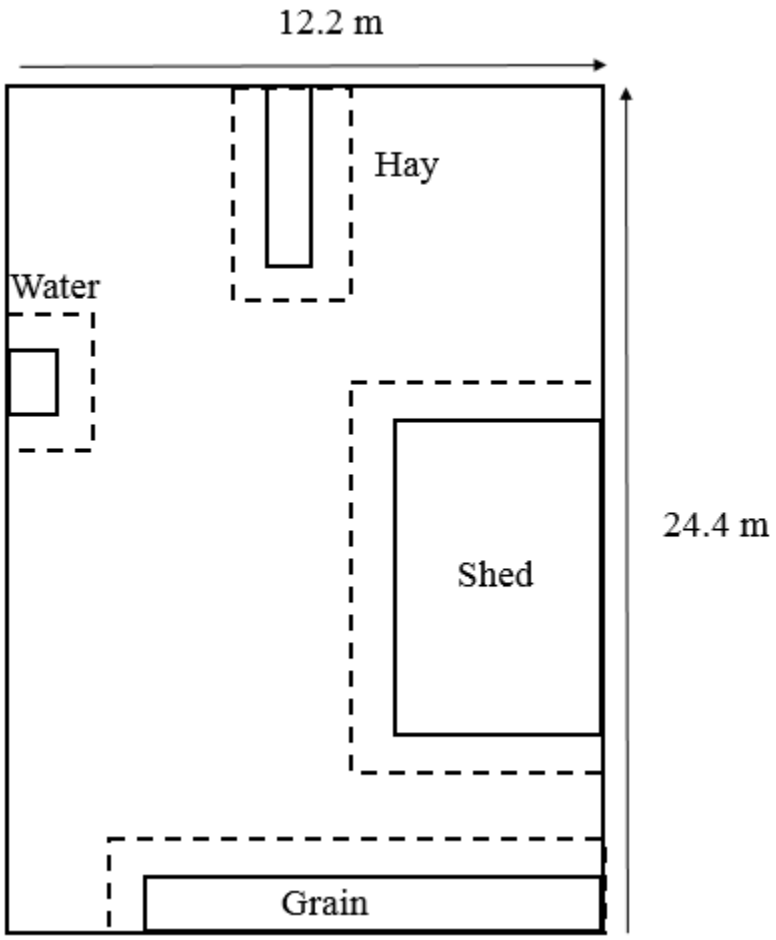
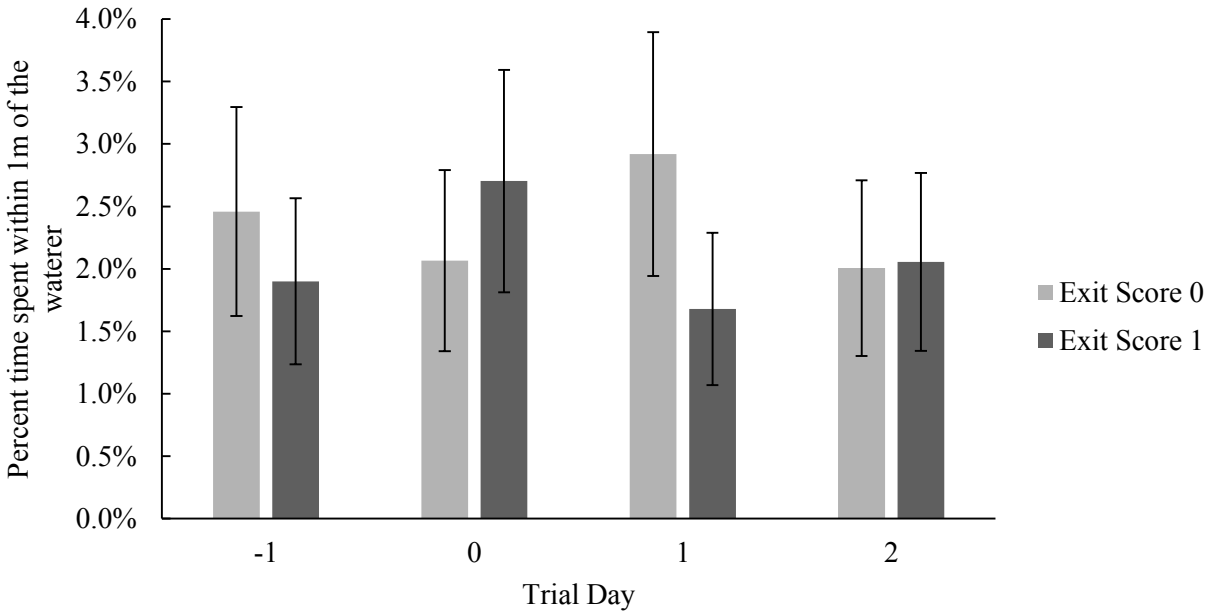
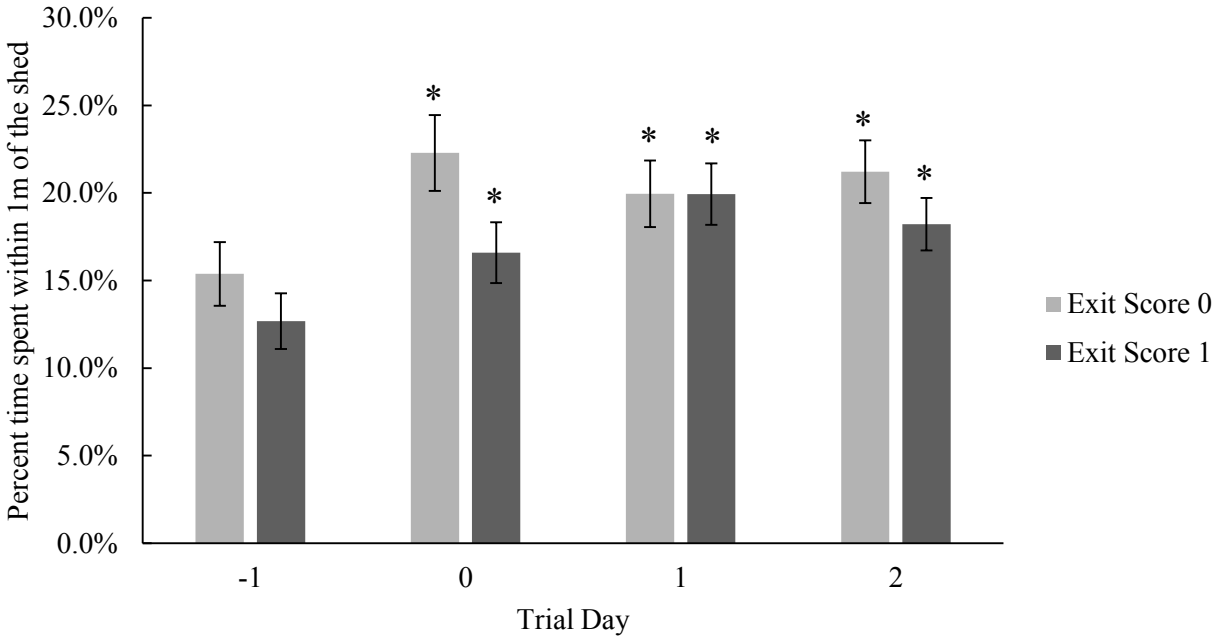


Figure 4.2 – a) Percent of time calves spent within 1 m of the waterer (mean ± SEM) over time according to exit score. Although a significant interaction between trial day and exit score was detected ($P < 0.05$), pairwise comparisons of day -1 (pre-handling baseline) and each subsequent day within each score were not significant at $P < 0.01$; b) Percent of time calves spent within 1 m of the shed (mean ± SEM) over time. A significant interaction between trial day and exit score was detected ($P < 0.05$) and all pairwise comparisons of day -1 and each subsequent day within each score were significant at $P < 0.01$; c) Percent of time calves spent within 1 m of the hay rack (mean ± SEM) over time according to vocalization score. Although a significant interaction between trial day and vocalization score was detected ($P < 0.05$), pairwise comparisons of day -1 and each subsequent day within each score were not significant at $P < 0.01$. Handling occurred on trial day 0, and trial days 1 and 2 were the 2 days post-handling. An asterisk above an individual study day indicates that the pairwise comparison between that study day and the pre-handling baseline day (day -1) was significantly different ($P < 0.01$) within the respective temperament score.

a)



b)


30.0%
25.0%
20.0%
15.0%
10.0%
5.0%
0.0%
Percent time spent within 1m of the shed
*
*
*
*
*
*
-1
0
1
2
Trial Day
Exit Score 0
Exit Score 1

c)

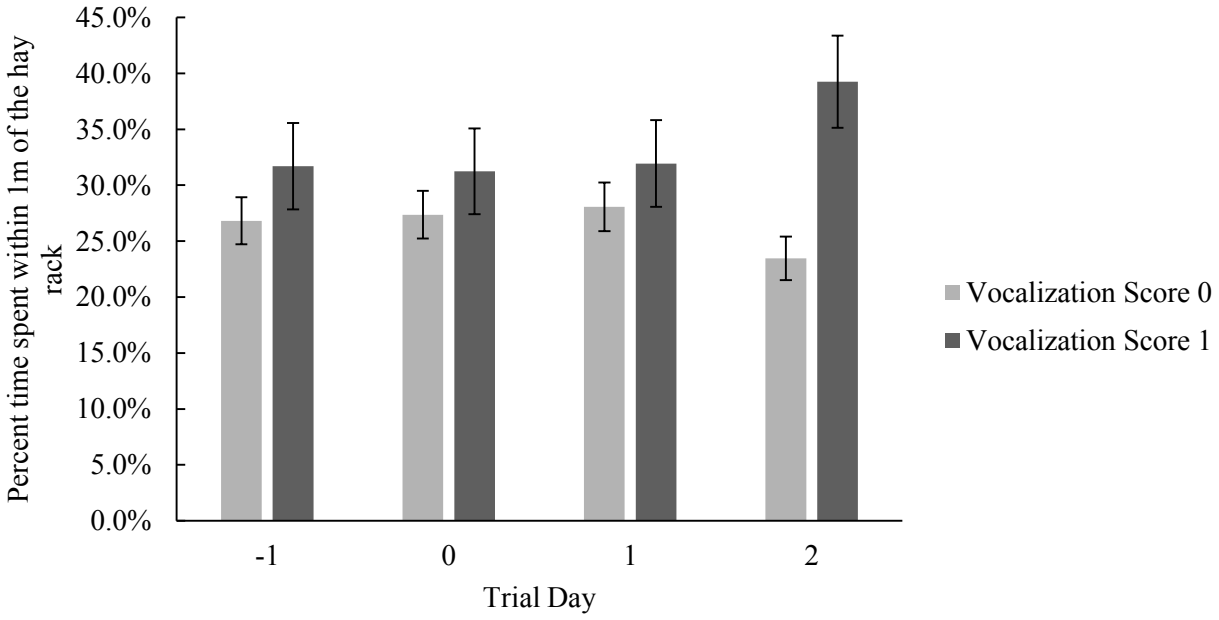
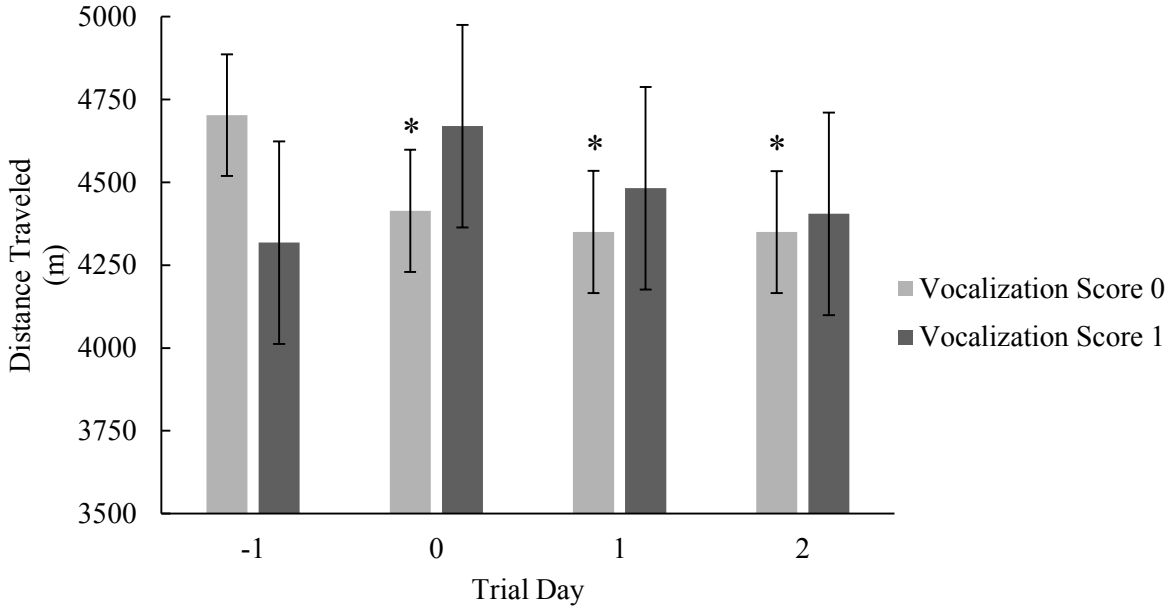

45.0%
40.0%
35.0%
30.0%
25.0%
20.0%
15.0%
10.0%
5.0%
0.0%
Percent time spent within 1m of the hay rack
-1
0
1
2
Trial Day
Vocalization Score 0
Vocalization Score 1

Figure 4.3 – Distance traveled (mean ± SEM) over time as modified by both a) vocalization score and b) chute score. Both interactions were significant at $P < 0.05$ but only calves that did not vocalize during handling (vocalization score 0) exhibited significantly lower ($P < 0.01$) distance traveled on the day of handling (day 0) and the two post-handling days (days 1 and 2) when compared to the pre-handling baseline day (day -1). An asterisk above an individual study day indicates that the pairwise comparison between that study day and the pre-handling baseline day (day -1) was significantly different ($P < 0.01$) within the respective temperament score.

a)



b)

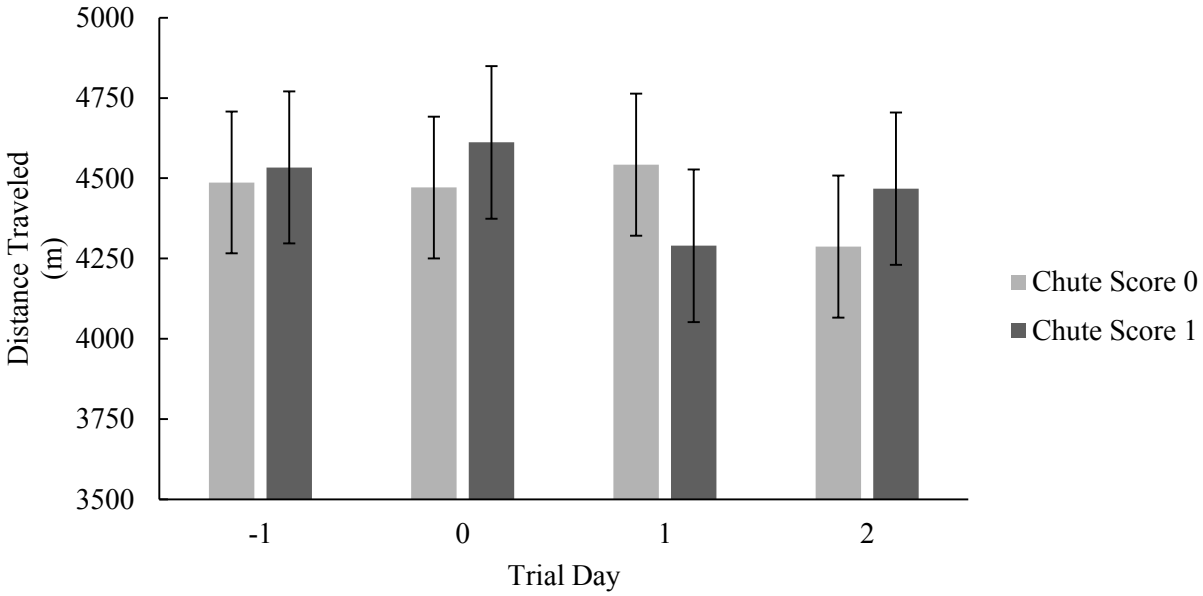

5000
4750
4500
4250
4000
3750
3500
Distance Traveled (m)
-1
0
1
2
Trial Day
Chute Score 0
Chute Score 1

Table 4.1 – Description of the three temperament scores used in this trial. Vocalizations included all vocal sounds of any pitch, duration, volume, or frequency made by the calf.

Score	0	1
Chute	Stood calmly in the squeeze chute.	Did not stand calmly in the squeeze chute.
Exit	Walked out of the squeeze chute.	Did not walk out of the squeeze chute.
Vocalization	Did not vocalize in the squeeze chute.	Vocalized ≥ once while in the squeeze chute.

Table 4.2 – Descriptive weather data for each replicate obtained from the Manhattan Regional Airport weather station in Manhattan, Kansas, USA.

	Date	Min Temperature (˚C)	Max Temperature (˚C)	Min Humidity (%)	Max Humidity (%)	Mean Wind Speed (KPH)	Precipitation (cm)
Replicate 1	6/12/2014	13.3	25.0	43	93	12.9	0.79
	6/13/2014	10.0	26.1	37	96	9.7	0
	6/14/2014	16.7	30.6	55	78	30.6	0
	6/15/2014	17.2	28.3	51	93	11.3	1.96
	6/16/2014	21.1	31.7	59	93	24.1	0
Replicate 2	6/19/2014	22.2	31.1	55	93	12.9	0.03
	6/20/2014	19.4	33.9	49	93	11.3	0
	6/21/2014	20.6	33.3	44	79	12.9	0
	6/22/2014	20.0	33.9	39	93	12.9	0.74
	6/23/2014	18.3	28.3	58	100	8.0	0.15

Table 4.3 – Total number of calves that received each score within each replicate for which real-time location system (RTLS) or accelerometer behavior data was analyzed. Although from the same initial population, due to equipment malfunctions, illness, or lameness, the individual calves represented within each row of the table are not identical. Numbers in parentheses indicate the percentage of all calves within the replicate that received each score.

	Replicate	Chute Score 0	Chute Score 1	Exit Score 0	Exit Score 1	Vocalization Score 0	Vocalization Score 1	Total calves per replicate
RTLS	1	28 (63.6)	16 (36.4)	19 (43.2)	25 (56.8)	37 (84.1)	7 (15.9)	44
	2	24 (54.4)	20 (45.5)	26 (59.1)	18 (40.9)	41 (93.2)	3 (6.8)	44
Accelerometer	1	27 (60.0)	18 (40.0)	19 (42.2)	26 (57.8)	37 (82.2)	8 (17.8)	45
	2	24 (54.5)	20 (45.5)	26 (59.1)	18 (40.9)	41 (93.2)	3 (6.8)	44

Chapter 5 - The impact of oral meloxicam prior to transportation on inflammatory mediators and immune function of arriving feedlot cattle

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Abstract

The objective of this study was to investigate the effects of meloxicam administration prior to shipping on the maintenance of leukocyte function and the reduction of inflammation during and after a long-distance transportation event in cattle. Yearling mixed-breed beef steers (n = 60; 309.5 kg ± 5.3 kg) were randomly assigned to be either transported (TR) or non-transported controls (NTC) and received either 1 mg/kg meloxicam orally (TR-MEL, n = 20 and NTC-MEL, n = 10) or a placebo orally (TR-CON, n = 20 and NTC-CON, n = 10) in an unbalanced 2 x 2 factorial design. All NTC cattle were transported from Mississippi to Kansas on day -17 of the study and allowed to acclimate while the TR steers remained in Mississippi. On day -1, both TR and NTC steers were randomly administered either MEL or placebo and the TR steers were transported to Kansas from Mississippi and arrived on day 0. Blood samples were obtained from all steers on days -1, 0, and 3 for analysis of markers of leukocyte function and inflammation. Bronchoalveolar lavage samples were also obtained from the TR steers on days 0 and 3 for analysis of annexin A1 levels. Ocular thermographic images of TR steers were taken on days -1, 0, and 3. Rectal temperature probes continuously monitored temperature of TR steers after treatment with MEL or CON through day 2 post-transport. No clinical cases of bovine respiratory disease were observed during the study. Plasma MEL concentrations were greater in TR-MEL steers on day 0 than NTC-MEL steers ($P < 0.01$). Substance P concentrations were lower in NTC-MEL steers compared to all other groups ($P < 0.01$) with no significant effect of study day. Sampling time was associated with rectal temperature, ocular temperature, cortisol, haptoglobin, neutrophil L-selectin expression, neutrophil phagocytosis intensity, percentage of active neutrophils, red blood cell count, hematocrit, mean corpuscular hemoglobin concentration, total leukocyte count, and polymorphonuclear leukocyte count ($P <$

0.05). Steers in the TR group had greater ($P < 0.01$) values of haptoglobin, neutrophil L-selectin intensity, and polymorphonuclear leukocyte count than NTC steers. The concentration of Hp-MMP 9 was greater in TR-CON steers compared to NTC-CON steers on day 0 ($P < 0.01$). In this study, meloxicam did not have a statistically significant effect on the variables measured in these healthy steers and only some leukocyte and inflammatory markers differed in transported versus non-transported steers.

1. Introduction

Stress is a complex physiological response to either single or multiple stimuli that, depending on severity and duration, have both positive and negative effects on various physiological parameters in cattle.[1,2] Although stress can potentially negatively impact the immune system, the mere presence of stress, either physiological or psychological, does not always lead to clinical disease.[2]

Across the beef production system, cattle experience varied stressors throughout their lives including weaning, handling for basic processing or other procedures, commingling at auction markets or other facilities, and transportation between locations in the production system.[3] Cattle are frequently transported multiple times within their lifetime[3] for varying distances. Long-distance transportation is considered a significant source of stress in cattle and has been associated with increased bovine respiratory disease (BRD) morbidity risk.[4,5] Several studies have indicated that long-distance transportation results in changes in various measures of inflammation and immunity along with appreciable differences in weight, hydration status, and other alterations in normal physiological status.[6-8] While most studies agree that long-distance

transportation, overall, negatively impacts cattle, not all measured variables have provided consistent results between studies and, for some variables, considerable individual variability has been reported.[9] This may be due to the fact that transportation of cattle is stressful on multiple levels and involves more than just the distance traveled and time spent in the truck. Handling and restraint prior to or immediately following transport, weather conditions experienced during transit, temperament of individual cattle, and other variables may impact the overall level of stress an individual animal experiences.[10]

Although many advances have been made in therapeutic and preventative strategies[11], BRD remains a significant disease cattle face today. Antimicrobials, applied both therapeutically and preventatively in cattle at high risk for BRD, are commonly used to combat BRD. In an effort to reduce the need for antibiotic use in food animals, adjunct therapies to reduce morbidity associated with BRD are being explored. Meloxicam is a non-steroidal anti-inflammatory drug (NSAID) that mainly acts by inhibiting the contribution of cyclooxygenase-2 (COX-2) to the inflammatory response.[12,13] Although still considered an extra-label drug use (ELDU), Coetzee *et al*. showed that oral meloxicam administered to ruminating calves at a dose of 1 mg/kg resulted in high bioavailability and a mean plasma half-life of approximately 28 h indicating that analgesia may continue for several days following a single oral administration.[14] Given that stress and transport can influence certain measures of inflammation and leukocyte responses the authors hypothesized that meloxicam administered at 1 mg/kg, prior to transportation, may help mitigate some of the negative effects of long-distance transport. Therefore, the objective of this study was to investigate the effects of meloxicam administration prior to shipping on the

maintenance of leukocyte function and the reduction of inflammation during and after a long-distance transportation event in cattle.

2. Materials and Methods

2.1. Study Population

This study protocol was approved by the Kansas State University (Protocol # 3335) and the Iowa State University (Protocol # 5-12-7355-B) Institutional Animal Care and Use Committees. Sixty mixed-breed beef steers (average weight: 309.5 kg ± 5.3 kg) from the Mississippi State University's (MSU) Brown Loam Branch Experiment Station were used in this study. Steers were born on the research station during the spring of 2013 and were between approximately 15.2 and 17.8 months of age at the start of the study (16.7 months ± 0.08 months). All animals were castrated within approximately 24 h of birth and received a clostridial vaccine (Ultrabac 8, Zoetis, Florham Park, New Jersey, USA), a 5-way modified live respiratory vaccine (Pyramid 5, Boehringer Ingelheim, St. Joseph, MO, USA), *Mannheimia haemolytica* Toxoid vaccine (Presponse SQ, Boehringer Ingelheim, St. Joseph, MO, USA), and pour-on anthelmintic (Eprinex, Merial Inc., Duluth, GA, USA) at pre-weaning and weaning. Weaning occurred 28 days after pre-weaning and approximately 8 months prior to the start of the study according to standard Mississippi State University protocols. Steers for this study were gathered off grass, brought to a pen near the working facility, and provided *ad libitum* grass hay and water approximately 2 days prior to transportation.

Prior to the beginning of the study, the 60 steers were randomly assigned to either be transported (TR; n = 40) or to serve as non-transported controls (NTC; n = 20). Half the steers in

the TR group were randomly assigned to receive 1 mg/kg meloxicam (Aurobindo Pharma USA Inc. Dayton, NJ, USA; NDC 65862-098-05) orally (TR-MEL; n = 20) and half the steers in the TR group were randomly assigned to receive a lactose placebo orally (TR-CON; n = 20). Similarly, half the NTC steers were randomly assigned to receive 1 mg/kg meloxicam orally (NTC-MEL; n = 10) and half the NTC steers were randomly assigned to receive a lactose placebo orally (NTC-CON; n = 10). Boluses containing MEL were filled with lactose powder so that they were indistinguishable from the CON boluses and then numbered for each calf to keep personnel administering the boluses blinded to treatment. This resulted in an unbalanced 2 x 2 factorial study design with transport and meloxicam status as the two treatment factors (Figure 5.1).

2.2. Study Timeline, Treatment Administration, and Sampling Schedule

Steers within the NTC group were transported overnight from the MSU Brown Loam Branch Experiment Station on day -18 and arrived at the KSU LARC facility on day -17 where they were allowed to acclimate to the facility and recover from transport. Steers within the TR group remained at the MSU Brown Loam Branch Experiment Station until day -1 when they were also transported overnight to the KSU LARC and arrived on day 0 of the study. The entire transportation event for both TR and NTC steers involved a distance of approximately 1,300 kilometers covered over approximately 15 h. Prior to transportation of the TR steers, both the NTC and TR steers received their MEL or CON treatment synchronously at their respective locations in Mississippi or Kansas on day -1 with subsequent sampling occurring on day 0 and day 3 (Figure 5.2).

2.3. Study Population Management and Health Monitoring

Within each of the 4 groups, cattle were randomly assigned to one of 4 open-air, dirt-floor group housing pens (297 m^2 per pen) at the Kansas State University Large Animal Research Center (KSU LARC) facility such that each pen (n = 15 steers/pen) held a relatively equal distribution of steers from each of the four groups. Throughout their time spent at KSU LARC, cattle were provided with access to grass hay and water *ad libitum*, a grain ration provided twice daily that was appropriate for their age and weight, and access to a three-sided shelter.

Prior to the transportation of the NTC and the TR steers, a trained observer confirmed that all steers were visually healthy. For 28 days after transportation to KSU, all steers were observed twice daily by the same veterinarian (SFC) for signs of BRD or other health issues and each calf was assigned a Clinical Illness Score (CIS) twice daily for BRD according to the following modified scale[15]: CIS 1 – Normal calf; CIS 2 – Moderate signs of BRD: moderate depression, and/or cough; CIS 3 – Severe signs of BRD: severe depression, labored breathing, and/or cough; CIS 4 – Moribund and/or recumbent. Steers that received a CIS of 2 or greater during the previous evening or current morning were pulled in the morning and a rectal temperature was taken. In order to qualify as a BRD case, steers had to have received a CIS of 2 or greater and also have a rectal temperature of ≥ 40 ˚C when they were examined. Steers diagnosed with BRD or other health issues were treated by the attending veterinarian(s) according to protocols comparable to current industry standards. Any calf requiring antimicrobial treatment remained in the study population but if treatment included NSAIDs of

any kind, the animal was considered off trial from that point on and was removed from the study population.

2.4. Rectal Temperature Probes

Rectal temperature monitoring devices were placed in the TR steers on day -2 prior to transportation as described previously[16] with minor modification. Specifically, a smaller temperature sensor was used (25.4 mm in length, 8.3 mm in diameter, and 3.3 grams (Starr-Oddi DST micro-T, MeterMall USA, Marysville, OH, USA;[17]) and therefore a smaller probe container that attached to the end of the plastic cross-linked polyethylene (PEX) tubing was used. Probes were monitored twice daily for proper placement and signs of swelling or discomfort and removed if needed. Rectal temperature monitoring devices allowed for the continuous recording of the TR steers' rectal temperature at 5-min intervals which were then averaged by hour prior to analysis. Rectal temperature data were then averaged for each calf by every 6 h period starting after administration of meloxicam or placebo prior to transport, continuing through day 2 post-arrival. Devices were removed on day 3 during sampling.

2.5. Ocular Thermography

Each steer within the TR group had a digital thermographic image taken on days -1, 0, and 3 of the medial canthus of the left eye. Each image was translated to temperature readings through software calibrated internally within the camera (Thermacam Research Pro 2.8 SR-1, FLIR Systems, Nashua, NH, USA). Images were evaluated to determine the maximum temperature around the medial canthus of the eye and these data were then exported to statistical software for analysis.

2.6. Venipuncture and Blood Variables

Steers were restrained in a manual squeeze chute and blood was obtained via jugular venipuncture on days -1 (prior to transport and meloxicam treatment), 0 (arrival at KSU LARC), and 3 to obtain whole blood, plasma, and serum for measurement of various markers of inflammation and immune function into the following tubes: lithium heparin tube (plasma cortisol and meloxicam concentrations), serum tube (Hp-MMP 9), regular EDTA tube (hematology), and sodium heparin tube (plasma haptoglobin, neutrophil L-selectin intensity, and neutrophil phagocytic and oxidative burst activity). Blood for analysis of substance P was first collected into a blank tube and then 6 mL of blood were immediately drawn off via needle and syringe and carefully added to a previously prepared EDTA tube spiked with 300 μL of EDTA containing 0.9 mg of benzamidine. All laboratory personnel remained blinded to treatment during analysis of all variables. At MSU, the 40 TR calves were split into approximately equal groups for sampling on day -1; sampling of each group took approximately 60 minutes. At KSU, the 20 NTC calves were likewise split into equal groups for sampling on day -1 which took between 30 and 40 minutes per group. Blood samples on day 0 and day 3 were obtained over the course of approximately 6 hours; every attempt was made to limit the amount of time calves spent in the tub, alley, and squeeze chute to less than 30 minutes so as to minimize the effects of handling stress on the various blood variables.

Cortisol

Cortisol was analyzed using a commercially available kit (MP Biomedical, LLC, Santa Ana, CA, USA) using the method previously described.[18] The optimal detection range was 3 to

500 ng/mL. The radioimmunoassay's inter- and intra-assay variabilities were 11.1 and 12.6 %, respectively as calculated in Excel (Microsoft, Redmond, WA).

Substance P

Plasma concentrations for substance P were analyzed using a radioimmunoassay double antibody system with a primary antibody against substance P (1:20,000, product number: H-061-05, lot# 1286-1, Phoenix Pharmaceuticals, Burlingame, CA) and 125 I-substance P (20,000 cpm, PerkinElmer Inc., Waltham, Massachusetts, USA). Methods of analysis were consistent with those previously described.[18] Protease inhibitors EDTA (13 mM) and benzamidine (1 mM) were utilized in the assay. The range of detection for substance P was between 10 and 160 pg/mL, with a coefficient of variation for intra-assay variability of 9.59% and an inter-assay variability of 16.28%.

Plasma Meloxicam Concentration

Plasma concentrations of MEL were determined using high-pressure liquid chromatography (Surveyor MS Pump and Autosampler, Thermo Scientific, San Jose, CA, USA) with mass spectrometry detection (TSQ Quantum Discovery MAX, Thermo Scientific, San Jose, CA, USA) as previously described[18] and samples for each animal were performed at the same time to limit variability. The standard curve applied to each run for bovine plasma was linear from 1 to 20,000 ng/mL. The correlation coefficient exceeded 0.97 and all measured standard curve samples were within 25% of the designated values with most of the samples being less than 15% different. The accuracy of the assay for MEL in bovine plasma was 93.3 ± 7.5 % of

the actual concentration while the coefficient of variation was 3.8 % determined on 4 sets of replicates for each of the following concentrations: 100 and 1000 ng/mL.

Hp-MMP 9

The Hp-MMP 9 ELISA was performed as previously described.[19,20] The capture antibody was a monoclonal anti-bovine MMP 9 (clone 10.1; native bovine neutrophil MMP 9 antigen) and wells were blocked by the addition of 300 μL of SuperBlock T20 (TBS) (Thermo Scientific, Pierce, Rockford, IL, USA). All plates used in this study were prepared at the same time to minimize plate-to-plate variation.

The serum standards prepared for use in this study were from a cow that was ill and had concentrations of Hp-MMP 9 of 913 ng/mL as quantified in prior studies.[19] This serum, stored in aliquots, was thawed on ice and sonicated for 1 minute intervals a total of 3 times, vortexed, and serially diluted so that each standard had 228, 114, 57, 28, 14, 7, and 3.5 ng/mL. Blank wells contained all reagents, along with serum from a healthy steer, diluted 1:10 in Tris-buffered saline (pH 7.5) to which 0.05% Tween-20 was added (Tris-buffered saline (TBS) 1X Cold Spring Harbor Protocols, 2009; doi:10.1101/pdb.rec11830). Affinity chromatography and Hp-MMP 9 ELISA of the healthy steer's serum demonstrated it was below the limit of detection for Hp-MMP 9 (Lakritz J., unpublished observations).

Serum samples from experimental animals were diluted 1:10 in the aforementioned TBS + Tween 20 buffer prior to analysis by ELISA. Diluted standards and serum samples from experimental animals (100 μL) were placed into wells of a 96 well plate in duplicate (16

standards, 40 serum samples/plate) for 2 h on a plate shaker at room temperature. After washing pre-diluted rabbit-anti-bovine Haptoglobin-HRP conjugate (Immunology Consultants Laboratory, Portland, OR, USA RHPT-10A; 1:5,000 dilution), were allowed to bind to haptoglobin that is bound to MMP 9 in the wells on a plate shaker for 1 h. Then, after washing wells 5 times in TBS (pH 7.5) with 0.05% Tween-20, 100 μL of TMB substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA; 50-76-11) was added/well and color development was allowed for 20 minutes. After 20 minutes, 100 μL 0.1 N hydrochloric acid was added/well to stop the enzymatic reaction. Standard and sample absorbance was determined on a micro-plate reader at 450 nM. All samples whose absorbance at 450 nm was greater than the highest standard were re-diluted to 1:50 with the aforementioned TBS + Tween 20 buffer and re-assayed (56 samples total). Sample concentrations were determined by linear regression of the known standard concentration versus absorbance value, using the intercept and slope calculated from the linear regression and corrected for the dilution of the sample (10-fold or 50-fold).

Hematology, haptoglobin, neutrophil L-selectin intensity, neutrophil phagocytic and oxidative burst activity

Analysis of hematology and neutrophil activity parameters prior to transportation (day -1) was not possible due to laboratory constraints requiring analysis within 24 h and therefore samples were only obtained on days 0 and 3. Following collection, sodium heparin tubes were kept at room temperature and processed within 24 h of sampling. EDTA tubes were placed on ice and transported to the laboratory within several hours of sampling. Plasma haptoglobin values, neutrophil phagocytic and oxidative burst activity, neutrophil L-selectin intensity, and hematology values were obtained as described previously.[21,22]

2.7. Bronchoalveolar Lavage and Annexin A1

Bronchoalveolar lavage (BAL) samples could not be obtained prior to transportation (day -1) and were only obtained from TR steers on study days 0 and 3. The procedure for collection of BALs was modified from previous studies[23,24] using a total of 200 mL of 0.9% sterile saline introduced and then aspirated in 2 separate aliquots of 120 ml and 80 mL respectively. The collected fluid was filtered through sterile gauze into 50 mL conical tubes and placed on ice until centrifugation for 10 min at 500 x g. The supernatant was then frozen at -80˚C and annexin A1 levels were measured in concentrated samples of bronchoalveolar lavage fluid as previously described[25], with some modifications as follows. Samples were concentrated by centrifugal filtration with a 3 kDa molecular weight-limit device (Amicon Ultra-15, Millipore, Billerica, MA, USA), purified (Ready Prep 2-D Cleanup Kit; Bio-Rad Laboratories, Hercules, CA, USA), and protein concentrations were measured (Nanodrop 2000C, Thermo Scientific, Rockford, IL, USA). Two samples of concentrated BAL fluid that had intermediate annexin A1 levels were used as standards; these were loaded on each gel to facilitate comparison of test samples between gels and blots. Samples containing 10 μg of protein were loaded on sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gels (TGX Stain-Free Fast Cast Acrylamide solutions, Bio-Rad Laboratories, Hercules, CA, USA) and separated at 200 V for 1 h, then proteins were visualized (ChemiDoc, Bio-Rad Laboratories (Canada), Ltd, Mississauga, ON, Canada) and total protein in each lane was quantified by densitometry (Image Lab software, Bio-Rad Laboratories, Hercules, CA, USA). Proteins were transferred to a membrane (TransBlot Turbo PVDF Membrane, Bio-Rad Laboratories (Canada), Ltd, Mississauga, ON, Canada) that was used for western blot analysis. The membrane was blocked with 5% BSA for 1 h, then incubated with rabbit anti-

human annexin A1 antibody (1:375; H00000301-D01P, Novus Biologicals, Oakville, ON, Canada) overnight at 4˚C, washed, and incubated with peroxidase-conjugated goat anti-rabbit immunoglobulin as secondary antibody (1:3000; DakoCytomation, Glostrup, Denmark), for 30 minutes, washed for 1 h, and visualized by chemiluminescence (Clairity ECL western blotting substrate, Bio-Rad Laboratories (Canada), Ltd, Mississauga, ON, Canada). Bands (37 kDa), corresponding to that previously shown for annexin A1[25] were quantified by densitometry (Image Lab software, Bio-Rad, Laboratories, Hercules, CA, USA). Data are shown as the density of the band on the western blot adjusted for the background density of the blot, normalized to the corresponding adjusted total protein value obtained by densitometric analysis of the SDS-PAGE gel. Quantitative data were confirmed by visual inspection of the blots and gels. All laboratory personnel remained blinded to treatment during analysis.

2.8. Statistical Analysis

Parametric statistical procedures were performed by fitting generalized linear mixed models (GLMM) using the GLIMMIX procedure in SAS (Version 9.4, SAS Institute, Cary, NC, USA) to evaluate statistical associations between treatment effects (transportation and meloxicam status) and all outcome variables except Hp-MMP 9. A Gaussian distribution, identity link, and residual pseudo-likelihood estimation were specified for these models. Continuous outcomes analyzed in this paper included rectal temperature (˚C), ocular temperature (˚C), cortisol concentration (ng/mL), plasma meloxicam concentration (ng/mL), neutrophil L-selectin intensity (GMFI), red blood cell count (cells/μL), mean cell hemoglobin concentration (g/dL), and hemoglobin (g/dL). Outcomes that required a natural log transformation included substance P (pg/mL), haptoglobin concentration (μg/mL), oxidative burst intensity (GMFI),

phagocytosis intensity (GMFI), white blood cell count (cells/μL), lymphocyte count (cells/μL), polymorphonuclear leukocyte cell count (cells/μL), and monocyte cell count (cells/μL). Results from these models are presented as back-transformed means and 95% confidence intervals. GLMMs were also used to model categorical outcomes. Mean cell volume (fl) was modeled as a dichotomous outcome variable (normal or low) based on normal laboratory values of 40-65 fl and fit using a binary distribution, logit link, and residual pseudo-likelihood estimation. Other outcome variables including the percentage phagocytosing/oxidative neutrophils and hematocrit were fit using a beta distribution.

A random intercept for pen (a 4-level categorical variable) and a random covariance structure for unequally spaced repeated measures for calf were tested and incorporated whenever appropriate. When only two post-transport repeated measures were available (day 0 and day 3), a simple random intercept for calf was used.[26] Given the 2 x 2 factorial nature of the trial design, models included main effects for transportation (a 2-level categorical variable; TR and NTC), meloxicam status (a 2-level categorical variable; MEL and CON), study day (a 2 to 3-level categorical variable depending on the outcome), and the two-way interaction of transportation by meloxicam status, whenever data were available for all treatment groups. For models where outcomes were recorded in the TR group only (i.e., rectal temperature, ocular temperature, and annexin A1 data), models included fixed effects for meloxicam status and study day or time with a meloxicam status by study day or time two-way interaction term. Additionally, for the model where annexin A1 volume was the outcome variable, a natural log transformation of the response and an additional random intercept for blot number were utilized. Unless noted otherwise, model estimates are presented as model-adjusted means ± standard error of the means. An analysis of

either studentized or Pearson residual plots was performed for all models depending on the outcome distribution, and residuals ≥ |3| were further evaluated as potential outliers or influential observations. Significance of fixed effects was determined by a *P*-value of ≤ 0.05. To reduce the likelihood of Type I errors, a more stringent *P*-value of ≤ 0.01 was used to identify significance in all effects with more than one pair-wise comparison.

Residuals of the model for Hp-MMP 9 concentration (ng/mL) were not normally distributed or homoscedastic and despite several attempts, no amenable transformations normalized the residuals or improved the heteroscedasticity. Therefore, a Kruskal-Wallis test comparing the four treatment groups (TR-MEL, TR-CON, NTC-MEL, and NTC-CON) within the 2 x 2 factorial design followed by the Steel-Dwass method for multiple comparisons (α = 0.01) between days was performed for the Hp-MMP 9 outcome variable in JMP 12 (SAS Institute, Cary, NC, USA).

3. Results

3.1. Clinical illness

During the first 3 days following arrival, all steers had CIS of 1 (Normal) with no clinical signs of BRD. Throughout 28-day health monitoring period, several animals received a CIS of 2 but did not have a rectal temperature of ≥ 40 ˚C and so were not eligible to be classified as BRD cases. Therefore, no steers met the case definition for BRD during the 28 days after arrival at KSU. Two steers showed signs of lameness within the first 3 days of the study (n = 1 from TR-MEL and n = 1 from NTC-CON) but did not require treatment with NSAIDs and were therefore allowed to remain in the analysis for all variables reported in this study.

3.2. Rectal Temperature Probes

Five rectal temperature probes malfunctioned (TR-MEL: n = 3; TR-CON: n = 2) and did not provide data for this study. Several additional probes were removed from the dataset following their malfunction or removal. One probe had to be removed on arrival due to swelling (TR-CON), one probe fell out on arrival (TR-MEL), one probe fell out (TR-MEL) on study day 1, one probe was found partially misplaced from the rectum of a calf on the morning of day 2 (TR-MEL), and one probe fell out during the last 6 hour period of data collection (TR-MEL). Analysis of the remaining temperature probe data did not reveal a significant time by meloxicam status interaction but time, aggregated into 6 h time periods, was associated ($P < 0.01$) with the rectal temperature of study steers. However, there was no difference between rectal temperature in the TR-MEL or TR-CON steers ($P = 0.83$).

3.3. Ocular Thermography

Although no significant study day by meloxicam status interaction was detected, steers had greater ($P < 0.01$) ocular temperatures on day 0 (39.4 ˚C ± 0.08) compared to both day -1 (38.9 ˚C ± 0.08) and day 3 (38.8 ˚C ± 0.11). Meloxicam status did not have a significant effect on ocular temperatures.

3.4. Cortisol

There was no significant transport by meloxicam status interaction or significant effects of transport or meloxicam status on plasma cortisol concentration. However, significant differences were detected between study days (Figure 5.3).

3.5. Substance P

A transport by meloxicam status interaction was apparent ($P < 0.01$) with NTC-MEL steers having lower substance P concentrations (53.4 pg/mL ± 1.08) than TR-MEL (77.8 pg/mL ± 1.05), TR-CON (72.1 pg/mL ± 1.05), or NTC-CON (73.8 pg/mL ± 1.08) steers (Figure 5.4). Day of sampling did not have a significant effect on substance P concentrations in this study.

3.6. Hp-MMP 9

Prior to transport (day -1), there were no significant differences ($P = 0.57$) between the four treatment groups in terms of Hp-MMP 9 concentration. On day 0 (arrival), steers in the TR-CON group (377.8 ng/mL ± 140.65) had greater ($P < 0.01$) Hp-MMP 9 concentration compared to steers in the NTC-CON group (0 ng/mL ± 0). On day 3, an overall significant difference between the four treatment groups was detected ($P < 0.01$) but individual comparisons between groups were not significantly different at $P < 0.01$.

3.7. Hematology

The transport by meloxicam status interaction was not significantly different for any of the hematology variables. Main effects for transport status and meloxicam status were also not significantly different for red blood cell counts, hematocrit values, or mean cell hemoglobin concentrations (MCHC). Red blood cell counts were greater ($P < 0.01$) on day 0 (7,759,513 cells/μL ± 139,331) compared to day 3 (7,542,844 cells/μL ± 139,083). Similarly, hematocrit values were also greater ($P < 0.01$) on day 0 (30.2 % ± 0.46) compared to day 3 (29.4 % ± 0.46). Steers had lesser ($P < 0.01$) MCHC on day 0 (35.6 g/dL ± 0.22) compared to day 3 (36.6 g/dL ±

0.22). No significant effects for hemoglobin concentration or mean cell volume were observed in this study. Steers had greater ($P = 0.03$) total leukocyte counts on day 0 (11,485 cells/μL, 95% CI: [10,824; 12,187]) compared with day 3 (10,895 cells/μL, 95% CI: [10,271; 11,558]) but neither meloxicam status nor transport status had a significant effect on total leukocyte counts. Polymorphonuclear leukocyte counts were greater ($P < 0.01$) in TR steers (3,800 cells/μL, 95% CI: [3,406; 4,239]) compared to NTC steers (2,697 cells/μL, 95% CI: [2,310; 3,149]) and greater ($P < 0.01$) in all steers on day 0 (3,744 cells/μL, 95% CI: [3,348; 4,186]) compared to day 3 (2,737 cells/μL, 95% CI: [2,450; 3,058]). Meloxicam status did not have a significant effect on polymorphonuclear leukocyte counts. There were no significant interactions or main effects on lymphocyte or monocyte cell counts throughout this study. Descriptive statistics of all hematology values are provided (Table 5.1).

3.8. Haptoglobin

When haptoglobin concentrations were examined, a transport by meloxicam status interaction was not detected ($P = 0.46$). A transportation effect was detected ($P < 0.01$) with TR steers having greater haptoglobin concentrations (59.4 μg/mL, 95% CI: [41.9; 84.1]) than NTC steers (25.9 μg/mL, 95% CI: [15.7; 42.6]) but meloxicam status did not significantly affect haptoglobin concentrations. Additionally, day influenced haptoglobin concentrations ($P < 0.01$) with steers having different ($P = 0.01$) haptoglobin concentrations on day 0 (26.7 μg/mL, 95% CI: [18.2; 39.1]) and day 3 (47.1 μg/mL, 95% CI: [31.2; 71.1]). Neither day had significantly different haptoglobin concentrations when compared to day -1 (47.9 μg/mL, 95% CI: [31.4; 73.1]).

3.9. Neutrophil L-selectin

Although a significant transport by meloxicam status interaction was not detected, TR steers had greater ($P < 0.01$) neutrophil L-selectin intensity levels (58.6 GMFI ± 5.68) compared to NTC steers (48.0 GMFI ± 6.13). Steers had lesser ($P < 0.01$) neutrophil L-selectin intensity on day 0 (47.2 GMFI ± 6.24) compared to day 3 (59.4 GMFI ± 5.70). Meloxicam status did not significantly affect neutrophil L-selectin intensity levels.

3.10. Neutrophil Activity

No significant transport by meloxicam status interactions or main effects of transport or meloxicam status were detected for any of the measures of neutrophil activity. Phagocytosis intensity was lesser ($P < 0.01$) on day 0 (87.6 GMFI, 95% CI: [77.5; 99.0]) compared to day 3 (98.9 GMFI, 95% CI: [87.4; 111.8]) while the percentage of phagocytosing/oxidative burst neutrophils was greater ($P < 0.01$) on day 0 (65.5 % ± 2.23) compared to day 3 (55.7 % ± 1.59). No significant effects were detected on the intensity of neutrophil oxidative burst activity in this study.

3.11. Annexin A1

No significant interaction or main effects of time or meloxicam treatment were detected on annexin A1 levels within transported steers on arrival or on day 3 following transport.

3.12. Meloxicam Concentration

No study steers had detectable plasma meloxicam concentrations in any samples collected on day -1 (prior to meloxicam administration) and none of the TR-CON or NTC-CON

cattle had any detectable plasma meloxicam concentrations in any samples at any time point. Examination of plasma meloxicam concentrations in the NTC-MEL steers compared with the TR-MEL steers indicated a significant study day by transport interaction (Figure 5.5).

Discussion

This study provides the opportunity to examine the effect of long-distance transportation in the absence of some of the other common stressful events that cattle often experience in conjunction with transportation such as weaning, commingling, or time spent at a livestock auction. Although the animals studied herein were older, single-source, and were previously weaned, the TR cattle still experienced water and food deprivation during transport and both TR and NTC cattle were handled at the same time points both before and after transport. The effects of food or water deprivation during transport have been documented[27] and handling stress is considered by some authors to have a larger impact than the actual transportation event itself.[28,29] However, even considering the accumulation of several stressors in this study, no clinical BRD was observed in the entire set of steers across all of the treatment groups at any point during the entire monitoring period. Therefore, the results of this study also provide information regarding the effect of transportation in clinically healthy animals.

Prior work[14] indicated that the mean C_{max} of orally administered meloxicam is achieved at about 12 h post-administration and that the half-life is approximately 28 h on average. In this study, TR-MEL steers achieved approximately equivalent plasma meloxicam concentrations as found previously[18]; however, although the same doses of meloxicam were administered to both TR-MEL and NTC-MEL steers on day -1, TR-MEL steers had significantly greater meloxicam

concentrations compared to NTC-MEL steers when sampled on arrival. A plausible explanation is that perhaps transport-induced dehydration led to hemoconcentration and thus a seemingly elevated plasma meloxicam concentration in TR steers; however, all steers had clinically normal[30] hematocrits and all but two NTC-MEL steers had normal red blood cell counts. Another possible explanation is that a transportation-induced decreased in rumen fill altered the rate of passage in the gastrointestinal tract and absorption of meloxicam[13] due to less drug binding to forages in the rumen.

The only variable measured in this study affected by the application of meloxicam was substance P, a neuroactive peptide that is involved in the response of animals to pain and stress.[31] However, it is unclear why a decrease in circulating substance P was recorded in only NTC-MEL steers while the TR-MEL steers had concentrations that were not statistically different than the control steers. It is possible that the handling events on day -2 and day -1 produced some level of pain or stress in all 4 treatment groups that was mitigated by meloxicam in NTC-MEL steers but the additional discomfort associated with the long-distance transport event and rectal temperature probes in the TR steers exceeded meloxicam's ability to mitigate the substance P response in the TR-MEL steers. An alternative hypothesis for why the substance P concentrations in the transported and NTC-CON cattle were similar is that the transportation events and associated handling/sampling that were supposed to induce inflammation, discomfort, stress, and a corresponding elevation in substance P concentrations did not do so to an appreciable extent in this group of older transported steers; however, that does not explain the significant decrease in substance P concentrations found in the NTC-MEL steers and therefore is less likely than the initial explanation. Although it does not appear that meloxicam

administration affected the TR-MEL steers in this study, it is possible that meloxicam administration could affect substance P concentrations in higher risk steers (younger, recently weaned, extensively commingled, etc.) or calves that experienced any different combination of stressors differently and should be studied further.

Previous studies have reported increases in plasma cortisol concentrations following a stressful event such as transport[6,9,32] or transport following weaning[33], while others have reported decreases[27] or no differences.[34,35] It is probable that the relationship between stress and cortisol concentrations varies depending on the magnitude, type, length, or total amount of stress endured given that the responses to stress seem so variable.[10] However, separation of stress associated with transportation from stress incited by unloading, handling, and acquiring a blood sample remains challenging; limited research has been published separating out the many stressful events involved in transportation but some attempts have been made to do so.[33,36,37] The combination of the stress of handling and acquiring a blood sample experienced by the NTC cattle may have contributed to the lack of effect of transportation status on cortisol concentrations despite efforts made to minimize it in this study. A significant difference between day -1 cortisol concentrations and day 3 cortisol concentrations was observed; one contribution to the study day effect observed herein could be that steers were sampled in the late afternoon prior to transport on day -1 whereas sampling on day 0 and day 3 occurred early in the morning. However, cortisol concentrations were relatively high across all sampling time points including baseline indicating that some form of stress was experienced by all the cattle regardless of treatment group and thus making it difficult to identify any circadian differences in cortisol concentrations.[38]

Not only are there conflicting reports regarding the change, or lack thereof, of cortisol concentrations following a stressful event, there is also considerable controversy in the literature regarding the effect of glucocorticoids on the immune function of cattle. Some forms of stress have the potential to improve some aspects of the immune response[1] whereas others have observed that acute or prolonged stressors have a negative impact on immune function.[34,39] Indeed, it seems that the relationship between stress and the immune system is complex and complicated by factors such as genetics, the part of the immune system examined, and the type and chronicity of the stressor.[1,27] Despite experiencing a transportation event that should have been significantly stressful, the evaluation of the leukograms in this group of study animals did not indicate evidence of a full classic stress response (leukocytosis, lymphopenia, and mild neutrophilia) in transported cattle compared to non-transported cattle; the only part of the leukogram that was affected by transport group was the polymorphonuclear leukocyte count which, although greater in the TR cattle, remained within reference ranges.[30] While significantly greater total leukocyte counts and polymorphonuclear leukocyte counts were observed on day 0 compared to day 3, values on both days also remained within normal limits.[30] Additionally, although cattle showed a significant increase in red blood cell count and hematocrit and a decrease in MCHC on day 0 compared to day 3, again the variables remained within normal reference ranges for cattle[30] except for the hematocrit of a single calf. While the significant differences between sampling times observed in these variables do make sense physiologically, the lack of a day -1 baseline value for any of these variables and the fact that they all remained within normal limits makes interpretation of the significant differences between arrival and day 3 hematology and leukogram values difficult. As previously mentioned, it is possible that the

transportation event was not stressful enough to induce a classic stress leukogram in the class of cattle studied herein; a different response may be seen in other types of cattle.

The results from this study indicated that transported yearling steers had greater plasma haptoglobin concentrations than non-transported steers which is in contrast to previous work that found no difference in plasma haptoglobin concentration between groups.[34] However, Early and O'Riordan also found that haptoglobin concentrations did not differ significantly in their baseline and post-transport samples; this agrees with our results where neither day 0 nor day 3 samples were significantly different than baseline. However, this study did find that samples collected on day 3 had had significantly greater haptoglobin concentrations than on day 0 (arrival). This small delayed increase may be explained by the fact that haptoglobin is a late acute phase protein[40] and it was unlikely that an increase would be seen immediately following our overnight transportation event. However, other work in transported calves also found that haptoglobin was decreased at 4.5 and 9.75 h after the start of a 9 h transportation event but were no different than time 0 thereafter.[32] Additionally, it has been demonstrated that the individual animal acute-phase protein response to stress[41] and inducers of inflammation such as LPS[40] can vary considerably. Given the variability reported in the literature, it is possible that there is not a consistent amount of inflammation induced by transport or that varying factors between experiments such as length of transport, road conditions, environmental conditions, etc. are confusing the issue regarding haptoglobin concentrations in response to transportation or other stressful events.

It was expected that transported steers, even of a lower risk category such as the animals used in this study, would have greater cortisol, greater levels of inflammatory markers, decreased leukocyte function, and a stress leukogram. Although comparing results across studies examining transport stress is complicated by the variability in the study populations, transport events, and sample timing, the results observed in this study were still surprising and hint at the complexity of what constitutes a significant stress event in different groups of cattle. Despite efforts made to handle the cattle similarly across all treatment groups and to limit the amount of time each calf spent being handled, the time required to take samples on each of the study days was significant and may have induced unwanted variability. It is also possible that more frequent sampling timing[7,36] than what was employed herein is needed to detect the changes in physiology resulting from long-distance transportation in clinically healthy, lower-risk animals.

Overall, these results do not support the use of meloxicam as a means of mediating the effects of transport stress in single-source steers weaned for approximately 8 months. However, meloxicam may be beneficial in other populations of higher risk calves that have experienced additional stressors or inflammatory events[42]. Additional research needs to be performed in order to evaluate meloxicam's potential therapeutic effects in other populations as well as further exploring the complex interplay of different stress sources and their relationship with disease.

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Figure 5.1 – Random assignment of 60 steers to each of the study groups in an unbalanced, 2 x 2 factorial design. Within the transported (TR) and non-transported control (NTC) groups, half of the steers received 1 mg/kg meloxicam (MEL) or a lactose placebo (CON) orally.

		Transportation Status		
		TR	NTC	Total
Treatment Group	MEL	20	10	30
	CON	20	10	30
	Total	40	20	60

Figure 5.2 – Timeline of project including actions taken at both Mississippi State University's Brown Loam Branch Experiment Station (MSU; above timeline) and Kansas State University's Large Animal Research Center (KSU; below timeline). Non-transported control (NTC) steers were transported overnight from MSU to KSU on day -18 for acclimation prior to the beginning of the study while transported (TR) steers were kept at MSU until day -1. On day -2 and day -1, steers were handled at their respective locations and were given either 1 mg/kg meloxicam (MEL) or a lactose placebo (CON) orally on day -1.

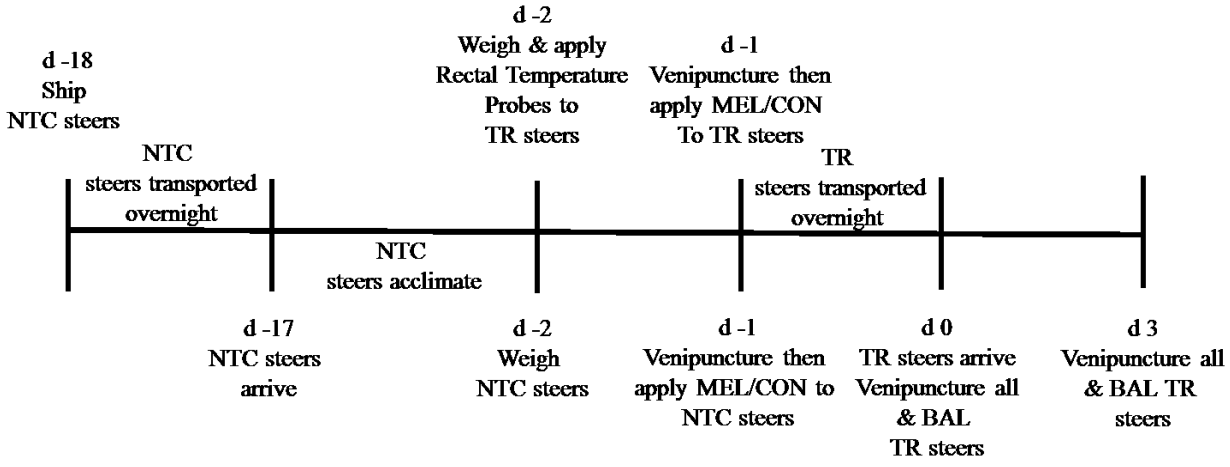


Figure 5.3 – Plasma concentration of cortisol (ng/mL) in steers prior to transport (day -1), on arrival (day 0), and on day 3 post-transport. There was no significant interaction or main effects of transportation or meloxicam status on cortisol concentrations. Columns with different letters were different ($P < 0.01$); data are presented as model-adjusted means ± SEM.

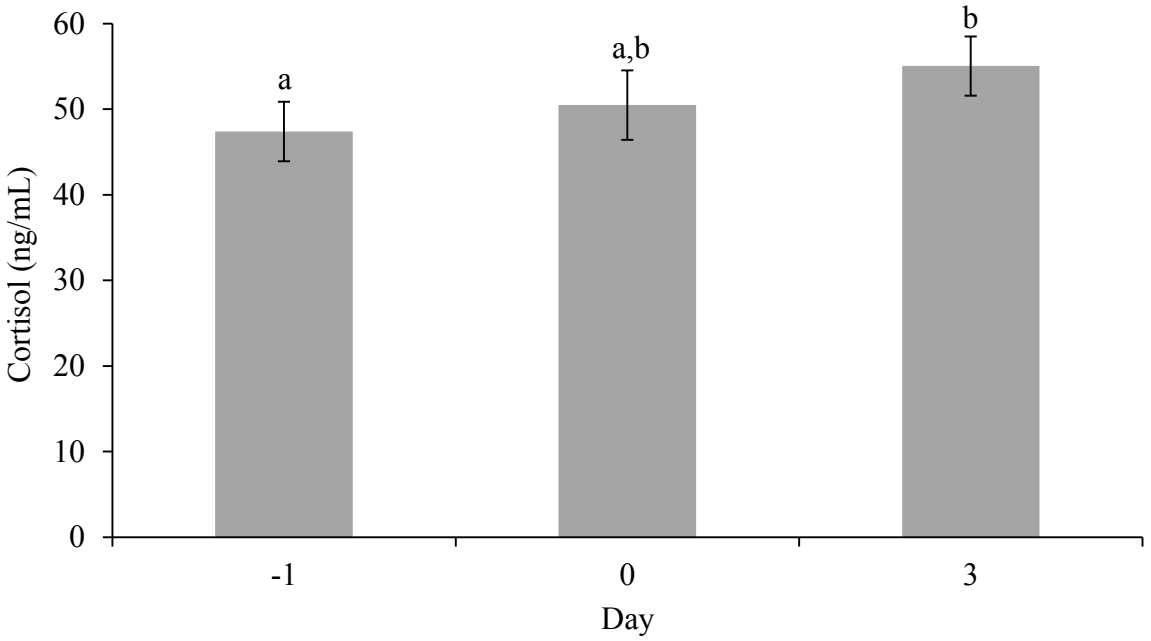


Figure 5.4 – Plasma concentration of substance P (pg/mL) in steers within each treatment group. NTC = non-transported controls; TR= transported; MEL = meloxicam; CON= lactose placebo. There was an interaction ($P < 0.01$) between transport group and meloxicam status with NTC-MEL steers having lesser ($P < 0.01$) substance P concentrations than all other steers. Data are presented as model-adjusted means and 95% confidence intervals.

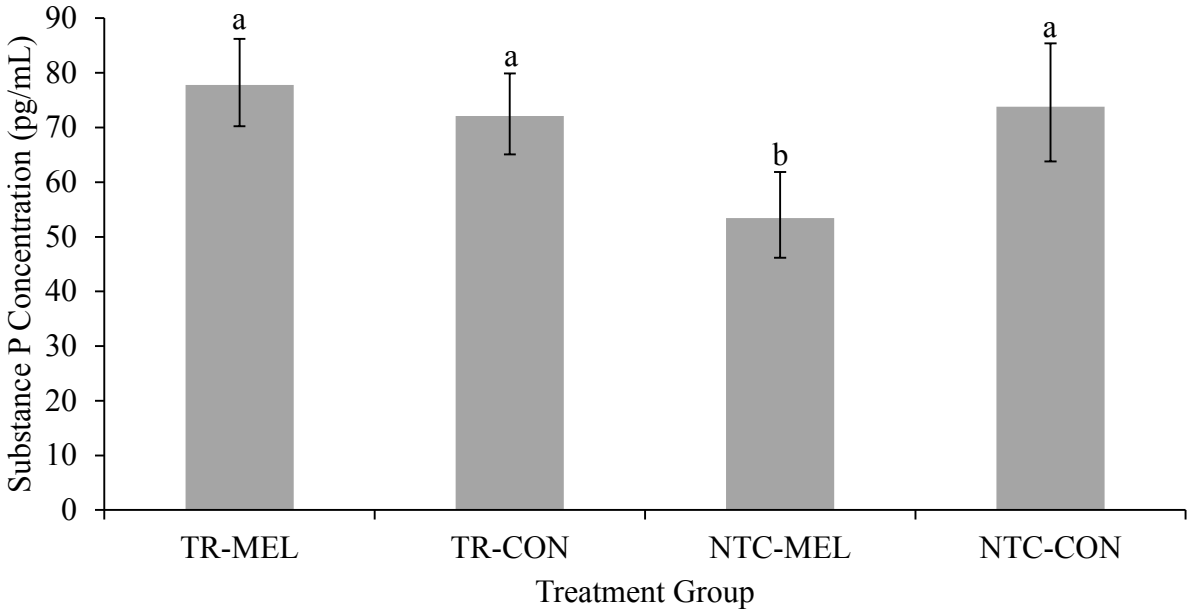
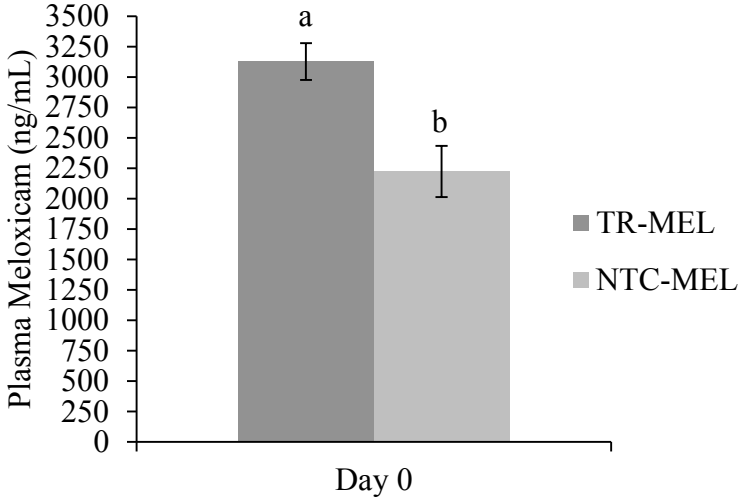


Figure 5.5 – Plasma meloxicam concentration (ng/ml) in transported (TR-MEL) and non-transported control (NTC-MEL) steers on a) day 0 and b) day 3 after receiving 1 mg/kg meloxicam orally on day -1. Steers in both the TR-CON and NTC-CON groups had plasma meloxicam concentrations below detectable limits for the duration of the trial and are not included in this figure. Different letters within each graph represent differences at $P < 0.01$; data are presented as model-adjusted means ± SEM.

a)



b)

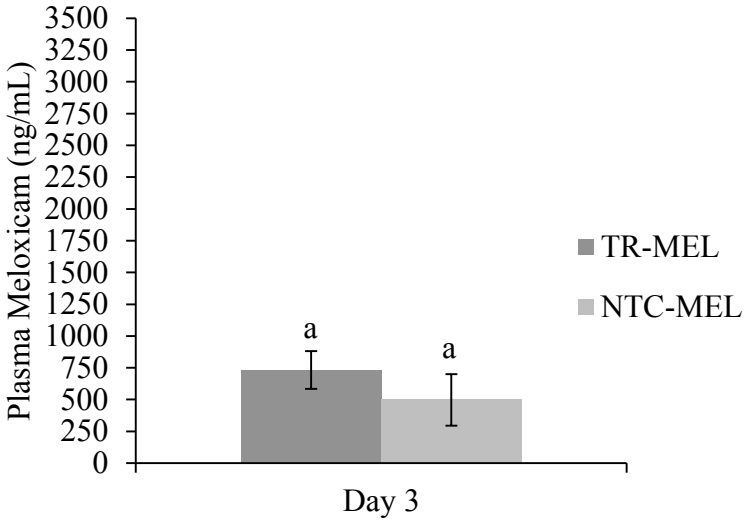


Table 5.1 – Descriptive statistics of all hematology and leukogram values broken down by study day for all transported (TR) and non-transported control (NTC) cattle that received either meloxicam (MEL) or a placebo control (CON). RBC = red blood cell count; HGB = hemoglobin concentration; HCT = hematocrit; MCV = mean corpuscular volume; MCHC = mean corpuscular hemoglobin concentration; WBC = total leukocyte count; PMN = polymorphonuclear leukocyte count; LYM = lymphocyte count; MON = monocyte count.

		NTC								TR							
		MEL				CON				MEL				CON			
	Day	**Mean ± SD**	**Median**	**Min**	**Max**	**Mean ± SD**	**Median**	**Min**	**Max**	**Mean ± SD**	**Median**	**Min**	**Max**	**Mean ± SD**	**Median**	**Min**	**Max**
RBC	0	7.75 ± 1.39	7.83	5.72	10.83	7.77 ± 1.05	7.47	6.31	9.79	7.73 ±0.99	7.77	6.00	9.57	7.86 ±0.81	7.69	6.36	9.46
(mm^3)	3	7.52 ± 1.25	7.41	6.08	10.29	7.69 ± 0.80	7.57	6.67	8.97	7.44 ± 1.11	7.37	5.27	9.53	7.58 ±0.85	7.38	5.94	9.40
HGB	0	10.47 ± 1.39	10.25	8.20	13.70	10.94 ± 1.25	10.95	8.70	13.10	10.64 ± 1.10	10.65	8.30	12.50	10.85 ±1.03	10.95	9.10	12.50
(g/dL)	3	10.65 ± 1.37	10.25	8.90	13.90	11.19 ± 0.92	11.20	9.80	12.50	10.54 ± 1.33	10.55	7.20	13.10	10.70 ± 1.00	10.80	8.80	12.90
HCT	0	29.42 ± 4.52	29.60	22.20	38.10	31.00 ± 3.61	30.30	27.10	37.60	29.76 ± 3.01	30.25	22.60	35.10	30.56 ± 3.05	30.65	25.70	35.40
(%)	3	28.96 ± 3.69	28.15	22.60	35.50	30.72 ± 2.27	30.10	27.30	34.30	28.74 ± 3.57	28.60	18.90	34.00	29.48 ± 3.24	29.55	24.00	38.80
MCV	0	38.21 ± 2.90	37.80	34.20	42.70	40.05 ± 2.05	40.25	36.70	42.90	38.73 ± 2.96	37.75	35.10	45.20	39.00 ± 2.94	39.65	34.30	44.30
(fl)	3	38.82 ± 3.51	37.80	34.20	43.60	40.11 ± 2.00	40.30	37.40	43.00	38.83 ± 3.02	38.25	35.50	45.40	39.02 ± 3.15	39.50	34.20	45.20
MCHC	0	35.79 ± 2.94	35.45	33.10	43.00	35.32 ± 1.62	35.25	32.10	37.40	35.78 ± 1.29	35.80	33.50	37.70	35.54 ± 1.72	35.40	31.90	38.90
(g/dL)	3	36.84 ± 1.62	36.55	34.30	39.40	36.43 ± 1.33	36.70	34.10	38.20	36.71 ± 1.55	37.00	34.10	39.20	36.34 ±1.29	36.30	33.20	38.90
WBC	0	10.91 ± 1.18	11.18	8.44	12.18	10.72 ± 2.14	10.37	8.83	16.46	12.19 ± 2.85	12.00	8.56	16.74	12.68 ± 2.36	12.36	8.92	18.48
(x 10^6/mL)	3	11.28 ± 2.03	11.06	8.00	14.47	10.40 ± 2.04	10.00	8.73	15.90	11.56 ± 3.27	12.40	7.09	20.90	11.74 ± 2.94	11.02	7.74	17.86
PMN	0	3.34 ± 0.72	3.34	2.55	4.65	2.95 ± 0.91	2.90	1.72	4.89	4.52 ± 1.94	4.34	1.92	8.73	5.18 ± 1.96	5.20	2.02	10.18
(x 10^6/mL)	3	2.83 ± 0.86	3.24	0.71	3.47	2.32 ± 0.92	2.49	1.06	4.03	3.50 ± 1.63	3.21	1.32	7.99	3.60 ± 1.93	3.01	1.64	9.40
LYM	0	5.60 ± 1.22	5.88	3.27	7.14	5.87 ± 1.22	5.69	4.35	8.51	5.76 ± 1.12	5.69	4.24	8.35	5.85 ± 1.19	5.89	3.71	8.18
(x 10^6/mL)	3	6.13 ± 1.75	5.71	4.04	8.91	5.94 ± 1.42	5.63	4.62	9.38	5.91 ± 1.33	5.48	4.00	8.60	6.09 ± 1.36	5.90	3.71	8.69
MON	0	1.47± 0.25	1.47	1.15	2.10	1.63 ± 0.38	1.53	1.36	2.65	1.68 ± 0.65	1.47	0.91	2.97	1.48 ± 0.43	1.56	0.78	2.32
(x 10^6/mL)	3	1.59 ± 0.27	1.60	1.16	2.11	1.67 ± 0.26	1.59	1.34	2.05	1.51 ± 0.64	1.36	0.66	3.04	1.53 ± 0.51	1.35	0.85	2.90

Chapter 6 - The effect of pre-shipment meloxicam on movement, feeding, and drinking behavior of transported and non-transported cattle

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Abstract

Objective

To monitor the effects of meloxicam administration pre-transport on the movement, feeding, and drinking behaviors of transported and non-transported calves.

Animals

High-risk beef steers (n=100).

Procedures

Transport (TR) experiment: steers were randomly assigned to receive either 1 mg/kg meloxicam (MEL, n=50) or a placebo orally (CON, n=50) then transported approximately 1,000 km overnight to a feedlot where they were processed and randomly assigned to 10-head pens within

treatment group (MEL=5 pens, CON=5 pens). Distance traveled, percent time spent near feed (PNF), and percent time spent near water (PNW) were recorded for each calf for 21 days post-arrival. Non-transported (NT) experiment: all pens received the opposite treatment (MEL or CON), were kept in their pens overnight, processed, and behaviors recorded for the next 21 days. Average daily gain (ADG) and feed efficiency (FE) were calculated for each experiment.

Results

During the TR experiment, meloxicam did not significantly affect distance traveled, PNF, or PNW but all behaviors did significantly vary by day ($P < 0.001$). Treatment did not affect TR FE ($P = 0.61$); TR ADG was descriptively similar between treatments. In NT cattle, the effect of day on distance traveled depended on meloxicam ($P < 0.01$) but there were no significant within-day comparisons between MEL and CON calves. Day was the only significant effect ($P < 0.001$) for PNF or PNW. Between treatment groups, ADG and FE were descriptively similar in NT cattle.

Conclusions and Clinical Relevance

This study did not demonstrate an effect of meloxicam administered prior to transportation on the behavior or performance variables measured in transported calves.

Abbreviations

BRD Bovine Respiratory Disease

CIS Clinical illness score

CON Control placebo-treated cattle

COX-2 Cyclooxygenase-2

MEL Meloxicam-treated cattle

NSAID non-steroidal anti-inflammatory drug

NT Non-transported cattle

TR Transported cattle

Introduction

While evidence exists that long-distance transportation increases BRD risk[1] and impacts various inflammatory, immunologic, and performance parameters in cattle[2,3], relatively few therapeutic strategies exist to mitigate the effects of long-distance transportation.[4] Meloxicam, a COX-2 inhibiting NSAID with high bioavailability and a relatively long mean plasma half-life of 28 hours[5], provides anti-inflammatory therapy for several days with a single oral bolus of 1 mg/kg. Work by Van Engen *et al.* has shown that meloxicam can affect some transportation-associated changes in immunity[2] and certain biomarkers of stress and inflammation.[6] Guarnieri Filho *et al.* recently found that repeated administration of meloxicam prior to loading, at unloading, and daily through the first week at the feedlot influenced certain performance parameters.[7] Therefore, the possibility exists for using an NSAID, such as meloxicam, to alleviate the adverse effects of long-distance transport.

Reductions in feed intake[8] in cattle that have been transported long distances have been reported, but specific examination of eating and drinking behavior following long-distance transport has not been well-studied. However, the development of real-time location monitoring systems (RTLS) have provided researchers with a non-invasive technology to study these behaviors after transportation.[9] Conventional receiving practices indicate that long-haul cattle, that are often shrunk[10] and have been standing for extended periods of time[11], should have access to hay and water soon after arrival[12] and have the opportunity to rest in a clean, dry location.[13] While these practices do seem beneficial and address the dehydration and shrink inevitably linked to long-distance transport, they do not address the impact of any potential inflammation or stress associated with long-distance transport on the behavior of newly-arrived calves. Development of an intervention that could lessen the impact of long-distance transport on cattle behavior and speed their recovery from such a stressful event could result in reduced BRD risk. Therefore, the objective of this project was to monitor the effects of meloxicam administration before transport on the movement, feeding, and drinking behaviors of transported and non-transported calves.

Materials and Methods

Study Population and Timeline

This study protocol was approved by the Kansas State University Institutional Animal Care and Use Committee (IACUC # 3591) and consisted of two separate experiments during which calves were either transported (TR) or not transported (NT). During the TR experiment (6/11/15 to 7/3/15), 100 high-risk crossbred beef steers were obtained from a livestock auction in Tennessee and transported approximately 1000 km overnight to a feeding facility in Missouri.

Prior to shipment, all calves were double tagged with commercial ear tags and randomly assigned to receive either 1 mg/kg meloxicam orally (MEL: n = 50) or a lactose placebo orally (CON: n = 50) and then randomly assigned within treatment group to pens (MEL: n = 5 pens and CON: n = 5 pens). On arrival in Missouri, calves were processed and received the following health interventions: a macrolide antibiotic[a], a 7-way clostridial vaccine[b], a 5-way respiratory vaccine[c], and an injectable macrocyclic lactone dewormer[d] according to label directions. Each calf was also fitted with a RTLS ear tag[e] in their right ear. Thermographic images of the eye and blood samples were also obtained on each calf (data not shown). Each 10-head pen (6.1 m x 23.4 m) contained inline bunks that provided a minimum of 0.61 m of bunk space per calf, automatic waterers, and shade. All calves were fed once daily and received a starter ration consisting mainly of hay for the first four days and then transitioned to a wet corn gluten, soy hull, shelled corn, and ground hay total mixed ration for the remainder of the trial.

The NT experiment (7/6/15 to 7/29/15) began with a "pre-transport" sampling and treatment application day (day -1) where each pen of cattle was given the opposite treatment that it had received during the TR experiment (Table 6.1). The treatment administration was followed by an overnight period where cattle remained in their home pens, a sampling day on day 0, and then 21 days of behavioral monitoring. The two experiments were separated by 2 full days where cattle were not considered on trial with the total time period between the first administered dose of meloxicam during the TR experiment and the second administered dose of meloxicam in the NT experiment encompassing 25 days. A detailed depiction of the study timeline and data collected on each study day can be found in 6.1.

Health Monitoring

Each calf was observed once daily by a trained individual blinded to treatment. Calves were assigned a clinical illness score (CIS) regarding signs of BRD according to the following modified scale[14]: CIS 1 – Normal; CIS 2 – Moderate illness, moderate depression, and/or cough; CIS 3 – Severe illness, severe depression, labored breathing, and/or cough; CIS 4 – Severe illness impairing ability to competitively access feed and water; CIS 5 – Moribund/recumbent. Any calf that received a CIS of 2 or greater was examined and a rectal temperature was taken; if the rectal temperature was ≥ 40˚C, treatment was applied according to a predetermined protocol and the calf was returned to the pen. Any calf showing signs of lameness or other illness was examined and treated according to common industry practices. If at any point in the study a calf required NSAID treatment, it was excluded from the study from that point on but remained in its pen provided it could access feed and water.

Behavioral monitoring

As previously mentioned, each calf was fitted with a RTLS tag[e] on arrival which allowed for continuous monitoring of movement, eating, and drinking behaviors during both experiments. These tags work by monitoring the location of calves within a two-dimensional grid with predetermined (x, y) coordinates establishing the perimeter of each pen and the location of the feed bunks and waterers within each pen. The tags track the location (x, y) of each animal every second and, by relating those coordinates to the locations of interest in the pen and the animal's previous location, can quantify distance traveled and time spent near those locations (e.g., within 1 m of feed and within 1 m of water). Data from the tags is sent to sensors located on the periphery of the pen. Although this RTLS has algorithms that use the behavioral data to identify

potentially diseased animals[15,16], the only data used from the tags in this study were the raw behavioral data. All behavioral data was aggregated first to the hourly level for each individual calf then to a pen level average on an hourly basis. The average pen-level hourly data was then aggregated into 24 hour trial days beginning at 0:00 each day. Therefore, the first full day of behavioral data began at 0:00 on day 1 of each experiment. Comparisons of behavior data were carried out using the daily average pen level data.

Performance data

In order to permit continuous behavioral monitoring for the full 21 days following arrival and to minimize handling events, calf weights were obtained prior to feeding on day 0 of the TR experiment and then again prior to feeding on day -1, day 0, and day 22 of the NT experiment (Figure 6.1). Therefore, the TR experiment average daily gain (ADG) and feed efficiency (FE) were based on performance data from TR day 0 (6/12/15) to day -1 of the NT experiment (7/6/15) while the NT experiment ADG and FE were based on performance data from NT day 0 (7/7/15) to NT day 22 (7/29/15).

Statistical Analysis

All data were evaluated on a pen-level basis as pen was the experimental unit. Within each experiment, generalized linear mixed models (GLMMs) were fit using the GLIMMIX procedure in SAS[f] to evaluate the effects of day, treatment, and a day by treatment interaction on behavioral outcomes including distance traveled, a continuous dependent variable fitted via a Gaussian distribution, and percent time spent near water (PNW) and percent time spent near feed (PNF) which were both continuous proportions fitted via beta distributions.

Studentized residual plots were evaluated for normality and homoscedasticity; residuals of ≥ |3| were further evaluated as potential outliers or influential observations. To account for the effect of daily repeated measures on each pen, which were not equally spaced due to missing data, a random intercept of pen within treatment and a random residual of pen within treatment with either an ante(1), sp(POW), or sp(EXP) covariance structure was incorporated into each model based on model fit. Fixed effects were considered significant when $P < 0.05$. When a treatment by study day interaction was significant, within-day comparisons between treatment groups were performed with a more conservative *P*-value of < 0.01. When study day was the only significant effect, pairwise comparisons were made between day 1 and all other study days with a *P*-value of < 0.01 considered significant. Results are presented as model-adjusted means ± SEM.

A GLMM for FE in the TR experiment was fit via a beta distribution and included treatment as a fixed effect and pen within treatment as a random intercept term. Results for the TR experiment FE are presented as model-adjusted means ± SEM. During the TR experiment, ADG was normally distributed but residual analysis revealed heteroscedasticity of the residuals. Therefore, analysis of ADG in the TR experiment was limited to descriptive statistics.

During the NT experiment, ADG was not normally distributed and the residuals could not be normalized via transformation. Additionally, the GLMM for FE in the NT experiment had convergence issues. Therefore, the analysis of performance data from the NT experiment was limited to descriptive statistics.

Results

Morbidity and Mortality

During the TR experiment, a total of 6 calves were diagnosed with BRD and 2 calves became lame; one calf (Pen 2) required re-treatment for BRD and one calf (Pen 4) required re-treatment for lameness. During the NT experiment, no calves were diagnosed with BRD and only one calf (Pen 3) was diagnosed with lameness and required re-treatment. The behavioral data from calves diagnosed with BRD or lameness was removed for the study day prior to diagnosis, the study day of diagnosis, and the study day after diagnosis in order to limit the effect the illness may have had on the behavioral outcomes in those pens.

Additionally, behavioral data from the following time frames during the TR experiment were also removed from the analysis due to the following calves leaving the pen perimeters: all calves on days 3 and 4; one calf from Pen 5 and two calves from Pen 6 on day 5; one calf from Pen 5 on days 5 and 6; and one calf from Pen 2 on day 21.

Behavioral data from one calf from Pen 6 was removed from the entire dataset due to a malfunctioning RTLS tag and from several other calves for specific days during the TR experiment (one calf in Pen 6 on day 1; one calf in Pen 3 and one calf in Pen 10 on day 2; and one calf in Pen 5 on day 14;). Additionally, several days were removed due to system malfunctions or data corruption in the TR experiment (day 18) or NT experiment (days 12, 19, 20, and 21). Therefore, the total number of days analyzed during the TR experiment was 18 and during the NT experiment was 17.

TR Experiment

The treatment by study day interaction was not significant for distance traveled (P = 0.70), PNW (P = 0.16), or PNF (P = 0.74) during the TR experiment. Likewise, the main effect for treatment was not significant for distance traveled (P = 0.94), PNW (P = 0.39), or PNF (P = 0.81) during the TR experiment. However, study day was significantly associated with distance traveled ($P < 0.001$), PNW ($P < 0.001$), and PNF ($P < 0.001$) with significant differences between study day 1 and some of the other study days within all 3 behaviors (Figure 6.2). Descriptive statistics for ADG and FE in the TR experiment can be found within Table 6.2. Treatment with meloxicam did not significantly affect FE during the TR experiment (P = 0.61).

NT Experiment

The treatment by study day interaction was significant ($P < 0.01$) for distance traveled during the NT experiment (Figure 6.3). The treatment by study day interaction was not significant for PNW (P = 0.30) or PNF (P = 0.44) during the TR experiment. The main effect for meloxicam treatment was not significant for PNW (P = 0.31), or PNF (P = 0.62) during the TR experiment. However, study day had an effect on time spent near the water ($P < 0.001$), and time spent near the feed ($P < 0.001$) with several significant pairwise comparisons between study day 1 and some of the other study days within each behavior (Figure 6.4). Descriptive statistics for FE and ADG in the NT experiment can be found within Table 6.3.

Discussion

Performance in TR and NT cattle was not affected by the single dose of meloxicam given in this study. In a study by Cooke *et al.*, flunixin meglumine, another NSAID with a shorter

plasma elimination half-life[17] than meloxicam, given before and after transportation also did not affect dry matter intake (DMI) or FE measurements.[18] However, other work by the same group found that multiple doses of meloxicam resulted in increased ADG, DMI, and FE in cattle transported for 24 hours.[7] It is possible that with a larger sample size in each treatment group or in a population of calves exposed to a different set of stressors in conjunction with long-distance transport, a significant difference based on NSAID treatment could be detected. Alternatively, in order to perceive performance benefits, more than a single dose of meloxicam prior to shipping may be required.

This study did find a significant effect of study day on eating and drinking behaviors in both TR and NT experiments but with no clearly identifiable trends related to known study events or management practices. The day-to-day variability in daily pen eating and drinking behaviors observed herein may reflect normal variation on a pen level or may be a result of cattle reacting to other stimuli that were not recorded or accounted for in this study such as ambient temperature, weather events, or the daily activities occurring throughout the yard. Cattle, as herd animals, often congregate near sources of feed or water even if they are not actively eating or drinking therefore our PNW and PNF variables, which are proxy measures of eating and drinking behavior, invariably include time spent actually eating and drinking as well as time when cattle are not engaging in the behavior itself. This lack of specificity regarding the actual behavior being performed during PNW and PNF may also have contributed to the lack of statistical differences observed between treatments in this study. However, differences in behavior following transportation have been documented by Theurer *et al.* who found that, on the day of transportation, transported heifers spent significantly more time within 0.3 m of the

hay feeder than their non-transported control group but detected no significant differences in time spent near the waterer or the feed bunk.

Behavioral differences between MEL and CON cattle may have been present prior to the initiation of behavioral data monitoring at 0:00 on day 1. Practical limitations of arrival data collection and sorting calves into pens precluded the inclusion of daily behavioral data from the first 18 hours following transportation as a full 24 hours was not available. However, in a study comparing transportation events of differing durations (5 hours, 10 hours, and 15 hours), Wariss *et al.* described similar average water consumption before and after transportation in all 3 groups and a potential decrease in average hay consumption in the first 2 days following transportation for 15 hours compared with the pre-transportation average.[11] Unfortunately, a lack of replication between groups precluded statistical comparisons of pre- and post-transport average water and hay consumption within that study.

Anecdotal reports from producers frequently indicate that long-haul cattle are often found resting soon after they are offloaded. In the TR experiment, study day significantly affected the distance traveled by calves with calves across both treatment groups being numerically less active on days 1, 2, 5, 6, and 7 following transportation compared with nearly all the other study days in the TR experiment. However, the TR experiment's analysis was complicated by the fact that behavioral data from two days, day 3 and day 4 following arrival, were lost due to cattle escaping the pens at some point in the evening on day 3 and so firm conclusions about activity following transportation is limited. When compared to the first day following transportation, calves had significantly higher activity levels on days 8 through 21 except for on day 11 which

had activity levels that were similar to day 1. One possible explanation is that day 11 corresponded with the placement of fresh sand under the shades in each pen which may have led to the decrease in activity observed on that day. Other work has shown that cattle behavior can vary following even a relatively innocuous handling event[19] and so careful consideration of the impact of routine feedlot management practices on cattle behavior and the potential it poses for inducing unwanted variability should be considered during behavioral research.

A difference in distance traveled over time in NT cattle that received MEL versus NT cattle that received CON was observed although MEL and CON pens did not have significantly different activity levels within each study day of the NT experiment. This was unsurprising as examination of the data revealed relatively large standard errors associated with the daily pen level distance traveled within treatment groups. A larger sample size may increase the precision of the estimate and reveal differences between NT MEL and CON cattle within study days but it is also possible that other factors such as weather or human interactions may have a stronger impact on cattle behavior.

It should be noted that, due to the design of the two experiments reported herein and the inherent differences in performance and behavior of cattle that have been effectively acclimated to a facility and their social groups versus freshly commingled and arrived from a sale barn, direct comparisons of the TR and NT results should not be made. As the primary purpose of this research was not to compare the effects of TR and NT but rather to compare the effects of MEL vs. CON within newly TR and acclimated NT cattle, this is not a limitation of the study but

rather a result of *a priori* trial design. Therefore, discussion of the results and our conclusions are limited to comparisons of treatment groups within each of the two experiments.

In this study population of TR calves, the results did not indicate that a single dose of meloxicam prior to long-distance transportation affected activity, feeding behavior, or drinking behavior. The lack of effect observed in this study may be related to the small sample size, the relatively shorter transportation event compared to other studies examining meloxicam, the delay in the start of behavioral monitoring, or that additional doses of meloxicam are needed to observe an effect on behavior. Additionally, meloxicam may affect behavior in other populations of calves that have experienced additional stressors such as weaning, castration, etc., in addition to long-distance transport. Regardless, it appears that the potential for meloxicam to positively impact transported cattle exists but more research is needed to further explore the possible benefits of meloxicam in mitigating the effects of long-distance transportation.

Footnotes

[a] Tulathromycin, Zoetis, Florham Park, New Jersey, USA

[b] UltraChoice 7, Zoetis, Florham Park, New Jersey, USA

[c] Bovi-Shield Gold 5, Zoetis, Florham Park, New Jersey, USA

[d] Moxidectin, Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO, USA

[e] REDI tag, MKW Electronics GmbH, Weibern, Austria

[f] SAS 9.4, SAS Institute, Inc., Cary, North Carolina, USA

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Figure 6.1 – Visual depiction of both the TR (transport) experiment (6/11/15 to 7/3/15) and the NT (non-transported) experiment (7/6/15 to 7/29/15). In the TR experiment, all calves (n=100) were randomly assigned to receive 1 mg/kg meloxicam (MEL) orally or a placebo control (CON) on day -1 prior to being transported (denoted by *) approximately 1,000 km to the research facility in Missouri. Calves were processed on arrival in Missouri on day 0, weighed, behavior tags were applied, and calves were randomly assigned to pens according to treatment (MEL: n = 5 pens; CON: n = 5 pens). Continuous behavioral monitoring occurred during the TR experiment through day 21. During the NT experiment, pens of cattle were given the opposite treatment they received during the TR experiment, returned to their pens overnight (denoted by **) and then monitored in a similar pattern as the TR experiment.

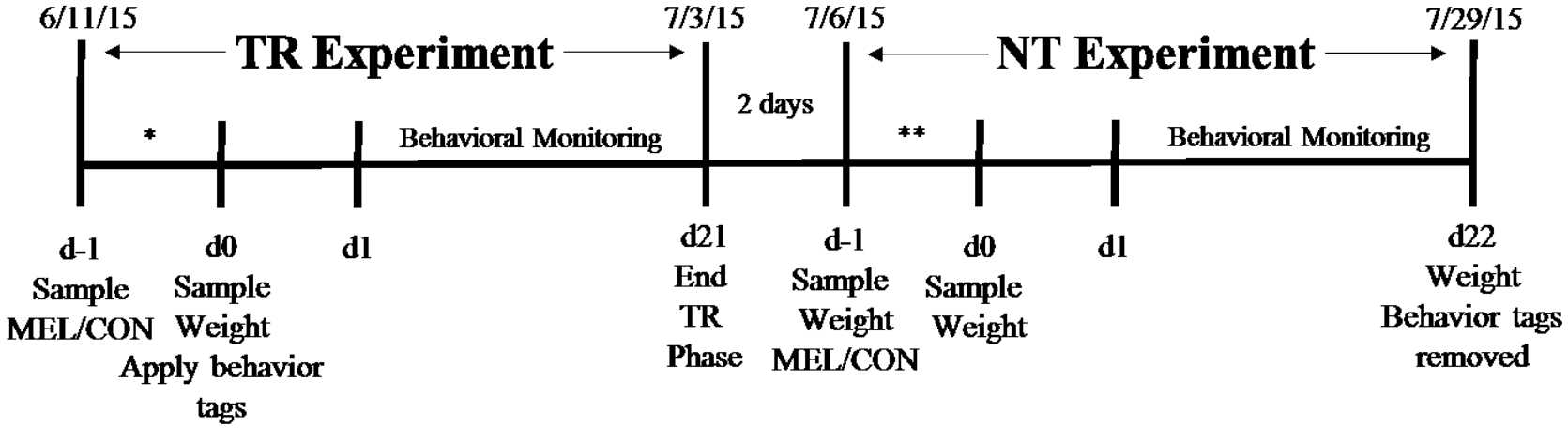
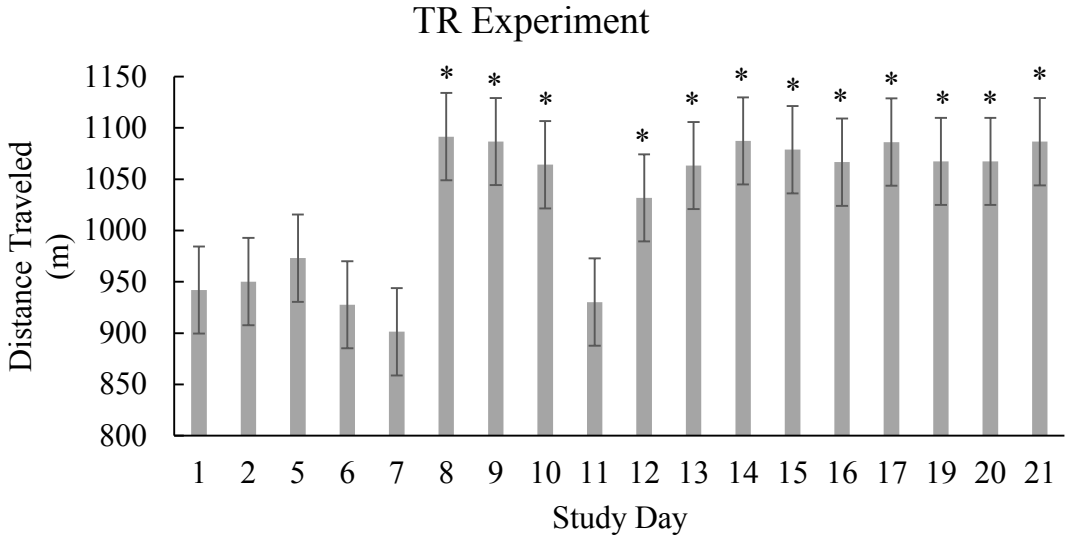
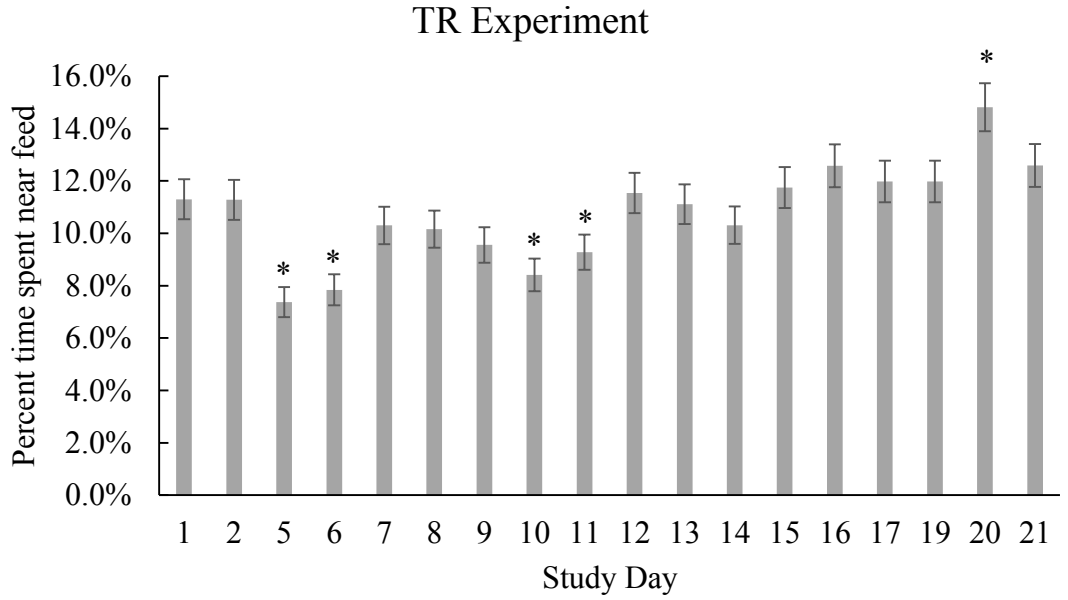


Figure 6.2 – a) Distance traveled (m), b) percent time spent near feed, and c) percent time spent near water during the TR experiment. Study day significantly affected all three behavioral outcomes ($P < 0.001$) and significant pairwise comparisons ($P < 0.01$) between day 1 of the TR experiment and all subsequent days are indicated by an asterisk. Data are presented as model adjusted means ± SEM.

a)



b)



c)

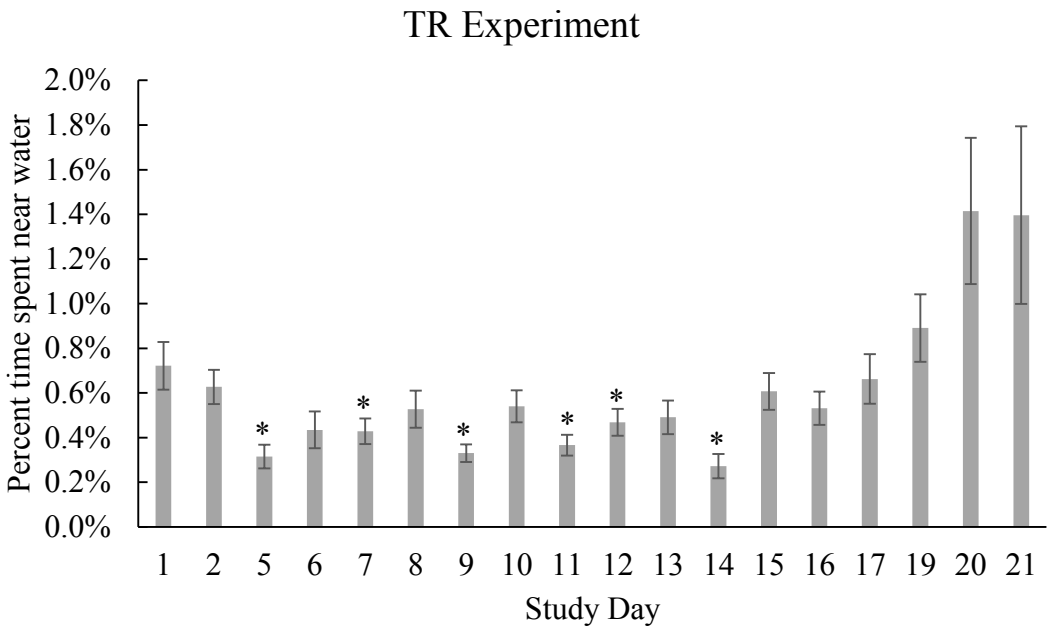

TR Experiment
Percent time spent near water
2.0%
1.8%
1.6%
1.4%
1.2%
1.0%
0.8%
0.6%
0.4%
0.2%
0.0%
1 2 5 6 7 8 9 10 11 12 13 14 15 16 17 19 20 21
Study Day

Figure 6.3 – Distance traveled (m) during the non-transported (NT) experiment was significantly affected by a study day by treatment interaction ($P < 0.01$). Significant comparisons ($P < 0.01$) of study day 1 compared to all other study days within each treatment group are denoted by an asterisk. No comparisons between MEL and CON were significant within any of the NT experiment study days ($P \geq 0.18$). Data are presented as model adjusted means ± SEM.

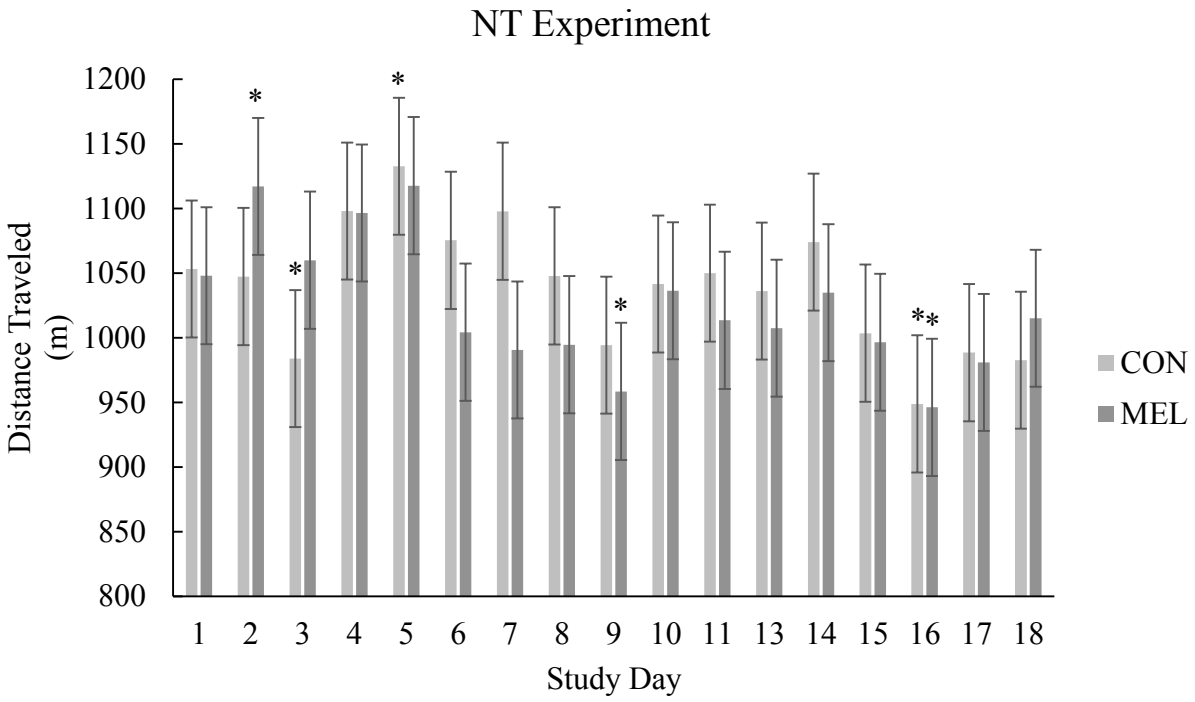
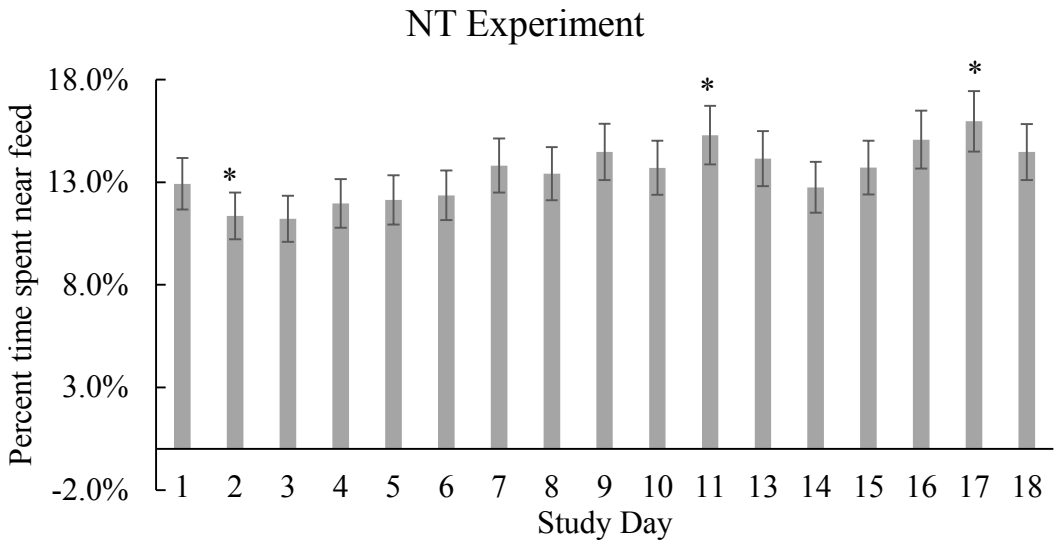


Figure 6.4 – a) Percent time spent near feed and b) percent time spent near water during the NT experiment. Study day significantly affected both behavioral outcomes ($P < 0.001$) and significant pairwise comparisons ($P < 0.01$) between day 1 of the NT experiment and all subsequent days are indicated by an asterisk. Data are presented as model adjusted means ± SEM.

a)



b)

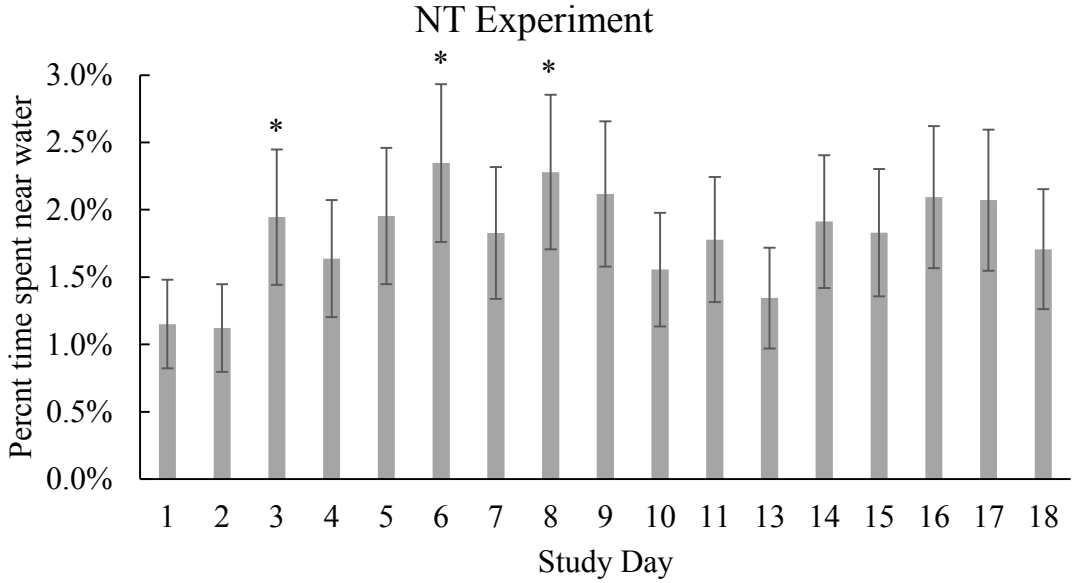


Table 6.1 – Treatments assigned to pens within both the transport (TR) and non-transport (NT) experiments. During each experiment, calves received either 1 mg/kg meloxicam orally (MEL) or a lactose placebo orally (CON) on day -1.

Pen	Experiment	
	TR	NT
1	CON	MEL
2	MEL	CON
3	MEL	CON
4	CON	MEL
5	CON	MEL
6	MEL	CON
7	MEL	CON
8	CON	MEL
9	CON	MEL
10	MEL	CON

Table 6.2 – Descriptive statistics of feed efficiency (FE) and average daily gain (ADG) from the transported (TR) experiment. Calves received either 1 mg/kg meloxicam orally (MEL) or a lactose placebo orally (CON) on day -1 of the TR experiment.

	Treatment Group	Mean ± SE	Median	SD	Min	Max
TR FE	CON	3.52 ± 0.12	3.42	0.28	3.21	3.82
	MEL	3.79 ± 0.39	3.41	0.88	2.97	4.98
TR ADG	CON	2.30 ± 0.08	2.21	0.18	2.14	2.53
	MEL	2.27 ± 0.21	2.41	0.47	1.68	2.73

Table 6.3 – Descriptive statistics of feed efficiency (FE) and average daily gain (ADG) from the non-transported (NT) experiment. Calves received either 1 mg/kg meloxicam orally (MEL) or a lactose placebo orally (CON) on day -1 of the NT experiment.

	Treatment Group	Mean ± SE	Median	SD	Min	Max
NT FE	CON	6.45 ± 0.37	6.96	0.84	5.45	7.15
	MEL	6.73 ± 0.42	6.94	0.94	5.20	7.56
NT ADG	CON	2.31 ± 0.17	2.16	0.38	1.97	2.76
	MEL	2.18 ± 0.14	2.01	0.32	1.93	2.68

Chapter 7 - Dissertation Conclusions

Overall, BRD remains a significant problem for the beef cattle industry and the complex interplay between risk factors such as transportation, commingling, and handling, the ecology of the causative and contributing organisms, and the variable responses of individual calves to those risk factors and organisms makes it a challenging disease to control. The purpose of the research contained within this dissertation was to explore multiple aspects of BRD – pathogen ecology and diagnosis, the impact of common events such as handling, and a possible method for mitigating the effects of long-distance transportation – and provide information that could improve BRD diagnosis and control.

Although our ability to differentiate between and within bacterial species has improved dramatically since the first BRD pathogens were cultured, the increased specificity and changing categorizations serve to highlight just how complex the pathogen side of the BRD epidemiologic triad is. Not only can diagnostic sample results obtained from different sites within the same animal vary but also the same tests applied to the same site in the same calf vary over time. Additionally, within the multiple pathogens potentially involved in BRD cases, there is genetic diversity that we have only recently begun to appreciate and when multiple isolates are taken from the same sample it is possible to see differences in antimicrobial susceptibility profiles and PFGE types. Although we have added to the body of knowledge regarding characterization and diagnosis of *Mannheimia haemolytica*, *Pasteurella multocida*, and *Histophilus somni*, there is still much research that needs to be done to further elucidate the ecology of these organisms within clinically ill and clinically health calves.

Our observations that even a relatively innocuous handling event can have an impact on cattle behavior for several days were surprising; logically, it would follow that a more noxious handling event such as when cattle receive vaccinations, implants, treatment for illness, or undergo diagnostic sampling for BRD would also have impacts on behavior and should be accounted for when performing behavioral research or incorporating behavior into diagnostic algorithms. Establishing just when behaviors return to baseline and improving the precision of our behavior estimates would be useful so more specific recommendations regarding adjustments could be made. Additionally, we demonstrated that cattle with different temperaments responded differently to the handling event; ascertaining and implementing objective, repeatable means of categorizing temperament will be important for future research in understanding cattle behavior and the role that temperament plays in determining the individual's response to stress.

Although long-distance transportation has been established as a significant risk factor for BRD during the feeding period, mitigating this form of stress has proven challenging. There is considerable variability in the literature regarding the effects of long-distance transportation on measures of stress, inflammation, and immune function. Part of the difficulty in addressing the stress of transportation is that there are often other factors, such as commingling, weaning, fasting, and dehydration that are experienced at the same time. Our two studies on the effect of pre-transport meloxicam administration did not reveal any benefits in the outcomes measured at a single 1mg/kg dose but it is possible that, in other study populations which experience more stress or inflammation than our populations did, a benefit might be seen. Additionally, there is some evidence that different dosing of meloxicam can provide benefits in highly stressed calves

that undergo long-distance transportation. Overall, more research is needed to clarify the role of meloxicam and its potential to mitigate the effects of long distance transportation.

Appendix A - Supplementary data

Table A.1 – Nasopharyngeal culture results from calves with clinical BRD (n = 28) that were sampled prior to treatment (day 0), 0.5, 1, or 5 days after treatment with gamithromycin according to sampling group. * = a mixture of gamithromycin susceptibility phenotypes was obtained from up to 12 *M. haemolytica*, 6 *P. multocida*, and 6 *H. somni* isolates tested from each positive sample.

Group 1				Group 2					Group 3				
Calf	Organism	Day 0	Day 5	Calf	Organism	Day 0	Day 0.5	Day 5	Calf	Organism	Day 0	Day 1	Day 5
	H. somni	+	-		*H. somni*	-	-	-		*H. somni*	-	-	+
104	*M. haemolytica*	+*	+	116	*M. haemolytica*	-	-	+	102	*M. haemolytica*	-	-	-
	P. multocida	-	-		*P. multocida*	+	-	-		*P. multocida*	-	+	+
	H. somni	-	-		*H. somni*	+*	+	+		*H. somni*	+	-	-
106	*M. haemolytica*	-	-	127	*M. haemolytica*	-	-	-	118	*M. haemolytica*	-	-	-
	P. multocida	+*	-		*P. multocida*	-	-	-		*P. multocida*	+	-	-
	H. somni	-	-		*H. somni*	-	-	-		*H. somni*	+	-	-
130	*M. haemolytica*	-	-	175	*M. haemolytica*	+	+*	+	126	*M. haemolytica*	-	-	-
	P. multocida	-	-		*P. multocida*	+	+	-		*P. multocida*	+	-	-
	H. somni	-	-		*H. somni*	-	-	-		*H. somni*	+	+	+
150	*M. haemolytica*	+	-	195	*M. haemolytica*	-	-	-	212	*M. haemolytica*	-	-	-
	P. multocida	-	-		*P. multocida*	+	+	+		*P. multocida*	+	+	+
	H. somni	-	-		*H. somni*	-	-	-		*H. somni*	-	-	-
174	*M. haemolytica*	+	-	204	*M. haemolytica*	+	-	-	218	*M. haemolytica*	-	-	-
	P. multocida	-	-		*P. multocida*	+	+	+		*P. multocida*	+	+	+
	H. somni	-	-		*H. somni*	-	-	-		*H. somni*	+	-	-
209	*M. haemolytica*	-	-	206	*M. haemolytica*	+	+	+*	224	*M. haemolytica*	-	-	-
	P. multocida	+	+		*P. multocida*	+	+	-		*P. multocida*	-	-	-
	H. somni	-	-		*H. somni*	-	-	-		*H. somni*	+	-	-
222	*M. haemolytica*	+	+*	213	*M. haemolytica*	-	-	-	240	*M. haemolytica*	-	-	+
	P. multocida	+	-		*P. multocida*	+	+	+		*P. multocida*	-	-	-
	H. somni	-	-		*H. somni*	+*	-	-		*H. somni*	-	-	-
229	*M. haemolytica*	+	-	225	*M. haemolytica*	-	-	-	241	*M. haemolytica*	+	-	-
	P. multocida	-	-		*P. multocida*	+	-	-		*P. multocida*	-	+*	-
	H. somni	-	-		*H. somni*	-	-	-		*H. somni*	+	-	-
262	*M. haemolytica*	-	-	236	*M. haemolytica*	-	-	+	245	*M. haemolytica*	-	-	+
	P. multocida	+*	+		*P. multocida*	+	-	-		*P. multocida*	-	-	-
					H. somni	-	-	-					
				243	*M. haemolytica*	+	+	+					
					P. multocida	+	+	-					

Table A.2 – Bronchoalveolar lavage culture results from calves with clinical BRD (n = 28) that were sampled either prior to (Day 0), 0.5, or 1 day post-treatment and 5 days post-treatment with gamithromycin according to sampling group. * = a mixture of gamithromycin susceptibility phenotypes was obtained from up to 12 *M. haemolytica*, 6. *P multocida*, and 6. *H somni* isolates tested from each positive sample.

Group 1				Group 2				Group 3			
Calf	Organism	Day 0	Day 5	Calf	Organism	Day 0.5	Day 5	Calf	Organism	Day 1	Day 5
	H. somni	+	-		*H. somni*	-	-		*H. somni*	-	-
104	*M. haemolytica*	-	+	116	*M. haemolytica*	-	-	102	*M. haemolytica*	-	-
	P. multocida	-	-		*P. multocida*	+*	-		*P. multocida*	-	+
	H. somni	+	-		*H. somni*	-	+		*H. somni*	-	-
106	*M. haemolytica*	-	-	127	*M. haemolytica*	-	-	118	*M. haemolytica*	-	-
	P. multocida	-	-		*P. multocida*	-	-		*P. multocida*	-	-
	H. somni	-	-		*H. somni*	-	-		*H. somni*	-	-
130	*M. haemolytica*	-	-	175	*M. haemolytica*	+	-	126	*M. haemolytica*	-	-
	P. multocida	+	-		*P. multocida*	+	-		*P. multocida*	-	-
	H. somni	-	-		*H. somni*	-	-		*H. somni*	+	+
150	*M. haemolytica*	-	-	195	*M. haemolytica*	-	-	212	*M. haemolytica*	-	-
	P. multocida	-	-		*P. multocida*	+	+		*P. multocida*	+	+
	H. somni	-	-		*H. somni*	-	-		*H. somni*	-	-
174	*M. haemolytica*	-	-	204	*M. haemolytica*	-	-	218	*M. haemolytica*	-	-
	P. multocida	-	-		*P. multocida*	+	+		*P. multocida*	-	-
	H. somni	-	+		*H. somni*	-	-		*H. somni*	-	-
209	*M. haemolytica*	-	-	206	*M. haemolytica*	+	-	224	*M. haemolytica*	-	-
	P. multocida	+	-		*P. multocida*	+	-		*P. multocida*	-	-
	H. somni	-	-		*H. somni*	-	-		*H. somni*	-	-
222	*M. haemolytica*	+	+	213	*M. haemolytica*	-	-	240	*M. haemolytica*	-	+
	P. multocida	+	-		*P. multocida*	+	+*		*P. multocida*	-	-
	H. somni	-	-		*H. somni*	-	-		*H. somni*	-	-
229	*M. haemolytica*	+	-	225	*M. haemolytica*	-	-	241	*M. haemolytica*	-	-
	P. multocida	-	-		*P. multocida*	-	-		*P. multocida*	-	-
	H. somni	-	-		*H. somni*	-	-		*H. somni*	-	-
262	*M. haemolytica*	-	-	236	*M. haemolytica*	-	+	245	*M. haemolytica*	-	+
	P. multocida	+	+		*P. multocida*	-	-		*P. multocida*	-	-
					H. somni	-	-				
				243	*M. haemolytica*	-	+*				
					P. multocida	-	-				

Table A.3 – Genetic subtype of *Mannheimia haemolytica* isolates obtained via paired BAL and NPS samples from calves when diagnosed with clinical bovine respiratory disease (day 0) and 0.5, 1, and 5 days post-treatment with gamithromycin. Day 1 paired samples are not shown since they all were negative for *M. haemolytica*. Cells in black indicate a paired sample was not obtained at that time point. Samples that were negative for *M. haemolytica* are indicted by a negative sign.

	Day 0		Day 0.5		Day 5	
Calf	BAL	NPS	BAL	NPS	BAL	NPS
102					-	-
104	-	1f			1f	1f
106	-	-			-	-
116			-	-	-	1f
118					-	-
126					-	-
127			-	-	-	-
130	-	-			-	-
150	-	1c, 2b			-	-
174	-	1e			-	-
175			1i	1i	-	1i
195			-	-	-	-
204			-	-	-	-
206			2c	2c	-	2c
209	-	-			-	-
212					-	-
213			-	-	-	-
218					-	-
222	2b	2b			2b	2b
224					-	-
225			-	-	-	-
229	2b	2b			-	-
236			-	-	2b	2b
240					2b	2b
241					-	-
243			-	2d	2d	2d
245					2b	2b
262	-	-			-	-