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Effect of *Mannheimia haemolytica* pneumonia on behavior and physiologic responses of calves during high ambient environmental temperatures[1]

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ABSTRACT: The objective of this study was to determine the effect of pneumonia during conditions of high (maximum ≥ 32°C) ambient temperatures on physiological and behavioral responses of calves. Eighteen black beef heifers averaging 240 kg were blocked by BW and randomly assigned to 1 of 2 treatment groups: 1) pneumonia induced by bronchoselective endoscopic inoculation with *Mannheimia haemolytica* (MH; $n = 10$) and 2) noninoculated controls (CN; $n = 8$). Nasal passage and rectal temperatures were measured every 2 h for 24 h after challenge and then twice daily for 9 d. Accelerometers, pedometers, and positioning devices monitored cattle behavior within the pen for 9 d after challenge. Blood samples were collected on trial d 0, 0.5, 1, 2, 3, 7, and 9 and were analyzed to determine the concentration of substance P, cortisol, haptoglobin, and metalloproteinase. All calves in the MH group were euthanized and necropsied on trial d 9. All MH calves became clinically ill postchallenge. A treatment × time interaction ($P < 0.05$) was evident for nasal and rectal temperatures, behavior, weight, and blood analysis. Rectal temperatures in MH were higher ($P < 0.01$) than CN during the period from 6 to 24 h after challenge. Conversely, nasal passage temperatures were less in MH calves compared with CN at 12 to 22 h after challenge. Calves in MH spent less time at the grain bunk, less time at the hay feeder, and more time lying down during the early pneumonia period compared with CN calves. Also, MH calves had significantly greater concentrations of blood biomarkers of pain (substance P) on d 0.5 ($P < 0.01$); stress (cortisol) on d 0.5 and 1 ($P < 0.01$); haptoglobin on d 0.5, 1, 2, 3, 7 ($P < 0.01$); and metalloproteinase on d 1, 2, and 3 ($P < 0.01$) compared with CN calves. At necropsy, all MH calves had right cranioventral bronchopneumonia (median lung lesions = 6.8%). *Mannheimia haemolytica* pneumonia caused significantly more changes in behavior and increased biomarkers during high (maximum ≥32°C) ambient temperatures compared with control calves. The results of this study may guide research in the development of objective assessment tools for management of cattle affected with bovine respiratory disease during extreme summer conditions.

Key words: behavior, bovine respiratory disease, endocrinology, heat stress, *Mannheimia haemolytica*, physiology

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INTRODUCTION

Bovine respiratory disease (**BRD**) complex is a common issue affecting feedlot cattle economic and performance outcomes (Galyean et al., 1999; Lechtenberg et al., 2011). *Mannheimia haemolytica* is the most common bacterial pathogen associated with BRD (Purdy et al., 1997). Improvement of the clinical case definition of BRD may lead to a more accurate disease diagnosis, resulting in improved management strategies and

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treatment (Apley, 2006; Hanzlicek et al., 2010a). Current methods for identification of calves with BRD are based on visual observations, but these methods have low sensitivity (61.8%) and specificity (62.8%; White and Renter, 2009). Behavior monitoring has been suggested for monitoring health of cattle (Weary et al., 2009). Remote monitoring tools have been used to detect changes in the health status of calves (Sowell et al., 1999; Buhman et al., 2000).

Heat stress has been associated with increased body temperature, respiratory rate, and decreased activity (Fuquay, 1981). High ambient temperatures result in decreased DMI (Hahn, 1999; Mader, 1999). Heat stress has an adverse effect on the animal wellness status (Silanikove, 2000). Cattle affected with BRD are expected to suffer more severely during periods of heat stress.

The behavioral and physiological responses of calves suffering from *M. haemolytica* pneumonia during periods of heat stress have not been described. The objective of this study was to determine the effect of *M. haemolytica* pneumonia on biothermal regulation, behavior, and physiological responses during extreme ambient temperatures. Our hypothesis was that calves challenged with *M. haemolytica* would have higher body temperatures, lethargic behavior, and increased cortisol, substance P, haptoglobin, and metalloproteinase compared with control calves. Conclusions of this study will be useful in improving diagnostic capabilities of identifying morbid animals in situations with high ambient temperatures.

MATERIALS AND METHODS

All study procedures were conducted with a protocol approved by the Institutional Animal Care and Use Committee (IACUC number 3039). Cattle were humanely handled throughout the research project and observed twice daily during the trial to monitor their health status. A protocol was in place stating that if a calf became severely ill [clinical illness score (**CIS**) = 4; see CIS definitions below] at any point during the study, the calf would be immediately humanely euthanized to alleviate unnecessary suffering.

Calf Selection and Trial Design

Eighteen black beef heifers averaging 240 kg (±13.1 kg) owned by Kansas State University were selected for this study. The heifers were observed twice daily for 30 d before study d 0 for clinical signs of illness. Calves were comingled and group housed throughout the trial in a 12.2 × 24.4 m pen and fed a receiving ration that included 2.3 kg of corn/d, 0.9 kg alfalfa/d, and ad libitum access to brome hay. A remote weather station (WS-2812, La Crosse Technology, La Crosse, WI) was placed at the research station to continuously monitor the environmental conditions where the calves were housed throughout the trial. Criteria for study initiation were forecasted high ambient temperature conditions including maximum daily temperature ≥32°C, average daily humidity ≥40%, and sun exposure.

Calves were blocked by BW and group participation from previous transportation stress trial (Theurer et al., 2013), then randomly allocated to a *Mannheimia haemolytica* (**MH**; $n = 10$) treatment group or control group (**CN**; $n = 8$). Endoscopic inoculations were conducted using bronchoselective endoscopy. This procedure allows selective placement of the endoscope and media into the right apical lung lobes via the tracheal bronchus. Calves in the CN were endoscopically challenged with 70 mL of phosphate buffer solution in the tracheal bronchus using a fiber-optic endoscope (length, 110 cm; diam., 6.6 mm; biopsy channel, 2.0 mm; VetVu Flexible Endoscope, Swiss Precision Products, Spencer, MA) as previously described (Hanzlicek et al., 2010a). Calves in the MH treatment group were challenged with 10 mL of *M. haemolytica* serotype A1 at a dosage rate of 1×10^9 cfu/mL and then flushed with 60 mL of phosphate buffered solution to give a total dosage volume of 70 mL deposited in the tracheal bronchus with a fiber-optic endoscope. *Mannheimia haemolytica* was prepared for inoculation as previously described (Mosier et al., 1995; Corrigan et al., 2007; Hanzlicek et al., 2010a). Challenge began at 0800 h on trial d 0. Calves challenged with *M. haemolytica* were necropsied 9 d after inoculation.

Temperature Monitoring

Radiofrequency biothermal sensors (Biothermal RFID Chip, Destron Technologies, Round Rock, TX) were implanted submucosally in the nasal passages of the left and right nares approximately 2 mm deep and 100 mm caudal to the alar cartilage. The radio-frequency transponders were initiated by an electronic reading device (Pocket Reader, Destron Technologies) that recorded temperature within ±0.1°C. A rapid equilibration thermometer (Pavia Rectal Temp, Pavia Sales Group Inc., Plymouth, MN) was used to measure rectal temperatures. A high-definition thermal sensor camera (ThermaCAM S65, FLIR Systems, Wilsonville, OR) was used to record the surface temperature of the right and left nares, nasal planum, and cornea at trial h 0, 4, 8, 12, 24, and 48. The temperatures of the left and right nares were then averaged to yield a single reading per calf per time point for temporal analysis.

Behavior Monitoring

Triangulation positioning transponder tags (Ubisense Series 7000 Compact Tag; Ubisense, Denver, CO) were

placed in the left ear of all calves to monitor behavior activity. These tags are a component of a remote triangulation monitoring system that evaluates calf position within the pen based on relative position compared with the sensors (Steggles and Gschwind, 2005). The compact design of the tags allowed attachment of each tag to a conventional ear tag button and placed with the sensor on the ear. The X and Y coordinates of the tag location were recorded and compared with the known X and Y locations of the previously mapped locations of the grain bunk, hay feeder, water, and shed using a data mining software program (Insightful Miner; Insightful Corporation Seattle, WA). A time stamp was documented by the computer every time the tag location was transmitted (average signal frequency of 1 s). Using criteria previously established, the amount of time at each location was calculated by subtracting the time stamp from the previous reading and then classifying the calf as being at the previous reading location (Theurer et al., 2012; White et al., 2012).

Accelerometers (GP1 SENSR; Reference LLC, Elkader, IA) and pedometers (NL-800; New-Lifestyles Inc., Lees Summit, MO) were placed within a protective neoprene sleeve that was attached to the lateral aspect of the metatarsus immediately proximal to the fetlock (Robert et al., 2009). The accelerometers use triaxial measurements, have an axis range of ±10 g, and record 100 samples per second (Reference LLC, 2007). The accelerometers were initialized with previously validated settings to measure cattle behavior (Robert et al., 2009), including 5-s recording intervals and recording X, Y, and Z acceleration, vector magnitude average, and vector magnitude maximum data. Vector magnitude and average force of gravity values were calculated by summing the *g* values and calf acceleration movement recordings and then dividing by 5-s intervals. The vector magnitude maximum is the greatest combined acceleration during the 5-s span.

Accelerometers were removed, downloaded to a computer, and reattached to the calf on trial d 6. Postural data were then processed using a data mining software program (Insightful Miner; Insightful Corporation Seattle, WA). The variables were used to classify the amount of time each calf spent standing, lying, or walking per 5-s epochs. The data from the accelerometers were then aggregated by hour.

Pedometers contained an accelerometer inside of them that monitored the number of steps each calf took on the basis of the up and down movement of the calf leg. The pedometer data were recorded at trial d 6 when the accelerometers were taken off and downloaded. All behavioral activity was continuously monitored for a period of 1 d before and for 9 d after challenge. Trial day consisted of a 24-h period beginning from the time the respiratory challenge initiated (0800 h).

Body Weight

All calves were individually weighed on trial d 0, 2, 3, 5, 7, and 9 to calculate percent change in BW. For statistical comparisons, changes in BW were analyzed using percent change in BW calculated by comparing current BW to the individual animal BW at the beginning of the trial before challenge.

Initial 24-h Monitoring Period

All calves were monitored before challenge (trial d 0, trial h 0) and then every 2 h for 24 h postchallenge. Rectal and nasal temperatures were recorded every 2 h for 24 h after endoscopic challenge (trial h 0).

Daily Monitoring Period

After the 24 h postchallenge period, calves were assessed twice daily at 0800 and 1600 h during the next 9 d. Rectal temperature, nasal temperature, heart rate, respiration rate, and clinical illness scores were collected from all calves twice each day. Clinical illness scores were assigned by the same individual trained in detection of clinical illness using an established classification method (White et al., 2012). This CIS system ranged from 1 to 4 with the following criteria used for each level: 1 = normal behavior, 2 = slight illness, mild depression, and/or a cough, 3 = moderate illness, severe depression, labored breathing, and/or cough, and 4 = severe illness, where animals may be moribund or have little response to human approach. All calves in the MH were humanely euthanized on trial d 9 by captive bolt (Koch Magnum 0.25 Stunner, KOCH Supplies Inc., Kansas City, MO) in accordance with the American Veterinary Medical Association guidelines (American Veterinary Medical Association, 2007).

Lung Lesion Scores

Lungs were harvested immediately after euthanasia and evaluated by a board-certified pathologist experienced in BRD assessment. This investigator assigned values to the extent of lung that was consolidated in each lung lobe. Lung scores were calculated by determining the total amount of lung that was infected and dividing it by the total lung volume using a previously described scoring system (Fajt et al., 2003). The total percent lung involvement was calculated with the following formula: (0.06 × right caudal apical/100) + (0.063 × right cranial apical/100) + (0.053 × left cranial apical/100) + (0.049 × left caudal apical/100) + (0.319 × left diaphragmatic/100) + (0.043 × intermediate/100) + (0.352 × right diaphragmatic/100) + (0.061 × accessory/100) = total lung score.

Blood Samples and Immunoassays

Blood samples (60 mL each) were collected from a jugular vein of all calves before challenge (trial d 0), at 12 h after challenge, and on trial d 1, 2, 3, 7, and 9. Samples were transferred to 6-mL serum clot activator and potassium EDTA tubes and centrifuged for 10 min at 1500 × *g* at room tempurature. Serum and plasma were harvested, placed in 2-mL cryovials, and frozen at –80°C until analyzed. Appropriate samples were assayed for cortisol, substance P, haptoglobin, and haptoglobin-metalloproteinase-9 complex.

A solid-phase competitive chemiluminescent enzyme immunoassay and automated analyzer system (Immulite, Siemens Medical Solutions, Los Angeles, CA) were used to analyze serum cortisol concentrations as previously described (Coetzee et al., 2007). All samples that had a serum cortisol concentration less than 5.5 nmol/L (the sensitivity of the machine was 5.5 nmol/L) were transformed to 5.5 nmol/L before statistical analysis.

Plasma substance P concentrations were analyzed by use of a commercial immunoassay kit (SP Correlate-EIA, ELISA kits, Assay Designs Inc., Ann Arbor, MI) that has been validated for use in bovine plasma (Coetzee et al., 2008). Samples were extracted by use of C-18 cartridges (Sep-Pak Vac 3cc C_{18} SPE, Waters Corp, Milford, MA). The immunoassay used a polyclonal antibody against substance P in the test sample. The substance P concentration in the sample was inversely proportional to the intensity of the color generated after incubation as determined at 405 nm on a microplate reader. All samples that had a substance P concentration outside of the standard curve were removed before analysis.

Total haptoglobin (**Hp**) concentrations in serum were determined using a commercially available bovine haptoglobin ELISA kit (Life Diagnostics, West Chester, PA). Briefly, all serum samples from calves were diluted 1:2,000 in sample buffer before aliquoting 100 μL to each well for Hp analysis. The serum was aliquoted into individual wells of the Hp ELISA plate precoated with anti-bovine Hp antibody. The plates were covered with plastic film and allowed to incubate at room temperature on an automated plate shaker. After incubation, the plates were washed with prediluted Hp ELISA wash buffer (3 times, 5 min each), and the prediluted horseradish peroxidase conjugated anti-bovine Hp antibody was added and incubated under plastic film for 30 min on the plate shaker at room temperature. The plates were washed again (as above) and 100 μL of tetramethylbenzidine (**TMB**) substrate were added to each well. Color development was monitored on an automated plate reader at 630 nm until the greatest standard concentration (250 ng/mL) achieved an absorbance of >0.500 AU. After development, the enzyme reaction was stopped by the addition of 100 μL of stop solution (1 *N* hydrochloric acid), and the well absorbance was determined at 450 nm. Serum concentrations were determined by first defining the concentration vs. absorbance relationship based on the standards (7.8, 15.6, 31.25, 62.5, 125, 250 ng/mL) and using the slope and intercept of the line to calculate serum concentrations based on their individual absorbance values. All serum sample concentrations were corrected for dilution (2,000-fold dilution).

The ELISA for bovine haptoglobin-matrix metalloproteinase-9 complex (**Hp-MMP 9**) was performed as described previously with a few changes (Bannikov et al., 2011). The ELISA is designed to exploit the binding of bovine MMP 9 to the well coated with anti-bovine MMP 9 monoclonal antibody. The serum samples were each diluted 1:5 with sample diluent (Tris-buffered saline (**TBS**) + 1% BSA + 0.05% Tween 20). Nonconjugated bovine MMP 9 MAb 10.1 as a capture antibody (100 μL, containing 2 μg per well in TBS + 0.1% BSA; Bovine Serum Albumin, Fraction V, Thermo Fisher Scientific, Pittsburgh, PA) was allowed to bind to the wells of a 96-well ELISA plate and incubated overnight at 4°C. Plates were blocked with TBS containing 2% BSA at 4°C for 120 min. After blocking the wells, calf serum samples and serum containing known concentrations of Hp-MMP 9 at a standard dilution were added (120 min at 21°C). Samples were washed 3 times for 5 min each with 300 μL TBS + 0.05% Tween 20, and rabbit anti-bovine Hp-horseradish peroxidase conjugate (100 μL, containing 0.1 μg per well in TBS + 0.1% BSA) was added. Wells were washed as before, and 100 μL of TMB were added to each well for detection of bound Hp-MMP 9. The wells were incubated for 20 min, and the reaction was stopped by addition of 100 μL of 1 *N* hydrochloric acid to stop the color reaction. The concentrations of Hp-MMP 9 were determined using the linear slope of the graphed line generated from an equation of the absorbance of the calibrators at 450 nm and the known concentration of these calibrators.

Statistical Analysis

Data were analyzed using statistical software packages (JMP; SAS Inst. Inc., Cary, NC). Descriptive statistics were used to evaluate the percentage of calves receiving a CIS of 2 by treatment group and trial day. Generalized mixed models were used to evaluate the potential relationships between continuous variables (rectal temperature, nasal passage temperature, heart rate, respiratory rate, number of steps, percentage change in BW, cortisol, substance P, haptoglobin, and Hp-MMP 9 complex concentrations), treatment group (MH or CN), trial hour and day, and the potential interaction between these variables. All analyses included random effects on each calf to account for repeated measures. Descriptive analysis

Table 1. Environmental conditions to which beef heifers were exposed by trial day

Day	Avg temperature, °C	Maximum temperature, °C	Minimum temperature, °C	Avg humidity, %	Maximum humidity, %	Minimum humidity, %	Maximum heat index, °C
0	28.9	34.8	21.0	67.4	92	46	34.9
1	32.0	39.7	22.9	60.9	92	37	40.5
2	35.0	45.1	29.0	42.8	61	22	46.6
3	30.7	36.0	25.3	59.0	77	41	36.2
4	26.0	31.7	22.6	78.6	89	59	31.6
5	28.6	36.0	22.4	75.8	94	49	36.3
6	31.9	39.7	24.6	68.0	92	39	40.2
7	35.1	45.1	23.9	50.2	85	22	45.7
8	34.4	43.9	27.0	45.9	76	20	45.8
9	30.0	33.8	26.1	71.1	85	41	36.9

was performed on rectal temperatures to determine the frequency of rectal temperatures above 39.5°C during the initial 24-h monitoring period and the frequency of nasal temperatures above 39°C by treatment group during the entire trial (Mills et al., 1971; Radostits, 2001).

Analysis of behavioral data used model effects, including treatment (MH and CN), trial day (d -1 through 9), and the potential interaction between treatment group and trial day. Statistical models were constructed in a stepwise procedure by including all potential effects and removing nonsignificant ($P > 0.05$) effects. A first-order autoregressive correlation structure was defined to account for the repeated measures on calves over time in all behavioral analyses (Agresti, 1996). Type 3 likelihood ratio statistics were used to test for associations of effects, and comparisons with a P-value < 0.05 were considered statistically significant. Potential differences between treatment groups within individual trial days were evaluated using t tests. To account for multiple comparisons, a P value < 0.01 was considered statistically significant for all comparisons between CN and MH calves within hour or day for all data.

RESULTS

Environmental conditions met the desired thresholds throughout the study period except for d 4, on which maximum ambient temperature was 31.8°C (Table 1). At trial initiation, the mean (±SD) BW of calves in the CN were 241.3 ± 13.6 kg, and in the MH they were 242.1 ± 12.4 kg. All MH calves had a CIS of 2 at 24 h after challenge; however, only 2 calves had a CIS of 2 at 12 h after challenge. The percentage of MH calves with a CIS of 2 varied between 10% and 70% on subsequent evaluation periods (Fig. 1). No calf was classified greater than CIS 2 during the trial. One CN calf was classified as CIS 2 on trial d 5, and a different CN calf was classified as CIS 2 on trial d 6. Otherwise, CN calves were clinically normal at all other time points.

Analysis of the average nasal passage temperature and average rectal temperature determined a significant interaction ($P < 0.05$) between trial hour and treatment group during the intensive monitoring period, with MH being greater than CN beginning 6 h postchallenge (Fig. 2). Calves in the MH had greater average rectal temperatures at trial h 6 through 24 and lower average nasal temperatures at trial h 14 and 18 compared with CN calves. Five of the calves in the CN exceeded the 39.5°C rectal temperature threshold, and all 10 calves in the MH group exceeded the 39.5°C cutoff value during the initial 24-h monitoring period. Rectal temperatures had a significant ($P < 0.05$) interaction between treatment group and trial day, with calves in the MH group having a greater average rectal temperature on trial d 0 and 1 compared with CN calves (Fig. 3). Average nasal passage temperature did not have any significant ($P > 0.10$) interaction by trial day and treatment group, but the main effect of trial day was significant ($P < 0.05$;

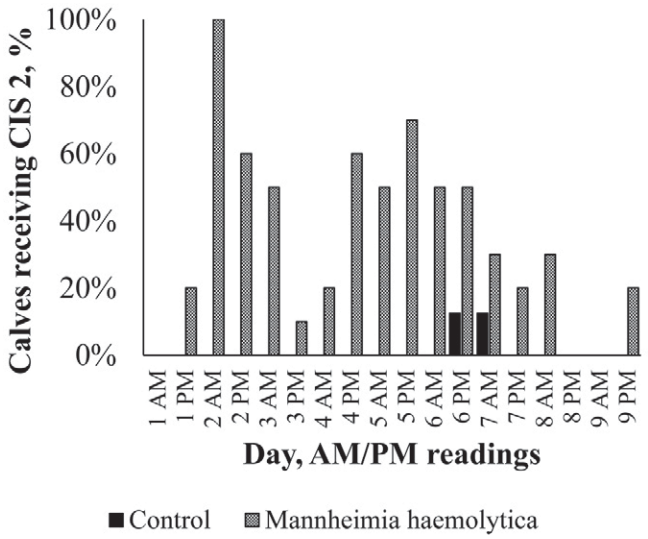

Figure 1. Percentage of calves by treatment group of control calves ($n = 8$) and calves challenged with *Mannheimia haemolytica* ($n = 10$) receiving a clinical illness score of 2 (mild illness) by trial day after endoscopic inoculation on d 0. Calves were observed twice daily (morning and evening) throughout the trial

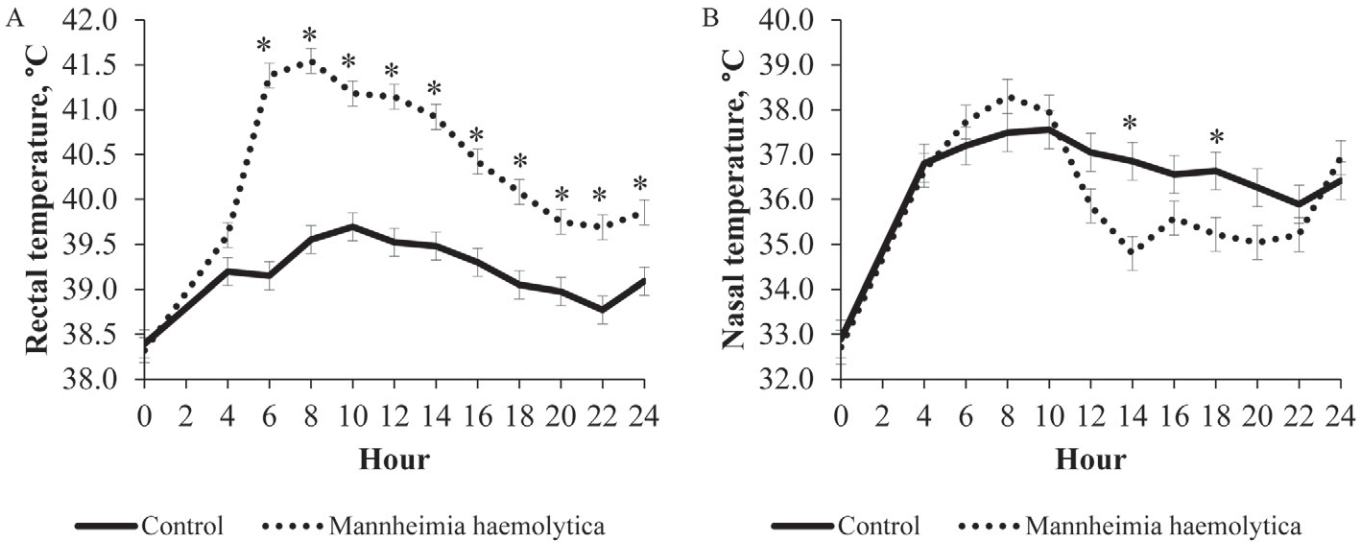

Figure 2. Model-adjusted least squares mean (±SE) (A) rectal temperature and (B) nasal temperature in beef heifers by trial hour and treatment group of control calves and calves challenged with *Mannheimia haemolytica* during initial 24-h monitoring period. The model included effects for trial hour and repeated measures on individual calves. *Significant differences ($P < 0.01$) between treatment group within trial hour. The interaction between trial hour and treatment group was significant ($P < 0.05$)

Fig. 3). Nine (90%) of the calves in the MH group and 5 (62.5%) calves in the CN group exceeded 39°C nasal temperature during the trial. An interaction ($P < 0.05$) was found between treatment group and trial hour for average nasal planum surface temperature as measured by thermography with MH greater than CN throughout the monitoring period. Calves in the MH group had higher average nasal planum temperature compared with the CN calves at trial h 8 and 48 (Fig. 4). A treatment group effect ($P < 0.05$) was found for average nares surface temperature, with MH calves having a higher average surface temperature (32.0 ± 0.4°C) compared with CN calves (31.2 ± 0.4°C). No significant relationships were identified between treatment group and the physiological variables of heart rate and average respiratory rate.

There was a significant ($P < 0.05$) interaction between treatment group (CN or MH) and trial day for the amount of time calves spent within 0.3 m of the grain bunk, hay feeder, water, and shed. Calves in the MH group spent less time near the grain bunk on trial d 1; less time at the hay feeder on trial d 0, 1, and 2; more time at the water on trial d 4 and less time near the water on trial d 5; and less time near the shed on trial d 4 compared with CN calves (Fig. 5). After challenge (study d 0), MH calves spent less time near the grain bunk and within the shed compared with CN calves. There was

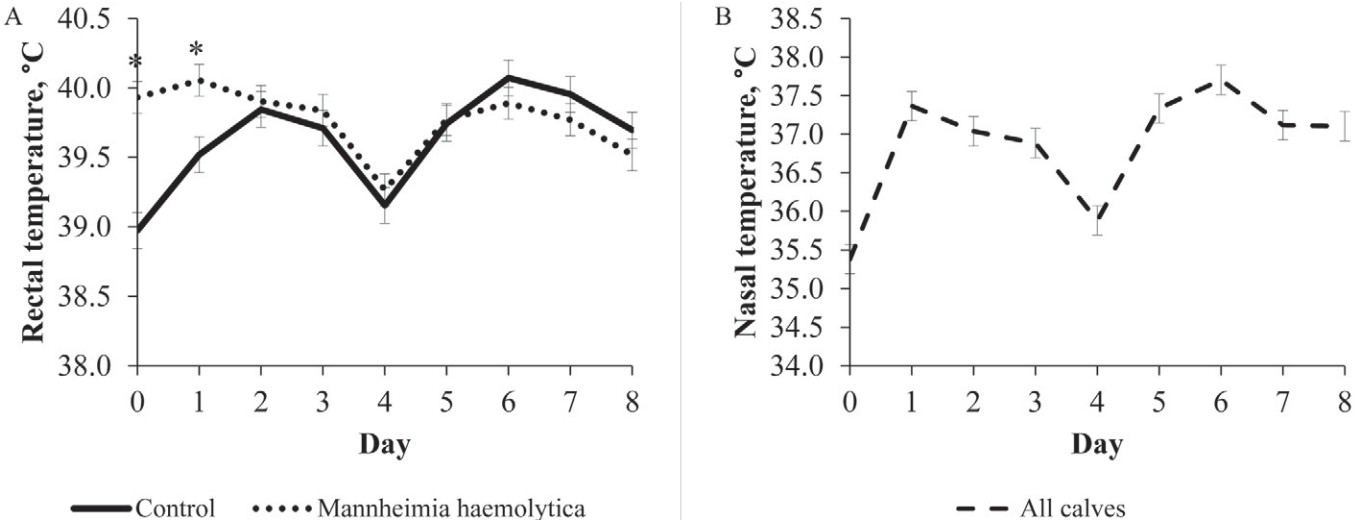

Figure 3. Model-adjusted least squares mean (±SE) (A) rectal temperature and (B) nasal temperature in beef heifers by trial day and treatment group of control calves and calves challenged with *Mannheimia haemolytica* during daily monitoring period. The model included effects for trial day and repeated measures on individual calves. *Significant differences ($P < 0.01$) between treatment group within trial day. Rectal temperature had a significant ($P < 0.05$) interaction between trial day and treatment group. The interaction between trial day and treatment group was not significant ($P > 0.10$) in nasal temperature

an interaction ($P < 0.05$) between treatment group and trial day for posture behavior and the number of steps taken daily. Calves in the MH group spent more time lying down on d 0 through 8 compared with CN calves (Fig. 6). Pedometer analysis determined a significant ($P < 0.05$) interaction between treatment group and trial day (Fig. 7). Evaluation of the average percent change in BW determined a significant interaction ($P < 0.05$) between treatment group and trial day. Calves challenged with *M. haemolytica* had significantly more BW loss postchallenge when weighed on trial d 2, 3, 5, 7, and 9 compared with CN calves (Fig. 8).

Average cortisol, substance P, haptoglobin, and Hp-MMP 9 concentrations revealed a significant ($P < 0.05$) interaction between treatment group and trial day (Fig. 9). Calves challenged with *M. haemolytica* had greater average serum cortisol concentrations at trial d 0.5 and 1. Substance P was significantly increased in MH calves on trial d 0.5 but had decreased average concentrations at d 7 compared with the CN group. Calves in the MH group had greater average haptoglobin concentrations on trial d 0.5, 1, 2, 3, and 7 compared with CN calves. Average

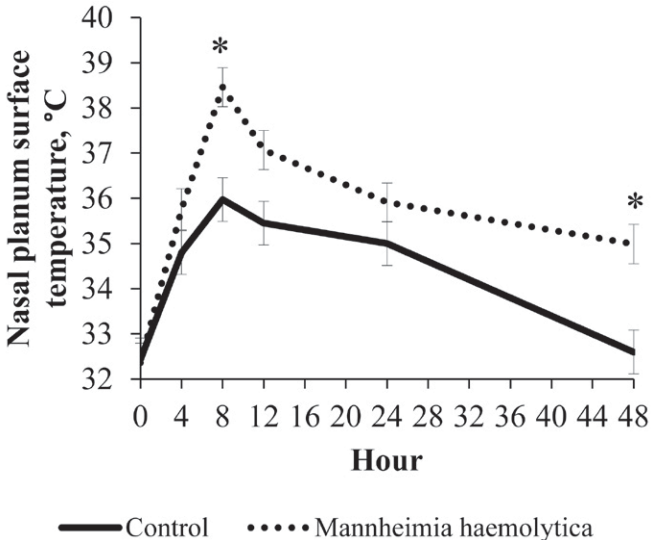

Figure 4. Model-adjusted least squares mean (±SE) nasal planum surface temperature in beef heifers by trial hour and treatment group of control calves and calves challenged with *Mannheimia haemolytica*. The model included effects for trial hour and repeated measures on individual calves. *Significant differences ($P < 0.01$) between treatment group within trial hour. The interaction between trial hour and treatment group was significant ($P < 0.05$)

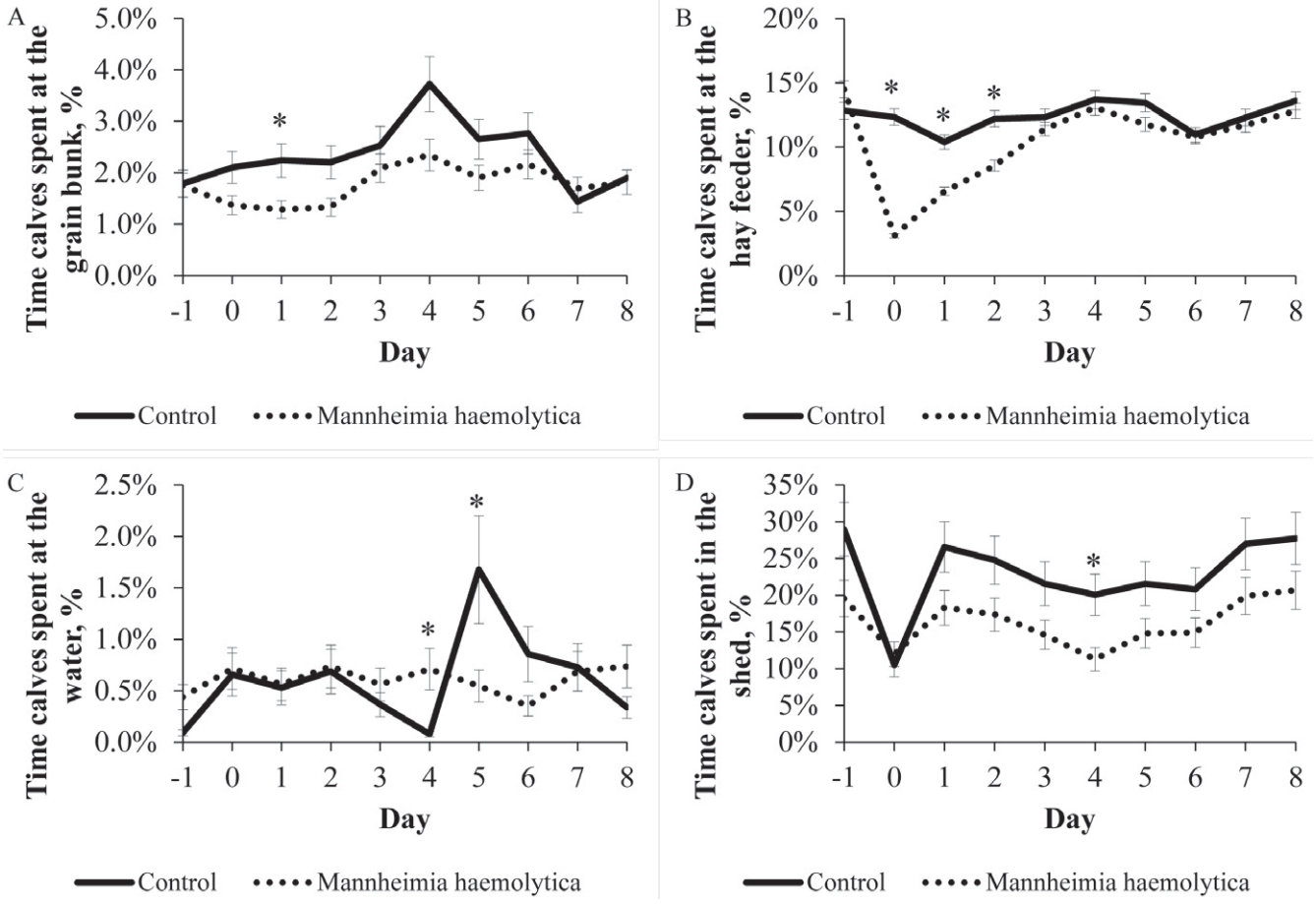

Figure 5. Model-adjusted least squares mean (±SE) percent of time calves spent within 0.3 m of the (A) grain bunk, (B) hay feeder, (C) water, and (D) shed in beef heifers by trial day and treatment group of control calves and calves challenged with *Mannheimia haemolytica*. The model included effects for trial day and repeated measures on individual calves. *Significant differences ($P < 0.01$) between treatment group within trial day. The interaction between trial day and treatment group was significant ($P < 0.05$)

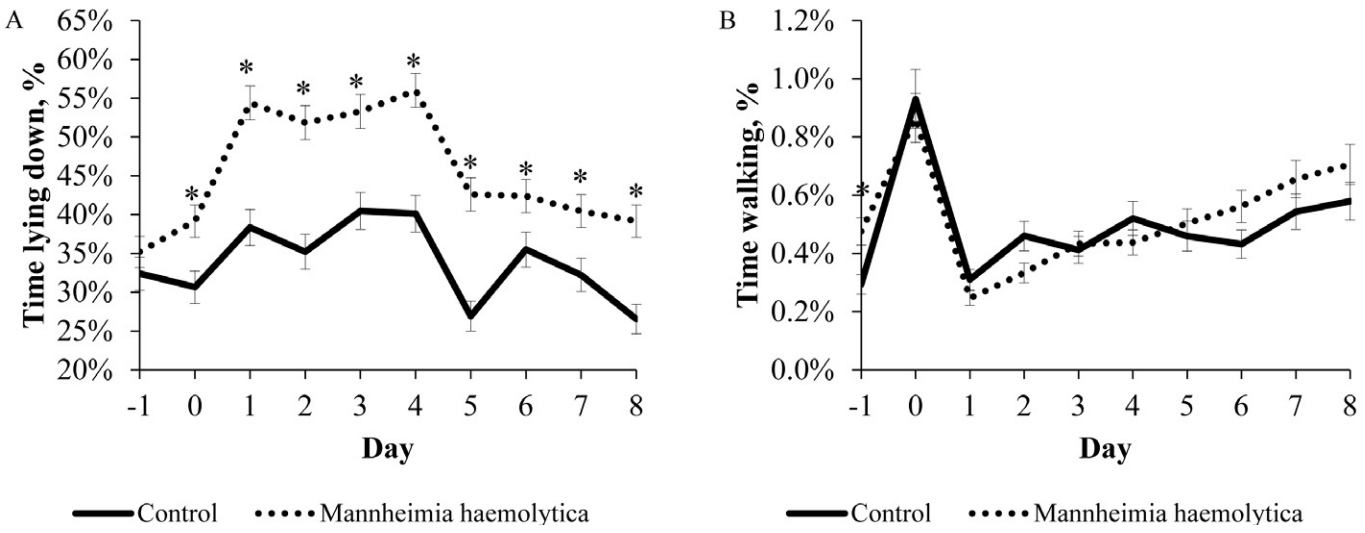

Figure 6. Model-adjusted least squares mean (±SE) percent of time calves spent (A) lying down and (B) walking in beef heifers by trial day and treatment group of control calves and calves challenged with *Mannheimia haemolytica*. The model included effects for trial day and repeated measures on individual calves. *Significant differences ($P < 0.01$) between treatment group within trial day. The interaction between trial day and treatment group was significant ($P < 0.05$)

HP-MMP 9 concentration was greater in MH calves on trial d 2 and 3 compared with CN calves.

At necropsy, all lung sets from calves challenged with *M. haemolytica* contained cranioventral gross lesions consistent with *M. haemolytica* bronchopneumonia. Pathological lesions were present in the right apical and intermediate lung lubes and consisted of dense fibrinous to immature fibrous adhesions, enlarged interlobular septa, parenchymal necrosis, and atelectasis. Pulmonary lung scores ranged from 5.04% to 9.71% (median lung score of 6.8%; Table 2). Bacterial cultures of lung at necropsy were positive for *M. haemolytica* in 9 out of 10 calves.

DISCUSSION

The results of this study augment the clinical case definition, physiological, and behavioral changes that occur in calves exposed to *M. haemolytica* during conditions of high ambient temperature. The challenge model to induce *M. haemolytica* disease has been previously described and successfully established BRD as confirmed by postchallenge CIS and necropsy examination (Hanzlicek et al., 2010a). The challenge model used here was successful in establishing BRD in calves based on the lung lesions at necropsy. This allowed us to focus on extreme environmental conditions as the primary stressor. In our model, bronchoselective inoculation of the tracheal bronchus is effective at confining the developing pneumonia to the right apical lung lobes. Similar to field cases of BRD, all calves had cranioventral bronchopneumonia.

Increased rectal temperature in MH calves 6 h after challenge was expected and similar to published literature (Corrigan et al., 2007; Confer et al., 2009; Burciaga-Robles et al., 2010b). Calves challenged with *M. haemolytica* had rectal temperatures greater than 42°C during the first 24 h after challenge. However, after d 1 MH rectal temperatures were not different from CN. Others have shown that rectal temperatures return to normal by 1 to 3 d postinfection (Ames et al., 1985; Vestweber et al., 1990; Hewson et al., 2011). These transient responses may be a result of endotoxin or other pyrogenic features of *M. haemolytica*. The CN calves also had rectal temperatures that exceeded the upper limit of the normal rectal temperature reference range (39.5°C; Radostits, 2001). This provided evidence of heat stress within these cattle and the need for reference range correction when using rectal temperature as a diagnostic tool for BRD during summer conditions.

Nasal mucosal temperature was measured to determine how ambient and body temperature effect nasal mucosal temperature. One possible explanation for interaction in nasal passage temperature between treatment group and trial hour may be contributed to endotoxin release and subsequent peripheral vasoconstriction in nasal mucosae. Also, we were able to show that nasal mucosal temperature exceeds the critical thermal limit (39°C) for temperature sensitive vaccines in MH calves (Mills et al., 1971; Pastoret et al., 1980). Contrary to studies evaluating nares and nasal planum temperature that reported that nares temperatures are considerably and consistently below this limit (Mills et al., 1971), we were able to document nasal passage mucosal temperature exceeded the critical thermal limit in 5 out of 10 calves suffering BRD during high ambient temperatures. This may need to be considered when administering intranasal modified live vaccines as these temperatures may inactivate the MLV vaccine and result in vaccine failures.

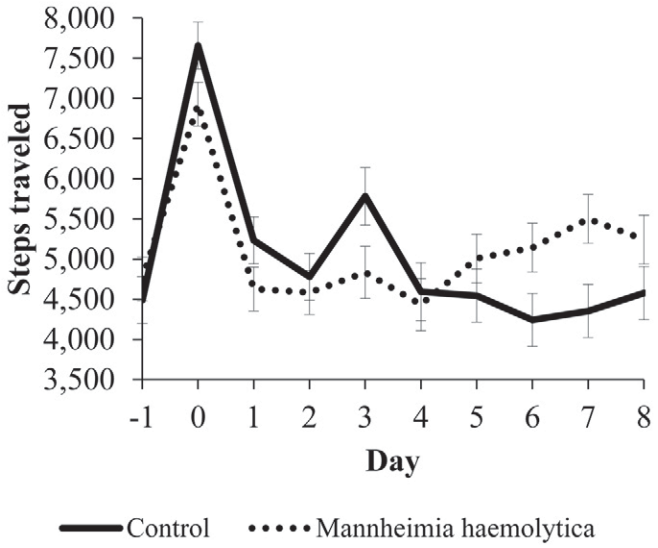

Figure 7. Model-adjusted least squares mean (±SE) number of steps traveled in beef heifers by trial day and treatment group of control calves and calves challenged with *Mannheimia haemolytica*. The model included effects for trial day and repeated measures on individual calves. *Significant differences ($P < 0.01$) between treatment group within trial day are denoted. The interaction between trial day and treatment group was significant ($P < 0.05$)

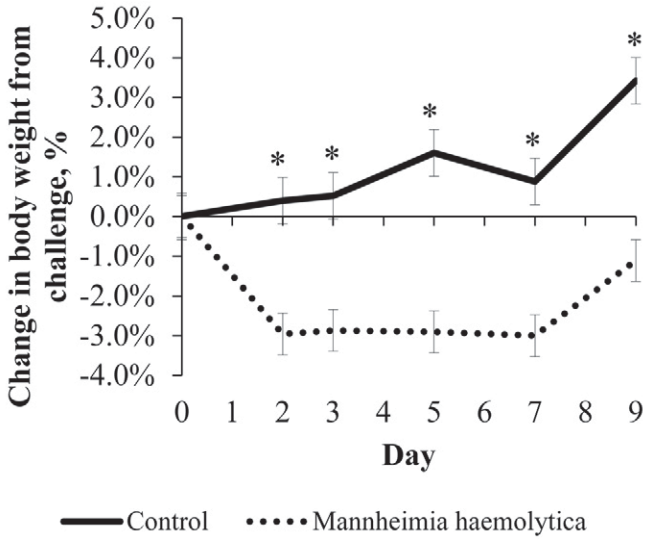

Figure 8. Model-adjusted least squares mean (±SE) percent change in BW in beef heifers by trial day and treatment group of control calves and calves challenged with *Mannheimia haemolytica*. The model included effects for trial day and repeated measures on individual calves. *Significant differences ($P < 0.01$) between treatment group within trial day. The interaction between trial day and treatment group was significant ($P < 0.05$)

Previous research has shown that *M. haemolytica* administration caused increases in both heart rate and respiratory rate (Friend et al., 1977) and that respiratory rate is expected to be associated with the extent of lung consolidation (Reeve-Johnson, 2001). In the current trial heart rate and respiratory rate were not different between treatment groups. Calves in this experiment were housed in extremely high ambient temperatures throughout the trial. Therefore, increases in heart rate and respiratory rate in response to heat stress may have obscured our ability to use these physical examination tools as discriminators of BRD (Fuquay, 1981; Hahn, 1999).

Although infrared thermography has been suggested as an effective animal welfare monitoring tool in cattle, extreme ambient conditions likely diminish the effectiveness of surface thermography for detection of BRD (Stewart et al., 2005; Gomez et al., 2011). In this study, we were only able to detect an interaction between treatment group and time in nasal planum surface temperature. These thermography temperatures differed from nasal passage mucosal temperatures in that calves challenged with *M. haemolytica* had higher surface temperatures throughout the trial.

Behavior of animals is commonly analyzed to determine animal well-being (Gonyou, 1994). Behavioral changes lasted longer than temperature differences in our study. The lethargy associated with BRD was evident in grain bunk and hay feeder activity of MH compared with CN calves. Similar to previous studies, MH calves spent less time near the grain bunk early on during the pneumonia period (Sowell et al., 1998, 1999; Hewson et al., 2011). However, although we were able to detect a difference between treatment groups on d 1, calves spent a relatively small amount of time per day at the grain bunk.

Accelerometers were useful in detecting differences in MH and CN calves based on the percent of time lying down. The percent of time calves spend lying may be correlated to lethargy or depression. Lying behavior is expected to be 1 indicator of animal well-being. Previous trials indicated that calves challenged with *M. haemolytica* spent more time lying down (Hanzlicek et al., 2010a,b). In MH calves, lying time was inversely related to grain bunk and hay feeder behavioral activity. In our study, pedometers were not useful for discrimination between MH and CN calves. This differs from previous research in which pedometers detected decreased number of steps traveled after challenge with *M. haemolytica* (Hanzlicek et al., 2010a). The increase in percentage of time spent walking and number of steps traveled on d 0 may be attributed to the frequent movement that occurred during the intensive monitoring period.

Change in BW in calves suffering BRD is of short-term economic importance for the cattle industry. Burciaga-Robles et al. (2010a) determined that calves challenged with *M. haemolytica* had less ADG up to 4 d after challenge, but they did not demonstrate a difference in BW between calves challenged with *M. haemolytica* and nonchallenged calves. We detected a difference between treatment groups, possibly because of the larger sample size of calves challenged with *M. haemolytica* and control calves and the analysis of percent change in BW rather than average BW.

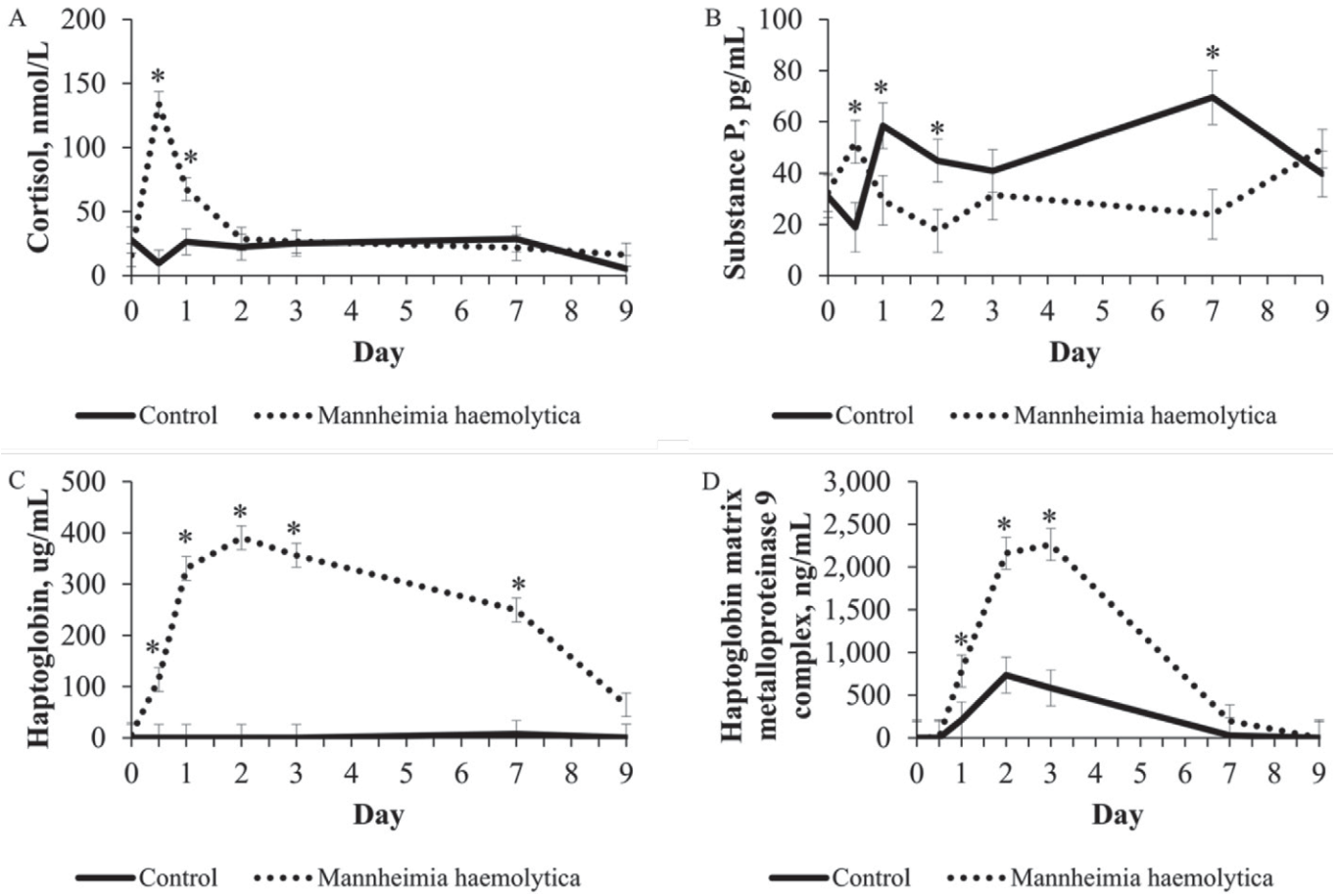

Figure 9. Model-adjusted least squares mean (±SE) (A) cortisol, (B) substance P, (C) haptoglobin, and (D) matrix metalloproteinase-9 concentrations in beef heifers by trial day and treatment group of control calves and calves challenged with *Mannheimia haemolytica*. The model included effects for trial day and repeated measures on individual calves. *Significant differences ($P < 0.01$) between treatment group within trial day. The interaction between trial day and treatment group was significant ($P < 0.05$)

Serum biomarkers may be effective diagnostic tools for early diagnosis of BRD or for use in assigning risk categories for calves moving to feedlots. Cortisol is widely used as an indicator of stress in livestock. Serum cortisol concentrations increased rapidly in the MH group after challenge with MH. This was paralleled by increases in rectal temperatures. Surprisingly, serum cortisol concentrations rapidly declined despite the progressing pneumonia. This differs from previous research in which cortisol concentrations did not change after challenge with *M. haemolytica* (Corrigan et al., 2007). Hewson et al. (2011) demonstrated highly variable concentrations of cortisol after challenge and could not determine a difference between control calves and calves challenged with *M. haemolytica* until 5 d after challenge, when control calves had greater concentrations.

Substance P has been used as a specific biomarker to quantify pain in cattle (Coetzee et al., 2008; DeVane, 2001). The rapid increase in substance P concentrations after challenge was expected, but the increase in substance P in control calves after trial d 2 was not anticipated, and the cause of the change is unknown. Substance P is a volatile neuropeptide that may be influenced by a wide variety of conditions, including the heat stress that both MH and CN groups encountered; however, substance P may not be a specific indicator for disease.

Haptoglobin was the most specific and reliable biomarker for discriminating MH and CN calves as CN had near 0 µg/mL haptoglobin concentrations throughout the trial, whereas MH calves had significantly greater haptoglobin concentrations. These results agree with previous reports where haptoglobin was shown to increase after exposure to *M. haemolytica* (Ganheim et al., 2003; Burciaga-Robles et al., 2010b). More research needs to be performed to establish more precise reference ranges for calves with BRD to use haptoglobin concentrations as a diagnostic tool in the field.

Rapid recruitment and accumulation of white blood cells, especially neutrophils, occur with the onset of BRD (Slocombe et al., 1985). Metalloproteinases are expressed by alveolar macrophages within 12 h of lipopolysaccharide treatment. Degranulated neutrophils release preformed matrix metalloproteinase and Hp-MMP 9 complex into tissue or blood (Lemjabbar et al.,

Table 2. Pulmonary pathology as a percentage of each lung lobe and total long volume from calves challenged with *Mannheimia haemolytica*[1]

Calf ID	Right cranial apical lobe	Right caudal apical lobe	Right diaphragmatic lobe	Accessory lobe	Intermediate lobe	Left cranial apical lobe	Left caudal apical lobe	Left diaphragmatic lobe	Total lung score
1	20	90	5	0	30	0	0	0	9.71
2	40	90	0	0	10	0	0	0	8.35
3	0	100	0	0	0	0	0	0	6.00
4	0	100	0	0	10	0	0	0	6.43
7	80	0	0	0	0	0	0	0	5.04
9	20	90	0	0	10	0	0	0	7.09
11	10	90	0	0	20	0	0	0	6.89
18	80	5	0	0	0	0	0	0	5.34
19	100	0	0	0	10	0	0	0	6.73
20	100	10	0	0	5	0	0	0	7.12
Avg	45	57.5	0.5	0	9.5	0	0	0	6.87

[1]Percentage pulmonary lesions were calculated by the following formula based on the percentage of each lung lobe to the total lung volume: (0.06 × right caudal apical/100) + (0.063 × right cranial apical/100) + (0.053 × left cranial apical/100) + (0.049 × left caudal apical/100) + (0.319 × left diaphragmatic/100) + (0.043 × intermediate/100) + (0.352 × right diaphragmatic/100) + (0.061 × accessory/100) = total lung score

1999; Lakritz et al., 2004; Lubbers et al., 2007). In a previous trial using endotoxin to initiate an inflammatory response, calves showed increased changes in both haptoglobin and Hp-MMP 9 (Lakritz et al., 2004). In our study, *M. haemolytica* was deposited directly into the lung, allowing exact temporal relationships to be assessed during the period from insult to onset of pneumonia. This model of naturally occurring disease allows assessment of the immune response to localized injury as opposed to that typically seen in a systemic response when administering endotoxin. The haptoglobin concentrations in the CN group were well below the reference range established by Young et al. (1996) for clinically healthy calves. The increase in Hp-MMP 9 but not an increase in haptoglobin concentrations in CN calves indicates there was only a local inflammatory response from the pulmonary macrophages and not a systemic response because haptoglobin concentrations remained low in the CN calves. The frequent handling of the calves during the first 24 h postchallenge may have caused a transient increase in Hp-MMP 9 complex and thus may not allow us to detect a difference between treatment groups at 12 h after challenge. These results warrant future research analyzing the effectiveness of Hp and Hp-MMP 9 as effective diagnostic tools for diagnosing BRD.

Limitations of this trial include the use of a challenge model to induce BRD in calves rather than evaluating naturally occurring animals affected by BRD as in a feedlot situation. Lungs from the CN group were not evaluated at necropsy because this was not the primary objective and there were no clinical signs in the CN group indicating the need for euthanasia of the CN calves. None of the MH calves had a CIS > 2, but calves with a CIS of 2 would have been deemed morbid enough to treat in production systems, and our goal was to look at changes relative to early BRD. Using a challenge model to induce BRD allowed the calves to not have other confounding stressors, and the challenge model may be less virulent than naturally occurring BRD. However, using a challenge model allowed us maximum temporal control for when calves became infected, thus allowing controlled monitoring along an established timeline for the development of BRD. The challenge model also puts a conservative estimate on the difference between the treatment groups during periods of high ambient temperature.

Conclusions

In this study, we evaluated cattle with and without experimental *M. haemolytica* infection during high ambient environmental conditions. The influence of increasing temperature during the day may be related to increased rectal temperature in the CN group during the initial 24-h monitoring period. These effects were magnified in the MH group. Evaluating a combination of behavioral, physiological, and clinical variables provides a more comprehensive look at the host and its response to these conditions compared with using single variables.

Results of this study may guide future research on management techniques to mitigate risk for disease in cattle during extreme summer conditions. The tools used herein demonstrate the value of multimodal assessment in disease research. Multimodal tools can enhance our ability to determine the effect of treatments with regard to changes in behavior and physiology. Development of practical interpretation of these tools may be used as a diagnostic tool for detection of BRD. This study further demonstrates the ability of *M. haemolytica* to be used in BRD challenge models where control of temporality, severity, and extent are desired. Results from this study demonstrate the ability of behavioral detection systems

to identify morbid calves and augment clinical case definition of BRD caused by *M. haemolytica*.

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