Investigation of pain and analgesic strategies in food animals

by

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B.S., University of Missouri, 2015
M.S., Colorado State University, 2018

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Anatomy and Physiology
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Abstract

Food animals are stoic by nature and have long been subject to evolutionary pressure to mask pain to avoid becoming prey. Quantifying pain via biomarkers allows researchers to capture changes that are not easily detected by the human eye. Negative public perception of pain associated with routine husbandry practices such as dehorning and castration is growing, increasing the need for the development of practices to relieve pain and suffering in livestock. Pre-emptive analgesia can be applied in advance of the painful stimulus, which reduces sensitization of the nervous system to stimuli that could amplify pain. Further research into the development of appropriate behavioral and physiological pain assessment tools is needed to objectively quantify pain and search for the most effective analgesic strategies.

In this dissertation, the diagnostic sensitivity and specificity of pain biomarkers is investigated across different collection time points and varying painful procedures and conditions including surgical castration, dehorning, lameness, abdominal surgery, and bovine respiratory disease using receiver operating characteristic (ROC) curves. The analgesic effects of flunixin transdermal on pain biomarkers, performance and health indicators are evaluated in feeder calves. Pain associated with induced bacterial pneumonia, scoop and hot-iron dehorning, surgical castration, and hot-iron branding is characterized. The effectiveness of bupivacaine liposome suspension, administered prior to dehorning and castration, to alleviate pain is compared to a multi-modal approach of lidocaine and meloxicam. The effect of meloxicam on pain biomarkers following hot-iron branding is explored. The pharmacokinetics of flunixin meglumine administered intramuscularly and meloxicam administered orally or intramuscularly in juvenile tilapia is described.

Results comparing nonsteroidal anti-inflammatory drug (NSAID) analgesic effects to uncontrolled pain consistently yielded good diagnostic accuracy for plasma cortisol, hair cortisol and infrared thermography. Biomarkers that yielded good diagnostic accuracy for predicting calves with significant lung lesions in the first 72 h after bovine respiratory disease (BRD) onset include, right front stride length, gait velocity, visual analog scale score, clinical illness score, average activity level, step count and rectal temperature. After 72 post-induction, prostaglandin E_2 metabolite, gait distance, cortisol, infrared thermography, right front force, average activity level, step count and serum amyloid A yielded the best diagnostic accuracy for predicting the severity of lung lesions.

Results suggest there were no significant advantages in performance, health, or activity measured by accelerometers from the co-administration of flunixin meglumine transdermal and tildipirosin. However, calves co-administered flunixin meglumine transdermal and tildipirosin did have lower visual analog scale scores indicating that less pain was apparent the first 36 hours post-drug application relative to calves only administered tildipirosin. A combination of reduced activity levels, decreased force on calves' right front limb, and increased visual analog scale pain scores all support that bacterial pneumonia in cattle is painful. Differences in right front force were observed in calves challenged with *M. haemolytica* and treated with flunixin transdermal and those given a placebo, indicating that flunixin transdermal may attenuate specific pain biomarkers in cattle with BRD.

Our results indicate that pain from cautery dehorning can last up to 120 h post-dehorning indicated by changes in nociception and gait analysis. Evidence provided in the current study demonstrates that pain from surgical castration can last up to 120 h post-castration, indicated by changes in ocular temperature, gait analysis, and prostaglandin E_2 metabolite concentrations.

These data show that administration of bupivacaine liposome suspension as a local anesthetic block at the time of dehorning and castration was as effective at controlling pain as a multi-modal approach of lidocaine and meloxicam.

Results suggest that lidocaine + meloxicam reduces cortisol and prostaglandin metabolite concentrations at certain timepoints more than ethanol + lidocaine or bupivacaine liposome suspension + lidocaine administered as a local infiltration and cornual block prior to scoop dehorning followed by cauterization. The treatments administered in the present study did not significantly extend the duration of analgesia beyond the currently recommended multi-modal approach, including local anesthesia and systemic analgesia such as lidocaine and meloxicam. Results suggest that sex influences certain pain biomarkers specifically nociceptive threshold and cortisol concentration, with males having a higher nociceptive threshold and lower cortisol responses.

These results show that infrared thermography, mechanical nociceptive threshold, lying time, step count, visual analog scale score and wound scoring all support that hot-iron branding cattle is painful and investigation into analgesic strategies is needed. Oral meloxicam administration reduced infrared thermography differences from the branding and control site and reduced lying bouts. Breed and sex effects were observed across a wide range of biomarkers.

In juvenile Nile tilapia, flunixin administered intramuscularly reached sufficient plasma concentrations to potentially have an analgesic effect, while meloxicam administered either intramuscularly or orally at the given dosage likely did not have an effect due to the relatively low plasma concentration. The feasibility of dosing individual fish is limited in commercial operations but may be relevant to settings where individual fish are more valuable and are

handled on occasion. Development of an effective granular formulation of an NSAID would be more likely to be integrated into commercial operations.

In conclusion, these results indicate that ROC analysis can be an indicator of the predictive value of biomarkers associated with pain and inflammation. The need for long-acting analgesic options for cattle that demonstrate pain alleviation across multiple biomarkers is apparent and would be beneficial to alleviating pain from routine husbandry procedures like dehorning, castration, and branding, as well as painful disease conditions such as bovine respiratory disease. Breed and sex effects were observed across a wide range of biomarkers and should be investigated in future pain studies. Further studies investigating different drug concentrations and dosage regimens of meloxicam, as well as clinical efficacy of flunixin and meloxicam in Nile tilapia are warranted to provide effective options for pain control in fish.

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In this dissertation, the diagnostic sensitivity and specificity of pain biomarkers is investigated across different collection time points and varying painful procedures and conditions including surgical castration, dehorning, lameness, abdominal surgery, and bovine respiratory disease using receiver operating characteristic (ROC) curves. The analgesic effects of flunixin transdermal on pain biomarkers, performance and health indicators are evaluated in feeder calves. Pain associated with induced bacterial pneumonia, scoop and hot-iron dehorning, surgical castration, and hot-iron branding is characterized. The effectiveness of bupivacaine liposome suspension, administered prior to dehorning and castration, to alleviate pain is compared to a multi-modal approach of lidocaine and meloxicam. The effect of meloxicam on pain biomarkers following hot-iron branding is explored. The pharmacokinetics of flunixin meglumine administered intramuscularly and meloxicam administered orally or intramuscularly in juvenile tilapia is described.

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Dedication

I would like to dedicate this dissertation to my family, my parents Phillip and Heather Martin and my siblings Joel and Audrey Martin, thank you for your support and encouragement. To the long line of educators and agriculturalists in my family who valued higher education and sacrificed for the next generations to have the opportunity to learn. To the many mentors who encouraged me to pursue graduate school and continue to advance my education at the University of Missouri and Colorado State University. To the group of Holstein calves that made much of this work possible; they made the ultimate sacrifice for the betterment of conditions for cattle everywhere. Finally, to my Lord and Savior Jesus Christ, who is the true author and creator, for through Him all things are possible.

Chapter 1 - Literature Review

INTRODUCTION

Freedom from pain, injury and disease is one of the most important aspects of food and companion animal husbandry outlined in the Brambell Report (Brambell, 1965). Cattle undergo elective procedures such as castration and dehorning in the United States which cause pain. They may also experience pain from conditions such as lameness or post-operatively, for example, after a cesarean section. In a survey of urban citizens, dehorning without pain mitigation was viewed as contentious and not supported (Cardoso et al., 2017).

Bovine practitioner use of analgesia varies by procedure or condition and age of the animal; furthermore, analgesia use also varies by practitioner. In a study of cattle practitioners, nearly half felt they had inadequate knowledge in pain recognition and 65% noted most of their knowledge was from experience in practice (Huxley and Whay, 2006). One factor contributing to the low adoption rate of pain mitigation protocols on U.S. farms is the lack of U.S. food and drug administration (FDA)-approved analgesic drugs. In a survey published in 2021, 89.5% of U.S. veterinarians reported that federal regulations limit their ability to use analgesic drugs (Johnstone et al., 2021). Moreover, 50% of respondents attributed a reduction in their analgesic use to concern about administering drugs that lack FDA approval. However, as the American Association of Bovine Practitioners (AABP) and American Veterinary Medical Association (AVMA) recommend in their policies on castration and dehorning (AABP, 2019; AVMA, 2019), veterinarians are permitted to prescribe analgesic drugs for extra-label purposes under the Animal Medicinal Use Clarification Act of 1994 (AMDUCA) (FDA, 1994).

PAIN

Pain results from mechanical, chemical or thermal stimulation of nerve endings containing nociceptors (Hudson et al., 2008). Pain can be categorized as either adaptive or maladaptive. Adaptive pain increases survival potential by protecting the animal from further injury and promoting survival; conversely, maladaptive pain is disease that results in pain long after the initiating causes have been removed (Anderson and Edmondson, 2013). Adaptive pain is often from surgical procedures such as castration or dehorning. Maladaptive pain could be from septic arthritis or a claw amputation for deep digital dermatitis (Anderson and Edmondson, 2013).

Pain and uncontrolled inflammation can suppress immune function and allow for infection, while slowing the rate of wound healing. Understanding these pain pathways is important for selecting the appropriate analgesia strategy; furthermore, drug selection should minimize side effects such as changes in the distribution of blood flow and take into account how the animal is being managed to restore function in a timely manner and minimize adverse effects (Anderson and Edmondson, 2013).

Human research has shown that male subjects have higher pain thresholds and tolerance, are less discriminative between painful sensations, and the nonsteroidal anti-inflammatory drug (NSAID) ibuprofen has been shown to be less effective in women (Walker and Carmody, 1998; Vallerand and Polomano, 2000). The need for further investigation into whether these differences exist in food animals among intact males, castrated males, and females is apparent to better characterize and alleviate pain.

Pain Biomarkers

Cortisol. Cortisol is a corticosteroid hormone that is commonly used as an indicator of acute stress responses, as well as pain (Glynn et al., 2013). Cortisol can be analyzed from blood, saliva, hair, urine, and feces. Cortisol collection from some of these media requires invasive procedures and does not capture long-term increases in circulating concentrations from blood, saliva, or feces (Burnett et al., 2014). Hair cortisol has been found to be a reliable alternative for measuring chronic stress. Cortisol responses measured via blood, saliva, urine, or feces are often characterized by a rapid rise in concentration following a procedure that peaks, rapidly declines, and then plateaus. Circulating cortisol concentrations are influenced by circadian rhythms, differences in breed and temperament, and collection procedures (Coetzee, 2011).

Prostaglandin E_2 metabolite. Prostanoids are synthesized from arachidonic acid via the cyclooxygenase (COX) pathway with prostaglandins associated with COX-2 being mainly responsible for pain and inflammation (Radi, 2009). Nonsteroidal anti-inflammatory drugs (NSAIDs) reduce prostaglandin E2 synthesis both in the spinal cord and periphery by inhibiting COX enzymes and thus reducing inflammatory pain (Kawabata, 2011).

Substance P. Substance P is an 11-amino acid prototypic neuropeptide that regulates the excitability of dorsal horn nociceptive neurons and is present in areas of the neuroaxis involved in the integration of pain, stress, and anxiety (Coetzee, 2011). Substance P not only plays a role in transmitting and integrating nociceptive signals, it also influences the memory of pain through anxiety and depression (Parent et al., 2012).

Infrared Thermography. Infrared thermography is a measurement of radiated electromagnetic energy and can be described as a stream of photons that travel in a wave-like pattern and move at the speed of light (Stewart et al., 2005). A change in eye temperature can be

associated with epinephrine release and vasodilation in response to pain which causes an increase in blood flow (Stewart et al., 2010; Coetzee, 2011).

Mechanical Nociceptive Threshold. Determining mechanical nociception threshold (MNT) via a pressure algometer can establish the minimal amount of pressure that produces a pain response and has been used to evaluate the effects of analgesia (Heinrich et al., 2009; Heinrich et al., 2010). Persistent injury or intense stimulation may sensitize the pain processing system leading to a decreased nociceptive threshold and/or an exaggerated perception to stimuli at the site of the painful lesion and can also affect locations distant from the primary painful lesion (Basbaum et al., 2009).

Kinematic Gait Analysis. A commercially available floor mat–based system can be used to assess variables such as stride length and weight distribution (Coetzee et al., 2017). Kinematic gait analysis systems have been deployed to assess pain associated with lameness in cattle and swine, castration in cattle, and parturition in cattle (Pairis-Garcia et al., 2015; Kleinhenz et al., 2018; Kleinhenz et al., 2019). Lameness or changes in limb function caused by trauma, disease, or pain is manifested from slight unnoticeable gait changes to complete avoidance of the lame limb and can be captured through gait analysis (Quinn et al., 2007).

Accelerometer Activity. Real-time data collection from remote monitoring devices via radio frequency and WiFi can quantify activity, rumination, and eating and drinking behavior (Richeson et al., 2018). Accelerometers depend on the piezoelectric effect, in which a microscopic crystal structure, constructed of either quartz or ceramic, generates a voltage and sends an electrical impulse to a processor chip within the accelerometer that records a movement and/or posture change in the three-plane axis (Reed, 2015). Accelerometers have been shown to

be very accurate compared to video analysis when classifying cattle behavior into one of three activities: standing, walking, or lying (Robert et al., 2009).

Computerized Lung Score. Lung scores are assigned by a computerized stethoscope that measures and analyzes the sounds of the lungs and heart using a machine-learning algorithm that assigns a score of 1 through 5 and estimates lung health at the time of clinical disease identification with increasing severity as scores rise (Nickell et al., 2020). A lung health estimate of 1 indicates that the lung tissue of the respective calf is relatively healthy; conversely, a lung health estimate of 5 reflects severely compromised lung tissue.

Visual Analog Scale. The Visual Analog Scale (VAS) originated from the field of psychology and Woodforde and Merskey (1972) are thought to be the first to use the VAS to score pain with the descriptors of "no pain at all" on one end of the scale, and "my pain could not be any worse" on the other end of the scale. Use of the VAS began with human patients indicating their degree of pain (Haefeli and Elfering, 2006) and has evolved into a behavior evaluation tool used in animal research.

Electrodermal Activity. Electrodermal activity is the measure of resistance between two electrodes that changes based upon sympathetic outflow (Coetzee, 2013a). A correlation has been described between pain perception and skin conductance along with a latency period in response to stimuli that should be accounted for (Susam et al., 2018).

Pain Behavior Scoring. Gleerup et al. (2015) provides a cow pain scale for facial expressions and pain behaviors based on attention to surroundings, head and ear position, facial expression, response to approach, and back position. de Oliveira et al. (2014) created a composite scale that includes locomotion, interactive behavior, activity, appetite, and miscellaneous behaviors like licking the surgical wound. Specific pain behaviors reported

include a greater incidence of tail flicks, kicks, falls in the chute and vocalizations following hot-iron branding (Schwartzkopf-Genswein et al., 1998); increased head shaking, ear flicking, head rubbing, lying time, and vocalizations following dehorning (Stilwell et al., 2009); and increased tail swishing and standing with legs extended, and less normal lying behavior following castration (Ting et al., 2003).

Wound Scoring. Wound scoring has been done following dehorning on a daily basis with scores assigned as follows: 0 = no wound present (completely healed), 1 = minor redness around the wound, 2 = inflamed around the wound with seepage, and 3 = inflamed around the wound with major drainage (Neely et al., 2014). Wound scoring following branding has been done on a weekly basis with score of 1 representing all of the initial scab being present, 2: a majority of the brand covered by a scab, 3: minority of the initial scab present, 4: initial scab gone and tissue becoming re-pigmented, 5: secondary scabbing present and majority of tissue re-pigmented, a score of 6 representing no presence of scabbing and 100% re-pigmentation (Tucker et al., 2014). Wound scoring has also been done following castration on a five point scale ranging from the incision beginning to scab over to no longer being able to see the incision and being fully healed (Mintline et al., 2014).

ANALGESIA

Agents that may provide analgesia in cattle include local anesthetics, NSAIDs, opioids, α2-agonists, and N-methyl-D-aspartate receptor antagonists (Coetzee, 2013b). One way to more effectively administer analgesia is via pre-emptive analgesia strategies which inhibit or minimize pain before it occurs and are more effective than administration after the noxious stimulus occurs (Anderson and Muir, 2005). Another effective analgesic strategy is a multimodal approach via

administration of a combination of drugs from different pharmacologic classes (Anderson and Edmondson, 2013).

Local Anesthetics

Local anesthetics are the most common pre-emptive analgesics used; lidocaine is often used to prevent incisional pain or before dehorning. Local anesthetics inhibit sodium channels but must disassociate in an alkaline environment; this is important because infected or injured tissues create a more acidic environment and result in less effective anesthesia (Anderson and Muir, 2005). Practitioners can use local anesthetics to perform a local block, ring block, peripheral nerve block, or regional block (Anderson and Muir, 2005). In many instances, a solution that has both rapid onset and prolonged duration of analgesia is optimal. Lidocaine, the original amino-amide local anesthetic that is most widely used in veterinary practice, has a limited duration of action, and bupivacaine, which is among the most potent and long-acting of the amino-amides with a higher pKa value than lidocaine, has a longer duration of action, but is thought to have a later onset (Best et al., 2015). An extended-release liposomal formulation of bupivacaine was approved for dogs in 2016 and cats in 2018, with administration prior to surgery providing up to 72 hours of pain control (FDA, 2016, 2018). Liposomal bupivacaine has been shown to have an increased duration of action and a delayed peak plasma concentration when compared to bupivacaine hydrochloride (Tong et al., 2014).

Systemic Analgesia

Analgesic drugs available for systemic use in cattle include NSAIDs, α2-agonists and opioids (Hudson et al., 2008). Administration of NSAIDs causes analgesia and has anti-inflammatory effects by reducing prostaglandin synthesis via cyclo-oxygenase (COX) enzyme inhibition (Ochroch et al., 2003). Administration of an NSAID as part of multimodal therapy for

pain is very common. Alpha2-agonists can produce sedation and chemical restraint, as well as analgesia in cattle (Coetzee, 2013b). Finally, opioids are another systemic analgesic that activate potassium channels and inhibit voltage-gated calcium channels; however, they can also result in negative side effects such as nausea, decreased gastrointestinal motility, and respiratory depression (Coetzee, 2013b).

NSAIDS

Meloxicam. Meloxicam is an NSAID with preferential cyclooxygenase-2 activity (Smith, 2013) that is a practical analgesic option for producers due to its long half-life of 27 hours (Coetzee et al., 2009). Meloxicam has not been shown to mitigate signs of acute distress (Coetzee et al., 2012) but has been shown to reduce the prolonged stress response in calves following dehorning (Heinrich et al., 2009). Meloxicam has been shown to reduce painful behaviors and physiological pain responses up to 72 hours following castration (Olson et al., 2016). Meloxicam has shown promise in reducing physiologic pain biomarkers following a combination of castration and branding procedures (Meléndez et al., 2018b), and has been evaluated in cattle branded on the jaw (Moreno Berggren, 2019). In Nile tilapia, meloxicam administered intramuscularly or intravenously at a dose of 1 mg/kg was rapidly eliminated suggesting that clinically relevant concentrations may be difficult to maintain (Fredholm et al., 2016).

Flunixin. Flunixin transdermal is another NSAID that has been effective in reducing prostaglandin E2 concentrations. Studies suggest that the anti-inflammatory effects of topical flunixin may last up to 48 hours (Thiry et al., 2017). When flunixin is administered topically it is rapidly absorbed and has a longer half-life relative to intravenous administration (Kleinhenz et al., 2016). Transdermal flunixin administered at the time of dehorning did not provide substantial

analgesia based on infrared thermography, mechanical nociceptive threshold, cortisol and substance P concentrations (Kleinhenz et al., 2017). When administered at the time of surgical castration, transdermal flunixin reduced plasma cortisol concentrations and mitigated the stress response for 8 h but provided negligible analgesic effects on substance P, IRT and gait analysis (Kleinhenz et al., 2018). Flunixin has been found to be an ineffective water treatment but has been determined to be effective when injected intra-peritoneally at a dose of 2.2 mg/kg in channel catfish (Brown et al., 1986).

MISCONCEPTIONS

In a survey by (Hudson et al., 2008), four misconceptions among practitioners were observed: (1) Age of the animal – Young animals are often assumed to feel less pain than adults which is invalid, (2) Pain restricts movement and thus prevents further tissue damage – Analgesia should still be administered and the animal should be housed in a small area rather than relying on pain, (3) Analgesia masks deterioration of the animal's condition – Other clinical signs should be used to monitor disease progression, (4) Corticosteroids are effective analgesics – They are potent anti-inflammatory agents but produce much less profound analgesia. Veterinarians may assume that farmers are unwilling to pay the costs associated with analgesia but many owners may be more prepared to pay than the clinician realizes (Hudson et al., 2008).

AMDUCA

In ruminants, the use of analgesia must be done in consideration of drug residues and in accordance with the Animal Medicinal Drug Use Clarification Act (AMDUCA) (FDA, 1994, 1996). "Extra-label drug use (ELDU) requires that some specific conditions be met which include: (1) ELDU is allowed only by or under the supervision of a veterinarian, (2) ELDU is allowed only for US Food and Drug Administration (FDA)–approved animal and human drugs;

(3) ELDU is only permitted when the health of the animal is threatened and not for production purposes; (4) ELDU in feed is prohibited, and (5) ELDU is not permitted if it results in a violative drug residue in food intended for human consumption," p. 12 (FDA, 1994; Coetzee, 2013b).

PAINFUL HUSBANDRY PROCEDURES

Dehorning

Dehorning or disbudding is a routine management procedure performed on a high percentage of dairies in the United States, with the majority of calves being dehorned by 12 wk of age using a hot-iron (Fulwider et al., 2008; USDA-APHIS-NAHMS, 2018). Even when the procedure is performed in calves less than 4 wk old, hot-iron dehorning causes behavioral, physiological and neuroendocrine changes and a physical form of restraint is still required (Faulkner and Weary, 2000; Stock et al., 2013). Hot-iron dehorning causes an initial increase in cortisol concentrations corresponding to the acute pain of tissue damage and stress of restraint, which does not return to baseline levels for at least 24 h after the procedure, indicating the presence of a prolonged inflammatory response (Stafford and Mellor, 2005; Heinrich et al., 2009). Previous research reports changes in cortisol (Glynn et al., 2013), MNT (Heinrich et al., 2010), ocular temperature (Stewart et al., 2008), and pain behaviors (Heinrich et al., 2010) all indicative of pain following dehorning.

Castration

Castration is a routine management procedure performed on beef and dairy operations in the United States. Dairy calves are on average 7.5 weeks old when castrated and the majority of beef calves are castrated prior to being sold after weaning (USDA-APHIS-NAHMS, 2018, 2020). All methods of castration (surgical or band) have been shown to produce behavioral,

physiologic and neuroendocrine changes associated with pain and distress (Coetzee, 2011). Previous research reports changes in ocular temperature (Stewart et al., 2010; Kleinhenz et al., 2018), gait analysis (Kleinhenz et al., 2018), behavior (Laurence et al., 2016; Meléndez et al., 2018a), and cortisol concentrations (Kleinhenz et al., 2018; Meléndez et al., 2018c) following surgical castration. Calves surgically castrated exhibited swelling for up to 14 days following the procedure, indicating the presence of a prolonged inflammatory response (Marti et al., 2017). Wound healing ranged from 35-56 days for calves to become completely healed following surgical castration (Marti et al., 2018).

Branding

Hot-iron branding permanently identifies cattle via thermal injury to the skin and results in tissue damage which is considered painful. In the most recent USDA survey, 75% of large cattle operations of 200 head or more used hot iron brands (USDA, 2019). Branding is generally used for the purposes of identifying imported cattle, disease control, theft prevention, and permanent identification on open range when cattle are not within enclosures. Hot-iron branding results in second or third degree burn injuries described as initial tissue damage causing cell death, inflammation of the tissue leading to a local edema and the invasion of inflammatory cells, and re-epithelialization of the damaged wound (Laycock et al., 2013). Previous studies have quantified pain from hot-iron branding (Lay Jr et al., 1992; Schwartzkopf-Genswein et al., 1997) and have begun to investigate analgesic strategies (Tucker et al., 2014; Meléndez et al., 2018b; Moreno Berggren, 2019). Tucker et al. (2014) showed that only 67% of brand wounds were fully healed 10 weeks after hot-iron branding.

NEW FRONTIERS IN ANALGESIC USE

BRD

Bovine respiratory disease (BRD) is the most common and costliest disease affecting the cattle industry (DeDonder et al., 2010; Peel, 2020), with BRD death loss alone costing upwards of $907 million annually in the United States (USDA, 2017). The most commonly isolated organism from BRD-affected lung tissue is an opportunistic pathogen, *Mannheimia haemolytica* (Griffin et al., 2010). *Mannheimia haemolytica* is considered a commensal organism that, when cattle experience stress, can proliferate into the nasopharynx and translocate to the lung, where it causes fibrinous pleuropneumonia (Rice et al., 2007). Symptoms of BRD include nasal and ocular discharge, depression, anorexia, and increased respiratory rate with dyspnea. Clinical outcomes may range from hardly noticeable to death (Griffin et al., 2010). Pleuritic chest pain resulting from bacterial pneumonia is commonly reported in human medicine (Boyd et al., 2006). Percussion of the thoracic wall has been reported to elicit signs of pain and painful respiration has been documented from pleuropneumonia in cattle, with these findings published over twenty years ago (Ter Laak, 1992; Braun et al., 1997).

Aquaculture

Aquaculture is one of the largest growing sectors of the world food supply. Between 1961 and 2016, the average annual increase in global food fish consumption (3.2%) outpaced population growth (1.6%) and exceeded that of meat from all terrestrial animals combined (2.8%) (FAO, 2018). In 2013, global aquaculture production accounted for about 50 million of the 125 million metric tons of seafood produced annually for human consumption (Kite-Powell et al., 2013). Ornamental fish make up another large sector with around 27 million ornamental fish being traded each year (Townsend, 2011). Evidence of pain perception in fish is supported

by a similar sensory system, evidence of adverse behavioral and physiological responses and normal behavior being suspended during a potentially painful event (Sneddon, 2006). Administration of morphine to fish has been shown to significantly reduce pain-related behaviors and opercular beat rate, showing evidence that morphine can act as an analgesic in fish (Sneddon, 2003). Research into post-operative analgesia investigating the use of an opioid or NSAID in ornamental fish (*Cyprinus carpio)* showed promise but has not been expanded to different species (Harms et al., 2005). Fish that are subject to tissue damaging, invasive procedures, traumatic injury or aggression may require that pain and discomfort be reduced by the use of an analgesic such as an NSAID; yet, validation of analgesic protocols is very limited and a great deal of species variation exists making extrapolation difficult (Sneddon, 2012; Chatigny et al., 2018). The Farm Animal Welfare Committee base their guidelines for farmed fish welfare on the "Five Freedoms", therefore, the use of analgesia in aquaculture could potentially play a role in allowing for freedom from discomfort, pain, injury, and distress (FAWC, 2014).

CONCLUSIONS

Effective analgesia use starts with identifying pain using a combination of biomarkers, then requires using an effective agent and method of administration, taking into account the type of condition or procedure, and its compliance with AMDUCA. Finally, it should consider the age, breed, and sex of the animal with attention to withdrawal time periods. Opportunities to prevent the onset of pain and limit the deviation from physiological norms with analgesia should be taken to prevent negative animal welfare outcomes.

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Chapter 2 - Assessment of the diagnostic sensitivity and specificity of pain biomarkers in cattle using receiver operating characteristic (ROC) curves

This chapter has been submitted for publication to the Journal of Dairy Science.

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ABSTRACT

Biomarkers are commonly used to assess pain and analgesic drug efficacy in livestock. However, the diagnostic sensitivity and specificity of these biomarkers for different pain conditions over time have not been described. Receiver operating characteristic (ROC) curves are graphical plots that illustrate the diagnostic ability of a test as its discrimination threshold is

varied. The objective of this analysis was to use area under the curve (AUC) values derived from ROC analysis to assess the predictive value of pain biomarkers at specific time points. The biomarkers included in the analysis were plasma cortisol, salivary cortisol, hair cortisol, infrared thermography (IRT), mechanical nociceptive threshold (MNT), substance P, kinematic gait analysis and a visual analog scale for pain. A total sample size of 7,992 biomarker outcomes collected from seven pain studies involving pain associated with castration, dehorning, lameness, and abdominal surgery were included in the analysis. Each study consisted of three treatments: pain, no pain, and analgesia. All statistics were performed using a statistical software. Results comparing analgesic effects to pain consistently yielded good diagnostic accuracy (AUC > 0.7; 95% CI: 0.40 to 0.99) for plasma cortisol (time points: 1.5, 2, 3, 4, 6, and 8 h); hair cortisol (time point: 62 d); and IRT (time point: 72 h). Results yielded the best diagnostic accuracy (AUC > 0.75) at 2 h for plasma cortisol following castration and dehorning, 12 h for IRT following castration, 24 h for plasma cortisol following lameness induction and 48 h for IRT following dehorning when comparing analgesia versus pain. Results yielded the best diagnostic accuracy (AUC > 0.75) at 1 h for plasma cortisol following castration and dehorning; at 8 h for IRT following castration; at 24 h for VAS following castration and plasma cortisol and substance P following lameness induction; and 48 h for IRT and MNT following dehorning, when comparing pain versus no pain. These results indicate that ROC analysis can be an indicator of the predictive value of pain biomarkers and certain time points seem to yield good diagnostic accuracy while many do not.

Keywords: Analgesia, Biomarker, Pain, ROC

INTRODUCTION

Pain results from mechanical, chemical or thermal stimulation of nerve endings containing nociceptors (Hudson et al., 2008). Cattle undergo elective procedures such as castration and dehorning which cause pain. They may also experience pain from conditions such as lameness or post-operatively, for example, after a cesarean section. In a survey of urban citizens, dehorning without pain mitigation was viewed as contentious and not supported (Cardoso et al., 2017). The American Veterinary Medical Association (AVMA) policy on castration and dehorning states that because these procedures cause pain and discomfort, the use of medications to alleviate pain should be used under the Animal Medicinal Drug Use and Clarification Act (AMDUCA) (AVMA, 2019). In the United States, approved analgesic drugs for use in livestock are limited to non-steroidal anti-inflammatory drugs (NSAIDS). Flunixin meglumine, as a transdermal formulation, is the only US Food and Drug Administration (FDA) approved analgesic to specifically control pain associated with foot rot in cattle (FDA, 2017). In the U.S., there are no analgesics approved to control pain in cattle from castration, dehorning, or surgery.

Biomarkers are commonly used to assess pain and analgesic drug efficacy in livestock. During the drug development process, one of the components when submitting data to the FDA for biomarker qualification, is the characterization of the relationships between the biomarker, the clinical outcomes, and the treatment (Amur et al., 2015). Some biomarkers currently used to assess pain include cortisol, substance P, infrared thermography (IRT), mechanical nociceptive threshold (MNT), kinematic gait analysis and visual analog scale pain assessment. These biomarkers quantify the animal's response to stress and inflammatory pain; however, some are more invasive than others, some are influenced by restraint, and they can change due to stress

rather than pain which may be difficult to quantify. This brings into question which biomarkers yield good diagnostic accuracy at varying time points throughout the stress response following a painful procedure or condition.

The diagnostic sensitivity and specificity of pain biomarkers for different painful conditions in cattle over time have not been described. Receiver operating characteristic (ROC) curves are graphical plots that illustrate the diagnostic ability of a test as its discrimination threshold is varied. The plot of true positive (sensitivity) verses false positive (1-specificity) across possible cut-off values generates a ROC curve (Hajian-Tilaki, 2013). The area under the ROC curve (AUC) can be used to measure discriminative ability. Additionally, the AUC value can be compared between ROC curves (Ekelund, 2012). The objective of this analysis was to use AUC values derived from ROC analysis to assess the predictive value of pain biomarkers at specific time points.

MATERIALS AND METHODS

Study Design

A total of 7,992 biomarker outcomes were included in the analysis. These outcomes were collected from 519 animals enrolled in seven different studies involving pain in cattle associated with castration, dehorning, lameness, and abdominal surgery (**Table 1**) (Kleinhenz et al., 2017; Meléndez et al., 2017; Kleinhenz et al., 2018; Meléndez et al., 2018; Kleinhenz et al., 2019a; Kleinhenz et al., 2019b; Martin et al., 2020). The biomarkers included in the analysis were plasma cortisol, salivary cortisol, hair cortisol, infrared thermography (IRT), mechanical nociceptive threshold (MNT), substance P, kinematic gait analysis and visual analog scale (VAS). Biomarker outcomes for each of the seven studies are outlined in **Table 1** with collection

time points outlined in **Table 2**. Each of the studies were reviewed and approved by the Institutional Animal Care and Use Committee at Iowa State University (Log # 5-15-8016-B, 6-15-8039-B, 7–16–8314-B, and 8-14-7845-B), at Kansas State University (IACUC# 4002), the University of Calgary (ACC14-0159), and the Lethbridge Research Centre Animal Care Committee (ACC# 1410 and 1428).

All the data were collected between 2016 and 2019. Data from multiple castration studies were combined for the substance P outcome. All the cattle enrolled into the castration studies were castrated surgically. Each study consisted of three treatments: pain, no pain, and analgesia. Analgesics used were non-steroidal anti-inflammatory drugs (NSAIDs)- transdermal flunixin meglumine or meloxicam. Baseline time points are not included in the ROC analysis due to the lack of comparison between pain, no pain, and analgesia.

Physiological and Behavioral Parameters

Plasma Cortisol. A total of 1,564 plasma cortisol samples made up this data set. The samples were obtained as described by Kleinhenz et al. (2017). Blood was obtained by jugular catheter or by jugular venipuncture using a syringe (Kleinhenz et al., 2018; Kleinhenz et al., 2019a). The blood was immediately transferred to a blood tube containing sodium heparin and was centrifuged at 3,000 g for 10 min. The plasma was pipetted into cryovials, placed on dry ice, and stored at –80 °C until analysis. Cortisol concentration were determined using a commercially available radioimmunoassay (MP Biomedicals, Santa Ana, CA).

Salivary Cortisol. A total of 523 salivary cortisol samples made up this data set. As described in Meléndez et al. (2018), briefly, saliva samples were collected with a cotton swab, immediately stored in a plastic tube, and frozen at –20 °C for further cortisol analysis using an enzyme immunoassay kit (Salimetrics, State College, PA).

Hair Cortisol. A total of 175 hair cortisol samples made up this data set. As described in Meléndez et al. (2018), briefly, hair from the forehead was clipped and stored in plastic bags at room temperature for further cortisol analysis. Cortisol was quantified using an enzyme-linked immunosorbent assay (Salimetrics, State College, PA).

Infrared Thermography. A total of 724 IRT measures made up this data set. Infrared images of the medial canthus of the eye for castration and dehorning; and images from lame feet were included in the analysis. Mean, maximum and temperature differentials between the left and right feet from the lameness study were included in the analysis. Infrared thermography images were obtained using a research-grade infrared camera (FLIR SC 660; FLIR Systems AB, Danderyd, Sweden). The IRT camera was calibrated prior to being used with the ambient temperature and relative humidity. As described in Kleinhenz et al. (2017) and Kleinhenz et al. (2018), an image of the lateral aspect of the head was obtained so that the image contained the medial canthus of the eye. As described in Kleinhenz et al. (2019b), images of the foot were obtained at a 45° angle, 1 m from the coronary band and 3 images at each time point were averaged for analysis, and the difference between the temperatures of the left and right hind feet (left hind minus right hind) were determined for each time point. Infrared images were analyzed using research grade computer software (FLIR ExaminIR, Inc., North Billerica, MA).

Mechanical Nociception Threshold. A total of 1,065 MNT measures made up this data set. As described in Kleinhenz et al. (2017), briefly, using a hand held pressure algometer (Wagner Instruments, Greenwich, CT), a force was applied perpendicularly at a rate of approximately 1 kg of force per second at 2 locations (lateral and caudal) adjacent to the horn bud. A third control location between the eyes was used to evaluate MNT. A withdrawal response was indicated by an overt movement away from the applied pressure algometer and

values were recorded by a second investigator to prevent bias. Locations were tested 3 times in sequential order, and the values were averaged for statistical analysis.

Substance P. A total of 1,402 substance P samples made up this data set. As described in Kleinhenz et al. (2017), 200 µg benzamidine was added to EDTA blood tubes (BD Vacutainer, Franklin Lakes, NJ) 48 h prior to the start of the studies. During sample collection, 6 mL of blood were added to the spiked EDTA tube. The samples were immediately placed on ice, centrifuged within 30 min of collection, and the plasma was placed into cryovials. The cryovials were stored at -80°C until analysis. Substance P levels were determined using the methods described by Van Engen et al. (2014a) using non-extracted plasma. A similar method was employed by Meléndez et al. (2018), but 200 µg benzamidine was not added to the blood tubes prior to the start of the study.

Kinematic Gait Analysis. A total of 239 gait analysis readings made up this data set. As described in Kleinhenz et al. (2019b), a commercially available kinematic gait system (MatScan, Tekscan, Inc., South Boston, MA) was used to record gait and biomechanical parameters. The system was calibrated, using a known mass, daily and before each use of the computer software to ensure accuracy of the measurements at each time point. Video synchronization was used to ensure consistent gait between and within cows at each time point. Using research specific software (HUGEMAT Research 5.83, Tekscan, Inc.), force, contact pressure, and impulse in the affected feet were assessed.

Visual Analog Scale. A total of 2,300 VAS scores made up this data set. As described in Martin et al. (2020), a daily VAS pain assessment was conducted by two trained evaluators blinded to treatment allocations. The VAS used was a 100 mm (10 cm) line anchored by descriptors of "No Pain" on the left (0 cm) and "Severe Pain" on the right (10 cm). Five

parameters were used to assess pain: depression, tail swishing or flicking, stance, head carriage, and foot stomping or kicking. No pain was characterized by being alert and quick to show interest, no tail swishing, a normal stance, head held above spine level, and absence of foot stomping. Severe pain was characterized by being dull and showing no interest, more than three tail swishes per minute, legs abducted, head held below spine level, and numerous stomps. The evaluator marked the line between the 2 descriptors to indicate the pain intensity. A millimeter scale was used to measure the score from the zero anchor point to the evaluator's mark. The mean VAS measures of the two evaluators were combined into one score for statistical analysis.

Receiver Operating Characteristic Curve Determination. All statistics were performed using statistical software (JMP Pro 14.0, SAS Institute, Inc., Cary, NC). Receiver operating characteristic curves were created for each time point, with AUC values comparing uncontrolled pain × no pain and uncontrolled pain × analgesic use, with uncontrolled pain as the positive control. The biomarker outcome was plotted as the x-coordinate and the status (uncontrolled pain, no pain, analgesic use) was plotted as the y-coordinate. Bootstrapping via fractional weights was used to generate confidence intervals for each AUC value. AUC values ≥ 0.7 were considered to yield good diagnostic accuracy (Yang and Berdine, 2017). Specific cut-off values were selected based upon optimized specificity and sensitivity values. Positive predictive values with confidence intervals were calculated for each AUC value (MedCalc Software Ltd, Ostend, Belgium).

RESULTS

Plasma Cortisol. Plasma cortisol AUC values comparing analgesic use verses uncontrolled pain are outlined in **Table 3,** with rankings in **Table 5**. The timepoints that yielded good diagnostic accuracy for surgical castration, dehorning, and lameness when comparing

analgesic use to uncontrolled pain are outlined below. Surgical castration study results for plasma cortisol (**Fig. 1.A**) yielded good results (AUC > 0.7; 95% CI: 0.40 to 0.99) at 1, 1.5, 2, 3, 4, 6, 8, and 12 h with cut-off values decreasing from the 1.5 h time point (cut-off values: 17.51, 22.94, 11.70, 5.88, 8.99, 4.65, 2.52, and 4.58 ng/ml, respectively). Dehorning study results for plasma cortisol (**Fig. 1.B**) yielded good results (AUC > 0.7; 95% CI: 0.40 to 0.95) at 1.5, 2, 3, 4, 6, 8, 48 and 72 h with cut-off values fluctuating (cut-off values: 11.32, 9.78, 5.43, 4.12, 5.59, 9.69, and 2.60 and 15.57 ng/ml, respectively). Lameness study results for plasma cortisol (**Fig. 1.C**) yielded good results (AUC > 0.7; 95% CI: 0.42 to 0.99) at 1.5, 2, 3, 4, 6, 8, 16, 24, 32, 40, 48, 56, 64, 72, 80, 96, and 120 h with cut-off values decreasing after 40 h (cut-off values: 5.16, 10.16, 8.61, 9.85, 16.47, 11.43, 7.64, 9.92, 6.07, 11.19, 4.43, 4.11, 6.25, 5.33, 7.33, 4.81, and 3.00 ng/ml, respectively).

Plasma cortisol AUC values comparing no pain verses uncontrolled pain are outlined in **Table 4**, with rankings in **Table 6.** The timepoints that yielded good diagnostic accuracy for surgical castration, dehorning, and lameness when comparing no pain to uncontrolled pain are outlined below. Surgical castration study results for plasma cortisol yielded good results (AUC > 0.7; 95% CI: 0.42 to 0.99) at 0.5, 1, 1.5, 2, 3, 4, and 6 h with cut-off values decreasing from 2 h (cut-off values: 30.34, 17.51, 5.92, 20.04, 14.01, 11.94, and 1.71 ng/ml, respectively). Dehorning study results for plasma cortisol yielded good results (AUC > 0.7; 95% CI: 0.42 to 0.99) at 0.17, 0.33, 0.5, 0.67, 0.83, 1, 1.5, 6 and 48 h with cut-off values decreasing over time (cut-off values: 15.89, 16.36, 16.07, 14.50, 13.62, 9.82, 6.47, 7.83, and 3.78 ng/ml, respectively). Lameness study results for plasma cortisol yielded good results (AUC > 0.7; 95% CI: 0.55 to 0.95) at 24 h with a cut-off value of 12.08 ng/ml. Positive predictive values for castration, dehorning and

lameness are outlined in **Tables 7, 8, and 9**, respectively. Cut-off values for castration, dehorning and lameness are outlined in **Tables 11, 12, and 13**, respectively.

Salivary Cortisol. Salivary cortisol AUC values comparing analgesic use verses uncontrolled pain are outlined in **Table 3.** Surgical castration study results for plasma cortisol yielded poor diagnostic accuracy (AUC < 0.7; 95% CI: 0.35 to 0.82) at all the time points examined (6, 13, 20, 34, 48, 62 d).

Salivary cortisol AUC values comparing no pain verses uncontrolled pain are outlined in **Table 4.** Surgical castration study results for salivary cortisol yielded poor diagnostic accuracy (AUC < 0.7; 95% CI: 0.36 to 0.73) at all the time points examined (6, 13, 20, 34, 48, 62 d). Positive predictive values and cut-off values for castration are outlined in **Table 7** and **Table 11**, respectively.

Hair Cortisol. Hair cortisol AUC values comparing analgesic use verses uncontrolled pain are outlined in **Table 3**, with rankings in **Table 5.** Surgical castration study results comparing analgesic use verses uncontrolled pain yielded good diagnostic accuracy (AUC > 0.7; 95% CI: 0.51 to 0.85) for hair cortisol at time point 62 d with a cut-off value of 10.33 pg/ml.

Hair cortisol AUC values comparing no pain verses pain are outlined in **Table 4.** Surgical castration study results comparing no pain verses uncontrolled pain yielded poor diagnostic accuracy (AUC < 0.7; 95% CI: 0.44 to 0.71) for hair cortisol at all the time points examined. Positive predictive values and cut-off values for castration are outlined in **Table 7** and **Table 11**, respectively.

Infrared Thermography. Ocular infrared thermography AUC values comparing analgesic use verses uncontrolled pain are outlined in **Table 3**, with rankings in **Table 5.** The timepoints that yielded good diagnostic accuracy for surgical castration, dehorning, and

abdominal surgery when comparing analgesic use to uncontrolled pain are outlined below. Surgical castration study results for IRT yielded good results (AUC > 0.7; 95% CI: 0.40 to 0.97) (**Fig. 2.A**) at 12 and 72 h with cutoff values of 38.6 and 37.3 °C, respectively. Dehorning study results for IRT yielded good results (AUC > 0.7; 95% CI: 0.53 to 0.89) at 48 h with a cut-off value of 37.5°C. Abdominal surgery study results for IRT (**Fig. 2.B**) yielded good results (AUC > 0.7; 95% CI: 0.48 to 0.89) at 3 and 72 h with cut-off values of 33.2 and 35.1°C, respectively.

Ocular infrared thermography AUC values comparing no pain verses uncontrolled pain are outlined in **Table 4**, with rankings in **Table 6.** The timepoints that yielded good diagnostic accuracy for surgical castration, dehorning, and abdominal surgery when comparing no pain to uncontrolled pain are outlined below. Surgical castration study results for IRT yielded good results (AUC > 0.7; 95% CI: 0.44 to 0.94) at 6, 8 and 48 h with cut-off values of 37.7, 38 and 37.5°C, respectively. Dehorning study results for IRT yielded good results (AUC > 0.7; 95% CI: 0.58 to 0.91) at 48 h with a cut-off value of 37.6°C. Abdominal surgery study results for IRT yielded good results (AUC > 0.7; 95% CI: 0.47 to 0.92) at 24 h with a cut-off value of 35.3°C. Positive predictive values for castration, dehorning and abdominal surgery are outlined in **Tables 7, 8, and 10**, respectively. Cut-off values for castration, dehorning and abdominal surgery are outlined in **Tables 11, 12, and 14**, respectively.

Mechanical Nociception Threshold. Mechanical nociception threshold AUC values comparing analgesic use verses uncontrolled pain are outlined in **Table 3.** Dehorning study results for MNT yielded poor diagnostic accuracy (AUC < 0.7; 95% CI: 0.40 to 0.65) at all the time points examined (6, 25, and 49 h).

Mechanical nociception threshold AUC values comparing no pain verses uncontrolled pain are outlined in **Table 4**, with rankings in **Table 6.** Dehorning study results for MNT yielded

good diagnostic accuracy (AUC > 0.7; 95% CI: 0.75 to 0.95) at time points 6, 25, and 49 h with cut-off values decreasing over time (cut-off values: 1.05, 0.89, and 0.78 kg F, respectively). Positive predictive values and cut-off values for dehorning are outlined in **Table 8** and **Table 12**, respectively.

Substance P. Substance P AUC values comparing analgesic use verses uncontrolled pain are outlined in **Table 3**. Surgical castration, dehorning, lameness, and abdominal surgery study results comparing analgesia verses pain yielded poor diagnostic accuracy (AUC < 0.7; 95% CI: 0.28 to 0.88) for substance P at all the time points examined (1, 2, 3, 4, 6, 8, 12, 18, 24, 48, 72, 96, 120, 144, 312, 480, 816, 1152, and 1488 h).

Substance P AUC values comparing no pain verses uncontrolled pain are outlined in **Table 4**, with rankings in **Table 6.** The timepoints that yielded good diagnostic accuracy for surgical castration, dehorning, lameness, and abdominal surgery when comparing no pain to uncontrolled pain are outlined below. Surgical castration and dehorning study results for substance P yielded poor diagnostic accuracy (AUC < 0.7; 95% CI: 0.20 to 0.88) at all the time points examined (1, 2, 3, 4, 6, 8, 12, 18, 24, 48, 144, 312, 480, 816, 1152, and 1488) except for dehorning at 72 h (0.70; 95% CI: 0.44 to 0.90) with a cut-off value of 118.80 pg/mL. Lameness study results for substance P yielded good results (AUC > 0.7; 95% CI: 0.71 to 0.95) at 8, 24, and 120 h with cut-off values of 77.56, 84.52, and 82.36 pg/mL, respectively. Abdominal surgery study results for substance P yielded good results (AUC > 0.7; 95% CI: 0.45 to 0.91) at 3, 6, and 48 h with cut-off values of 84.17, 93.78, and 79.79 pg/mL, respectively. Positive predictive values for castration, dehorning, lameness, and abdominal surgery are outlined in **Tables 7, 8, 9 and 10,** respectively. Cut-off values for castration, dehorning, lameness, and abdominal surgery are outlined in **Tables 11, 12, 13 and 14,** respectively.

Kinematic Gait Analysis. Gait AUC values comparing analgesic use verses uncontrolled pain are outlined in **Table 3**, with rankings in **Table 5.** The timepoints that yielded good diagnostic accuracy for lameness when comparing analgesic use to uncontrolled pain are outlined below. Area yielded good results (AUC > 0.7; 95% CI: 0.47 to 0.89) at 24 h with a cut-off value of 0.02 cm^2.

Gait AUC values comparing no pain verses uncontrolled pain are outlined in **Table 4**, with rankings in **Table 6.** The timepoints that yielded good diagnostic accuracy for lameness when comparing no pain to uncontrolled pain are outlined below. Area yielded poor results (AUC < 0.7; 95% CI: 0.32 to 0.90) at all the time points examined (8, 16, 48, 72, 96, and 120 h) except 24 h (0.73; 95% CI: 0.46 to 0.90) with a cut-off value of 0.16 cm^2. Force yielded poor results (AUC < 0.7; 95% CI: 0.33 to 0.83) at all the time points examined (8, 16, 24, 48, 72, 96, and 120 h). Impulse yielded good results (AUC > 0.7; 95% CI: 0.50 to 0.90) at 96 h with a cut-off value of 150.28 kg*s. Pressure yielded poor results (AUC < 0.7; 95% CI: 0.29 to 0.72) at all the time points examined (8, 16, 24, 48, 72, 96, and 120 h). Positive predictive values and cut-off values for lameness are outlined in **Table 9** and **Table 13**, respectively.

Visual Analog Scale. Visual analog scale AUC values comparing analgesic use verses uncontrolled pain are outlined in **Table 3.** Surgical castration study results comparing analgesic verses uncontrolled pain yielded poor diagnostic accuracy (AUC < 0.7; 95% CI: 0.43 to 0.74) for VAS scores at all the time points examined (1, 2, 3, 4, 5, and 6 d).

Visual analog scale AUC values comparing no pain verses uncontrolled pain are outlined in **Table 4**, with rankings in **Table 6.** Surgical castration study results comparing no pain verses uncontrolled pain yielded good diagnostic accuracy (AUC > 0.7; 95% CI: 0.65 to 0.81) for VAS

scores at 1 and 4 d with cut-off values of 7.5 and 3.0. Positive predictive values and cut-off values for castration are outlined in **Table 7** and **Table 11**.

DISCUSSION

It is essential that non-invasive measures of acute and chronic stress are developed for assessment of animal welfare (Stewart et al., 2005). One avenue is the development of robust biomarkers to objectively quantify pain and evaluate analgesic treatment regimen efficacy during routine elective animal husbandry procedures such as castration and dehorning (Coetzee, 2011). Cortisol is a corticosteroid hormone that is commonly used as an indicator of acute stress responses, as well as pain (Glynn et al., 2013). Cortisol responses are often characterized by a rapid rise in concentration following a procedure that peaks, rapidly declines, and then plateaus; however, many limitations exist and the stress from restraint may impact cortisol response. Studies have shown that plasma cortisol concentrations are influenced by the different procedural methods and have high individual variability (Stafford et al., 2002), some animals have low responses likely due to higher pain thresholds (Stafford and Mellor, 2005). Difficulties exist in obtaining true baseline measurements, missing rapid response times, accounting for the impact of circadian rhythms on hormone levels, differences in breed and temperament, and the nature of blood sampling which may itself cause a stress response (Coetzee, 2011). Measures that can be taken to try and overcome these challenges include assigning calves to a control treatment that experience the stress of restraint, collecting samples at the same time each day to account for circadian rhythms, accounting for time of day in the statistical model, and sampling frequently during the acute phase response. The AUC values included in this analysis indicated that plasma cortisol can yield good diagnostic accuracy for many procedures immediately post-procedure and can continue to yield sound results for multiple days following lameness induction,

specifically. Plasma cortisol yielded good diagnostic accuracy when identifying pain, as well as analgesic effects. Salivary cortisol data included in the analysis were collected days after the surgical castration procedure rather than hours, and as a result seemed to yield poor diagnostic accuracy. It would be beneficial for future analyses for salivary cortisol to be collected closer to the time of the procedure. Hair cortisol data included in the analysis yielded promising results but was only measured twice following surgical castration due to the nature of hair growth, so data was limited.

Infrared thermography is a measurement of radiated electromagnetic energy and can be described as a stream of photons that travel in a wave-like pattern and move at the speed of light (Stewart et al., 2005). A change in eye temperature can be associated with epinephrine release and vasodilation in response to pain which causes an increase in blood flow (Stewart et al., 2010; Coetzee, 2011). A decrease in eye temperature has been observed following castration in calves (Stewart et al., 2010); however, a lack of analgesic effect may suggest that temperature change is more indicative of stress and isn't as accurate as a sole indicator of pain (Glynn et al., 2013). The AUC values included in this analysis indicated that IRT may yield good diagnostic accuracy at time points later (castration and surgery: 72 h) than plasma cortisol (castration: 1-12 h) and yield good diagnostic results when identifying pain, as well as analgesic effects.

Determining mechanical nociception threshold via a pressure algometer can establish the minimal amount of pressure that produces a pain response and has been used to evaluate the effects of analgesia (Heinrich et al., 2009; Heinrich et al., 2010). Downfalls of MNT determination include a high degree of intra-individual variation and inappropriate selection of a non-painful control site (Raundal et al., 2014). Inter-observer reliability has been shown to increase when MNT values are averages rather than taking single measurements (Tapper et al.,

2013). The AUC values included in this analysis indicated that MNT yielded better diagnostic accuracy for identifying pain, rather than analgesic effects.

Substance P is an 11-amino acid neuropeptide that is present in areas of the neuroaxis that are involved in integrating pain and stress (Coetzee, 2011). Coetzee et al. (2008) suggested that substance P measurement may discriminate between a stressful event, which will cause a cortisol response, and a more specific nociceptive stimulus. Substance P concentrations have been found to be significantly higher in castrated calves compared to controls and in lame cattle with laminitis (Bustamante et al., 2015). Lack of differences in substance P concentrations between calves administered analgesia and negative controls at the time of surgical castration (Coetzee et al., 2008) may be a good explanation in the present study for the poor diagnostic accuracy when identifying analgesic effects, but good diagnostic accuracy when identifying pain.

Kinematic gait analysis in cattle has not been well-characterized in the literature (Schulz et al., 2011). A commercially available floor mat–based system can be used to assess variables such as stride length and weight distribution (Coetzee et al., 2017). Kinematic gait analysis systems have been shown to detect lameness (Kleinhenz et al., 2019a), as well as changes in weight distribution after castration and calving (Kleinhenz et al., 2018; Kleinhenz et al., 2019c). Approval for the use of transdermal flunixin for control of pain due to foot rot was achieved through gait analysis using a floor-based pressure mat system (FDA, 2017). One downfall of the system is that if an animal stops or slows down while walking across the pressure mat, the measurement if often lost (Maertens et al., 2011). The AUC values included in this analysis indicated that kinematic gait analysis may not yield good diagnostic accuracy for experimentally induced lameness at the time points examined. The use of gait analysis in naturally occurring clinical lameness has not been well described in the literature.

Visual analog scale assessment is a method of evaluating pain intensity based on behavioral parameters. For the data included in the present analysis, the parameters used to assess pain were depression, tail swishing, stance, head carriage, and foot stomping (Martin et al., 2020). Some disadvantages exist when using subjective visual assessment; behavioral observations may lack sensitivity because of individual animal variation or because many behaviors are socially facilitated, and prey species are also generally stoic in response to pain (Currah et al., 2009). The AUC values included in this analysis indicated that VAS assessment yielded better diagnostic accuracy when identifying pain, rather than analgesic effects.

The data included in the present analysis was combined from studies where only non-steroidal anti-inflammatory drugs (NSAIDs) were used for analgesia. Results likely may have differed if a local anesthetic was also included in the protocol as they have been shown to decrease the acute stress response (Winder et al., 2018). Non-steroidal anti-inflammatory drugs prevent inflammation by inhibiting cyclooxygenase enzymes that produce prostaglandins. Use of an NSAID alone does not effectively reduce the acute stress associated with many elective procedures but provides analgesic and anti-inflammatory effects into the post-operative period (Coetzee, 2011). The mean half-life for topical flunixin has been observed to be 6.42 h (Kleinhenz et al., 2016) and approximately 26 h for meloxicam (Heinrich et al., 2010). Differences in duration of analgesic effect may have had an impact on the diagnostic accuracy of analgesia versus pain comparisons, with AUC values likely decreasing as the analgesic effect wore off.

When assessing pain, choosing which biomarkers would be of interest and the appropriate time points for sample collection is reliant upon many factors that influence the stress response. Stress is an adaptive pattern of behavioral, neural, endocrine, immune,

hematological, and metabolic changes focused on restoring homeostasis (Anderson and Muir, 2005). First, calves respond to the pain of the procedure, as well as the physical restraint (Faulkner and Weary, 2000). Following a procedure such as dehorning or castration, an acute painful response is observed followed by a period of inflammatory pain (Stock et al., 2013). Acute pain is capable of producing an acute stress response by activating the sympathetic nervous system and secretion of glucocorticoids (Anderson and Muir, 2005). Duration of stress response for different procedures and conditions likely varies, along with the amount of pain the animal is experiencing at different time points as well. The influence of analgesia on this response to try and restore homeostasis is dependent upon whether the analgesia is administered prior to the procedure, the mechanism of action and duration of the analgesic regimen, and the duration of the stress response. Finally, biomarker selection that will result in good diagnostic accuracy should consider whether the objective is to identify pain or quantify the effects of analgesia.

CONCLUSIONS

Results from the present study comparing NSAID analgesic effects to uncontrolled pain consistently yielded good diagnostic accuracy for plasma cortisol, hair cortisol and infrared thermography. Results yielded the best diagnostic accuracy (AUC > 0.75) at 2 h for plasma cortisol following castration and dehorning, 12 h for IRT following castration, 24 h for plasma cortisol following lameness induction and 48 h for IRT following dehorning when comparing analgesia versus pain. Results yielded the best diagnostic accuracy (AUC > 0.75) at 1 h for plasma cortisol following castration and dehorning; at 8 h for IRT following castration; at 24 h for VAS following castration and plasma cortisol and substance P following lameness induction; and 48 h for IRT and MNT following dehorning, when comparing pain versus no pain. The

objectives of the study, length of the stress response, and duration of the analgesic regimen administered should all be considered when selecting biomarkers to assess painful procedures and conditions. These results indicate that ROC analysis can be an indicator of the predictive value of pain biomarkers and certain time points seem to yield good diagnostic accuracy while many do not. These results can be used to guide refinement of future research regarding painful procedures and analgesic efficacy.

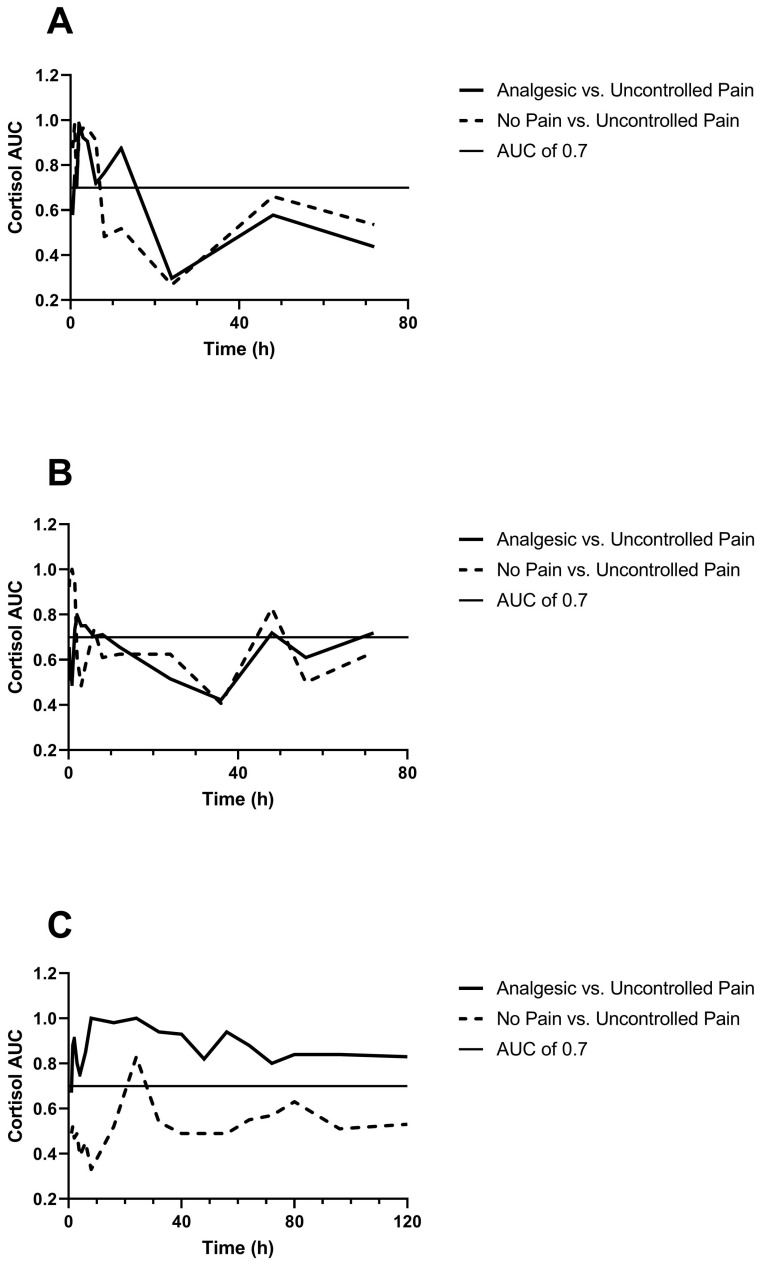

Figure 2.1. Area under the curve (AUC) values for plasma cortisol concentrations following 1.A) surgical castration; 1.B) hot iron dehorning; and 1.C) lameness induction

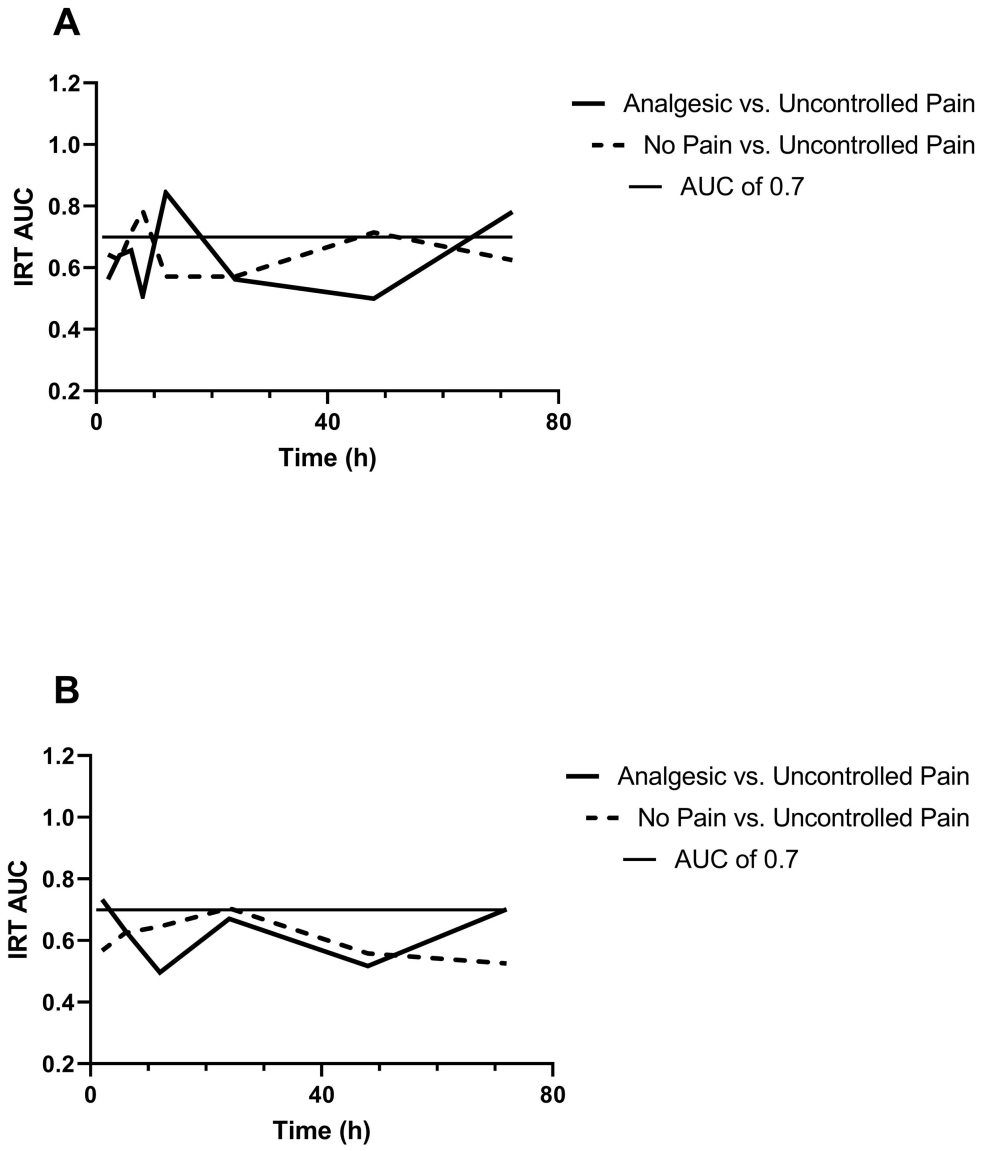

Figure 2.2 Area under the curve (AUC) values for ocular infrared thermography temperatures following 2.A) surgical castration and 2.B) abdominal surgery

Table 2.1 Cattle pain studies included in the receiver operating characteristic (ROC) curve analysis

Reference	Procedure or Condition	Analgesic regimen	Outcome Parameters
(Kleinhenz et al., 2017)	Dehorning	Transdermal flunixin meglumine 3.33 mg/kg	Plasma cortisol IRT[1] MNT[2] Substance P
(Meléndez et al., 2017)	Surgical Castration	SC Meloxicam 0.5 mg/kg	Salivary cortisol Hair cortisol Substance P
(Kleinhenz et al., 2018)	Surgical Castration	Transdermal flunixin meglumine 3.33 mg/kg	Plasma cortisol IRT Substance P
(Marti et al., 2018; Meléndez et al., 2018)	Surgical Castration	SC Meloxicam 0.5 mg/kg	Salivary cortisol Substance P
(Kleinhenz et al., 2019a)	Lameness	Transdermal flunixin meglumine 3.33 mg/kg	Plasma cortisol Gait analysis Substance P
(Kleinhenz et al., 2019b)	Abdominal surgery	IV flunixin meglumine 2.2 mg/kg	IRT Substance P
(Martin et al., 2020)	Surgical Castration	Transdermal flunixin	VAS[3] score

	meglumine 3.33 mg/kg

[1]IRT = infrared thermography

[2]MNT = mechanical nociceptive threshold

[3]VAS = visual analog scale

Table 2.2 Collection time points for each biomarker

Biomarker	Time point
Plasma cortisol, h	0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 24, 48, 72
Salivary cortisol, d	6, 13, 20, 34, 48, 62
Hair cortisol, d	34, 62
IRT[1], h	1, 2, 3, 4, 6, 8, 12, 24, 48, 72
MNT[2], h	6, 25, 49
Substance P, h	1, 2, 3, 4, 6, 8, 12, 18, 24, 48, 72, 96, 120, 144, 312, 480, 816, 1152, 1488
Kinematic Gait Analysis, h	8, 16, 24, 48, 72, 96, 120
VAS[3], d	1, 2, 3, 4, 5, 6

[1]IRT = infrared thermography

[2]MNT = mechanical nociceptive threshold

[3]VAS = visual analog scale

Table 2.3 AUC values for analgesia vs pain by collection time point* for each biomarker (N = 7,992 outcomes)

Procedure	Biomarker	Time point														
		h													d	
		1	2	3	4	6	8	12	16	24	48	72	96	120	34	62
Castration	Plasma cortisol	0.75	0.98	0.91	0.90	0.72	0.72	0.87		0.45	0.62	0.51				
	IRT[1]		0.620		0.65	0.67	0.61	0.84		0.62	0.58	0.77				
	Substance P		0.61		0.68	0.61	0.59	0.60		0.63	0.61	0.61				
	Hair cortisol														0.67	0.71
	Salivary cortisol														0.58	0.58
	VAS[3]									0.63	0.58	0.54	0.62	0.56		
Dehorning	Plasma cortisol	0.67	0.79	0.75	0.74	0.71	0.72	0.67		0.61	0.72	0.72				
	IRT[1]	0.59	0.58		0.58	0.68		0.62		0.57	0.75					
	MNT[2]					0.51				0.56	0.57					
	Substance P	0.62	0.62		0.62			0.62		0.61	0.61	0.62				
Lameness	Plasma cortisol	0.67	0.90	0.79	0.75	0.85	1.00		0.97	1.00	0.82	0.80	0.83	0.82		
	Substance P	0.61	0.65				0.64			0.60	0.62	0.59	0.61	0.60		
	Pressure Mat - Area						0.60		0.63	0.70	0.64	0.61	0.58	0.59		
	Pressure Mat - Force						0.62		0.63	0.64	0.61	0.62	0.59	0.60		
	Pressure Mat - Impulse						0.64		0.59	0.62	0.60	0.60	0.67	0.58		
	Pressure Mat - Pressure						0.58		0.62	0.61	0.58	0.59	0.60	0.60		
Abdominal surgery	IRT[1]			0.73		0.63		0.57		0.67	0.57	0.70				

Substance P	0.66	0.64	0.58	0.59	0.56	0.61	

*Timepoints that overlapped across multiple studies are reported in the table, some timepoints not taken on the hour are not reported in the table

[1]IRT = infrared thermography

[2]MNT = mechanical nociceptive threshold

[3]VAS = Visual Analog Scale

Table 2.4 AUC values for no pain vs pain by collection time point* for each biomarker (N = 7,992 outcomes)

		Time point																
Procedure	Biomarker	h													d			
		1	2	3	4	6	8	12	16	24	48	72	96	120	144	20	34	62
Castration	Plasma cortisol	0.98	0.92	0.97	0.97	0.91	0.62	0.62		0.56	0.68	0.60						
	IRT[1]		0.66		0.64	0.73	0.79	0.62		0.63	0.72	0.65						
	Substance P		0.66		0.69	0.61	0.69	0.63		0.62	0.63	0.63						
	Hair cortisol																0.56	0.58
	Salivary cortisol														0.57	0.59	0.54	0.56
	VAS[3]									0.75	0.70	0.65	0.72	0.61	0.63			
Dehorning	Plasma cortisol	1.00	0.64	0.58	0.61	0.74	0.64	0.65		0.65	0.83	0.65						
	IRT[1]	0.58	0.60		0.60	0.59		0.58		0.64	0.78							
	MNT[2]					0.81				0.88	0.92							
	Substance P	0.63	0.58		0.62			0.64		0.62	0.62	0.70						
Lameness	Plasma cortisol	0.58	0.59	0.59	0.60	0.58	0.58		0.57	0.83	0.60	0.61	0.61	0.60				
	Substance P	0.68	0.66				0.77			0.83	0.68	0.67	0.68	0.71				
	Pressure Mat - Area						0.63		0.65	0.73	0.67	0.59	0.59	0.62				
	Pressure Mat - Force						0.61		0.62	0.62	0.66	0.60	0.61	0.61				
	Pressure Mat - Impulse						0.62		0.64	0.62	0.63	0.61	0.74	0.70				

	Pressure Mat - Pressure			0.60	0.61	0.61	0.57	0.59	0.60	0.60	
Abdominal surgery	IRT[1]	0.64	0.64	0.66		0.72	0.60	0.61			
	Substance P	0.72	0.70	0.64		0.62	0.72	0.67			

*Timepoints that overlapped across multiple studies are reported in the table, some timepoints not taken on the hour are not reported in the table

[1]IRT = infrared thermography

[2]MNT = mechanical nociceptive threshold

[3]VAS = Visual Analog Scale

Table 2.5 AUC rankings > 0.7 for analgesia vs pain by collection time point for each biomarker

Procedure	Biomarker	Ranking											
		1		2		3		4		5		6	
		AUC	Time point	AUC	Time point	AUC	Time point	AUC	Time point	AUC	Time point	AUC	Time point
Castration	Plasma cortisol	0.98	2	0.91	3	0.90	4	0.87	12	0.75	1	0.72	6
	IRT[1]	0.84	12	0.77	72								
	Hair cortisol	0.71	62										
Dehorning	Plasma cortisol	0.79	2	0.75	3	0.74	4	0.72	8	0.72	48	0.72	72
	IRT[1]	0.75	48										
Lameness	Plasma cortisol	1.00	24	0.97	16	0.90	2	0.85	6	0.83	96	0.82	48
	Area	0.70	24										
Abdominal surgery	IRT[1]	0.73	3	72	0.70								

[1]IRT = infrared thermography

Table 2.6 AUC rankings > 0.7 for no pain vs pain by collection time point for each biomarker

Procedure	Biomarker	Ranking											
		1		2		3		4		5		6	
		AUC	Time point	AUC	Time point	AUC	Time point	AUC	Time point	AUC	Time point	AUC	Time point
Castration	Plasma cortisol	0.98	1	0.97	3	0.97	4	0.92	2	0.91	6		
	IRT[1]	0.79	8	0.73	6	0.72	48						
	VAS[3]	0.75	24	0.72	96	0.70	48						
Dehorning	Plasma cortisol	1.00	1	0.83	48	0.74	6						
	MNT[2]	0.92	48	0.88	24	0.81	6						
	IRT[1]	0.78	48										
	Substance P	0.70	72										
Lameness	Plasma cortisol	0.83	24										
	Substance P	0.83	24	0.77	8	0.71	120						
	Impulse	0.74	72	0.70	120								
	Area	0.73	24										
Abdominal surgery	IRT[1]	0.72	24										
	Substance P	0.70	6	0.72	2	0.72	48						

[1]IRT = infrared thermography

[2]MNT = mechanical nociceptive threshold

[3]VAS = Visual Analog Scale

Table 2.7 Positive predictive value percentages for no pain vs pain by collection time point* for castration

Biomarker	Time point																
	h														d		
	1	2	3	4	6	8	12	16	24	48	72	96	120	144	20	34	62
Plasma cortisol	100.00	100.00	100.00	100.00	79.78 to 92.72	63.33 to 77.54	74.75 to 95.72		100.00	71.15 to 89.95	100.00						
IRT[1]		66.37 50.95 to 78.94		100.00	74.75 to 91.08	85.55 to 97.43	100.00		100.00	74.75 to 91.08	79.78 36.13 to 96.50						
Substance P		71.15 40.48 to 89.95		100.00	59.68 to 75.92	100	66.37 to 83.54		66.37 to 88.50	100.00	58.66 to 70.82						
Hair cortisol																33.61	37.89
																27.07 to 40.85	27.47 to 49.56
Salivary cortisol														41.81	40.46	74.89	32.20
														22.51 to 63.99	26.89 to 55.67	25.04 to 96.38	26.70 to 38.24
VAS[2]									61.70	56.83	60.29	63.54	55.50	68.20			

		54.37 to 68.52	49.71 to 63.68	51.19 to 68.73	55.24 to 71.11	44.53 to 65.96	50.88 to 81.62	

*Timepoints that overlapped across multiple studies are reported in the table, some timepoints not taken on the hour are not reported in the table

[1]IRT = infrared thermography

[2]VAS = Visual Analog Scale

Table 2.8 Positive predictive value percentages for no pain vs pain by collection time point* for dehorning

Biomarker	h										
	1	2	3	4	6	8	12	16	24	48	72
Plasma cortisol	100.00	61.54 to 73.24	66.67 18.27 to 94.71	61.54 to 73.24	100.00	75.00 28.06 to 95.85	61.54 to 73.24		63.64 45.48 to 78.59	83.33 42.53 to 97.12	71.43 40.17 to 90.30
IRT[1]	60.64 41.15 to 55.28	59.09 52.12 to 100		60.75 44.12 to 58.06	61.90 52.13 to 84.71		57.52 49.83 to 78.69		65.00 48.89 to 67.50	71.23 58.69 to 100	
MNT[2]					84.62 77.49 to 89.78				91.43 84.97 to 95.27	95.10 89.13 to 97.87	
Substance P	80.00	80.00		62.50			100.00		62.50	100.00	83.33

36.04 to 96.60	36.04 to 96.60	36.99 to 83.55	36.99 to 82.55	42.53 to 97.12

*Timepoints that overlapped across multiple studies are reported in the table, some timepoints not taken on the hour are not reported in the table

[1]IRT = infrared thermography

[2]MNT = mechanical nociceptive threshold

Table 2.9 Positive predictive value percentages for no pain vs pain by collection time point* for lameness

Biomarker	Time point												
	h												
	1	2	3	4	6	8	12	16	24	48	72	96	120
Plasma cortisol	66.37	57.50	56.31	55.61	100.00	55.61		58.50	77.18	100.00	81.85	65.27	65.27
	45.49	37.81	43.61	50.47		50.47		50.83	56.21		37.71	37.74	53.12
	to	to	to	to		to		to	to		to	to	to
	82.35	75.08	68.23	60.64		60.64		65.77	89.91		97.11	85.35	75.71
Substance P	66.99	61.70				71.73			100.00	66.99	66.99	66.99	87.12
	51.36	51.78				53.60				51.36	51.36	51.36	49.62
	to	to				to				to	to	to	to
	79.60	70.74				84.78				79.60	79.60	79.60	97.89
Pressure Mat - Area						72.97		69.23	85.71	83.33	55.56	100.00	61.54
						41.83		50.61	46.62	41.32	47.83		44.45
						to		to	to	to	to		to
						91.02		83.17	97.63	97.26	63.02		76.19
Pressure Mat - Force						61.17		80.00	62.50	66.67	55.56	57.14	66.67
						40.58		34.93	50.12	46.83	47.83	42.42	31.86
						to		to	to	to	to	to	to
						78.41		96.75	73.44	81.95	63.02	70.71	89.54
Pressure Mat - Impulse						67.74		64.29	64.29	100.00	61.54	69.23	66.67

	43.36 to 85.21	48.36 to 77.58	48.36 to 77.58		44.45 to 76.19	50.61 to 83.17	51.83 to 78.80
Pressure Mat - Pressure	64.29	66.67	63.64	53.85	58.33	62.50	63.64
	29.92 to 88.36	40.60 to 85.40	42.53 to 80.54	37.88 to 69.06	40.03 to 74.60	34.96 to 83.79	42.53 to 80.54

*Timepoints that overlapped across multiple studies are reported in the table, some timepoints not taken on the hour are not reported in the table

Table 2.10 Positive predictive value percentages for no pain vs pain by collection time point* for abdominal surgery

Biomarker	Time point h										
	1	2	3	4	6	8	12	16	24	48	72
IRT[1]			80.32		78.90		100.00		87.69	87.72	100.00
			56.48		64.19				55.67	53.71	
			to		to				to	to	
			92.77		88.64				97.58	97.78	
Substance P			89.08		88.62		78.90		75.37	90.18	100.00
			57.47		56.06		64.19		66.38	60.68	
			to		to		to		to	to	
			98.01		97.94		88.64		82.59	98.20	

*Timepoints that overlapped across multiple studies are reported in the table, some timepoints not taken on the hour are not reported in the table

[1]IRT = infrared thermography

Table 2.11 Cutoff values for no pain vs pain by collection time point* for castration

Biomarker	Units	Time point															
		h													d		
		1	2	3	4	6	8	12	16	24	48	72	96	120	144	20	34	62
Plasma cortisol	ng/ml	17.51	20.04	14.01	11.94	1.71	7.89	8.30		36.83	2.66	5.35						
IRT[1]	°C		37.6		38.5	37.7	38.0	36.8		38.2	37.5	37.3						
Substance P	pg/ml		82.42		105.26	77.08	91.63	80.16		77.36	98.33	91.09						
Hair cortisol	pg/ml																14.87	10.33
Salivary cortisol	ng/ml														1.96	2.57	0.75	2.41
VAS[2]	1-10 cm									7.5	5.5	7.5	3.0	3.5	4.5			

*Timepoints that overlapped across multiple studies are reported in the table, some timepoints not taken on the hour are not reported in the table

[1]IRT = infrared thermography

[2]VAS = Visual Analog Scale

Table 2.12 Cutoff values for no pain vs pain by collection time point* for dehorning

	Units	h										
		1	2	3	4	6	8	12	16	24	48	72
Plasma cortisol	ng/ml	9.82	4.96	8.64	13.59	7.83	18.14	8.03		8.67	3.78	10.51
IRT[1]	°C	38.6	37.6		38.5	40.5		39.1		37.5	37.6	
MNT[2]	kg F					1.05				0.89	0.78	
Substance P	pg/ml	100.07	109.81		107.35			84.75		99.47	76.19	118.80

*Timepoints that overlapped across multiple studies are reported in the table, some timepoints not taken on the hour are not reported in the table

[1]IRT = infrared thermography

[2]MNT = mechanical nociceptive threshold

Table 2.13 Cutoff values for no pain vs pain by collection time point* for lameness

Biomarker	Units	Time point h												
		1	2	3	4	6	8	12	16	24	48	72	96	120
Plasma cortisol	ng/ml	7.55	14.72	16.66	20.48	8.83	25.55		32.31	12.08	17.45	17.12	10.89	2.75
Substance P	pg/ml	71.98	69.23				77.56			84.52	66.16	76.15	75.94	82.36
Pressure Mat - Area	cm^2						0.02		0.02	0.02	0.02	0.02	0.01	0.02
Pressure Mat - Force	kg						191.94		149.22	162.08	173.6	250.97	248.64	174.83
Pressure Mat - Impulse	kg*sec						120.1		182.81	115.09	179.63	124.56	150.28	139.57
Pressure Mat - Pressure	kg/cm^2						4.2		3.7	3.6	4.1	2.8	3.8	4.0

*Timepoints that overlapped across multiple studies are reported in the table, some timepoints not taken on the hour are not reported in the table

Table 2.14 Cutoff values for no pain vs pain by collection time point* for abdominal surgery

		Time point										
Biomarker	Units	h										
		1	2	3	4	6	8	12	16	24	48	72
IRT[1]	°C			33.7		31.1		31.9		35.3	35.4	31.2
Substance P	pg/ml			84.17		93.78		93.40		101.25	79.79	69.10

*Timepoints that overlapped across multiple studies are reported in the table, some timepoints not taken on the hour are not reported in the table

[1]IRT = infrared thermography

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Chapter 3 - Assessment of diagnostic accuracy of biomarkers to assess lung consolidation in calves with induced bacterial pneumonia using receiver operating characteristic (ROC) curves

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ABSTRACT

Bovine respiratory disease (BRD) is the most economically significant disease for cattle producers in the United States. Cattle with advanced lung lesions at harvest due to BRD during the production cycle have reduced average daily gain, yield grades and carcass quality outcomes.

The identification of biomarkers that accurately predict the extent of lung lesions would benefit livestock producers in determining a prognosis in BRD cases. Receiver operating characteristic (ROC) curves are graphical plots that illustrate the diagnostic ability of a biomarker as its discrimination threshold is varied. Previously we used the area under the ROC curve (AUC) to identify cortisol, hair cortisol and infrared thermography imaging as having acceptable (AUC > 0.7) diagnostic sensitivity and specificity for detecting pain in cattle. Herein, we used ROC curves to assess the sensitivity and specificity of biomarkers associated with lung lesions after experimentally induced bovine respiratory disease (BRD) in calves. Our hypothesis was that biomarkers associated with pain and inflammation assessed at specific timepoints after induction of BRD could be used to predict lung consolidation at necropsy. Lung consolidation of >10% was retrospectively assigned at necropsy as a true positive indicator of BRD. Calves with a score of <10% were considered negative for BRD. The biomarkers included in the analysis were cortisol concentrations; infrared thermography (IRT); mechanical nociceptive threshold (MNT); substance P; kinematic gait analysis; a visual analog scale (VAS); clinical illness score (CIS); computerized lung score (CLS); average activity levels; prostaglandin E_2 metabolite concentrations (PGEM); serum amyloid A concentrations and rectal temperature. A total sample size of 5,122 biomarker outcomes were collected from 26 calves, eighteen of which were inoculated with *M. haemolytica*. All statistics were performed using statistical software. Results comparing calves with significant lung lesions to those without yielded the best diagnostic accuracy (AUC > 0.75) for right front stride length at 0 h; gait velocity at 32 h; VAS, CIS, average activity and rumination levels, step count and rectal temperature, all at 48 h; PGEM at 72 h; gait distance at 120 h; cortisol at 168 h; and IRT, right front force and serum amyloid A, all at 192 h. These results indicate that ROC analysis can characterize the predictive value of

biomarkers associated with pain and inflammation in cattle with induced bacterial pneumonia. AUC values for VAS score, average activity levels, step count, and rectal temperature seemed to yield very good diagnostic accuracy (AUC > 0.75) at multiple timepoints while average and percent change MNT values, substance P concentrations, and CLS did not (all AUC values < 0.75).

Keywords: bovine respiratory disease, lung lesion, pain, biomarkers

INTRODUCTION

Bovine respiratory disease (BRD) is the most economically significant disease for cattle producers in the United States with 16.2 percent of cattle placed on feed being affected. Fibrinous pleuropneumonia is typically produced by *M. haemolytica* and is the most common form of acute pneumonia in weaned, stressed beef cattle (Andrews and Kennedy, 1997). The presence of pleuritis is an indication of the aggressiveness of the lung infection and the extent of infection and inflammation; lipopolysaccharide and leukotoxin are the two factors responsible for most of the destructive lesions of *M. haemolytica* infection. (Panciera and Confer, 2010). In studies quantifying lung lesions at slaughter, 62-67% lung lesion incidence was recorded (Schneider et al., 2009; Tennant et al., 2014; Blakebrough-Hall et al., 2020).

A biomarker is a defined molecular, histologic or physiological characteristic “that is measured as an indicator of normal biological processes, pathogenic processes, or responses to an exposure or intervention, including therapeutic interventions” (FDA, 2017). The relationship between clinical illness score, computerized lung score, rectal temperature, facial thermography, behavior biomarkers and lung lesion scores has been examined in past studies to detect the

presence of BRD (White et al., 2008; DeDonder et al., 2010; Baruch et al., 2019). However, which of these biomarkers yield good diagnostic accuracy when predicting lung lesions at varying timepoints throughout the disease state has not been examined.

Receiver operating characteristic (ROC) curves are graphical plots that illustrate the diagnostic ability of a test as its discrimination threshold is varied. The plot of true positive (sensitivity) verses false positive (1-specificity) across possible cut-off values generates a ROC curve (Hajian-Tilaki, 2013). The area under the ROC curve (AUC) can be used to measure discriminative ability. Additionally, the AUC value can be compared between ROC curves (Ekelund, 2012). The objective of this analysis was to use AUC values derived from ROC analysis to assess the predictive value of BRD biomarkers to evaluate the presence of lung lesions after experimentally induced bacterial pneumonia.

MATERIALS AND METHODS

This study was reviewed and approved by the Institutional Animal Care and Use Committee at Kansas State University (IACUC# 4465).

Study Design

Twenty-six calves, 6-7 months of age, weighing an average of 185 ± 4 kg were enrolled onto the study. Upon arrival to the study site, calves were affixed with a 3-axis accelerometer ear-tag (Allflex Livestock Intelligence, Madison, WI) to quantify activity. At 24 h prior to the start of the study, 18 calves were inoculated with a strain of *Mannheimia haemolytica* using bronchoalveolar lavage (BAL) as described by Theurer et al. (2013). The right apical lung lobes were inoculated using broncho-selective endoscopy. A 10 mL dose of *M. haemolytica* serotype A1 at 1 x 10^9 cfu/mL was used to inoculate each calf, then the endoscope was flushed with 60 mL of phosphate buffered solution to achieve a total volume of 70 mL. The 8 control calves

received 70 mL of phosphate buffered solution as described above. At any point while on study, if the rectal temperature of a calf was > 40.3 ºC, tildipirosin was administered at a dosage of 4 mg/kg. Eight calves that were inoculated with *M. haemolytica* were administered flunixin transdermal at a dosage of 3.33 mg/kg at 0 and 24 h relative to disease onset.

Disease onset was considered to be 24 h following inoculation and was considered timepoint 0 h. Biomarker variables were collected at -48 prior to disease onset, 0 (disease onset), 4, 8, 24, 32, 48, 72, 96, 120, 144, 168, and 192 h relative to disease onset, in addition to the 3-axis accelerometer ear-tags continuously collecting data throughout the study. A total of 5,122 biomarker outcomes were collected which included: infrared thermography (IRT) imaging, kinematic gait analysis, mechanical nociception threshold (MNT), visual analog scale (VAS) score, clinical illness score (CIS), computerized stethoscope (Whisper Veterinary Stethoscope, Merck Animal Health, Madison, NJ) lung score (CLS), average activity levels, rectal temperature, and blood sampling for cortisol, substance P, prostaglandin E_2 metabolite (PGEM), and serum amyloid A (SAA) analysis. All trained evaluators were blinded to treatment for the duration of the study. Following the 192 h collection, calves were euthanized and transported to the Kansas State Veterinary Diagnostic Laboratory for necropsy and lung lesion scoring. The postmortem examination was performed by a board-certified veterinary pathologist (KMA) to determine lung lesions and a lung lesion score was assigned based on lung consolidation. The lung lesion score was determined using methods described by Fajt et al. (2003). Briefly, the extent of consolidated lung divided by the total lung volume was determined. A lung score >10% was used as a positive indicator of BRD for the sake of this analysis. The biomarkers and sample collection timepoints are outlined in **Table 1.**

Physiological and Behavioral Parameters

Serum Cortisol. A total of 312 cortisol samples made up the data set. The samples were obtained as described by Kleinhenz et al. (2017). Blood was obtained by jugular venipuncture using a syringe (Kleinhenz et al., 2018; Kleinhenz et al., 2019a) and was immediately transferred to a blood tube and centrifuged at 3,000 g for 10 min. The serum was pipetted into cryovials, placed on dry ice, and stored at –80 °C until analysis. Cortisol concentrations were determined using a commercially available radioimmunoassay (MP Biomedicals, Santa Ana, CA).

Infrared Thermography. A total of 312 IRT images made up the data set. Mean and percent change from baseline values from images of the medial canthus of the left eye were included in the analysis. Infrared thermography images were obtained using a research-grade infrared camera (Fluke TiX580, Fluke Corp, Everett, WA). The IRT camera was calibrated prior to being used with the ambient temperature and relative humidity. As described in Kleinhenz et al. (2017) and Kleinhenz et al. (2018), an image of the lateral aspect of the head was obtained so that the image contained the medial canthus of the eye. Infrared images were analyzed using research grade computer software (SmartView v. 4.3, Fluke Thermography, Plymouth, MN).

Mechanical Nociception Threshold. A total of 208 MNT measures made up the data set. Using a hand held pressure algometer (Wagner Instruments, Greenwich, CT), force was applied perpendicularly at a rate of approximately 1 kg of force per second at 1 location on each side of the ribs of each calf over the 6th intercostal space for a total of 2 locations, as described in (Williams et al., 2020). A withdrawal response was indicated by an overt movement away from the applied pressure algometer and values were recorded by a second investigator to prevent bias. Locations were tested 3 times in sequential order, and the values were averaged for statistical analysis.

Substance P. A total of 312 substance P samples made up the data set. As described in Kleinhenz et al. (2017), benzamidine hydrochloride (final concentration of 1mM) was added to EDTA blood tubes (BD Vacutainer, Franklin Lakes, NJ) 48 h prior to the start of the studies. During sample collection, 3 mL of blood was added to the spiked EDTA tube. The samples were immediately placed on ice, centrifuged within 30 min of collection, and the plasma was placed into cryovials. The cryovials were stored at -80°C until analysis. Substance P levels were determined using the methods described by Van Engen et al. (2014) using non-extracted plasma.

Kinematic Gait Analysis. A total of 2,184 gait readings made up the data set. As described in Kleinhenz et al. (2019b), a commercially available kinematic gait system (Walkway, Tekscan, Inc., South Boston, MA) was used to record gait and biomechanical parameters. The gait system was calibrated, using a known mass, daily and before each use of the computer software to ensure accuracy of the measurements at each time point. Video synchronization was used to ensure consistent gait between and within calf at each time point. Using research specific software (Walkway 7.7, Tekscan, Inc.), force, contact pressure, and impulse were assessed.

Visual Analog Scale. A total of 312 VAS scores made up the data set. As described in Martin et al. (2020a), a daily VAS pain assessment was conducted by two trained evaluators blinded to treatment allocations. The VAS used was a 100 mm (10 cm) line anchored by descriptors of "No Pain" on the left (0 cm) and "Severe Pain" on the right (10 cm). Five parameters were used to assess pain: depression, tail swishing or flicking, stance, head carriage, and foot stomping or kicking. No pain was characterized by being alert and quick to show interest, no tail swishing, a normal stance, head held above spine level, and absence of foot stomping. Severe pain was characterized by being dull and showing no interest, more than three

tail swishes per minute, legs abducted, head held below spine level, and numerous stomps. The evaluator marked the line between the 2 descriptors to indicate the pain intensity. A millimeter scale was used to measure the score from the zero anchor point to the evaluator's mark. The mean VAS measures of the two evaluators were combined into one score for statistical analysis.

Clinical Illness Score. A total of 312 CIS measures made up the data set. A CIS was assigned by two trained evaluators blinded to treatment allocations. The CIS consisted of: (1) is a normal healthy animal, (2) slightly ill with mild depression or gauntness, (3) moderately ill demonstrating severe depression/labored breathing/nasal or ocular discharge, and (4) severely ill and near death showing minimal response to human approach. If either evaluator scored a calf >1, that score was used for statistical analysis, with 1 being considered normal and greater than 1 considered abnormal.

Computerized Lung Score. A total of 312 computerized lung scores made up the data set. A computerized stethoscope (Whisper, Merck Animal Health, De Soto, KS) was used to analyze lung and heart sounds via a machine-learning algorithm that assigns a lung score from 1-5, with 1 being normal and 5 being severely compromised lung tissue (Nickell et al., 2020). The bell of the lung stethoscope was placed approximately two inches caudal and dorsal to the right elbow of each calf, and lung sounds were recorded for 8 s. If the recording was deemed acceptable by the computer program, the score was recorded.

Average Activity and Rumination. A total of 208 average activity and rumination scores made up the data set. A 3-axis accelerometer ear-tag (Allflex Livestock Intelligence, Madison, WI) was used to quantify activity and rumination throughout the study. The average number of active and ruminating minutes over 60 min time periods for the study duration was then calculated.

Step Count. A total of 208 step counts made up the data set. IceTag (IceRobotics Ltd, South Queensferry, Edinburgh, Scotland UK) accelerometers were placed on the left rear leg of each calf for the duration of the study. Accelerometer ID was paired with calf ID prior to placement onto the calf. Accelerometers were removed at the conclusion of the study and data was downloaded from the accelerometers for analysis.

Prostaglandin E_2 Metabolite. A total of 78 samples were analyzed for prostaglandin E_2 metabolites (PGEM). Prostaglandin E_2 metabolites were analyzed using a commercially available ELISA kit (cat. no. 514531, Cayman Chemical, Ann Arbor, MI) following manufacturer specifications with minor modifications. Sample input was adjusted to 375 µL with 1.5 mL ice-cold acetone added for sample purification. Samples were incubated at -20°C for 30 min., then centrifuged at 3,000 x g for 5 min. Supernatant was transferred to clean 13 x 100 mm glass tubes and evaporated using a CentriVap Concentrator (cat. no. 7810014, Labconco, Kansas City, MO) overnight (approx. 18h). Samples were reconstituted with 375 µL of appropriate kit buffer. A 300 µL aliquot of the reconstituted sample was derivatized with proportionally adjusted kit components. Manufacturer protocol was then followed. Samples were diluted 1:2 and ran in duplicate. Absorbance was measured at 405 nm after 60 min of development (SpectraMax i3, Molecular Devices, San Jose, CA). Sample results were excluded if the raw read exceeded the raw read of the highest standard (Standard 1; 50 pg/mL) or was below the lowest acceptable standard. The lowest acceptable standard was defined for each individual plate and was identified by excluding standards that had a ratio of absorbance of that standard to the maximum binding of any well ($\%B/B_0$) of $\geq 80\%$ or $\leq 20\%$. Any individual sample outside the standard curve, with a $\%B/B_0$ outside the 20-80% range, or a CV > 15% were re-analyzed.

Serum Amyloid A. A total of 312 serum amyloid A samples made up the data set. Serum Amyloid A concentrations were determined in serum samples using an ELISA assay (Phase Range Multispecies SAA ELISA kit; Tridelta Development Ltd. Cat no. TP802). Manufacturer specifications were followed and samples were diluted as necessary. Absorbance was measured at 450 nm on a SpectraMax i3 plate reader (Molecular Devices). Raw data was analyzed using MyAssays Desktop software (version 7.0.211.1238) for concentration determination. Standard curves were plotted as a 4-parameter logistic curve. Samples with a coefficient of variation (CV) > 15% were re-analyzed.

Rectal Temperature. A total of 260 rectal temperature samples made up the data set. Rectal temperatures were taken by placing a digital thermometer (180 Innovations, Lakewood, CO) against the wall of the rectum until a temperature reading was produced on the screen of the thermometer.

Receiver Operating Characteristic Curve Determination. All statistics were performed using statistical software (JMP Pro 14.0, SAS Institute, Inc., Cary, NC). Receiver operating characteristic curves were created for each timepoint, with AUC values comparing >10% lung lesions to <10%, with >10% as the positive control. The biomarker was plotted as the x-coordinate and the status (>10% or <10%) was plotted as the y-coordinate. Bootstrapping via fractional weights was used to generate confidence intervals for each AUC value. AUC values ≥ 0.7 were considered to yield good diagnostic accuracy (Yang and Berdine, 2017). Specific cut-off values were selected based upon optimized specificity and sensitivity values. Positive and negative predictive values with confidence intervals were calculated for each AUC value (MedCalc Software Ltd, Ostend, Belgium).

RESULTS

Five calves received tildipirosin as an intervention and all had lung lesion scores > 10%. Seven of the eight calves who received flunixin had lung lesion scores > 10%. The biomarker parameter AUC values are outlined in **Table 2** with rankings in **Table 3.**

Average Activity and Rumination. BRD study results comparing calves with significant lung lesions to those without yielded good diagnostic accuracy (AUC > 0.7; 95% CI: 0.70 to 0.90) for average activity at 0 h (cutoff: 27.67 min, PPV: 88.48%, NPV: 82.41%), 24 h (cutoff: 23 min, PPV: 100%, NPV: 38.98%), 48 h (cutoff: 23.33 min, PPV: 89.96%, NPV: 83.39%), 72 h (cutoff: 23.58 min, PPV: 90.44%, NPV: 100%), 96 h (cutoff: 21.58 min, PPV: 100%, NPV: 46.76%), 120 h (cutoff: 22.75 min, PPV: 94.10%, NPV: 66.75%), 144 h (cutoff: 25.75 min, PPV: 86.32%, NPV: 100%) and 168 h (cutoff: 19.55 min, PPV: 100%, NPV: 43.85%) (**Fig. 1**). BRD study results comparing calves with significant lung lesions to those without yielded good diagnostic accuracy (AUC > 0.7; 95% CI: 0.72 to 0.82) for average rumination at 48 h (cutoff: 23.92 min, PPV: 100%, NPV: 43.85%) and 120 h (cutoff: 42.67 min, PPV: 88.19%, NPV: 55.65%).

Step Count. BRD study results comparing calves with significant lung lesions to those without yielded good diagnostic accuracy (AUC > 0.7; 95% CI: 0.73 to 0.85) for average step count at 0 h (cutoff: 1606 steps, PPV: 88.75%, NPV: 61.32%), 48 h (cutoff: 1075 steps, PPV: 90.44%, NPV: 100%), 72 h (cutoff: 1271 steps, PPV: 90.44%, NPV: 100%), 96 h (cutoff: 1136 steps, PPV: 75.81%, NPV: 35.17%), 120 h (cutoff: 1352 steps, PPV: 90.44%, NPV: 100%), 144 h (cutoff: 1383 steps, PPV: 90.44%, NPV: 100%), and 168 h (cutoff: 878 steps, PPV: 85.63%, NPV: 79.19%) (**Fig. 2**).

Rectal Temperature. BRD pain study results comparing calves with significant lung lesions to those without yielded good diagnostic accuracy (AUC > 0.7; 95% CI: 0.71 to 0.90) for rectal temperature at 0 h (cutoff: 39.17 °C, PPV: 86.49%, NPV: 53.94%), 24 h (cutoff: 39.67 °C, PPV: 92.83%, NPV: 50.10%), 48 h (cutoff: 38.94 °C, PPV: 93.73%, NPV: 60.09%) and 72 h (cutoff: 39.33 °C, PPV: 100%, NPV: 46.76%).

Visual Analog Scale. BRD study results comparing calves with significant lung lesions to those without yielded good diagnostic accuracy (AUC > 0.7; 95% CI: 0.72 to 0.84) for VAS score at 0 h (cutoff: 8.5, PPV: 92.28%, NPV: 53.94%), 48 h (cutoff: 6, PPV: 90.44%, NPV: 100%), 120 (cutoff: 9.5, PPV: 100%, NPV: 41.27%), 144 h (cutoff: 7, PPV: 88.85%, NPV: 62.59%), 168 h (cutoff: 1, PPV: 91.64%, NPV: 42.95%) and 192 h (cutoff: 8.5, PPV: 84.16%, NPV: 57.24%) (**Fig. 3**).

Serum Cortisol. BRD study results comparing calves with significant lung lesions to those without yielded good diagnostic accuracy (AUC > 0.7; 95% CI: 0.76 to 0.81) for cortisol at 96 h (cutoff: 1.17 ng/mL, PPV: 92.06%, NPV: 67.44%) and 168 h (cutoff: 3.82 ng/mL, PPV: 100%, NPV: 66.36%).

Kinematic Gait Analysis. BRD study results comparing calves with significant lung lesions to those without yielded good diagnostic accuracy (AUC > 0.7; 95% CI: 0.70 to 0.82) for right front stride length at 0h (cutoff: 122.4 cm, PPV: 92.26%, NPV: 71.55%), 24 h (cutoff: 110.5 cm, PPV: 100%, NPV: 45.42%), 72 h (cutoff: 113.9 cm, PPV: 88.14%, NPV: 55.21%) and 96 h (cutoff: 125.8 cm, PPV: 81.84%, NPV: 100%).

BRD study results comparing calves with significant lung lesions to those without yielded good diagnostic accuracy (AUC > 0.7; 95% CI: 0.75 to 0.76) for right front force at 192 h (cutoff: 89.89 kg, PPV: 88.63%, NPV: 59.95%).

BRD study results comparing calves with significant lung lesions to those without yielded good diagnostic accuracy (AUC > 0.7; 95% CI: 0.73 to 0.74) for right front impulse at 192 h (cutoff: 45.83 kg*sec, PPV: 88.63%, NPV: 59.95%).

BRD study results comparing calves with significant lung lesions to those without yielded good diagnostic accuracy (AUC > 0.7; 95% CI: 0.73 to 0.74) for right front pressure at 144 h (cutoff: 7.8 kg/cm^2, PPV: 84.39%, NPV: 100%) and 192 h (cutoff: 4.8 kg/cm^2, PPV: 100%, NPV: 41.13%).

BRD study results comparing calves with significant lung lesions to those without yielded good diagnostic accuracy (AUC > 0.7; 95% CI: 0.73 to 0.79) for gait distance at 120 h (cutoff: 163.2 cm, PPV: 88.63%, NPV: 59.95%) and 144 h (cutoff: 122.4 cm, PPV: 83.67%, NPV: 77.85%).

BRD study results comparing calves with significant lung lesions to those without yielded good diagnostic accuracy (AUC > 0.7; 95% CI: 0.73 to 0.79) for gait velocity at 32 h (cutoff: 95.4 cm/s, PPV: 93.83%, NPV: 50.10%) and 48 h (cutoff: 98.3 cm/s, PPV: 84.16%, NPV: 57.24%).

Prostaglandin E_2 Metabolite. BRD study results comparing calves with significant lung lesions to those without yielded good diagnostic accuracy (AUC > 0.7; 95% CI: 0.70 to 0.78) for PGEM concentration at 0 h (cutoff: 31.26 pg/mL, PPV: 87.23%, NPV: 60.96%), 72 h (cutoff: 28.4 pg/mL, PPV: 100%, NPV: 41.27%) and 192 h (cutoff: 17.54 pg/mL, PPV: 88.19%, NPV: 55.65%) (**Fig. 4**).

Clinical Illness Score. BRD pain study results comparing calves with significant lung lesions to those without yielded good diagnostic accuracy (AUC > 0.7; 95% CI: 0.72 to 0.78) for

CIS at 48 h (cutoff: 1, PPV: 92.83%, NPV: 50.10%) and 96 h (cutoff: 1, PPV: 91.64%, NPV: 42.95%).

Infrared Thermography. BRD study results comparing calves with significant lung lesions to those without yielded good diagnostic accuracy (AUC > 0.7; 95% CI: 0.70 to 0.75) for IRT at 0 h (cutoff: 31.06 °C, PPV: 87.23%, NPV: 60.96%), 32 h (cutoff: 33.89 °C, PPV: 100%, NPV: 38.98%) and 192 h (cutoff: 29.17 °C, PPV: 37.45%, NPV: 80.06%).

Mechanical Nociception Threshold. BRD study results comparing calves with significant lung lesions to those without yielded good diagnostic accuracy (AUC > 0.7; 95% CI: 0.70 to 0.75) for MNT percent change from baseline at 8 h (cutoff: 0.23 kg F, PPV: 88.19%, NPV: 55.65%).

Substance P. BRD study results comparing calves with significant lung lesions to those without yielded good diagnostic accuracy (AUC > 0.7; 95% CI: 0.70 to 0.71) for substance P at 0 h (cutoff: 388.52 pg/mL, PPV: 90.38%, NPV: 42.26%).

Computerized Lung Score. BRD pain study results comparing calves with significant lung lesions to those without yielded good diagnostic accuracy (AUC > 0.7; 95% CI: 0.70 to 0.71) for CLS at 96 h (cutoff: 1, PPV: 84.16%, NPV: 57.24%).

Serum Amyloid A. BRD pain study results comparing calves with significant lung lesions to those without yielded good diagnostic accuracy (AUC > 0.7; 95% CI: 0.70 to 0.71) for SAA concentrations at 192 h (cutoff: 55.36 μg/mL, PPV: 89.44%, NPV: 71.51%).

DISCUSSION

Cattle with advanced lung lesions at harvest have been shown to have reduced average daily gain; hot carcass weight; kidney, pelvic and heart fat; 12th rib fat; calculated yield grades;

marbling scores and percentage choice carcasses (Schneider et al., 2009; Tennant et al., 2014; Blakebrough-Hall et al., 2020), all resulting in economic losses. This creates a need for the development of robust biomarkers to objectively predict BRD early on and apply an intervention to prevent the further development of lung lesions.

Biomarkers previously used as diagnostic tools for BRD diagnosis include cortisol concentrations (Theurer et al., 2013; Foote et al., 2017), infrared thermography (Baruch et al., 2019), computerized lung score (Zeineldin et al., 2016; Baruch et al., 2019; Nickell et al., 2020), acute phase protein levels (Abdallah et al., 2016), clinical illness score (Amrine et al., 2013), and activity and behavior measurements (Hanzlicek et al., 2010; White et al., 2012; Theurer et al., 2013; Pillen et al., 2016; Tomczak et al., 2019).

Accelerometers have been used to monitor animal behavior without the presence of human evaluators which can reduce subjectivity (White et al., 2008). Our results showed the highest AUC values to be associated with average activity levels and step count indicating that they may yield the best diagnostic accuracy. Diagnostic accuracy remained strong throughout the duration of the study, indicating that activity levels and step count may be viable options for quantifying differences early in the BRD disease process as well as during the disease progression.

Rectal temperatures yielded good diagnostic accuracy, particularly between 24 and 72 h after disease onset. In previous BRD challenge studies, rectal temperature differed between days when animals were diseased when compared with healthy (Baruch et al., 2019) and calves exhibited fever as part of the sickness response on d 3-7 (Toaff-Rosenstein et al., 2016).

Visual analog scale assessment is a method of evaluating pain intensity based on behavioral parameters. Our results revealed good diagnostic accuracy for VAS values out to 9

days following inoculation which may be due to the nature of BRD causing more chronic changes that VAS scoring captured 7-9 days post inoculation. Cutoff values did not decrease throughout the study, supporting the idea that calves were painful for the study duration.

Cortisol results from the present study revealed higher AUC values later in the study indicating that there may be meaningful differences in cortisol levels more chronically and less acutely when identifying calves with lung lesions. These results are similar to our previous findings for AUC values for lameness which is also a more chronic condition (Kleinhenz et al., 2019a). Cutoff values were lower than cutoff values from previous findings, indicating that BRD may not cause as acute or magnified of a cortisol response as some procedures such as castration and dehorning.

Kinematic gait analysis in cattle has not been well-characterized in the past but is becoming more prevalently used as a diagnostic tool (Pairis-Garcia et al., 2015; Kleinhenz et al., 2018; Kleinhenz et al., 2019c). A commercially available floor mat–based gait system can be used to assess variables such as gait distance and weight distribution (Coetzee et al., 2017). Our results yielded good diagnostic accuracy for right front stride length. In the present study the right apical lung lobes were inoculated indicating that gait measurements more specific to the area of trauma may yield better results.

Bacterial infections such an pneumonia cause the activation of monocytes and macrophages and release of inflammatory mediators such as prostaglandin E_2 (Idoate et al., 2015). Our results revealed good diagnostic accuracy for PGEM concentrations with cutoff values decreasing over the duration of the study. Concentrations of PGEM were only quantified at three timepoints throughout the study which all yielded good diagnostic accuracy, at 0, 72, and

192 h indicating that PGEM concentrations maintained good diagnostic accuracy throughout the study.

Clinical illness scores yielded good diagnostic accuracy 48 and 96 h after BRD onset but did not continue to yield good diagnostic accuracy throughout the study duration. Amrine et al. (2013) found that interobserver agreement for assigning CIS was low and CIS accuracy relative to pulmonary consolidation varied based upon the severity of consolidation.

The AUC values from this analysis did not indicate a clear pattern for when IRT may yield good diagnostic accuracy. An acute epinephrine release would be more likely to occur shortly after an acutely painful procedure such as dehorning relative to respiratory disease event. Environmental factors along with distance from the animal can be very influential upon IRT readings (Church et al., 2014). Sampling timepoints being at different points throughout the day resulted in varying ambient temperatures that likely influenced results.

Determining mechanical nociception threshold via a pressure algometer can establish the minimal amount of pressure that produces a response. The AUC values indicated poorer diagnostic accuracy relative to previous findings when MNT was used in a dehorning study (Kleinhenz et al., 2017). Our results also revealed higher cutoff values relative to dehorning cutoff values. This may be due to calves being more sensitive to force around their horn buds compared to calves being relatively less sensitive to force in their thoracic region.

Our results showed poor diagnostic accuracy for substance P when identifying calves with lung lesions. Previous cattle pain studies did not find a difference in substance P levels between calves likely experiencing pain from procedures such as dehorning and castration compared to sham calves who likely were not in pain (Kleinhenz et al., 2017; Kleinhenz et al., 2018). Higher cutoff values were observed in our results relative to previous findings indicating

that BRD may result in higher substance P levels relative to castration and dehorning studies (Kleinhenz et al., 2017; Kleinhenz et al., 2018).

Computerized lung scores only seemed to yield good diagnostic accuracy at 96 h after BRD onset. Baruch et al. (2019) when examining the association between computerized lung score relative to lung consolidation found a trend towards significance with a difference only between normal and moderate acute scores. This analysis did not differentiate between mild and moderate acute CLS. This analysis used a cutoff of <10% consolidation while (Baruch et al., 2019) found a mean of 13.7% lung consolidation to be associated with a normal CLS score.

Our results yielded poor diagnostic accuracy for serum amyloid A concentrations which was likely due to all calves having elevated SAA levels regardless of lung lesions. Higher levels of SAA were observed in the present study compared to levels in previously recorded naturally occurring BRD in calves (Joshi et al., 2018). In the present study, the cutoff values were most elevated at 8 h and steadily decreased for the remainder of the study, indicating that a SAA response was observed.

The impact on animal well-being from BRD is considerable (Hanzlicek et al., 2010). Pleuritic chest pain as a result of bacterial pneumonia is commonly reported in human medicine (Boyd et al., 2006); however, published literature regarding pain associated with lung lesions as a result of bacterial pneumonia in cattle is lacking. The biomarkers quantified in the present study varied in their specificity to BRD, objectivity, and association with pain. Through previous ROC analysis we identified cortisol, hair cortisol and infrared thermography imaging as having acceptable (AUC > 0.7) diagnostic sensitivity and specificity for detecting pain in cattle (Martin et al., 2020b). In the present study, average activity levels yielded good diagnostic accuracy for predicting lung lesions and were objective, but not specific to pain. Visual analog scale scores

yielded good diagnostic accuracy and were somewhat specific to pain but were not specific to BRD and were subjective. Rectal temperatures yielded good diagnostic accuracy and were objective but were not specific to BRD or pain. Identifying biomarkers that are (1) predictive of lung lesions and (2) specific to pain will require further investigation into diagnostic tools and biomarkers to quantify pain from BRD.

CONCLUSIONS

In the first 72 h after onset of BRD, right front stride length, gait velocity, VAS, CIS, average activity level, step and rectal temperature yielded the best diagnostic accuracy (AUC > 0.75) for predicting calves with significant lung lesions (>10% consolidation) at necropsy compared to those with < 10% lung lesions. After 72 post-induction, PGEM, gait distance, cortisol, IRT, right front force, average activity level, step count and serum amyloid A yielded the best diagnostic accuracy (AUC > 0.75) for predicting the severity of lung lesions. These results indicate that ROC analysis can characterize the predictive value of biomarkers associated with pain and inflammation in cattle with induced bacterial pneumonia. AUC values for VAS score, average activity levels, step count and rectal temperature seemed to yield very good diagnostic accuracy (AUC > 0.75) at multiple timepoints while average and percent change MNT values, substance P concentrations, and CLS did not (all AUC values < 0.75). These results can be used to guide refinement of the optimal timepoints and biomarkers for the diagnosis of significant lung lesions after BRD.

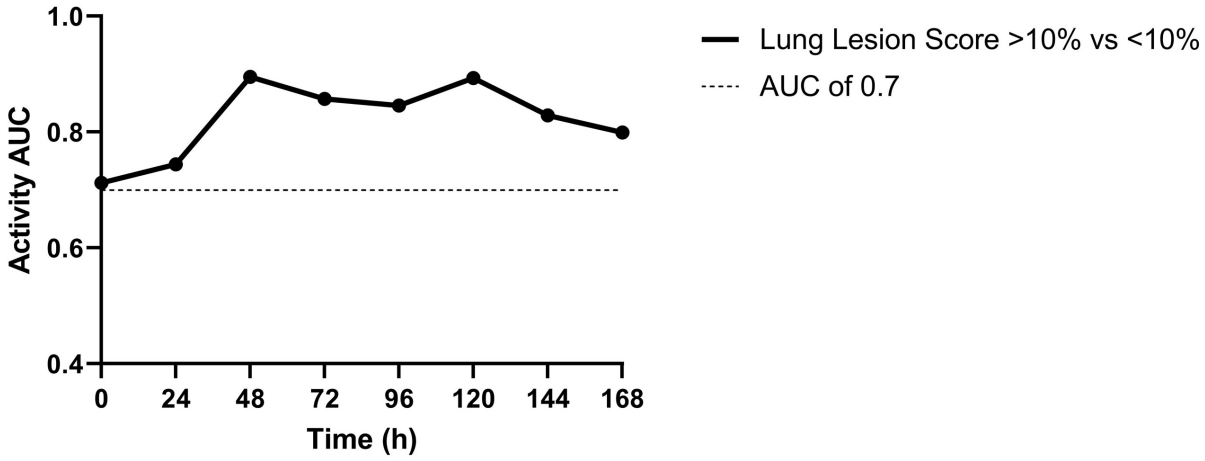

Figure 3.1 Area under the curve (AUC) values for average activity levels

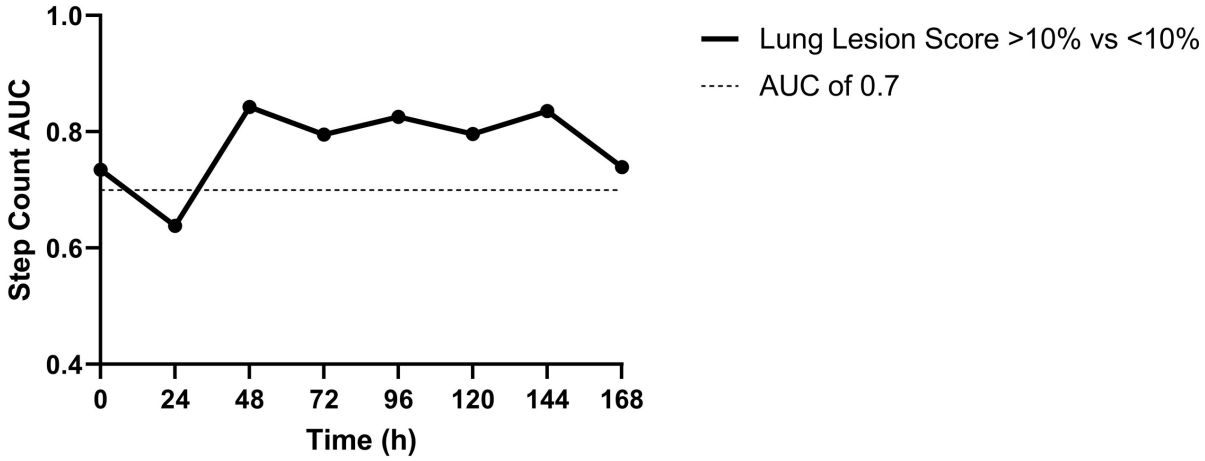

Figure 3.2 Area under the curve (AUC) values for average step count

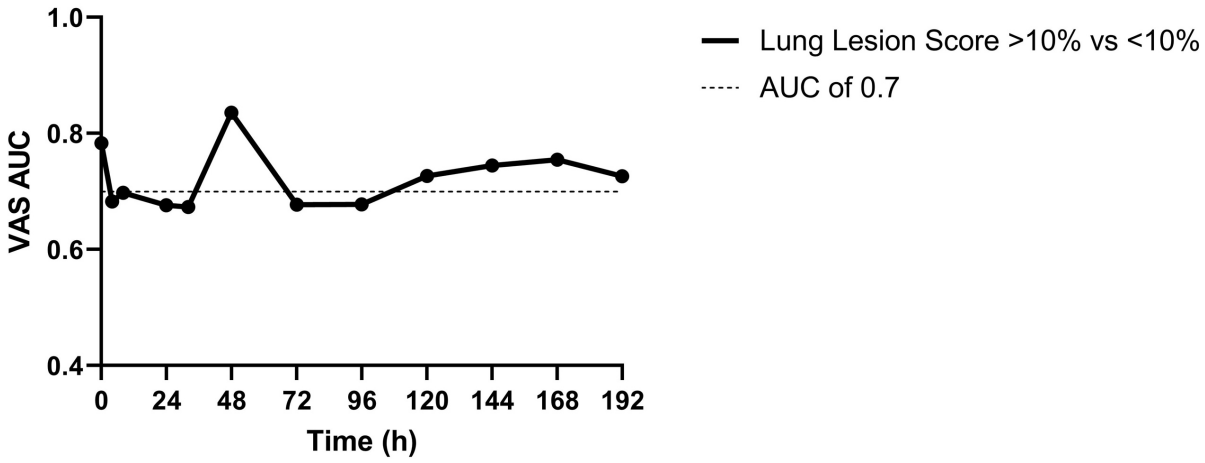

Figure 3.3 Area under the curve (AUC) values for visual analog scale (VAS) scores

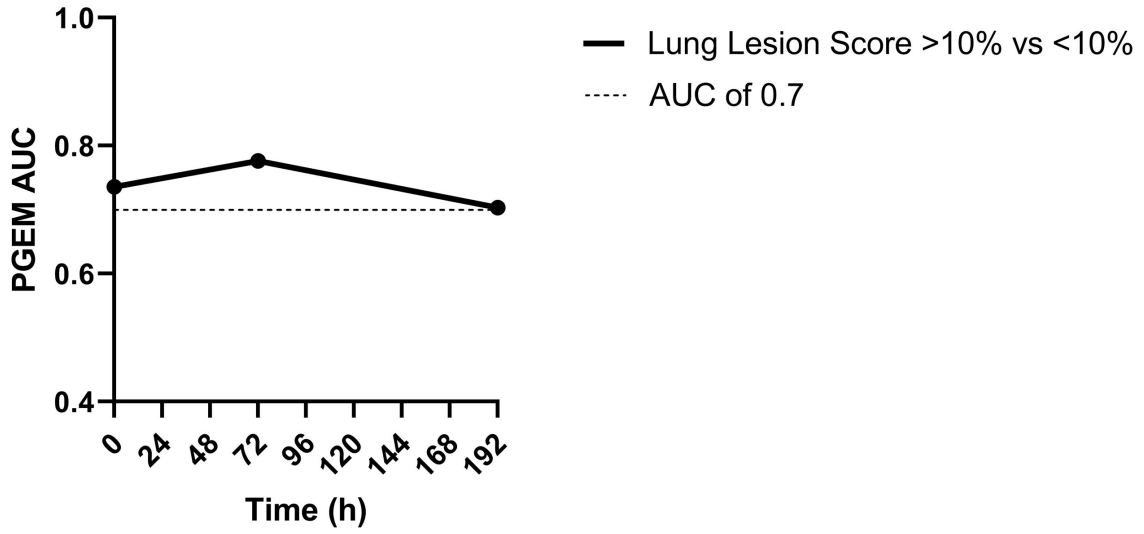

Figure 3.4 Area under the curve (AUC) values for prostaglandin E_2 metabolite (PGEM) concentration

Table 3.1 Collection timepoints for each BRD biomarker with 0 h being time of disease onset

Biomarker	Timepoint
Cortisol, h	0, 4, 8, 24, 32, 48, 72, 96, 120, 144, 168, 192
IRT[1], h	0, 4, 8, 24, 32, 48, 72, 96, 120, 144, 168, 192
MNT[2], h	8, 24, 72, 192
Substance P, h	0, 4, 8, 24, 32, 48, 72, 96, 120, 144, 168, 192
Kinematic gait analysis, h	0, 4, 8, 24, 32, 48, 72, 96, 120, 144, 168, 192
VAS[3], h	0, 4, 8, 24, 32, 48, 72, 96, 120, 144, 168, 192
CIS[4], h	0, 4, 8, 24, 32, 48, 72, 96, 120, 144, 168, 192
Computerized lung score, h	0, 4, 8, 24, 32, 48, 72, 96, 120, 144, 168, 192
Average activity, h	0, 24, 48, 72, 96, 120, 144, 168
Average rumination, h	0, 24, 48, 72, 96, 120, 144, 168
Step count, h	0, 24, 48, 72, 96, 120, 144, 168
PGEM[5], h	0, 72, 192
Serum amyloid A, h	0, 4, 8, 24, 32, 48, 72, 96, 120, 144, 168, 192
Rectal temperature, h	0, 8, 24, 32, 48, 72, 96, 120, 144, 168

[1]IRT = infrared thermography

[2]MNT = mechanical nociceptive threshold

[3]VAS = visual analog scale

[4]CIS = clinical illness score

[5]PGEM = prostaglandin E_2 metabolites

Table 3.2 AUC values for lung scores <10% vs. lung scores < 10% by collection timepoint for each biomarker

Biomarker	h											
	0	4	8	24	32	48	72	96	120	144	168	192
Cortisol	0.64	0.59	0.63	0.60	0.66	0.63	0.64	0.77	0.69	0.68	0.81	0.67
IRT[1]	0.74	0.64	0.69	0.59	0.71	0.64	0.58	0.69	0.66	0.69	0.67	0.75
MNT[2] avg			0.60	0.69			0.67					0.64
MNT percent change			0.74	0.60			0.58					0.66
Substance P	0.71	0.69	0.63	0.68	0.65	0.64	0.61	0.61	0.62	0.59	0.64	0.59
Rf[3] stance time	0.61	0.62	0.59	0.62	0.62	0.62	0.61	0.60	0.67	0.60	0.61	0.66
Rf stride length	0.81	0.64	0.67	0.79	0.62	0.65	0.72	0.70	0.62	0.63	0.60	0.61
Rf force	0.65	0.63	0.65	0.60	0.58	0.64	0.64	0.60	0.61	0.61	0.58	0.76
Rf impulse	0.59	0.62	0.61	0.65	0.61	0.60	0.59	0.59	0.64	0.62	0.59	0.73
Rf pressure	0.62	0.64	0.62	0.65	0.62	0.56	0.61	0.68	0.61	0.72	0.64	0.74
Gait distance	0.60	0.65	0.66	0.62	0.69	0.61	0.64	0.62	0.78	0.73	0.59	0.68
Gait velocity	0.58	0.61	0.62	0.68	0.77	0.70	0.66	0.67	0.62	0.60	0.59	0.61
VAS[4]	0.78	0.68	0.70	0.68	0.67	0.84	0.68	0.68	0.73	0.75	0.75	0.73
CIS[6]	0.65	0.58	0.60	0.59	0.60	0.77	0.62	0.72	0.64	0.68	0.64	0.67
Computerized lung score	0.59	0.58	0.58	0.68	0.61	0.58	0.56	0.71	0.64	0.57	0.64	0.56
Average activity	0.71			0.74		0.90	0.86	0.85	0.89	0.83	0.80	
Average rumination	0.63			0.58		0.81	0.68	0.68	0.73	0.68	0.69	
Step count	0.74			0.64		0.84	0.80	0.83	0.80	0.84	0.74	
PGEM[6]	0.74						0.78					0.70
Serum Amyloid A	0.62	0.59	0.53	0.62	0.59	0.62	0.63	0.61	0.61	0.66	0.63	0.71
Rectal Temperature	0.72		0.69	0.78	0.63	0.90	0.82	0.66	0.61	0.57	0.66	

[1]IRT = infrared thermography

[2]MNT = mechanical nociceptive threshold

[3]Rf = right front

[4]VAS = Visual Analog Scale

[5]CIS = clinical illness score

[6]PGEM = prostaglandin E_2 metabolites

Table 3.3 AUC value rankings > 0.7 for each biomarker by collection timepoint (h) with 0 h being the time of disease onset

	Ranking											
Biomarker	1		2		3		4		5		6	
	AUC	Timepoint	AUC	Timepoint	AUC	Timepoint	AUC	Timepoint	AUC	Timepoint	AUC	Timepoint
Average activity	0.90	48	0.89	120	0.86	72	0.85	96	0.83	144	0.80	168
Rectal temperature	0.90	48	0.82	72	0.78	24	0.72	0				
Step count	0.84	48	0.84	144	0.83	96	0.80	72	0.80	120	0.74	168
VAS[4]	0.84	48	0.78	0	0.75	144	0.75	168	0.73	120	0.73	192
Cortisol	0.81	168	0.77	96								
Rf[3] stride length	0.81	0	0.79	24	0.72	72	0.70	96				
Gait distance	0.78	120	0.73	144								
PGEM[6]	0.78	72	0.74	0	0.70	192						
CIS[6]	0.77	48	0.72	96								
IRT[1]	0.77	192	0.71	32								
Gait velocity	0.77	32	0.70	48								
Rf force	0.76	192										
MNT percent change	0.74	8										
Rf pressure	0.74	192	0.72	144								
Rf impulse	0.73	192										
Substance P	0.71	0										
Computerized lung score	0.71	96										
Serum Amyloid A	0.71	192										

[1]IRT = infrared thermography

[2]MNT = mechanical nociceptive threshold

[3]Rf = right front

[4]VAS = Visual Analog Scale

[5]CIS = clinical illness score

[6]PGEM = prostaglandin E_2 metabolites

Table 3.4 Cutoff values for lung scores <10% vs. lung scores < 10% by collection timepoint for each biomarker

Biomarker	Units	h											
		0	4	8	24	32	48	72	96	120	144	168	192
Cortisol	ng/mL	9.72	14.54	2.84	11.75	1.8	6.12	5.02	1.17	2.64	2.69	3.82	1.03
IRT[1]	°C	31.06	34.56	34.5	31.11	33.89	30.61	27.89	30.22	32.22	28.11	27.89	29.17
MNT[2] avg	kg F			1.84	1.30			1.97					1.09
MNT percent change	%			0.23	-0.34			0.40					0.21
Substance P	pg/mL	388.52	409.71	473.29	374.61	129.31	360.21	344.13	185.86	113.19	368.81	146.49	484.05
Rf[3] stance time	s	0.71	0.94	0.62	0.47	0.80	0.68	0.85	0.86	0.81	1.14	0.67	0.87
Rf stride length	cm	122.4	127.5	112.2	110.5	120.7	125.8	113.9	125.8	115.6	113.9	110.5	112.2
Rf force	kg	99.35	82.5	105.27	81.01	69.36	95.62	80.73	82.73	94.44	94.18	99.41	89.89
Rf impulse	kg*sec	60.24	71.21	50.74	32.58	46.8	35.61	62.69	50.85	35.69	50.17	36.62	45.83
Rf pressure	kg/cm^2	4.7	7.3	5.2	5	6.2	5.8	4.8	4.8	4.6	7.8	5	4.8
Gait distance	cm	166.6	168.3	137.7	175.1	161.5	190.4	170.0	190.4	163.2	122.4	171.7	161.5
Gait velocity	cm/s	86.8	138.8	122.9	88.5	95.4	98.3	92.2	71.9	84.1	67.5	85.0	82.4
VAS[4]	1-10 cm	8.5	6.5	9.0	6.5	6.0	6.0	5.5	8.5	9.5	7.0	11.0	8.5
CIS[6]	0 = normal, ≥ 1 abnormal	1	1	1	1	1	1	1	1	1	1	1	1
Computerized lung score	0 = normal, ≥ 1 abnormal	1	0	0	1	0	0	0	1	1	0	1	0
Average activity	60 min avg	27.67			23.00		23.33	23.58	21.58	22.75	25.75	19.55	
Average rumination	60 min avg	37.33			13.17		23.92	44.50	37.33	42.67	33.08	26.64	
Step count	count	1606			1549		1075	1271	1136	1352	1383	878	

PGEM[6]	pg/mL	31.26						28.40					17.54
Serum Amyloid A	µg/mL	423.73	419.50	484.64	321.17	251.03	305.07	237.07	132.47	73.07	54.68	77.28	55.36
Rectal Temperature	°C	39.17		39.78	39.67	39.67	38.94	39.33	39.05	38.17	38.94	38.78	

[1]IRT = infrared thermography

[2]MNT = mechanical nociceptive threshold

[3]Rf = right front

[4]VAS = Visual Analog Scale

[5]CIS = clinical illness score

[6]PGEM = prostaglandin E_2 metabolites

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Chapter 4 - Comparison of the effect of tildipirosin administered alone or in combination with transdermal flunixin on the performance, health, activity and well-being of transported feedlot calves on arrival at the feedlot

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ABSTRACT

Long distance transportation can be a significant source of stress to cattle and is associated with increased risk of bovine respiratory disease (BRD). The administration of a nonsteroidal anti-inflammatory drug (NSAID) has been shown to reduce stress following long distance transport. The objective of this study was to compare performance, health, accelerometer activity, and well-being between calves receiving either tildipirosin (Zuprevo 18%; Merck Animal Health, Madison, NJ) alone or in combination with transdermal flunixin (BANAMINE Transdermal Pour-on Solution; Merck Animal Health, Madison, NJ) on arrival at the feedlot. Three hundred eighty-four polled, Continental x English and English crossbred bulls (n = 199) and steers (n = 185) were enrolled into one of two treatments: 1) tildipirosin administered in the neck as a single dose of 4 mg/kg only (PLBO) 2) tildipirosin in combination with transdermal flunixin applied to the dorsal midline at a dose of 3.3 mg/kg (FTD). Outcomes measured were average daily gain (ADG), dry mater intake (DMI), gain to feed, morbidity, mortality, accelerometer activity data, and a daily visual analog scale (VAS) assessment of well-being. Body weight (BW) was determined by weighing individual animals; ADG was calculated as initial BW – final BW ÷ total days on feed; DMI was calculated as daily pen feed allocation – feed remaining at next feeding ÷ number of calves in the pen; and gain to feed was calculated as pen level ADG ÷ pen level DMI. The VAS used was a 100 mm line anchored at each end by descriptors of “No Pain” or “Severe Pain”. Statistical analysis was performed using JMP 13 computer software using pen as the experimental unit, lot number as a random variable, and treatment as a fixed variable. There was no treatment effect on DMI (P = 0.51). During the first 14 d on feed, FTD calves had a lower ADG of 0.90 kg/d compared to 1.33 kg/d in the PLBO group (P = 0.05). There were no differences observed in morbidity and mortality between groups

(P = 0.29). There were no treatment differences from activity data (P = 0.19). The VAS assessment showed a significant time x treatment interaction (P < 0.001). During the first 36 hours after treatment administration, the FTD treated calves had lower VAS scores [6.23 (95% CI: 5.27 – 7.20) compared to 7.28 (95% CI: 6.32 – 8.24)] than PLBO (P < 0.05). Results suggest that FTD treated calves showed less signs of pain the first 36 h post-drug application relative to PLBO calves.

Key words: Anti-Inflammatory, Cattle, Drug, Nonsteroidal, Stress

INTRODUCTION

Negative effects of transporting calves are often a result of compounded stress due to weaning, social regrouping, and exposure to new pathogens (Fike and Spire, 2006). A strategy to reduce the acute-phase protein response elicited by transportation is to provide anti-inflammatory agents. Van Engen et al. (2014b) previously demonstrated that administration of the non-steroidal anti-inflammatory drug (NSAID) meloxicam PO at 1 mg/kg, reduced stress in calves following long distance transportation. Similarly, Cooke et al. (2013) demonstrated that flunixin meglumine administered intravenously at 1.1 mg/kg reduced the cortisol and acute-phase protein response elicited by road transport.

The incidence of bovine respiratory disease (BRD) is commonly associated with these stressors and is likely linked to changes in immune cell function and number. Inflammation from BRD within the lungs has a significantly negative affect upon performance parameters (Gifford et al., 2012). Most approaches to managing BRD are limited to vaccination and antibiotic use to decrease disease prevalence and severity (Penny, 2015). Developing strategies for physiological

biomarker identification, treatment methods, and predictive behaviors may help reduce BRD incidence (Van Engen and Coetzee, 2018).

Flunixin transdermal has been effective in reducing prostaglandin E2 concentrations. Studies suggest that the anti-inflammatory effects of topical flunixin may last up to 48 hours (Thiry et al., 2017). When flunixin is administered topically it is rapidly absorbed and has a longer half-life relative to intravenous administration (Kleinhenz et al., 2016). The impact of the co-administration of flunixin meglumine transdermal with an antimicrobial on arrival has not been investigated. If co-administration of flunixin transdermal with tildipirosin would improve the health and performance of high-risk calves on arrival at the feedlot, this would be beneficial to producers and veterinarians. The objective of this study was to compare performance, health, accelerometer activity and visual assessment of well-being between calves receiving either tildipirosin alone or in combination with flunixin meglumine transdermal at 3.3 mg/kg on arrival at the feedlot.

MATERIALS AND METHODS

Animals, Housing, and Treatments

The Institutional Animal Care and Use Committee of Kansas State University reviewed and approved the experimental protocol for this project (IACUC# 4002).

This study was conducted at the Kansas State University Stocker Unit near Manhattan, KS, between October 2017 and January 2018. Calves were assembled through market facilities in Tennessee and shipped 12 h to the Kansas State University Stocker Unit via 4 different truckloads over a 10 d period. Calves remained assigned to 4 lots respective of each truckload throughout the study. A total of 397 polled, Continental × English and English crossbred bulls and steers were received for potential enrollment into the study. One hundred ninety-nine bulls

and 185 steers were enrolled in the study, totaling 384 calves weighing an average of 218 kg. Calves displaying signs of illness or cryptorchidism were not enrolled. The study lasted 63 d from when calves arrived at the study site.

Upon arrival, calves were individually weighed and received pretreatment examinations which included: rectal temperature taken; ear notched for bovine viral diarrhea (BVDv) persistent infection testing (IDEXX Laboratories, Inc., Westbrook, ME); examined for health and physical abnormalities; and given an ear tag for visual identification and electronic identification (EID).

Calves were housed in 32 outdoor pens with dirt flooring of equal size (9.1 x 15.2 m) with 12 calves/pen. Diets were formulated to provide 1.32 Mcal NEg/kg DM and offered at 2.2% BW. All treatment groups were fed once daily at approximately 0700 and refusals were collected and weighed daily prior to feeding. The diet was formulated to contain 40% Sweet Bran (Cargill Animal Nutrition, Blair, NE) on a DM basis. Feed bunks in each pen were evaluated as required to allow for daily feed delivery adequate to ensure all calves had ad libitum access to feed without an excess of unconsumed feed accumulating in the feed bunk from 0 to 6 d. From 7 d to the end of the study (63 d) animals were fed according to bodyweight. Feeding was adjusting weekly based on the average animal bodyweight within the pen. Calves were fed once daily per normal procedures at the study site.

Approximately 12-24 h post-arrival, calves were vaccinated with a killed vaccine against *Clostridium chauvoei, septicum, novyi, haemolyticum, perfringens* Types C and D and *tetani* Calvary 9 (Calvary 9; Merck Animal Health, Madison, NJ) and a modified-live vaccine against infectious bovine rhinotracheitis (IBR), bovine viral diarrhea types 1 and 2 (BVDI-II), parainfluenza 3 (PI3), bovine respiratory syncytial virus (BRSV), and aid in the reduction and

severity of pneumonic pasteurellosis due to *Mannheimia haemolytica* (Vista Once SQ; Merck Animal Health, Madison, NJ). Calves were treated for internal parasites with 10% Fenbendazole (Safe-Guard, Merck Animal Health, Madison, NJ) administered at a dose of 5 mg/kg of BW. Implants (Ralgro; Merck Animal Health, Madison, NJ) were administered upon the initiation of this study in the right ear of each calf. All animals were revaccinated on d 14 (Vista Once SQ; Merck Animal Health, Madison, NJ).

Calves received as bulls were castrated at processing in accordance with standard industry practice. Briefly, the scrotum was cleaned and disinfected using a cloth towel saturated with dilute iodine. The skin was surgically incised using a sharp, disinfected Newberry knife. The testes and spermatic cords were then exteriorized by blunt dissection. Testicles were pulled and the tunica, fascia and blood vessels were stripped back as the testicles were pulled and removed.

Calves were blocked by sex upon arrival (bulls on arrival or steers on arrival) to ensure equal distribution of bulls and steers within each pen and by body weight, and then randomly assigned to one of two study treatments with 16 pens/treatment. The two treatments were: 1) Calves received tildipirosin at 4 mg/kg subcutaneously only (PLBO) 2) Calves received tildipirosin at 4 mg/kg in combination with flunixin meglumine transdermal at a target of 3.3 mg/kg (FTD).

Tildipirosin was administered as a single subcutaneous injection. Flunixin transdermal was administered at a target of 3.3 mg/kg with a mean dose of 3.45 mg/kg bodyweight (equivalent to 1 mL/15 kg bodyweight), ranging from 3.21 to 3.84 mg/kg. The volume dosed per calf was determined using the dosing gauge on the product packaging. Due to this method of dosing, calves in the FTD group were given a mean (± SEM) 25.47 ± 1.6 additional milligrams

of flunixin over their weight determined dose. A placebo was administered at the dose rate of 1 mL/15 kg in a similar manner as the flunixin transdermal. The placebo was made up of propylene glycol, isopropyl alcohol, and a red dye to mimic the test product in color, viscosity, and odor, as described in Kleinhenz et al. (2017). The flunixin transdermal and placebo were administered on dry skin. The entire dose was applied on the dorsal midline between the withers and tail head in accordance with label directions.

Measurements and Sample Collection

Outcome variables measured for the entire 63 d feeding period were individual animal weights, pen weights, daily feed delivered, morbidity and mortality. Additionally, visual analog scale (VAS) scores for pain assessment were obtained for the first 6 d. Accelerometer data were collected on a portion of the animals for the first 14 d to record animal activity. Individual animal weights were recorded on d 0, 14, and 63, and pen weights were determined by summing and averaging individual weights on these days.

Animals were observed twice daily for signs of morbidity that included overall depression, nasal and/or ocular discharge and anorexia. Any animal showing these signs was removed from the pen and taken to the hospital facilities where rectal temperature and a clinical illness score (CIS) were recorded. CIS were assigned as follows 1; normal healthy animal, 2; slightly ill with mild depression or gauntness, 3; moderately ill demonstrating severe depression/labored breathing/nasal or ocular discharge, and 4; severely ill and near death showing minimal response to human approach. Animals removed from the pen with a rectal temperature $\geq$ 40 °C and demonstrating a CIS $\geq$ 2 were treated following label instructions with the following compounds; at first morbidity animals received florfenicol (Nuflor: Merck Animal Health, Madison, NJ) administered as a single subcutaneous injection at a dose of 40 mg/kg body

weight; enrofloxacin (Baytril 100®: Bayer Animal Health, Shawnee, KS) was administered at second morbidity at 12.5 mg/kg subcutaneously; Oxytetracycline (300 PRO LA; Norbrook Animal Health, Overland Park, KS) was administered subcutaneously at 30 mg/kg bodyweight as a single dose on the third treatment. Following the third treatment, the animal was considered chronic and removed from the trial. A BRD post metaphylaxis interval (PMI) of 3 d after the use of tildipirosin on arrival, and a BRD post treatment interval (PTI) of 3 d after the use of both florfenicol and enrofloxacin was observed.

A daily VAS pain assessment was conducted on 3 calves received as steers and 3 calves received as bulls and castrated on arrival, per pen, by two trained evaluators blinded to treatment allocations. Calves were chosen using the RAND function in Microsoft Excel (Microsoft Excel® 2016, Microsoft Corporation, Redmond, WA). The VAS used was a 100 mm (10 cm) line anchored at each end by descriptors of "No Pain" or "Severe Pain". Five parameters were used to assess pain; depression, tail swishing or flicking, stance, head carriage, and foot stomping or kicking. No pain was characterized by being alert and quick to show interest, no tail swishing, a normal stance, head held above spine level, and absence of foot stomping. Severe pain was characterized by being dull and showing no interest, more than three tail swishes per minute, legs abducted, head held below spine level, and numerous stomps. The evaluator marked the line between the 2 descriptors to indicate the pain intensity. A millimeter scale was used to measure the score from the zero anchor point to the evaluator's mark. VAS assessments were taken every 12 h, starting 12 h after being processed onto the study and continuing for 6 d. The mean VAS measures of the two evaluators were combined into one score for statistical analysis.

IceTag (IceRobotics Ltd, South Queensferry, Edinburgh, Scotland UK) accelerometers were placed on 40 animals (10 per study lot x 4 lots) on the day of enrollment on at least one calf

per pen. Calves were chosen using the RAND function in Microsoft Excel (Microsoft Excel® 2016, Microsoft Corporation, Redmond, WA). Accelerometers were placed on the left rear legs. Accelerometer ID was paired with calf ID prior to placement onto the calf. Accelerometers were removed and collected at the time of revaccination (14 d) and returned to Dr. Ty Lawrence at West Texas A&M University for data download. Raw data were returned to study investigators for analysis. Steps, standing up, lying down and lying bouts, and motion index data were collected via accelerometers.

The motion index, steps and lying bouts were summed into 12 h increments starting at 0600 h of d 1 and ending at 0600 of d 14 for 28-12 h intervals. Standing time and lying time were analyzed together due to their interrelation and were summed on a 24 h increment to account for the recording method of the accelerometer. Step counts and motion index for each 12 h increment were log transformed for normality.

Calculations and Statistical Analysis

Statistical analysis was performed using computer software (JMP 13, SAS Institute, Cary, NC). Responses were analyzed using a mixed linear model with pen as the experimental unit using AR-1 as the covariance structure. Pen nested in a treatment group (FTD or PLBO) and lot were designated as a random effect with treatment, time (DOF), and treatment by time interaction as fixed effects. Pair-wise comparisons were done using Tukey-HDS tests. Responses measured included initial body weight, interim body weights, final body weights, gain, average daily gain (ADG), gain to feed, dry matter intake (DMI), morbidity, mortality, case fatality, removals, accelerometer activity data, VAS measures, and number of cattle that were pulled but not treated. Body weight (BW) was determined by weighing individual animals; ADG was calculated as initial BW – final BW ÷ total days on feed; DMI was calculated as daily pen feed

allocation – feed remaining at next feeding ÷ number of calves in the pen; gain to feed was calculated as pen level ADG ÷ pen level DMI. Statistical significance was set *a priori* at $P \leq 0.05$.

RESULTS

Performance

Performance data were calculated with data from the cattle that died or were removed from the study because of medical conditions that occurred during the 63 d feeding period excluded. During the 63 d feeding period, performance was not affected by treatment (Table 1); FTD calves had similar ADG and dry matter intake (DMI) to PLBO calves over the 63 d feeding period ($P = 0.94$ and $P = 0.51$, respectively). However, during the first 14 d on feed, calves treated with FTD had a lower ADG of 0.90 kg/d compared to 1.33 kg/d in the PLBO group ($P = 0.05$; Fig. 1). During the first 14 d on feed, calves treated with FTD also demonstrated a lower overall weight gain of 12.6 kg compared to 18.7 kg in the PLBO group ($P = 0.04$; Fig. 1). On d 14, the DMI for the FTD group was 3.92 kg/hd/d compared to 4.31 kg/hd/ ($P = 0.01$) in the PLBO group (Fig. 2). Additionally, there was a significant effect of days on feed for DMI ($P < 0.001$).

Health

The health data are summarized in Table 2. During the 63 d feeding period, health parameters were not affected by treatment. There were 168 calves identified for health evaluations over the course of the study and 118 calves treated for at least one treatment regimen, resulting in 30.73% overall morbidity. There was no effect of treatment on mean days to first pull ($P = 0.64$) or mean days to first treatment ($P = 0.29$). The overall chronic removal rate was 3.91% with 66.67% of the chronic removals due to BRD. The overall mortality rate was

4.95% with 89.47% of the mortalities due to BRD. One calf from each treatment group died as a result of the castration procedure (exsanguination).

VAS Pain Assessment

VAS pain assessment data is presented in Fig. 3. There was a significant time by treatment interaction ($P < 0.001$). During the first 36 h after treatment administration, the FTD treated calves had lower VAS measures [6.23 (95% CI: 5.27 – 7.20) compared to 7.28 (95% CI: 6.32 – 8.24)] than PLBO ($P \leq 0.05$). VAS measures for both groups decreased over time.

Accelerometer Activity Data

The standing time, lying time, steps and lying bouts of two animals were excluded from analysis as the accelerometer failed to record data. Accelerometer data is summarized in Table 3. There was an increase in the motion index (amount of movement) in the first 12 h and this is shown as a significant time effect ($P < 0.001$). After the first 12 h, the motion index had a diurnal pattern, but there were no differences in the motion index between treatment groups ($P = 0.94$). Similar to motion index, both treatment groups had an increased number of steps in the first 12 h, then the number of steps ranged between 250-597 steps per 12-h interval. There was no effect of treatment or treatment by time interaction for the number of steps. There was a significant time effect ($P = 0.01$) on number of lying bouts but there were no significant treatment effects ($P = 0.19$) or treatment by time interactions ($P = 0.68$). There were no differences in the amount of time standing or lying for each treatment group ($P = 0.86$) and no interaction with time ($P = 0.99$).

DISCUSSION

In the present study, the topical administration of flunixin meglumine transdermal in combination with tildipirosin did not significantly improve receiving performance compared to cattle that only received tildipirosin. These results are consistent with the findings of Cooke et al. (2013), who observed that injectable flunixin meglumine did not improve performance of feeder cattle. Gifford et al. (2012) observed that with a greater incidence of clinical signs of BRD, comes a decrease in ADG and body weight. In the present study, no significant differences were observed in health measurements between treatment groups. Conversely, failure to decrease clinical signs of BRD may be attributable to the lack of significant differences in performance between treatment groups.

Although ADG and DMI were similar over the 63 d feeding period, calves who received flunixin meglumine had lower ADG and DMI on d 14. González et al. (2010) observed a reduction in intake but not ADG after flunixin meglumine and xylazine co-administration following band castration over a 6 wk period. However, Coetzee et al. (2012) did not observe any effect of meloxicam administration on ADG or DMI of surgically castrated calves over a 28 d period, indicating that a reduction in intake may be specific to certain NSAIDs and not others. Whether or not this effect is profound enough to be biologically relevant should be considered.

There were no observed differences in morbidity or mortality due to BRD between the two treatment groups. However, since both treatment groups received tildipirosin, it may have reduced the incidence of BRD from what it would have been without metaphylaxis. Tildipirosin has been observed to lower the hazard of being affected with BRD and/or otitis (Teixeira et al., 2017) and has been shown to be more effective than tilmicosin at lowering first-pull treatment rates for BRD (Donkersgoed and Merrill, 2013). Since the calves in the present study were

considered high-risk, metaphylaxis was used throughout the study in the interest of animal well-being.

Pain is defined as an aversive sensory experience associated with actual or potential tissue damage; it results in physiologic, neuroendocrine, and behavioral changes that indicate a stress response in the animal (Molony and Kent, 1997). Postoperative inflammatory pain should be treated by using an NSAID (Huber et al., 2013). In the present study, visual analog assessment results indicated that calves co-administered flunixin transdermal and tildipirosin showed less signs of pain the first 36 hours post-drug application. Several types of pain responses can be recognized: 1) those that modify the animal's behavior to avoid the reoccurrence of the experience; 2) those that protect the animal such as withdrawal reflexes; 3) those that minimize pain and assist with healing such as lying or standing still; 4) those that elicit help or to stop another animal or human from inflicting more pain such as vocalization or posture (Molony and Kent, 1997). Our results indicate that the administration of an NSAID postoperatively reduced visual signs of pain in calves up until 36 hours after administration. Kleinhenz et al. (2018) observed that administration of transdermal flunixin reduced plasma cortisol concentration and mitigated the stress response in calves for 8 hours when given at the time of castration. However, negligible effects on the pain biomarkers of substance P, ocular infrared thermography, and gait analysis were observed (Kleinhenz et al., 2018). NSAIDs alone are not effective in reducing the acute distress associated with castration, but their analgesic and anti-inflammatory effects do extend into the postoperative period (Coetzee, 2013).

Assessing pain by monitoring animal behavior can be difficult and often subjective. One means of measuring changes in behavior outcomes without the presence of human evaluators is through accelerometers- which reduce subjectivity and outside influence (White et al., 2008).

Currah et al. (2009) observed that stride length and the number of steps taken by calves after castration can be good measures of pain, with castrated calves taking fewer steps and showing less activity. Calves treated with flunixin were observed to have decreased stride lengths after drug application, but significantly longer stride lengths 4 and 8 hours post-drug application compared to calves not administered flunixin (Currah et al., 2009). It has also been observed that calves spent significantly more time standing after castration than prior to castration (White et al., 2008). In the present study, both treatment groups were observed to have an increased number of steps in the first 12 hours when compared to the following 13 d. No significant differences were observed between treatments in the amount of time standing, time lying, steps, motion index, or lying bouts. Accelerometer activity data seems to be a promising way to more objectively evaluate animal behavior without human intervention and warrants further investigation.

CONCLUSION

Results of this study suggest there were no significant advantages in performance, health, or activity measured by accelerometers from the co-administration of flunixin meglumine transdermal and tildipirosin. However, calves co-administered flunixin meglumine transdermal and tildipirosin did have lower visual analog scale scores indicating that less pain was apparent the first 36 hours post-drug application relative to calves only administered tildipirosin.

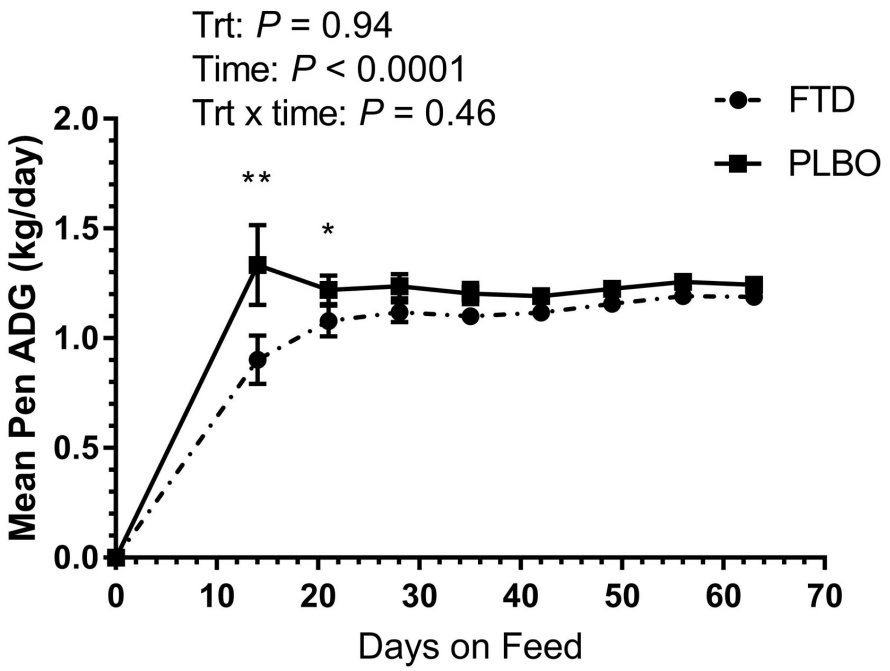

Figure 4.1 Mean pen average daily gain (ADG) for calves treated with tildipirosin in combination with transdermal flunixin (FTD) or tildipirosin alone (PLBO) at arrival.

** **P ≤ 0.05**

* **P ≤ 0.10**

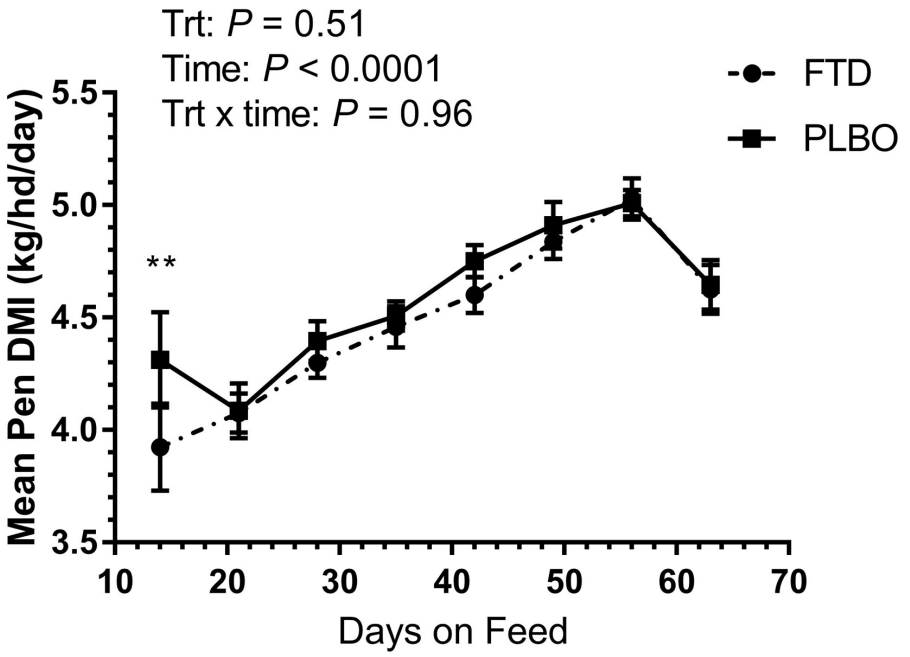

Figure 4.2 Mean pen dry matter intake (DMI) for calves treated with tildipirosin in combination with transdermal flunixin (FTD) or tildipirosin alone (PLBO) at arrival.

** **P ≤ 0.05**

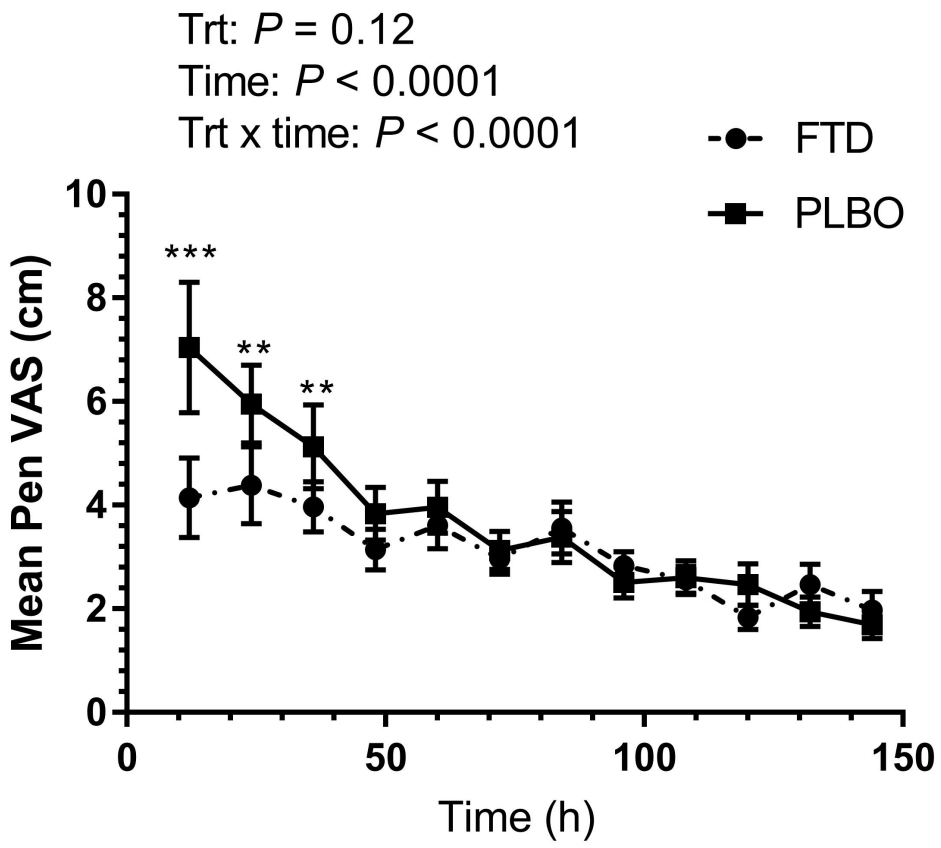

Figure 4.3 Mean pen VAS assessment over time for calves treated with tildipirosin in combination with transdermal flunixin (FTD) or tildipirosin alone (PLBO) at arrival.

*** **P < 0.001**

** **P ≤ 0.05**

Table 4.1 Mean performance summary in kilograms for calves treated with tildipirosin in combination with transdermal flunixin (FTD) or tildipirosin alone (PLBO) at arrival.

	Period	FTD	PLBO	SEM	*P*-value
Item	d	kg	kg		
Number of pens		16	16		
Number of bulls		100	99		
Number of steers		92	93		
BW[1]	0	221.7	221.3	3.52	0.95
BW	14	234.3	240.0	3.52	0.42
BW	63	296.7	299.7	3.52	0.60
BW Gain	0 - 14	12.6[a]	18.7[b]	1.99	0.04
BW Gain	14 - 63	62.4	59.6	2.26	0.39
BW Gain	0 - 63	75.0	78.4	1.73	0.18
ADG[2]	0 - 14	0.90[a]	1.33[b]	0.15	0.05
ADG	14 - 63	1.29	1.14	0.10	0.30
ADG	0 - 63	1.10	1.10	0.05	0.94
DMI[3]	0 - 14	3.92[a]	4.31[b]	0.13	0.01
DMI	14 - 63	4.59	4.61	0.08	0.55
DMI	0 - 63	5.70	5.77	0.08	0.51
G:F[4]	0 - 14	0.23	0.31	0.04	0.18
G:F	0 - 63	0.27	0.27	0.01	0.69

[a, b] Performance within days on trial with different superscripts are significantly different ($P \leq 0.05$)

[1]BW = body weight

[2]ADG = average daily gain

[3]DMI = dry matter intake

[4]G:F = gain to feed ratio

Table 4.2 Health summary for calves treated with tildipirosin in combination with transdermal flunixin (FTD) or tildipirosin alone (PLBO) at arrival.

	FTD	FTD	FTD	PLBO	PLBO	PLBO
Item	n	%	d	n	%	d
Total morbidity		33.3			27.6	
BRD[1] 1st Treatment	61	31.8		51	26.6	
BRD 2nd Treatment	25	13.0		21	10.9	
BRD 3rd (Chronic)	4	2.1		6	3.1	
1st Treatment Success Rate		56.3			58.8	
BRD Observations not Treated		14.6			12.0	
Days to First BRD Pull			12.4			11.9
Treated for Lameness	4	2.1		3	1.6	
Other Treatment	1	0.5		2	1.0	
BRD Mortality	10	5.2		7	3.6	
Non-BRD Mortality	1	0.5		1	0.5	
Overall Mortality	11	5.7		8	4.2	
BRD Removal	4	2.1		6	3.1	
Non-BRD Removal	2	1.0		3	1.6	
Overall Removal	6	3.1		9	4.7	

There were no significant differences between treatment groups ($P \leq 0.05$)

[1]BRD = bovine respiratory disease

Table 4.3 Accelerometer summary for calves treated with tildipirosin in combination with transdermal flunixin (FTD) or tildipirosin alone (PLBO) at arrival.

Item	FTD	PLBO	SEM	*P*-value
Motion Index	1749	1814	112	0.96
Standing, min	603.4	609.8	12.96	0.78
Lying, min	841.5	834.8	12.96	0.72
Lying, bouts	8.44	7.00	0.52	0.08
Steps	422.1	396.1	13.2	0.25

There were no significant differences between treatment groups ($P \leq 0.05$)

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Chapter 5 - Assessment of pain associated with bovine respiratory disease and its mitigation with flunixin meglumine in cattle with induced bacterial pneumonia

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ABSTRACT

Pleuritic chest pain due to bacterial pneumonia is commonly reported in human medicine. However, studies investigating pain associated with bovine respiratory disease (BRD) are lacking. The objectives of this study were to assess if bacterial pneumonia elicits a pain response in calves with experimentally induced BRD and to determine the analgesic effects of transdermally administered flunixin. Twenty-six calves, 6-7 months of age, with no history of BRD were enrolled into 1 of 3 treatment groups: (1) experimentally induced BRD + transdermal

flunixin at 3.3 mg/kg twice, 24 h apart (BRD + FTD); (2) experimentally induced BRD + placebo (BRD + PLBO); and (3) sham induction + placebo (CNTL + PLBO). Calves enrolled into the BRD groups were inoculated with *Mannheimia haemolytica* using bronchoalveolar lavage. Outcome variables were collected from -48 to 192 hours post-treatment and included serum cortisol concentrations; infrared thermography (IRT); mechanical nociceptive threshold (MNT); substance P; kinematic gait analysis; a visual analog scale (VAS); clinical illness score (CIS); computerized lung score (CLS); average activity and rumination levels; prostaglandin E_2 metabolite concentrations (PGEM); plasma serum amyloid A concentrations and rectal temperature. Each outcome parameter was evaluated using either a generalized logistic mixed model for categorical variables or a generalized linear mixed model for continuous variables. Right front force differed by treatment ($P = 0.01$). Calves in the BRD + PLBO group had lower mean force applied to the right front limb (85.5 kg) compared to BRD + FTD calves (96.5 kg) ($P < 0.01$). Average VAS differed significantly by a treatment by time interaction *(*$P = 0.01$). The mean VAS scores differed for the BRD + PLBO group at -48 (3.49 mm) compared to 168 and 192 h (13.49 and 13.64 mm, respectively) ($P < 0.01$). Average activity for the BRD + PLBO group was higher at -48 h (27 min/h) compared to 48, 72, 120 and 168 h (≤ 22.24 min/h) ($P < 0.01$). Average activity differed by a treatment by timepoint interaction ($P = 0.01$). Average activity for the BRD + FTD group was higher at -48 and 0 h (28.2 and 28.2 min/h, respectively) compared to 48, 72, 96 and 168 h (≤ 23.7 min/h) ($P < 0.01$). The results from calves in the BRD + PLBO group in the present study indicate that a combination of reduced activity levels, decreased force on the right front limb, and increased visual analog scale pain scores all support that bacterial pneumonia in cattle is painful. Differences in right front force were observed in calves challenged with *M. haemolytica* and treated with flunixin transdermal compared to those

given a placebo, indicating that flunixin transdermal may attenuate certain pain biomarkers in cattle with BRD. Lung lesion scores were negatively correlated with activity levels from 48 h to the end of the study, with step count from 72-96 h and with rumination levels at the end of the study period. To our knowledge, this is the first study to report changes in pain biomarkers associated with BRD. These findings suggest that analgesic drugs may improve the humane aspects of care for cattle with BRD.

Keywords: BRD, pain, biomarkers, lung lesion scores

INTRODUCTION

Bovine respiratory disease (BRD) is the most common and costliest disease affecting the cattle industry (DeDonder et al., 2010; Peel, 2020), with BRD death loss alone costing upwards of $907 million annually in the United States (USDA, 2017). The most commonly isolated organism from BRD-affected lung tissue is an opportunistic pathogen, *Mannheimia haemolytica* (Griffin et al., 2010). *Mannheimia haemolytica* is considered a commensal organism that, when cattle experience stress, can proliferate into the nasopharynx and translocate to the lung, where it causes fibrinous pleuropneumonia (Rice et al., 2007). Symptoms of BRD include nasal and ocular discharge, depression, anorexia, and increased respiratory rate with dyspnea. Clinical outcomes may range from hardly noticeable to death (Griffin et al., 2010). Pleuritic chest pain resulting from bacterial pneumonia is commonly reported in human medicine (Boyd et al., 2006). However, published literature regarding the association of pain and respiratory disease in cattle is lacking.

Cattle are stoic animals by nature and have long been subject to evolutionary pressure to mask pain (Hudson et al., 2008). Further research into the development of appropriate behavioral and physiological pain assessment tools is needed to objectively quantify pain and search for the most effective analgesic strategies (Schwartzkopf-Genswein et al., 2012). The analgesic class of nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit the cyclo-oxygenase enzymes, reduce inflammation, and decrease prostaglandin production, which attenuates the response of the peripheral and central components of the nervous system to noxious stimuli, and results in a reduction in response to pain (Ochroch et al., 2003). The objectives of this study were to assess if bacterial pneumonia elicits a pain response in calves with experimentally induced BRD and to determine the analgesic effects of flunixin meglumine transdermal in experimentally induced bacterial pneumonia.

MATERIALS AND METHODS

Animals, Housing, and Treatments

The Institutional Animal Care and Use Committee and the Institutional Biosafety Committee of Kansas State University reviewed and approved the experimental protocol for this project (IACUC# 4465 and IBC# 1499). This study was conducted at the Kansas State University Stocker Unit near Manhattan, KS, between October and December 2020. A total of 50 weaned and vaccinated male Holstein calves were received for potential enrollment in the study in June 2020. Calves were dehorned and castrated at Kansas State University. Twenty-six calves, 6-7 months of age, weighing an average of 185 ± 4 kg were then enrolled onto the study based on the following inclusion criteria: (1) healed from dehorning and castration procedures, (2) clear lung ultrasound, (3) negative bovine viral diarrhea (BVDv) test (IDEXX Laboratories, Inc., Westbrook, ME), and (4) no history of clinical signs of bovine respiratory disease.

Calves were housed in two outdoor pens with dirt flooring of equal size with 13 calves/pen. Once inoculated, calves were housed in a pen with solid paneling around the perimeter to prevent calves from touching noses with calves not currently on the study. Diets were formulated to meet or exceed the nutritional requirements set by the NRC (NRC, 2016), were formulated at 2.2% BW, and all calves were fed once daily per normal procedures at the study site. Calves were moved to the study site 2 weeks prior to the study start date to allow for an acclimation period. Upon arrival to the study site, calves were affixed with a 3-axis accelerometer ear-tag (Allflex Livestock Intelligence, Madison, WI) to quantify activity and daily rumination time.

After the acclimation period, prior to the start of the study, calves were weighed and randomly allocated to 1 of 3 experimental treatment groups (**Fig. 1**). Cattle were uniquely identified with individual ear tags prior to acclimation period and randomized utilizing the RAND function in Microsoft Excel (Microsoft Excel® 2016, Microsoft Corporation, Redmond, WA). The treatment groups were as follows: (BRD + FTD) – experimentally induced respiratory disease + transdermal flunixin administered topically at 3.3 mg/kg twice, 24 h apart, (BRD + PLBO) – experimentally induced respiratory disease + placebo, (CNTL + PLBO) – sham induction + placebo. Eight calves were assigned to each of the BRD + FTD and CNTL + PLBO groups and 10 calves were assigned to the BRD + PLBO group due to the higher risk of calves reaching the endpoint criteria in that treatment group. Calf was the experimental unit for the study. The study took place in two phases, with 13 calves in each phase, with each phase being housed in one pen. The phases were put into place due to the terminal nature of the study and the constraints of the veterinary diagnostic laboratory necropsy capacity. The study duration was 9 days/phase from calves being enrolled onto the study to the completion of the study phase.

At 48 h prior to the start of the study, baseline parameters were collected and IceTag (IceRobotics Ltd, South Queensferry, Edinburgh, Scotland UK) accelerometers were placed on the left rear leg of each calf for the duration of the study. At 24 h prior to the start of the study, calves in the BRD + FTD and BRD + PLBO treatment groups were inoculated with a strain of *Mannheimia haemolytica* using bronchoalveolar lavage (BAL) as described by Theurer et al. (2013). The right apical lung lobes were inoculated using broncho-selective endoscopy. A 10 mL dose of *M. haemolytica* serotype A1 at 1 x 10^9 cfu/mL was used to inoculate each calf, and then the endoscope was flushed with 60 mL of the phosphate-buffered solution to achieve a total volume of 70 mL. The CNTL + PLBO calves received 70 mL of phosphate-buffered solution as described above.

After inoculation, calves were monitored for disease onset using visual clinical illness scoring every 6 hours as previously described in Perino and Apley (1998). Clinical signs of illness included lethargy, anorexia, nasal or ocular discharge, tachypnea and fever (rectal temperature > 40 °C). At 24 hours post-inoculation, calves received one of the following treatments: Topical flunixin meglumine (Banamine Transdermal, Merck Animal Health, Madison, NJ) at 3.33 mg/kg (1 mL/15 kg) or topical placebo at 1 mL/15 kg. The placebo was made up of propylene glycol, isopropyl alcohol, and a red dye to mimic the test product in color, viscosity, and odor, as described in Kleinhenz et al. (2017). The flunixin transdermal and placebo were administered on dry skin. The entire dose was applied on the dorsal midline between the withers and tail head in accordance with label directions. The time of treatment was considered time 0 h for the study. Calves with a fever (rectal temperature > 40º Celsius) at any point in time throughout the study were administered a single subcutaneous injection of tildipirosin (Zuprevo 18%; Merck Animal Health, Madison, NJ) at a dose of 4 mg/kg.

Measurements and Sample Collection

Outcome variables were collected at -48, 0, 4, 8, 24, 32, 48, 72, 96, 120, 144, 168, and 192 h post-treatment (**Fig. 1**), in addition to the 3-axis accelerometer ear-tags and accelerometers continuously collecting activity and rumination data throughout the study. Outcome variables collected at the given timepoints included: rectal temperature, infrared thermography (IRT) imaging, kinematic gait analysis, mechanical nociception threshold (MNT), visual analog scale (VAS) score, clinical illness score (CIS), computerized stethoscope (Whisper Veterinary Stethoscope, Merck Animal Health, Madison, NJ) lung score (CLS), and blood sampling for serum cortisol, substance P, prostaglandin E_2 metabolite (PGEM) and serum amyloid A (SAA) analysis. All trained evaluators were blinded to treatment for the duration of the study. Following the 192 h collection, calves were euthanized and transported to the Kansas State Veterinary Diagnostic Laboratory for necropsy and lung lesion scoring.

The IRT images captured the medial canthus of the left eye using a research-grade infrared camera (Fluke TiX580, Fluke Corp, Everett, WA). Infrared images were analyzed using research-specific computer software (SmartView v. 4.3, Fluke Thermography, Plymouth, MN) to determine maximum and minimum temperatures. The difference between the temperature of the medial canthus baseline and the timepoints following were determined and used for statistical analysis.

A commercially available pressure mat gait analysis system (Walkway, Tekscan Inc., South Boston, MA) was used to record gait and biomechanical parameters. The pressure mat was calibrated using a known mass to ensure the accuracy of the measurements at each timepoint. Video synchronization was used to ensure consistent gait between and within calves at each

timepoint. Using research-specific software (Walkway 7.7, Tekscan Inc.), force, contact pressure, impulse, stance time, and stride length were assessed.

A hand-held pressure algometer (Wagner Instruments, Greenwich, CT) was used for MNT determination. A force was applied perpendicularly, at a rate of approximately 1 kg of force per second, at 1 location on both the left and right side of the ribs of each calf, over the 6th intercostal space for a total of 2 locations, as described in (Williams et al., 2020). A withdrawal response was indicated by an overt movement away from the applied pressure algometer. Locations were tested 3 times in sequential order, and the values were averaged for statistical analysis. A second investigator recorded the MNT values to prevent bias by the investigator performing the MNT collection. The collection of MNT values was recorded at only -48, 8, 24, 72, and 192 h timepoints to prevent sensitization of the calf.

A VAS score was assigned by two trained evaluators blinded to treatment allocations using methods adapted from Martin et al. (2020). The VAS used was a 100 mm (10 cm) line anchored at each end by descriptors of “No Pain” or “Severe Pain”. Seven parameters were used to assess pain: depression, tail swishing or flicking, stance, head carriage, spinal alignment, movement and ear carriage. No pain was characterized by being alert and quick to show interest, no tail swishing, a normal stance, head carriage above spine level, a straight spine, moving freely around the pen and ears forward. Severe pain was characterized by being dull and showing no interest, more than three tail swishes per minute, legs abducted, head held below spine level, a curved spine, reluctant to move, and ears down. The evaluator marked the line between the 2 descriptors to indicate the pain intensity. A millimeter scale was used to measure the score from the zero anchor point to the evaluator’s mark. The mean VAS scores of the two evaluators were combined into one score for statistical analysis.

A CIS was assigned by two trained evaluators blinded to treatment allocations. The CIS consisted of: (1) is a normal healthy animal, (2) slightly ill with mild depression or gauntness, (3) moderately ill demonstrating severe depression/labored breathing/nasal or ocular discharge, and (4) severely ill and near death showing minimal response to human approach (Perino and Apley, 1998). The CIS scores of the two evaluators were combined into one score for statistical analysis, if either evaluator scored >1 then a final score >1 was assigned, with 1 being considered normal and greater than 1 considered abnormal.

A computerized stethoscope (Whisper, Merck Animal Health, De Soto, KS) was used to analyze lung and heart sounds via a machine-learning algorithm that assigns a CLS from 1-5, with 1 being normal and 5 being severely compromised lung tissue (Nickell et al., 2020). The bell of the lung stethoscope was placed approximately two inches caudal and dorsal to the right elbow of each calf, and lung sounds were recorded for 8 s. If the recording was deemed acceptable by the computer program, the score was recorded.

Calves were affixed with a 3-axis accelerometer ear-tag (Allflex Livestock Intelligence, Madison, WI) to quantify activity and daily rumination time throughout the study. The average number of minutes spent active or ruminating over 60 min time periods for the study duration was then calculated. An IceTag (IceRobotics Ltd, South Queensferry, Edinburgh, Scotland UK) accelerometer was also placed on the left rear leg of each calf for the duration of the study. Accelerometer ID was paired with calf ID prior to placement onto the calf. Accelerometers were removed at the conclusion of the study and data was downloaded from the accelerometers for analysis. Steps, standing, lying, lying bouts, and motion index data were collected via the accelerometers.

Rectal temperatures were taken daily by placing a digital thermometer (180 Innovations, Lakewood, CO) against the rectum wall until a temperature reading was produced on the thermometer's screen.

Blood samples for serum cortisol, substance P, PGEM, and serum amyloid A determination were collected from the jugular vein via venipuncture. The whole blood samples were immediately transferred to tubes (Vacutainer, BD Diagnostics, Franklin Lakes, NJ) containing either no additive for cortisol determination or EDTA anticoagulant for PGEM determination. For substance P determination, benzamidine hydrochloride (final concentration of 1mM) was added to EDTA blood tubes 48 h prior to collection. Samples were immediately placed on ice after collection, centrifuged within 30 min of collection for ten minutes at 1,500 g, and serum and plasma were placed in cryovials via transfer pipette and stored at -80 °C.

Serum cortisol concentrations were determined using a commercially available radioimmunoassay (RIA) kit (MP Biomedicals, Irvine, CA) following manufacturer specifications with minor modifications; the standard curve was extended to include 1 and 3 ng/mL by diluting the 10 and 30 ng/mL manufacturer-supplied standards, 1:10 respectively. The standard curve ranged from 1 to 300 ng/mL. A low (25 ng/mL) and high (150 ng/mL) quality control (QC) were ran at the beginning and end of each set to determine inter-assay variability. Plain 12 x 75 mm polypropylene tubes were used as blank tubes to calculate non-specific binding. Input for standards, QCs, and samples was adjusted to 50 µL. Samples were incubated at room temperature for 30 minutes prior to the addition of I-125. Manufacturer instructions were then followed. Tubes were counted on a gamma counter (Wizard2, PerkinElmer, Waltham, MA) for 1 minute. The raw data file was then uploaded onto MyAssays Desktop software (version 7.0.211.1238, 21 Hampton Place, Brighton, UK) for concentration determination. Standard

curves were plotted as a 4-parameter logistic curve. Samples with a coefficient of variation (**CV**) > 18% were re-analyzed.

Substance P (SP) concentrations were determined through RIA. The standard curve, ranging from 20 to 1,280 pg/mL, was created by diluting synthetic SP (Phoenix Pharmaceuticals, Burlingame, CA) with RIA Buffer (50mM sodium phosphate dibasic heptahydrate, 13mM disodium EDTA, 150mM sodium chloride, 1mM benzamidine hydrochloride, 0.1% gelatin, 0.02% sodium azide; pH 7.4). A 100µL of samples, standards, and QCs were aliquoted into plain 12 x 75mm conical bottom tubes followed by 100 µL of Rabbit anti-SP primary antibody (1:20,000; Phoenix Pharmaceuticals). Iodine-125-SP tracer (custom iodination by PerkinElmer) was diluted with RIA buffer to 20,000 cpm, then 100 µL was added to the samples, standards, and QCs. Samples were then covered and stored at 4°C for 48 h. At the end of the 48 h incubation, samples were placed on ice and 100 µL of normal rabbit plasma (1:80) and goat anti-rabbit secondary antibody (1:40; Jackson ImmunoResearch, West Grove, PA) were added to each tube. Samples were then incubated at room temperature for 10 min, placed back on ice, and 100µL of blank bovine plasma was added to the standards and QCs. All tubes then had 1mL of 12% polypropylene glycol in 0.85% sodium chloride added. Samples were centrifuged at 3,000 x g for 30 min at 4°C and the supernatant aspirated. Tubes were counted on a gamma counter (Wizard2, PerkinElmer, Waltham, MA) for 1 minute. The raw data file was then uploaded onto MyAssays Desktop software for concentration determination. Standard curves were plotted as a 4-parameter logistic curve. Samples with a CV > 18% were re-analyzed.

Prostaglandin E_2 metabolites were analyzed using a commercially available ELISA kit (Cayman Chemical, Ann Arbor, MI) following manufacturer specifications with minor modifications. Sample input was adjusted to 375 µL with 1.5 mL ice-cold acetone added for

sample purification. Samples were incubated at -20°C for 30 min., then centrifuged at 3,000 x g for 5 min. The supernatant was transferred to clean 13 x 100mm glass tubes and evaporated using a CentriVap Concentrator (Labconco, Kansas City, MO) overnight (approx. 18h). Samples were reconstituted with 375 µL of appropriate kit buffer. A 300 µL aliquot of the reconstituted sample was derivatized with proportionally adjusted kit components. Manufacturer protocol was then followed. Samples were diluted at 1:2 and ran in duplicate. Absorbance was measured at 405 nm after 60 min of development (SpectraMax i3, Molecular Devices, San Jose, CA). Sample results were excluded if the raw read exceeded the raw read of the highest standard (Standard 1; 50 pg/mL) or was below the lowest acceptable standard. The lowest acceptable standard was defined for each individual plate and was identified by excluding standards that had a ratio of absorbance of that standard to the maximum binding of any well ($\%B/B_0$) of $\geq 80\%$ or $\leq 20\%$. Any individual sample outside the standard curve, with a $\%B/B_0$ outside the 20-80% range, or a CV > 15% were re-analyzed. Prostaglandin E_2 metabolites were analyzed at -48, 0, 72 and 192 h timepoints.

Serum Amyloid A (SAA) concentrations were determined in serum samples using an ELISA assay (Phase Range Multispecies SAA ELISA kit; Tridelta Development Ltd, Maynooth, Kildare, Ireland). Manufacturer specifications were followed and samples were diluted as necessary. Absorbance was measured at 450 nm on a SpectraMax i3 plate reader (Molecular Devices). Raw data was analyzed using MyAssays Desktop software for concentration determination. Standard curves were plotted as a 4-parameter logistic curve. Samples with a coefficient of variation (CV) > 15% were re-analyzed.

Flunixin (FLU; Sigma Aldrich, St. Louis, MO) and flunixin-d3 (FLU-d3, internal standard, Toronto Research Chemicals, North York, ON, Canada) stock solutions were prepared

at 1mg/mL in methanol and acetonitrile respectively and stored at -80°C. FLU standard curve and quality controls (QCs) were prepared fresh daily in negative control plasma. The standard curve ranged from 1 to 100 ng/mL. A 50ng/mL working solution of FLU-d3 was prepared daily by diluting the 1mg/mL stock in 0.1% formic acid in acetonitrile. Plasma collected in lithium heparin tubes was used for flunixin concentration determination. Samples were extracted via protein precipitation. Briefly, 100µL of sample, standards, QCs and blanks were aliquoted and 400µL of 50ng/mL FLU-d3 in 0.1% formic acid in acetonitrile was added. Samples were then centrifuged at 3,000 *x g* for 5 min. Supernatant was decanted into 75x100mm glass tubes, evaporated using a CentriVap system (Labconco), and reconstituted with 200µL of 25% acetonitrile in water. The reconstituted samples were transferred to clean microcentrifuge tubes and centrifuged at 10,000 *x g* for 7 min. An aliquot of 100µL was transferred to an autosampler vial with a glass insert (QsertVial, Waters Corp., Milford, MA) with pre-slit septum lids. Vials were loaded onto an Acquity H Class ultra-performance liquid chromatography (UPLC) system coupled with a Xevo TQ-S tandem mass spectrometer (MS/MS; Waters Corp.). Chromatographic separation was achieved using an Aquity UPLC BEH C18 column held at 40°C during analysis. Mobile phase A and B consisted of 0.1% formic acid in acetonitrile and 0.1% formic acid in 18.2 MΩ.cm water, respectively. Flow rate was set at 0.4mL/min with the following gradient during the 3 min run time: 30% A at 0-1.49 min, 100% A from 1.5 to 2 min, then 30% A at 2.01 min. The Xevo TQ-S MS/MS was equipped with an electrospray ionization (ESI) interface set in positive mode. The quantifying transition for FLU was *m/z* 297.27→279.24 and the qualifying transition was *m/z* 297.27→264.15. The quantifying transition for FLU-d3 was *m/z* 300.23→282.26. Data acquisition and analysis was performed using MassLynx and TargetLynx software, respectively (Waters Corp). The standard curve was linear from 1 to 100

ng/mL and the correlation coefficient was accepted if it was at least 0.975. Samples with flunixin concentrations outside the standard curve linear range were diluted at 1:500 with blank plasma and reanalyzed.

All calves were sedated with intramuscular xylazine (0.1 mg/kg) and humanely euthanized following the 192 h timepoint with a penetrating captive bolt stunner (CASH Special, FRONTMATEC Accles & Shelvoke Ltd., Minworth, Sutton Coldfield, UK) followed by intravenous injection of potassium chloride (120 mL). A pathologist (KMA), blinded to treatment groups, performed a postmortem examination to determine lung lesions and assign a lung lesion score based on lung consolidation. The lung lesion score was determined using methods described by Fajt et al. (2003). The equation used was as follows: total percentage lung consolidation = (0.053 × cranial segment of left cranial lobe %) + (0.049 × caudal segment of left cranial lobe %) + (0.319 × left caudal lobe %) + (0.043 × accessory lobe %) + (0.352 × right caudal lobe %) + (0.061 × right middle lobe %) + (0.060 × caudal segment of right cranial lobe %) + (0.063 × cranial segment of right cranial lobe %).

Calculations and Statistical Analysis

Study data were imported into a commercially available statistical software package (R: An open-source programming language and environment for statistical computing, version 4.0.5, R Foundation for Statistical Computing, Vienna, Austria) for descriptive analyses and hypotheses testing. Twenty-four-hour accelerometer readings were calculated from 8 am to 8 am, coinciding with sample collection timepoints. A scoring system was adapted from White et al. (2012), with the addition of a medium category, to categorize consolidated lung tissue scores as low ≤10%, medium >10% and ≤20%, and high >20%. Treatment for BRD based on pyrexia was dichotomized into a yes/no (0/1) category for analysis. The outcomes for CIS and CLS were

dichotomized with a score of 1 considered normal and >1 considered abnormal. Each outcome parameter was evaluated using either a generalized logistic mixed model for categorical variables or a generalized linear mixed model for continuous variables. Packages used for modeling outcomes of interest included lme4, lmerTest, and car (Bates et al., 2015; Kuznetsova et al., 2017; Fox and Weisberg, 2019). The effect of treatment, time and time x treatment interaction were evaluated using a linear mixed model that included the covariates of categorized lung score and dichotomized tildipirosin administration. A random intercept was included to account for lack of independence and hierarchical data structure of calf within study phase. Due to the lack of observations for plasma flunixin levels, the parameter was removed from models for them to converge. Model estimated means using the emmeans package are reported for continuous variables and probabilities are reported for categorical variables. A Tukey-Kramer familywise error-adjustment was used for multiple comparisons. The Hmisc and ggcorrplot packages were used to evaluate Pearson correlations between outcomes (Kassambara, 2019; Harrell, 2021). Statistical significance was set *a priori* at $P \leq 0.05$. The following pharmacokinetic parameters were calculated using non-compartmental methods using PK Solver (Zhang et al., 2010) in Excel: slope of the terminal phase (λ_z), terminal half-life (T ½), maximum plasma concentration (C_{max}); time to achieve peak concentration (T_{max}), area under the curve from the time of dosing ($Dosing_{time}$) to the last measurable (positive) concentration ($AUC_{0\text{-}last}$), and AUC from $Dosing_{time}$ extrapolated to infinity, based on the last observed concentration (obs) ($AUC_{0\text{-}\infty}$).

RESULTS

Outcome measure means by treatment are outlined in **Table 1**. Throughout the study duration, five calves received tildipirosin for BRD based on pyrexia (rectal temperature > 40º

Celsius). Two calves who received tildipirosin were in the BRD + FTD group, once calf was in the BRD + PLBO group, and 2 calves were in the CNTL + PLBO group.

IRT. Infrared thermography average temperature did not differ by treatment ($P = 0.91$), treatment by time interaction ($P = 0.99$), or lung category ($P = 0.29$), but did differ by timepoint ($P < 0.01$). Average temperature readings at 144, 168, and 192 h (27.4, 27.1, and 28.4°C, respectively) were less than readings from 0 to 96 h (≥ 30.1°C) ($P < 0.01$). After treatment, the highest readings were at 8 and 32 h (32.9 and 35.3°C, respectively).

Pressure/Force System. Right front stance time did not differ by treatment ($P = 0.07$), treatment by time interaction ($P = 0.85$), or lung category ($P = 0.64$) but did differ by timepoint ($P < 0.01$). Stance time means were longer at 72 and 96 h (0.916 and 0.905 s, respectively) relative to -48 to 24 h (≤ 0.728 s) ($P < 0.03$).

Right front stride length did not differ by treatment ($P = 0.11$) or treatment by time interaction ($P = 0.13$) but did differ by timepoint ($P < 0.01$) and lung category ($P = 0.01$). Calves had a shorter right front stride length at 72 and 96 h (114 and 112 cm, respectively) relative to -48 and 32 h (128 and 123 cm, respectively) ($P < 0.05$). Calves in the high lung lesion category had shorter mean right front stride lengths compared to calves in the low lung lesion category (112 and 128 cm, respectively) ($P < 0.01$).

Right front force did not differ by treatment by time interaction ($P = 0.87$) but did differ by treatment ($P = 0.01$), timepoint ($P = 0.01$), and lung category ($P = 0.01$). BRD + PLBO calves had significantly lower mean right front force (85.5 kg) compared to BRD + FTD calves (96.5 kg) ($P = 0.03$). Calves had significantly lower mean right front force at 144 h (82 kg) relative to 32 h (96.9 kg) ($P = 0.04$). Calves in the high lung lesion category had significantly lower mean

right front force (80.0 kg) compared to calves in the medium and low lung lesion categories (91.3 and 95.5 kg, respectively) ($P < 0.03$).

Right front impulse did not differ by treatment ($P = 0.22$), timepoint ($P = 0.11$), treatment by time interaction ($P = 0.66$) or lung category ($P = 0.20$). Right front pressure did not differ by treatment ($P = 0.41$), treatment by time interaction (P = 0.59) or lung category ($P = 0.16$) but did differ by timepoint ($P = 0.01$). At the 32 h timepoint, right front pressure was the least (5.23 kg/cm^2) and at 8 h it was the greatest (6.37 kg/cm^2) but did not differ significantly. Average right front limb gait analysis outcomes are described in **Fig. 2**.

Gait distance did not differ by treatment ($P = 0.86$), timepoint ($P = 0.06$), treatment by time interaction ($P = 0.32$) or lung category ($P = 0.23$). Gait velocity did not differ by lung category ($P = 0.07$) but differed by a treatment by time interaction ($P = 0.05$) (**Fig. 3**). The BRD + PLBO group had greater velocity at -48 h (139 cm/s) compared to 48, 72 and 144 h (89.9, 78.5 and 93.6 cm/s, respectively) ($P < 0.05$).

MNT. Average MNT did not differ by treatment *(*$P = 0.35$), treatment by time interaction *(*$P = 0.14$) or lung category ($P = 0.96$) but did differ by timepoint ($P < 0.01$). Baseline average MNT means at -48 h (2.00 kg F) were larger than average MNT values at 24, 72, and 192 h (1.59, 1.66, and 1.23 kg F, respectively) ($P < 0.03$). The percent change from baseline MNT values did not differ by treatment ($P = 0.73$), timepoint ($P = 0.61$), treatment by time interaction ($P = 0.68$) or lung category ($P = 0.84$).

VAS. Average VAS scores did not differ by lung category ($P = 0.07$) but differed by treatment by time interaction *(*$P = 0.01$) **(Fig. 4).** The mean VAS scores differed for the BRD + PLBO group at -48 (3.49 mm) compared to 168 and 192 h (13.49 and 13.64 mm, respectively) ($P < 0.01$).

CIS. Clinical illness score did not differ by treatment ($P = 0.96$), treatment by time interaction ($P = 0.57$) or lung score ($P = 0.10$) but did differ by timepoint ($P < 0.01$). The probability of a CIS >1 at 4 h (0.09) was less than the probability of CIS >1 at 120, 168 and 192 h (0.67, 0.87, and 0.91, respectively) ($P < 0.03$).

CLS. Computerized lung score did not differ by treatment ($P = 0.84$) or treatment by time interaction ($P = 0.98$) but did differ by timepoint ($P < 0.01$) and lung category ($P = 0.05$). Probabilities for calves having a CLS >1 at 8 and 24 h (0.17 and 0.20, respectively) were significantly less than at 32, 72, 96, 144 and 192 h (0.83, 0.83, 0.69, 0.78, and 0.83, respectively) ($P < 0.05$). The probability of calves having a CLS >1 was significantly higher for calves in the high lung lesion category (0.73) compared to the medium lung lesion category (0.50) ($P = 0.05$), there was not a difference between the high and low lung lesion categories ($P = 0.23$).

Accelerometers. Average activity differed by treatment by time interaction ($P = 0.01$) (**Fig. 5**) and lung category ($P = 0.01$). Average activity for the BRD + PLBO group was higher at -48 h (27 min) compared to 48, 72, 120 and 168 h (≤ 22.24 min/h) ($P < 0.01$). Average activity for the BRD + FTD group was significantly higher at -48 and 0 h (28.2 and 28.2 min/h, respectively) compared 48, 72, 96 and 168 h (≤ 23.7 min/h) ($P < 0.01$). Average activity was significantly less in the high lung lesion category (20.9 min/h) compared to the low (27.2 min/h) ($P < 0.01$).

Rumination activity did not differ by treatment ($P = 0.73$) or treatment by time interaction ($P = 0.50$) but did differ by timepoint ($P = 0.01$) and lung category ($P = 0.01$). Rumination activity at 96 h (41.5 min/h) was higher compared to 48 h (31.1 min/h) ($P < 0.04$). Calves in the high lung lesion category had lower rumination activity levels compared to calves in the low lung lesion category (28.4 and 43.8 min/h, respectively) ($P < 0.04$).

Motion index differed by timepoint ($P < 0.01$) and lung category ($P = 0.01$). Motion index at -48 and 0 h (6983 and 7195) was greater compared to 48 to 168 h (≤ 5887) ($P < 0.01$). Calves with high and medium lung lesion scores had a lower motion index (4246 and 4727, respectively) relative to calves with a low score (7256) ($P < 0.01$).

Standing time did not differ by treatment ($P = 0.52$), treatment by time interaction ($P = 0.95$), or lung category ($P = 0.23$) but did differ by timepoint ($P < 0.01$). Calves spent more time standing and less time lying at 0, 24 and 120 h (0.48, 0.50 and 0.52, respectively) compared to -48, 48, 72, 96 and 168 h (≤ 0.38) ($P < 0.01$).

Step count differed by timepoint ($P < 0.01$) and lung category ($P = 0.01$). Calves took more steps at -48, 0 and 24 h (1358, 1544, and 1350 steps, respectively) compared to 48, 72, 96, 144 and 168 h (≤1045 steps) ($P < 0.01$). Calves in the medium and high lung lesion categories took fewer steps (1009 and 924 steps, respectively) than calves in the low category (1471 steps) ($P < 0.02$).

Lying bouts differed by timepoint ($P = 0.01$) and lung category ($P = 0.02$). Calves took more lying bouts at -48 and 72 h (14.7 and 14.4 bouts, respectively) relative to 144 h (11.2 bouts) ($P < 0.03$). Calves in the high lung lesion category had fewer lying bouts (10.3 bouts) than calves in the low category (15.1 bouts) ($P < 0.04$), there was not a difference between the high and medium lung lesion categories ($P = 0.10$).

Rectal Temperature. Average rectal temperature did not differ by treatment ($P = 0.98$), treatment by time interaction ($P = 0.15$) or lung category ($P = 0.20$) but did differ by timepoint ($P < 0.01$). Average rectal temperature at 120, 144 and 168 h (38.7, 38.6, and 38.6°C, respectively) was lower than at 0, 8, 24, and 32 h (39.4, 39.7, 39.5 and 39.8°C, respectively) ($P < 0.04$).

Cortisol. Cortisol concentrations did not differ by treatment ($P = 0.19$), treatment by time interaction ($P = 0.10$) or lung category ($P = 0.76$) but did differ by timepoint ($P = 0.01$). Cortisol levels were higher at -48 h (7.19 ng/mL) compared to 72, 144 and 168 h (2.41, 2.33 and 1.77 ng/mL, respectively) ($P < 0.04$).

Substance P. Substance P concentrations did not differ by treatment ($P = 0.14$), timepoint ($P = 0.07$) treatment by time interaction ($P = 0.90$), or lung category ($P = 0.99$).

PGEM. Prostaglandin E_2 metabolite concentration differed by timepoint ($P < 0.01$) and lung category ($P = 0.01$). Concentrations of PGEM were lower at 72 and 192 h (20.8 and 21.2 pg/mL, respectively) relative to -48 and 0 h (36.0 and 38.8 pg/mL, respectively) ($P < 0.01$). Concentrations of PGEM were lower in the low lung lesion category (18.5 pg/mL) relative to the high lung lesion category (39.1 pg/mL) ($P < 0.01$).

SAA. Concentrations of SAA did not differ by treatment ($P = 0.59$), treatment by time interaction ($P = 0.24$) or lung category ($P = 0.44$) but did differ by timepoint ($P < 0.01$). Concentrations of SAA were higher at 4, 8, 24, and 32 h (310, 366, 369, 323 μg/mL) compared to -48 and 120 to 192 h (≤ 175 μg/mL) ($P < 0.03$).

Lung lesion score. Lung lesion scores ranged from 1% to 34%. In the control group, 37.5% of the calves received a lung lesion score >10%. Of the calves inoculated with *M. haemolytica*, 87.5% of the calves that received flunixin transdermal received a lung lesion score >10%, and 90% of the untreated calves who received a placebo had a lung lesion score >10%. Seventy-three percent of the calves on study had a medium or high lung lesion score.

Plasma flunixin concentrations. Mean plasma flunixin concentrations are outlined in **Fig. 6**. The limit of detection (LOD) and limit of quantification (LOQ) for the plasma flunixin analysis were 1.0 ng/mL and 1.46 ng/mL, respectively. The mean λ_z was 0.058 1/h, T ½ was

11.18 h, C_{max} was 238.05 ng/mL, T_{max} was 4.27 h, $AUC_{0\text{-}last}$ was 16,943.29 (h*ng/mL), and $AUC_{0\text{-}\infty}$ was 16,953.53(h*ng/mL). The range for T ½ was 9.54 to 24.06 h and for C_{max} was 29.23 to 2,590.37 ng/mL.

Correlation coefficients. Pearson correlation coefficients are shown as a heatmap in **Fig. 7**. Visual analog scale score showed evidence of being positively correlated with CIS (Pearson correlation coefficient: 0.69). Gait velocity showed evidence of being negatively correlated with right front stance time (Pearson correlation coefficients: -0.74 and -0.68, respectively) and positively correlated with right front stride length (Pearson correlation coefficient: 0.63). Average activity showed evidence of being positively correlated with motion index and step count (Pearson correlation coefficients: 0.78, 0.75, respectively).

Lung lesion score and activity average showed evidence of being negatively correlated at 48, 72, 96, 120, 144, and 168 h (Pearson correlation coefficients: -0.67, -0.69, -0.72, -0.66, -0.71, -0.69, respectively). Lung lesion score and step count showed evidence of being negatively correlated at 72 and 96 h (Pearson correlation coefficients: -0.62, -0.63). Lung lesion score and rumination average showed evidence of being negatively correlated at 168 h (Pearson correlation coefficient: -0.64).

DISCUSSION

Pleuritic chest pain resulting from bacterial pneumonia is commonly reported in human medicine (Boyd et al., 2006). Percussion of the thoracic wall has been reported to elicit signs of pain and painful respiration has been documented from pleuropneumonia in cattle, with these findings published over twenty years ago (Ter Laak, 1992; Braun et al., 1997). The BRD induction model used in the present study was expected to produce pleuritis and gross necropsy findings revealed pleuritis was present in inoculated calves with significant lung consolidation.

The evaluation of pain in cattle requires a multifaceted approach as there is no single biomarker for pain – which may contribute to the lack of literature regarding the association of pain and bovine respiratory disease. The results from the present study indicate that a combination of reduced activity levels, decreased force on calves' right front limb near the area of bacterial infection, and increased visual analog scale pain scores all support that bacterial pneumonia in cattle is painful.

Gait analysis seemed to be the most objective measure that detected treatment differences that may be a good indicator of BRD and specific to pain. Calves who received flunixin transdermal placed more force on their right front limb compared to calves inoculated with *M. haemolytica* who did not receive an NSAID, providing evidence that flunixin may have attenuated pain. In the present study, the right apical lung lobes were inoculated, thus indicating that gait measurements more specific to the area of disease pathology may yield better results. For the study duration, right front force decreased as BRD progressed indicating that calves placing less force on their right front limb may be more painful.

Visual analog scale pain scores showed the most pronounced increase in calves inoculated with *M. haemolytica* who did not receive an NSAID over the duration of the study compared to other treatment groups. Visual analog scale scoring is a more subjective outcome but may be more sensitive to pain relative to CIS based on treatment differences being detected by VAS that were not detected with CIS.

Activity levels significantly decreased for both treatment groups inoculated with *M. haemolytica* relative to controls indicating that reduced activity levels may be a good indicator of BRD onset but less specific to pain, similar to results reported by Hanzlicek et al. (2010). Pillen et al. (2016) reported reduced activity levels at least 6 days prior to BRD diagnosis with more

pronounced changes the day before disease identification. Sick animals often increase time of rest to reduce energy expenditure (Hart, 1988) and remotely quantifying activity levels may become a useful way to identify sick cattle earlier on in the disease process before more clinical signs manifest themselves.

Lung lesion scores were assigned by a pathologist (KMA) at the K-State Veterinary Diagnostic Lab following the 192 h sample collection and euthanasia. Calves with a high degree of lung consolidation were likely experiencing more thoracic pain than calves with very little lung consolidation; however, at what point this consolidation occurred throughout the disease process and duration of the study for each animal is not known. High lung lesion scores were associated with a lower mean right front stride length and force, CLS >1, lower average activity and rumination levels, lower motion index and step count, fewer lying bouts, and higher PGEM concentrations. Previous literature has found distance traveled (White et al., 2012) and computerized lung score (Baruch et al., 2019) were associated with lung lesion scores.

This study was conducted in late fall with changing weather and large temperature swings that created conditions likely to produce respiratory disease outbreaks in calves. All the calves on study received thoracic ultrasounds and had not shown signs of respiratory disease prior to being on study and were not allowed to touch noses with other calves on site. However, inoculating the control calves with saline via bronchoalveolar endoscopy seemed to create enough of a stressful event in the described conditions that the control calves may have experienced some degree of pain and distress, as well as the calves inoculated with *M. haemolytica*. The percentage of calves in the control group with a >10% lung lesion score likely influenced treatment differences and thus the authors will discuss overall changes in outcomes

across treatment groups due to 73% of the calves on study having medium or high lung lesion scores.

Throughout the duration of the study, the probability of CIS >1 gradually increased from baseline. Baruch et al. (2019) had similar results with baseline CIS scores differing from those during the bacterial phase of BRD infection. Mechanical nociceptive threshold values, right front stride length, substance P concentrations, motion index and step count all decreased from baseline over the study duration. Williams et al. (2020) introduced using mechanical nociceptive threshold in the thoracic region in cattle and established the location used in the present study as repeatable and feasible. Sensitivity to force in the thoracic region is the most tangibly direct measure of chest pain used in the present study and may have potential as an indicator of thoracic pain from BRD. Serum amyloid A values sharply increased at 0 h, plateaued and began decreasing towards baseline values at 72 h. These results agree with the findings of Petersen et al. (2004) that an elevation in serum amyloid A is observed in cattle with viral as well as bacterial respiratory infection. Cortisol values were very low throughout the duration of the present study. Theurer et al. (2013) found an increase in cortisol concentrations following *M. haemolytica* challenge, but Foote et al. (2017) did not find differences in cortisol due to BRD classification. The calves in the current study were well acclimated to human interaction and moving through the handling facility leading us to believe that the cortisol response to restraint may have been mitigated, and in this study, there was no clear cortisol response to *M. haemolytica* challenge.

During the treatment administration at 0 h, heavy rainfall occurred, and thus the authors chose to readminister the flunixin transdermal and placebo treatment at 24 h in both cohorts. A previous study has shown that rainfall immediately after treatment reduces flunixin transdermal

absorption (Altenbrunner-Martinek et al., 2020). While the objective of this study was not to describe flunixin transdermal pharmacokinetics (PK), the authors chose to outline the PK parameters following the first dose of flunixin transdermal administration due to the impacts of rainfall and the results were closely aligned with those of Altenbrunner-Martinek et al. (2020). The T_{max} for flunixin transdermal has been shown to be 2.14 h (Kleinhenz et al., 2016) and samples were not collected until 4 h following flunixin transdermal administration in the present study, thus impacting the T_{max} and C_{max}. The mean half-life of flunixin transdermal has been found to be 6.42 h by Kleinhenz et al. (2016) and 8-9 h in the PK studies conducted for Food and Drug Administration (FDA) approval (FDA, 2017) but was longer in the present study at 11.18 h. This longer half-life may have been due to the disease state or age of the animals (Leavens et al., 2014) or the number of observations considering a wide range in values was recorded.

The study duration was 192 h in order to capture the evolution of the BRD disease process over a series of days. The results from the current study indicate that many pain outcomes had not returned to baseline levels at the end of the study, pointing to the need for longer-acting analgesic therapies to alleviate pain from BRD. Part of the analgesic approval process requires effectiveness studies in which clinical endpoints must be chosen (Smith, 2019). Therefore, correlations between biomarkers become important when choosing which endpoints to evaluate. Correlations in the present study were strong between visual analog scale score and CIS, both of which are subjective measures evaluated by a trained observer. Lung lesion scores were negatively correlated with activity levels from 48 h to the end of the study, with step count from 72-96 h and with rumination levels at the end of the study period. Further investigation into biomarker correlations and which combination of biomarkers is most appropriate for quantifying pain from BRD is warranted.

CONCLUSIONS

The results from the present study indicate that a combination of reduced activity levels, decreased force on calves' right front limb, and increased visual analog scale pain scores all support that bacterial pneumonia in cattle is painful. Differences in right front force were observed in calves challenged with *M. haemolytica* and treated with flunixin transdermal compared to those given a placebo, indicating that flunixin transdermal may attenuate specific pain biomarkers in cattle with BRD. Lung lesion scores were negatively correlated with activity levels from 48 h to the end of the study, with step count from 72-96 h and with rumination levels at the end of the study period. Investigating biomarkers associated with pain from BRD and its alleviation with analgesic compounds warrants further research to prevent pain and suffering in cattle.

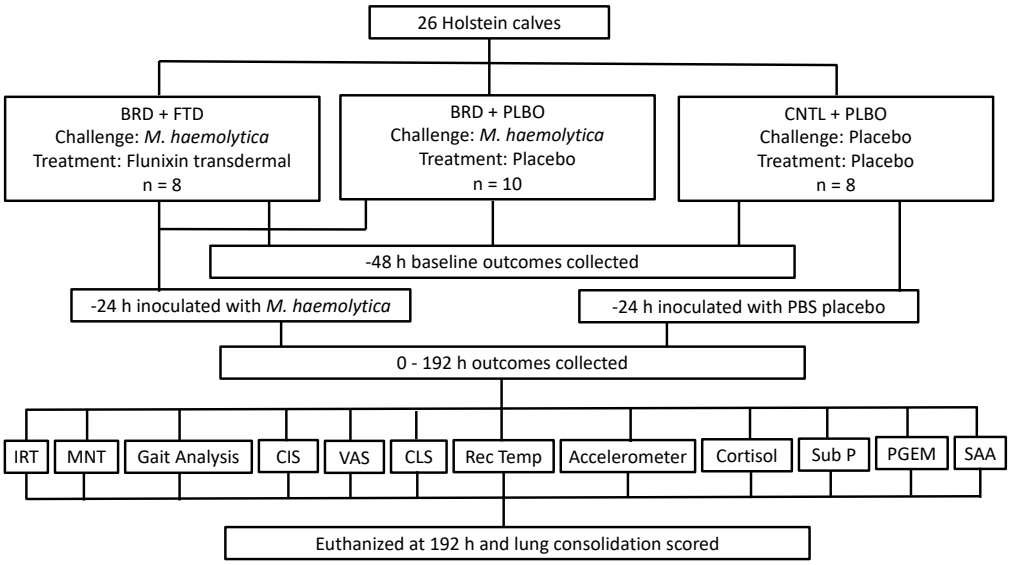

Figure 5.1 Flow chart outlining the timing of study events.

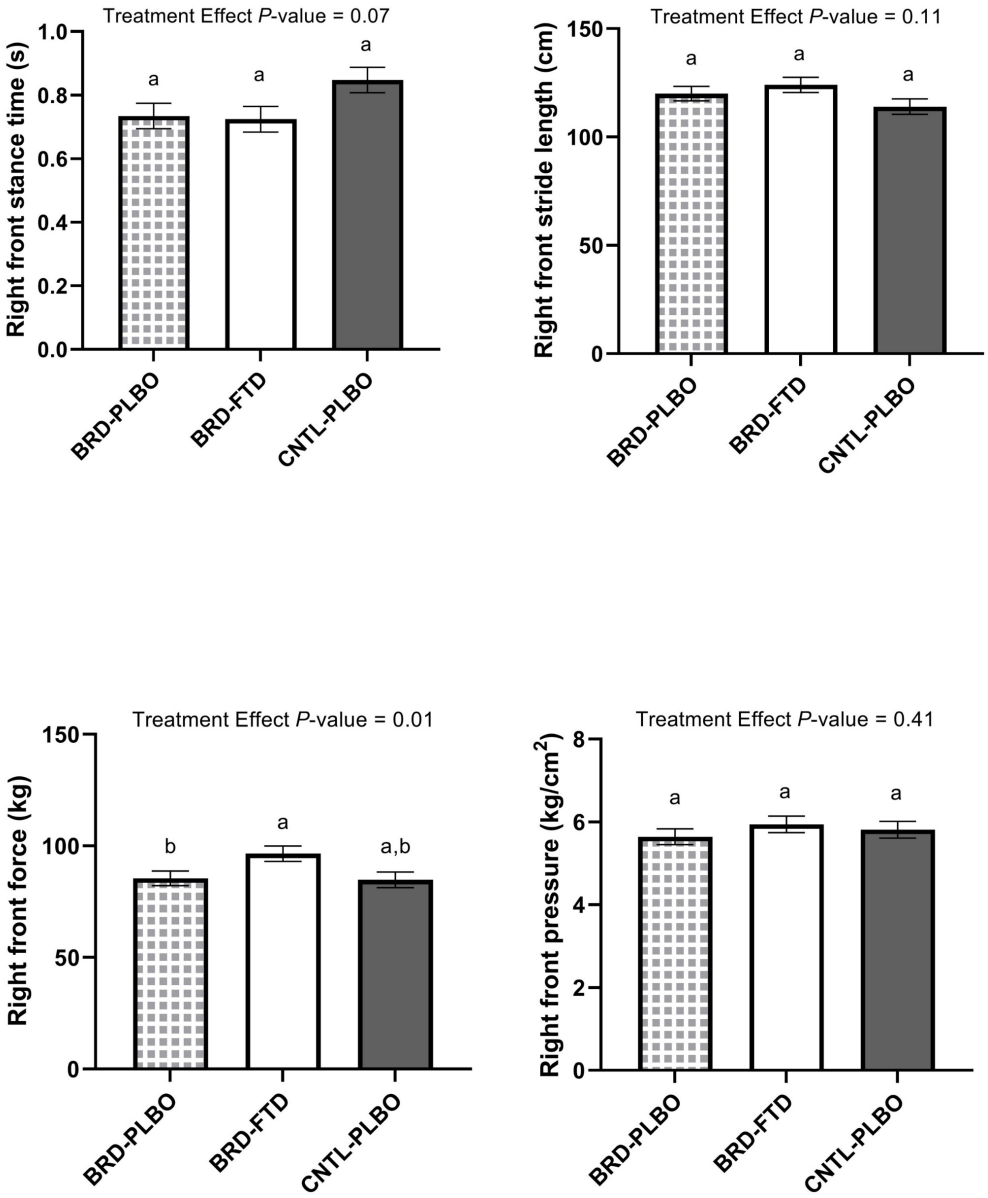

Figure 5.2 Mean values ± SEM for right front gait analysis outcomes by treatment.

[a,b]Different superscripts indicate significant differences between treatment groups ($P \leq 0.05$)

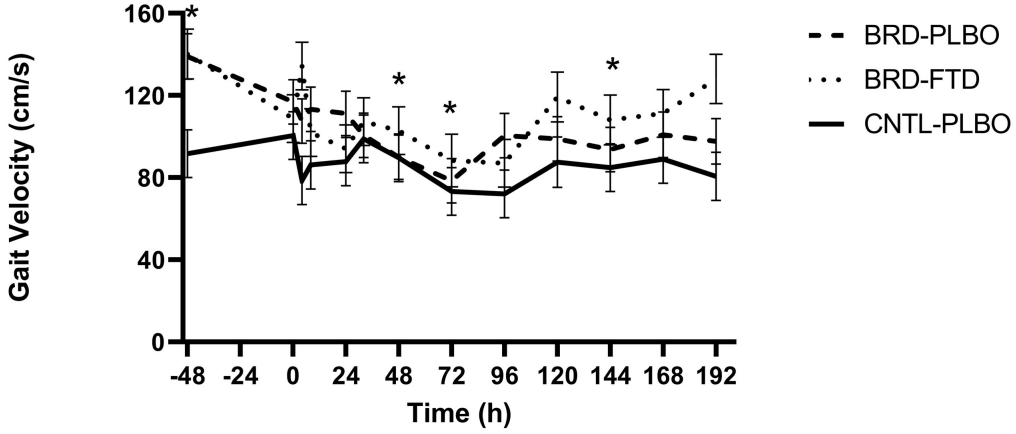

Figure 5.3 Mean values ± SEM for gait velocity over time by treatment group.

***Denotes timepoints where a statistically significant difference ($P \leq 0.05$) was observed**

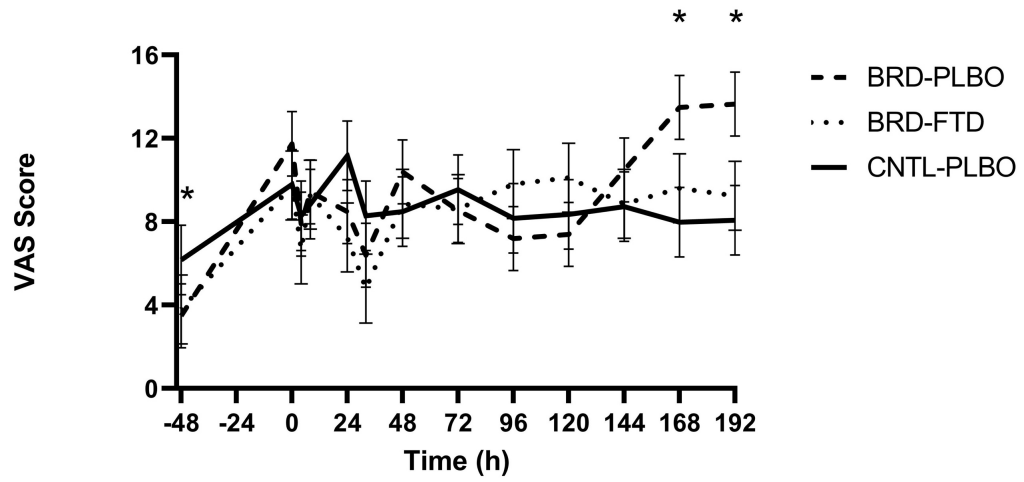

Figure 5.4 Mean values ± SEM for VAS (visual analog scale) over time by treatment group.

***Denotes timepoints where a statistically significant difference ($P \leq 0.05$) was observed**

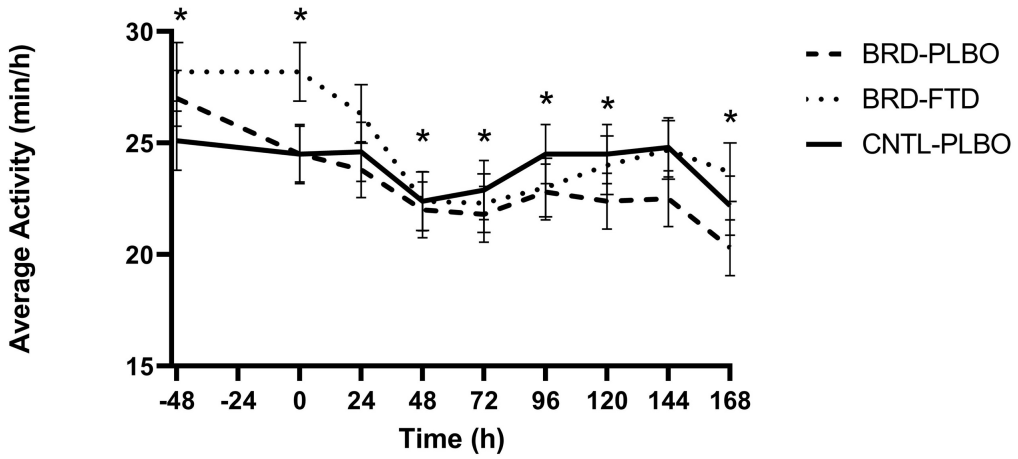

Figure 5.5 Mean values ± SEM for average activity levels over time by treatment group.

***Denotes timepoints where a statistically significant difference ($P \leq 0.05$) was observed**

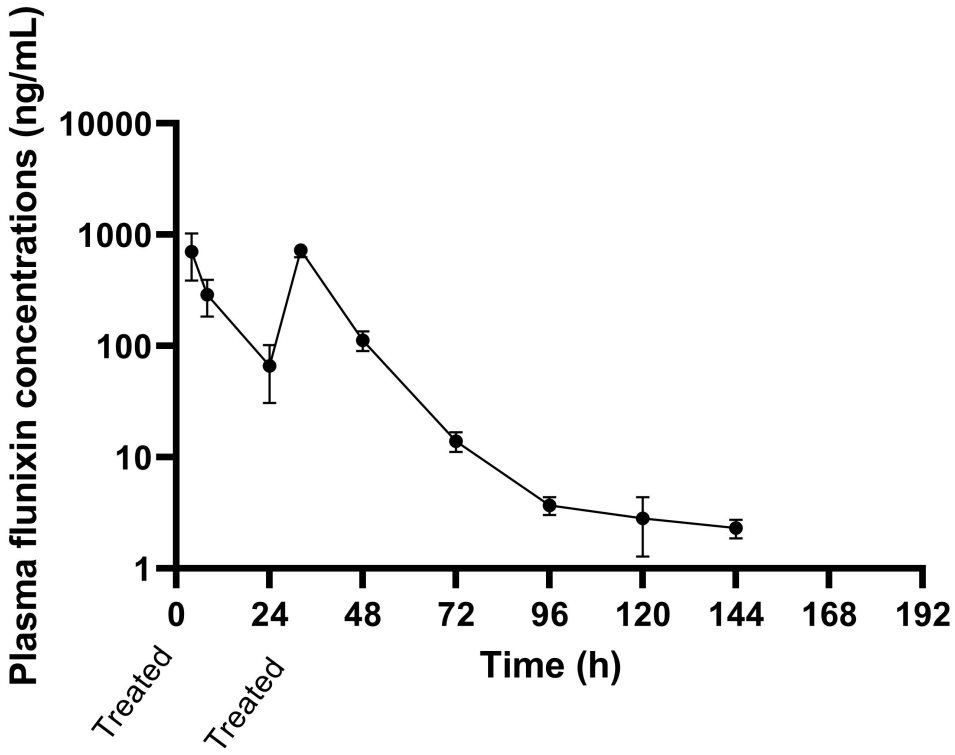

Figure 5.6 Arithmetic mean values ± SEM for plasma flunixin concentrations on a log scale over time in the BRD + FTD treatment group.

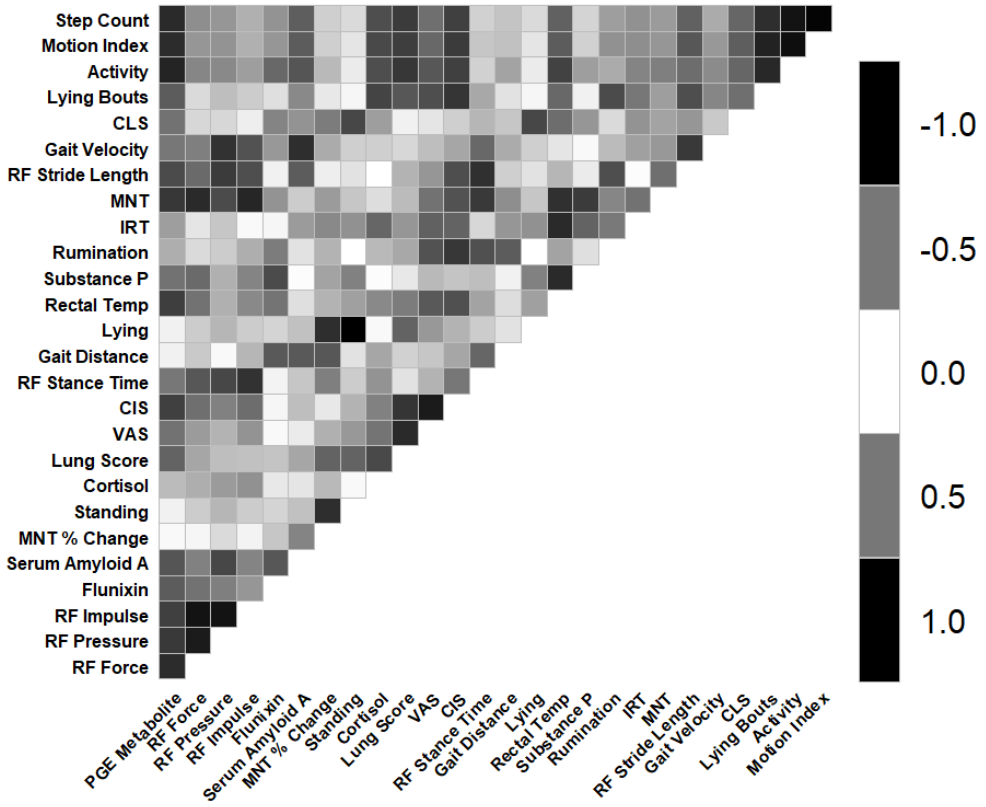

Figure 5.7 Pearson correlation coefficient for each outcome parameter collected.

Table 5.1 Least squares means and probabilities ± SEM for outcome variables by treatment group with associated *P*-values. Means are not reported for outcomes with significant interactions.

Outcome	Units	Treatment group least squares means			*P*-values		
		BRD + PLBO	BRD + FTD	CNTL + PLBO	Treatment	Timepoint	Treatment x Timepoint
IRT[1]	°C	30.3 ± 0.39	30.5 ± 0.40	30.3 ± 0.41	0.91	< 0.01*	0.99
Rf stance time	s	0.73 ± 0.04	0.72 ± 0.0414	0.85 ± 0.04	0.07	< 0.01*	0.85
Rf[2] stride length	cm	120 ± 3.37	124 ± 3.51	114 ± 3.54	0.11	< 0.01*	0.13
Rf[2] force	kg	85.5[a] ± 3.29	96.5[b] ± 3.42	84.8[a,b] ± 3.48	0.01*	0.01*	0.87
Rf[2] impulse	kg*sec	43.8 ± 3.16	49.3 ± 3.29	50.2 ± 3.34	0.22	0.32	0.66
Rf[2] pressure	kg/cm^2	5.64 ± 0.19	5.94 ± 0.20	5.81 ± 0.20	0.41	0.01*	0.59
Gait distance	cm	159 ± 3.39	159 ± 3.57	161 ± 3.60	0.86	0.06	0.32
Gait velocity	cm/s				0.004	0.52	0.05*
MNT[3] average	kg F	1.70 ± 0.11	1.77 ± 0.11	1.54 ± 0.12	0.35	< 0.01*	0.14
MNT[3] % change	%	0.06 ± 0.11	0.01 ± 0.11	-0.07 ± 0.11	0.73	0.61	0.68
VAS[4]	1-10 cm				0.45	0.56	0.01*
Average activity	min/h				0.24	0.01	0.01*
Average rumination	min/h	36.6 ± 3.62	38.7 ± 3.75	34.6 ± 3.82	0.73	0.01*	0.50
Motion index	index	5301 ± 540	5832 ± 616	5095 ± 573	0.60	< 0.01*	0.21
Standing time	proportion	0.40 ± 0.02	0.42 ± 0.02	0.42 ± 0.02	0.52	< 0.01*	0.95
Lying time	proportion	0.60 ± 0.02	0.58± 0.02	0.58 ± 0.02	0.64	< 0.01*	0.93
Steps	count	1112 ± 105	1209 ± 120	1084 ± 111	0.66	< 0.01*	0.08
Lying bouts	count	13.2 ± 1.20	13.9 ± 1.3	11.7 ± 1.27	0.46	0.03*	0.53
Rectal temperature	°C	39.1 ± 0.14	39.2 ± 0.15	39.2 ± 0.15	0.98	< 0.01*	0.15
Cortisol	ng/mL	4.83 ± 0.79	4.09 ± 0.86	3.22 ± 0.88	0.19	0.01*	0.10
Substance P	pg/mL	359 ± 64.3	361 ± 66.7	216 ± 67.9	0.14	0.07	0.90
PGEM[5]	pg/mL	29.5 ± 4.27	25.8 ± 4.43	32.3 ± 4.51	0.44	< 0.01*	0.84
SAA[6]	µg/mL	221 ± 54.3	279 ± 56.2	225 ± 57.2	0.59	< 0.01	0.24

		Treatment group probabilities of score being abnormal					
		BRD + PLBO	BRD + FTD	CNTL + PLBO			
CIS[7]	Score 1-4	0.156 ± 17.1	0.118 ± 13.4	0.148 ± 16.3	0.96	< 0.01*	0.57
CLS[8]	Score 1-5	0.626 ± 0.08	0.581± 0.09	0.565 ± 0.09	0.84	< 0.01*	0.98

[1]IRT = infrared thermography

[2]Rf = right front

[3]MNT = mechanical nociceptive threshold

[4]VAS = visual analog scale

[5]PGEM = prostaglandin E_2 metabolites

[6]SAA = serum amyloid A

[7]CIS = clinical illness score

[8]CLS = computerized lung score

*Denotes where a statistically significant difference ($P \leq 0.05$) was observed

[a,b]Different superscripts indicate significant differences between treatment groups ($P \leq 0.05$)

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Chapter 6 - Effect of bupivacaine liposome suspension administered as a cornual nerve block on indicators of pain and distress during and after cautery dehorning in dairy calves

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ABSTRACT

Dehorning is performed on a high percentage of dairies worldwide. Concern about the negative impact of dehorning on animal welfare has contributed to the development of new guidelines that require the use of pain management at the time of disbudding in the U.S. However, livestock producers are limited in how to address this requirement due to a lack of (1) approved analgesic drugs, (2) analgesic options that control pain for an extended duration, and (3) analgesic formulations that are practical for producers to administer. The objective of this study was to evaluate the effectiveness of bupivacaine liposome suspension, a novel, long-acting, local anesthetic formulation administered as a nerve block at dehorning, compared to current

industry standard analgesic approaches using lidocaine nerve blocks alone, or in combination with the non-steroidal anti-inflammatory drug (NSAID) meloxicam. Fifty male Holstein calves, 10-14 weeks of age were enrolled and randomly assigned to 1 of 5 treatment groups prior to cautery dehorning: 1) bupivacaine liposome suspension block, oral placebo (BUP); 2) lidocaine block, oral placebo (LID); 3) lidocaine block, oral meloxicam (1 mg/kg) (LID + MEL); 4) saline block, oral placebo (CON); and 5) saline block, oral placebo, sham dehorn (SHAM). Biomarkers were collected from 0 to 120 h post-dehorning and included infrared thermography, mechanical nociceptive threshold (MNT), pressure mat gait analysis, chute defense and behavior scoring, and blood sampling for serum cortisol and prostaglandin E_2 metabolites (PGEM). Responses were analyzed using repeated measures with calf nested in treatment designated as a random effect, and treatment, time, and their interaction designated as fixed effects. At 2 h post-dehorning, the BUP group had a higher MNT compared to the CON group. Furthermore, at 24 h post-dehorning, the BUP group had a higher MNT compared to the LID group. Gait distance differed significantly between treatment groups; the CON, LID, and LID + MEL groups had an increased gait distance relative to the SHAM group. The CON group exhibited a higher chute defense behavior score during the dehorning procedure compared with all other treatments. Furthermore, the CON group exhibited more ear flicks than the BUP and LID + MEL groups post-dehorning. At 4 h and 24 h after dehorning, the LID + MEL group had a lower average PGEM concentration compared to all other treatment groups. These data show that administration of bupivacaine liposome suspension as a cornual nerve block at the time of dehorning was as effective at controlling pain as a multi-modal approach of lidocaine and meloxicam.

Key Words: analgesia, cattle, dehorning, pain

INTRODUCTION

Dehorning or disbudding is a routine management procedure performed on a high percentage of dairies in the United States, with the majority of calves being dehorned by 12 wk of age using a hot-iron (Fulwider et al., 2008; USDA-APHIS-NAHMS, 2018). Even when the procedure is performed in calves less than 4 wk old, hot-iron dehorning causes behavioral, physiological and neuroendocrine changes and a physical form of restraint is still required (Faulkner and Weary, 2000; Stock et al., 2013). Hot-iron dehorning causes an initial increase in cortisol concentrations corresponding to the acute pain of tissue damage and stress of restraint, which does not return to baseline levels for at least 24 h after the procedure, indicating the presence of a prolonged inflammatory response (Stafford and Mellor, 2005; Heinrich et al., 2009). Following burn injury, edema forms in the interstitial space rapidly for the first 8 h, and then continues to form more slowly. This excessive or prolonged edema can exacerbate pain and impair wound healing (Rowan et al., 2015). There is no evidence indicating that farm animals are unable to experience pain (Viñuela-Fernández et al., 2007) and Mellor and Stafford (2004) suggest that newborn and young animals may experience pain more intensely than older animals.

Concerns about the negative impact of dehorning on animal welfare has contributed to the development of new guidelines in the U.S. that require the use of pain management at the time of disbudding (National Milk Producers Federation, 2020). The American Veterinary Medical Association (AVMA) recommends that pre-emptive analgesia be used to mitigate pain from dehorning procedures (AVMA, 2014) and the American Association of Bovine Practitioners (AABP) recommends that pain management be the standard of care to mitigate pain

from the dehorning procedure itself and the during the recovery and healing period (AABP, 2019). However, less than 15% of U.S. producers report always using local anesthesia or analgesia at the time of disbudding in calves < 2 months of age (Johnstone et al., 2021). In a survey of EU farms, pain relief for disbudding was only used on < 30% of farms (Cozzi et al., 2015). In an Ontario survey, 97% of veterinarians and 62% of producers reported using a local anesthetic and 48% of veterinarians and 24% of producers used a nonsteroidal anti-inflammatory drug (NSAID) for disbudding or dehorning (Winder et al., 2016). One factor contributing to the low adoption rate of pain mitigation protocols on U.S. farms is the lack of FDA-approved analgesic drugs (Robles et al., 2021). In a survey published in 2021, 89.5% of U.S. veterinarians reported that federal regulations limit their ability to use analgesic drugs (Johnstone et al., 2021). Moreover, 50% of respondents attributed a reduction in their analgesic use to concern about administering drugs that lack FDA approval. However, as AABP states in their dehorning guidelines (AABP, 2019), veterinarians are permitted to prescribe analgesic drugs for extra-label purposes under the Animal Medicinal Use Clarification Act of 1994 (AMDUCA) (FDA, 1994). Other factors contributing to the low adoption of pain mitigation protocols include a lack of analgesic options that control pain for an extended duration and a lack of analgesic formulations that are practical for producers to administer. Globally, whether pain control is mandatory for disbudding or dehorning based on regulations or program enrollment, along with analgesic drug availability, has led to a wide disparity in accepted standard practices.

A multimodal approach using local anesthetics and non-steroidal anti-inflammatory drugs (**NSAIDs**) is recommended as the most effective method for reducing pain in cattle following dehorning (Stock et al., 2013; Winder et al., 2018). In many instances, a solution that has both rapid onset and prolonged duration of analgesia is optimal – lidocaine, the original amino-amide

local anesthetic that is most widely used in veterinary practice, has a limited duration of action (20-40 min), and bupivacaine, which is among the most potent and long-acting of the amino-amides, has a longer duration of action (5-8 h), but a higher pKa value than lidocaine and is thus thought to have a later onset (30 min) (Best et al., 2015; Riviere and Papich, 2018). McMeekan et al. (1998) administered bupivacaine 20 min prior to dehorning and again a second time 4 h after the procedure and prevented a cortisol response for 8 h; yet the feasibility of administering a local anesthetic twice must be taken into consideration. A novel extended-release liposomal formulation of bupivacaine was approved for dogs in 2016 and cats in 2018, with administration prior to surgery providing up to 72 h of pain control (FDA, 2016, 2018). Liposomal bupivacaine has been shown to have an increased duration of action and a delayed peak plasma concentration when compared to bupivacaine hydrochloride (Tong et al., 2014). Meloxicam is an NSAID with preferential cyclooxygenase-2 activity (Smith, 2013) that is a practical analgesic option for producers due to its long half-life of 27 h (Coetzee et al., 2009). Meloxicam has not been shown to mitigate signs of acute distress (Coetzee et al., 2012) but has been shown to reduce the prolonged stress response in calves following dehorning (Allen et al., 2013). The objective of this study was to evaluate the effectiveness of bupivacaine liposome suspension, a novel, long-acting, local anesthetic formulation administered as a nerve block at dehorning, against current industry standard analgesic approaches predicated on using lidocaine nerve blocks alone, or in combination with the NSAID meloxicam.

MATERIALS AND METHODS

Animals, Housing, and Treatments

The Kansas State University Institutional Animal Care and Use Committee reviewed and approved the experimental protocol for this project (IACUC# 4259). This study was conducted at

the Kansas State University College of Veterinary Medicine in Manhattan, KS, in July and August 2020. Pain was anticipated for calves in the CON group as a result of dehorning. All calves were assessed 3 times daily for signs of excessive pain via behavior, as well as inappetence for a 120 h period after the procedure. A rescue analgesic protocol for administration of flunixin meglumine (2.2 mg/kg, IV, q 12 h) was in place if calves showed excessive lying, reluctance to rise, or inappetence following dehorning. A total of 50 male Holstein calves all weaned, vaccinated, horned and intact were received for potential enrollment onto the study in June 2020.

Calves were group housed in outdoor pens with open front run-in sheds of equal size for shelter. Pens had concrete flooring bedded with straw and were of size exceeding the guidelines for the *Guide for Care and Use of Agricultural Animals in Research and Teaching* (FASS, 2020). Calves were fed a grain diet formulated at 3.5% BW twice daily per normal procedures at the study site along with free choice hay. Calves were moved to the study site upon arrival and were given a 2 wk acclimation period; throughout the acclimation period calves were trained to be led with a halter and stand haltered for an extended period of time to facilitate biomarker collection.

After the acclimation period, prior to the start of the study, calves were weighed and averaged 92 kg (range: 67 -117 kg). Calves were 10-14 wk of age at the time of enrollment. Calves were randomly allocated to 1 of 5 experimental treatment groups and were cautery dehorned in randomly allocated groups of 10 for feasibility. Randomization was accomplished using the RAND function in Microsoft Excel (Microsoft Excel® 2016, Microsoft Corporation, Redmond, WA). The treatment groups were as follows: 1) bupivacaine liposome suspension block, oral placebo (**BUP**); 2) lidocaine block, oral placebo (**LID**); 3) lidocaine block, oral

meloxicam (1 mg/kg) (**LID + MEL**); 4) saline block, oral placebo (**CON**); and 5) saline block, oral placebo, sham dehorn (**SHAM**). Ten calves were assigned to each treatment group and 2 calves from each treatment group were assigned to each dehorning day (dehorning day 1, 2, 3, 4, or 5), so each treatment was equally represented on each dehorning day. Calf was the experimental unit for the study. The duration of each dehorning phase was 120 h, with baseline measures collected 24 h prior to calves being dehorned. The time of dehorning was considered the 0 h timepoint.

Calves were administered their treatment 10 minutes prior to the dehorning procedure. The local anesthetic block for dehorning consisted of a conventional cornual nerve block and a local block of the horn. For the cornual nerve block, 4-5 mL of local anesthetic was injected half-way between the lateral canthus of the eye and horn just ventral to the frontal crest on each side of the head. For the local block of the horn as described in Bates et al. (2019), 1 mL of local anesthetic was injected laterally and caudally to the horn (12 -14 mL total volume injected). At 5 minutes prior to dehorning, the local anesthetic block was tested by pricking the skin immediately adjacent to the horn with a hypodermic needle. If the animal responded to the needle stick (i.e., not anesthetized), the local anesthetic block was repeated. Calves were dehorned using a pre-heated (approximately 600 °C) propane dehorner (The Coburn Company, Inc., Whitewater, WI) placed on the horn tissue for approximately 15-20 seconds per horn. The sham dehorning procedure was the same as described for the dehorning procedure, except the dehorning iron was inactive (i.e. not turned on). The oral meloxicam tablets (Zydus Pharmaceuticals Inc., Pennington, NJ) were placed in a gelatin capsule (Torpac Inc., Fairfield, NJ) and were administered via a bolus gun at a dosage of 1 mg/kg. The oral placebo was lactose monohydrate powder (Thermo Fisher Scientific, Waltham, MA) which is the binder used in

meloxicam tablets and was also placed in a gelatin capsule (Torpac Inc.) and administered via a bolus gun.

Measurements and Sample Collection

Outcome variables were collected at -24, 0, 0.5, 1, 2, 4, 8, 24, 72, and 120 h post-treatment, with chute defense behavior being scored at 0 h (at the time of dehorning) and pressure mat gait analysis beginning at 4 h. Outcome variables collected included: infrared thermography (**IRT**), mechanical nociceptive threshold (**MNT**), gait analysis using a pressure mat, chute defense and behavior scoring, and blood sampling for serum cortisol and Prostaglandin E_2 metabolites (**PGEM**) (**Fig. 1**). All trained personnel that collected outcome variables and scored behavior were masked to treatment for the duration of the study.

Blood samples for serum cortisol and PGEM determination were collected from the jugular vein via venipuncture. The whole blood samples were immediately transferred to tubes (Vacutainer, BD Diagnostics, Franklin Lakes, NJ) containing either no additive or EDTA anticoagulant for cortisol and PGEM determination, respectively. Blood samples were then centrifuged for ten minutes at 3,000 g, collected serum and plasma was placed in cryovials via transfer pipette, and stored at -80 °C.

Infrared Thermography

The IRT images captured the medial canthus of the left eye using a research-grade infrared camera (Fluke TiX580, Fluke Corp, Everett, WA) using methods adapted from (Kleinhenz et al., 2017). Infrared images were analyzed using research-specific computer software (SmartView v. 4.3, Fluke Thermography, Plymouth, MN) to determine maximum and minimum temperatures.

Mechanical Nociceptive Threshold

A hand-held pressure algometer (Wagner Instruments, Greenwich, CT) was used for MNT determination using methods adapted from (Kleinhenz et al., 2017). A force was applied perpendicularly at a rate of approximately 1 kg of force per second at 5 locations, consisting of 2 points (1 laterally and 1 caudally) around each horn at the hair-horn junction and a point in the center of the forehead. A withdrawal response was indicated by an overt movement away from the applied pressure algometer. The calves were blindfolded to prevent any sudden movements and MNT values were recorded by a second investigator to prevent bias by the investigator performing the MNT collection.

Pressure Mat Gait Analysis

A commercially available pressure mat gait analysis system (Strideway, Tekscan, Inc., South Boston, MA) was used to record gait and biomechanical parameters. The mat was placed next to the chute area so that calves could be dehorned, bio samples collected, and then immediately walked across the mat. The pressure mat was calibrated, using a known mass, to ensure accuracy of the measurements at each timepoint. Video synchronization was used to ensure consistent gait between and within calves at each timepoint. Using research specific software (Strideway 7.7, Tekscan, Inc.), force, contact pressure, impulse, stance time, stride length, and gait distance were assessed.

Behavior

Chute defense behavior was scored at the time of dehorning using the scale adapted from (Grandin, 1993), also cited by Hoppe et al. (2010), summarized as (1) calm, no movement; (2) restless, shifting; (3) squirming, occasionally shaking of the chute; (4) continuous vigorous movement and shaking of the chute; and (5) rearing, twisting of the body, or violent struggling.

Video cameras (Sony Handycam HDR-CX405, Sony Corporation of America, New York, NY) were placed outside of the chute area or calf pens, based on the location of calves at each time point. Calves were video recorded the day prior to the dehorning procedure for 30 min, to collect baseline behavior data. Post-dehorning, calves were video recorded for 30 min at the following time points: 0, 1, 2, 4, 8, and 24 h. A random number generator (random.org) to randomize the videos across time point and calf ID. Four observers masked to treatment, time point, and the research question used a detailed ethogram (**Table 1**) and BORIS software (Behavioral Observation Research Interactive Software v 7.7.3, Torino, Italy) to score calf behavior. Focal-animal, continuous sampling was used for behavioral analysis. Pain behaviors (ear flicking, head shaking, head rubbing, tail flicking, and foot stamping) were classified as events and the occurrence of each behavior (i.e., count data) were collected. The remaining behaviors in the ethogram were classified as states and total duration (sec) of these behaviors across the observation period were collected. A total of 10,500 min (175 h) of behavior recordings were scored and analyzed for this study. The interclass correlation coefficient (**ICC**) for the inter-observer reliability between the four individuals was calculated using data collected by having all observers score the same calf in three different videos for 30 mins. The ICC was ≤ 0.9 between the four individuals indicating excellent reliability between observers.

Cortisol

Serum cortisol concentrations were determined using a commercially available radioimmunoassay (RIA) kit (MP Biomedicals, Irvine, CA) following manufacturer specifications with minor modifications. The standard curve was extended to include 1 and 3 ng/mL by diluting the 10 and 30 ng/mL manufacturer-supplied standards 1:10 respectively. The standard curve ranged from 1 to 300 ng/mL. A low (25 ng/mL) and high (150 ng/mL) quality

control (QC) were ran at the beginning and end of each set to determine inter-assay variability. Plain 12 x 75 mm polypropylene tubes were used as blank tubes to calculate non-specific binding. Input for standards, QCs, and samples was adjusted to 50 µL. Samples were incubated at room temperature for 30 minutes prior to the addition of I-125. Manufacturer instructions were then followed. Tubes were counted on a gamma counter (Wizard2, PerkinElmer, Waltham, MA) for 1 minute. The raw data file was then uploaded onto MyAssays Desktop software (version 7.0.211.1238, 21 Hampton Place, Brighton, UK) for concentration determination. Standard curves were plotted as a 4-parameter logistic curve. Samples with a coefficient of variation (**CV**) > 18% were re-analyzed. The project average for serum cortisol intra-assay CV was 20.95% and inter-assay CV was 20.81%.

Prostaglandin E_2 Metabolites

Prostaglandin E_2 metabolites were analyzed using a commercially available ELISA kit (cat. no. 514531, Cayman Chemical, Ann Arbor, MI) following manufacturer specifications with minor modifications. Sample input was adjusted to 375 µL with 1.5 mL ice-cold acetone added for sample purification. Samples were incubated at -20°C for 30 min., then centrifuged at 3,000 x g for 5 min. Supernatant was transferred to clean 13 x 100mm glass tubes and evaporated using a CentriVap Concentrator (cat. no. 7810014, Labconco, Kansas City, MO) overnight (approx. 18h). Samples were reconstituted with 375 µL of appropriate kit buffer. A 300 µL aliquot of the reconstituted sample was derivatized with proportionally adjusted kit components. Manufacturer protocol was then followed. Samples were diluted 1:2 and ran in duplicate. Absorbance was measured at 405 nm after 60 min of development (SpectraMax i3, Molecular Devices, San Jose, CA). Sample results were excluded if the raw read exceeded the raw read of the highest standard (Standard 1; 50 pg/mL) or was below the lowest acceptable standard. The lowest acceptable

standard was defined for each individual plate and was identified by excluding standards that had a ratio of absorbance of that standard to the maximum binding of any well (%B/B_0) of ≥ 80% or ≤ 20%. Any individual sample outside the standard curve, with a %B/B_0 outside the 20-80% range, or a CV > 15% were re-analyzed. The project average for PGEM intra-assay CV was 15.08% and inter-assay CV was 11.40%.

Calculations and Statistical Analysis

Mechanical nociceptive threshold was used to determine the number of calves needed per treatment group, as previously described (Heinrich et al., 2010). The study was designed to have power exceeding 0.80 assuming a difference in effect size (Δ) of 0.51, a standard error (σ) of 0.13, and a statistical inference level (α) of 0.05. Based on this calculation, a sample size of 10 animals per treatment group was determined. Concentrations of serum cortisol and PGEM were log-transformed for normality before statistical analysis. Responses (i.e. IRT, MNT, gait analysis, behavior, serum cortisol, and PGEM) were analyzed using repeated measures with calf as the experimental unit. Calves nested in a treatment group were designated as a random effect, with treatment, time, and treatment by time interaction designated as fixed effects. F-tests were utilized for testing significance of main effects and interactions. If significant overall differences were identified, pairwise comparisons were performed using the Tukey honestly significant difference (HSD) test. Statistics were performed using statistical software (JMP Pro 15.1.0 and Statistical Analysis System 9.4, SAS Institute, Inc., Cary, NC). Statistical significance was set a priori at $P \leq 0.05$. Data are presented as least squares means.

RESULTS

At 5 min prior to dehorning, after testing the local anesthetic block by pricking the skin immediately adjacent to the horn with a hypodermic needle, one instance of the calf responding

was reported. The entire local anesthetic block was repeated on both horns (12-14 mL administered) and the calf was dehorned 10 min later after not responding to the needle prick following the second administration of the local anesthetic block. The calf was assigned to the BUP treatment group.

Infrared Thermography

A total of 498 IRT images were scored, two images did not save properly to the IRT camera. No evidence was found for a treatment effect on IRT maximum ocular temperature (P = 0.72). The treatment groups had similar maximum ocular temperatures (**Table 2**). There was a significant time effect ($P < 0.0001$) with the highest ocular temperatures at 8 h (41.21°C; 95% CI: 40.79 to 41.63) being consistent with higher ambient temperatures midday. The lowest ocular temperatures (29.34 °C; 95% CI: 28.60 to 30.09) were recorded at 120 h.

Mechanical Nociceptive Threshold

A total of 1,750 MNT readings were recorded with 350 readings taken at each of 5 locations. There was evidence of a significant treatment by time interaction for mean MNT (KgF) ($P < 0.0001$) (**Table 2; Fig. 2**). At 2 h, the BUP group (1.41 KgF: 95% CI: 1.19 to 1.62) was less sensitive to force compared to the CON group (0.88 KgF: 95% CI: 0.67 to 1.10) (P = 0.0009). At 24 h, the BUP group (1.17 KgF: 95% CI: 0.95 to 1.38) was less sensitive to force compared to the LID group (0.81 KgF: 95% CI: 0.59 to 1.02) (P = 0.0201).

Pressure Mat Gait Analysis

A total of 6,578 data points were collected for gait analysis with 60 data points missing due to one failure to record along with calves not having an adequate number of full strides recorded. Gait distance (cm) differed significantly between treatment groups (P = 0.04); the CON, LID, and LID + MEL groups had an increased gait distance relative to the SHAM group

(CON: 200.59 cm; 95% CI: 191.17 to 210.02, LID 199.54 cm; 95% CI: 190.05 to 209.03, LID + MEL 195.78 cm; 95% CI: 186.35 to 205.20, SHAM 182.10 cm; 95% CI: 172.63 to 191.48) (**Table 2; Fig. 3**). There was a significant treatment by time interaction for mean stance time (s) ($P < 0.0001$) (Table 1). There was no evidence of a treatment effect for mean stride (cm) ($P = 0.31$), mean force (kg) ($P = 0.9499$), mean impulse (kg*s) ($P = 0.38$), or mean pressure (kg/cm^2) ($P = 0.18$) (**Table 2**).

Behavior

For the behavior data, 3,560 observations were collected and 1,845 were used for analysis due to calves being out-of-view. Calves were partially or fully obstructed from view and with 41±16% of the scoring period unavailable for scoring. There was evidence of a treatment effect for mean chute defense behavior score ($P < 0.0001$) (**Table 3; Fig. 4**). The CON group (CON score 4.7) exhibited a significantly higher mean chute defense behavior score relative to all other treatment groups (LID score 3.3, $P = 0.005$; BUP score 2.4, $P < 0.0001$; LID + MEL score 2.4, $P < 0.0001$; and SHAM score 1.6, $P < 0.0001$). Additionally, the LID group exhibited a significantly higher mean chute defense behavior score relative to the SHAM group ($P = 0.007$).

Drinking behavior differed by treatment ($P = 0.03$) (**Table 3; Fig. 5**). The BUP group spent significantly more time drinking across the observation period compared to the LID + MEL ($P = 0.03$) group. Eating behavior demonstrated a significant time effect ($P < 0.0001$). Calves spent significantly less time eating at 4 h (estimate: -1103.65, $P < 0.0001$), 8 h (estimate: -904.06, $P = 0.01$), and 24 h (estimate: -619.51, $P = 0.001$) compared to baseline measures at -24 h. There was no evidence of a treatment effect for eating ($P = 0.55$), grooming ($P = 0.64$), lying ($P = 0.54$), ruminating ($P = 0.66$), standing ($P = 0.66$), or walking ($P = 0.30$) activities (**Table 2**).

Ear flicking differed significantly by treatment ($P = 0.002$) (**Table 3; Fig. 6**). The CON group flicked their ears significantly more than the BUP (P = 0.01) and LID + MEL (P = 0.002) groups. There was no evidence of a treatment effect for the pain behaviors tail flicking ($P = 0.17$), foot stamping ($P = 0.64$), head rubbing ($P = 0.19$), or head shaking ($P = 0.10$) (**Table 3**).

Cortisol

A total of 442 data points were reported for cortisol concentrations due to 58 samples collected not having detectable cortisol concentrations. No evidence of a treatment effect was found for average cortisol concentrations (ng/mL) when log transformed ($P = 0.21$). The treatment groups had similar overall average cortisol concentrations (**Table 2; Fig. 7**). There was a significant time effect observed ($P < 0.0001$) with average concentrations being highest at 0 h (14.47 ng/mL; 95% CI: 13.14 to 15.80) and 0.5 h (15.36 ng/mL; 95% CI: 14.04 to 16.70).

Prostaglandin E2 Metabolites

A total of 250 PGEM samples were collected, analyzed and their concentrations reported. There was evidence of a significant treatment by time interaction for average PGEM concentrations (pg/mL) following log transformation ($P < 0.0001$) (**Table 2; Fig. 8**). At 4 h, the LID + MEL group had a lower average PGEM concentration (15.19 pg/mL: 95% CI: 6.05 to 24.33) compared to all other treatment groups (LID: 28.73 pg/mL; 95% CI: 19.59 to 37.86, BUP: 23.81 pg/mL; 95% CI: 14.67 to 32.95, CON: 23.85 pg/mL; 95% CI: 14.72 to 32.99, SHAM: 23.51 pg/mL ; 95% CI: 14.37 to 32.65) ($P < 0.0001$). At 24 h, the LID + MEL group had a lower average PGEM concentration (10.63 pg/mL: 95% CI: 1.49 to 19.77) compared to all other treatment groups (LID: 24.74 pg/mL; 95% CI: 15.61 to 33.88, BUP: 17.61 pg/mL; 95% CI: 8.47 to 26.74, CON: 30.17 pg/mL; 95% CI: 21.03 to 39.30, SHAM: 24.19 pg/mL; 95% CI: 15.05 to 33.32) ($P < 0.0001$).

DISCUSSION

Calves in the present study were 10-14 weeks of age which is older than what is recommended by the National Dairy FARM Program Animal Care Reference Manual Version 4 which requires that calves be disbudded by 8 weeks of age (National Milk Producers Federation, 2020). Results from the 2017 National Animal Health Monitoring Survey of cow-calf operations showed that only 44% of cow-calf operations dehorned horned calves before they left the operation, revealing that many horned animals have the potential to be dehorned after weaning (USDA-APHIS-NAHMS, 2020). The need for analgesic protocols appropriate for calves > 8 weeks of age exists and the data from the current study is translatable for older beef and dairy calves.

Evidence from the current study indicates that cautery dehorning resulted in changes in MNT, gait analysis, chute defense behavior, activity levels, and pain behaviors. These results are consistent with previous findings suggesting that dehorning causes physiological, neuroendocrine and behavioral changes, indicating a stressful or painful response in cattle (Stafford and Mellor, 2011; Stock et al., 2013). Previous research reports changes in cortisol (Glynn et al., 2013), MNT (Heinrich et al., 2010), ocular temperature (Stewart et al., 2008), and pain behaviors (Heinrich et al., 2010) following dehorning.

In the current study, treatment effects were observed for MNT, gait distance, chute defense behavior, drinking and ear flicking behavior, and PGEM concentrations. Changes from baseline levels in MNT and gait analysis were observed throughout the duration of the study—indicating that calves may be experiencing pain 120 h post-cautery dehorning. Previous studies have shown that pressure sensitivity increased around the horn bud for at least 3 weeks following disbudding (Adcock and Tucker, 2018) and that calves experienced ongoing pain three weeks

following disbudding (Adcock and Tucker, 2020). Wounds took between 52 and 72 d to re-epithelialize and were more painful than new epithelium throughout the healing process (Adcock and Tucker, 2018). The prolonged pain response for days following dehorning may be due to the nature of the injury. Burn injury from cautery dehorning causes excessive and prolonged edema which can exacerbate pain and slow wound healing (Rowan et al., 2015).

Ocular temperatures gradually increased for all treatment groups from 0 h to 8 h with a sharp increase from 2 h to 8 h consistent with higher ambient temperatures mid-day. Environmental factors as well as distance from the animal can be very influential upon IRT readings (Church et al., 2014). The study investigators maintained the same distance from each calf and avoided capturing images in direct sunlight. Ocular temperatures for all treatment groups returned to baseline levels at 24 h. These results are consistent with Stock et al. (2015) who also recorded a mean peak temperature at 8 h. There was not a statistically significant decrease in ocular temperature immediately following dehorning in any of the treatment groups. Previous studies have reported a decrease in ocular temperature during the 5 min following dehorning without use of a local anesthetic and between 2 and 3 h when a local anesthetic without an NSAID was administered (Stewart et al., 2008; Stewart et al., 2009). The lack of an analgesic effect on ocular temperature may be indicative that ocular temperature changes are reflective of a stress response and are not solely accurate as a pain indicator (Glynn et al., 2013).

Mechanical nociceptive threshold measures decreased for all treatment groups throughout the current study, with the sharpest decrease observed from 24 to 72 h for all treatment groups except SHAM. The SHAM group returned to baseline levels from 8 to 120 h indicating that calves on study did not become sensitized to MNT measurement. This also suggests that all dehorned treatment groups were still painful at the conclusion of the study period. Based on the

MNT results, the BUP treatment seemed most effective having the longest duration of pain control, with the CON and LID groups being most painful at 4 and 24 h, respectively. Findings from previous research have shown administration of meloxicam in addition to lidocaine resulted in calves being less sensitive to force at 24 h than calves only administered lidocaine (Allen et al., 2013). In the current study, at the end of the LID treatment duration, the LID group may have become more painful than the CON group due to the anesthetic blunting the body's natural inflammatory response to injury during its duration of action (Stafford and Mellor, 2005). In a meta-analysis of previous disbudding studies, higher cortisol concentrations were seen at 4 h in calves receiving a local anesthetic relative to controls, corresponding with when the local anesthetic duration of action ended (Winder et al., 2018), this cortisol spike was not observed in the present study.

Pressure mat gait analysis uses a floor-based mat that records and analyzes the contact force, contact area, and stance time for each limb. Gait distance increased the most from baseline at 4 h and then gradually decreased out to 120 h but did not return to baseline levels by the conclusion of the study period. The longer gait distance in the CON, LID, and LID + MEL groups may be due to calves experiencing more pain at the dehorning site, and as a result changing their head position which increased stride length. Mean stance time decreased from baseline levels at 4 h and remained shorter, not returning to baseline levels by the conclusion of the study period. Mean stride length increased from baseline levels at all timepoints except 24 h. Mean force and mean contact pressure decreased steadily over time from baseline out to 120 h. Gait analysis is a novel approach to evaluate pain in cattle. Recently, gait analysis was employed to support the approval of a transdermal formulation of flunixin, which is the first analgesic compound labeled for cattle to control pain associated with foot rot in the U.S. (FDA, 2017). The

pressure mat gait analysis has also been deployed to assess pain associated with lameness in cattle and swine, castration in cattle, and parturition in cattle (Pairis-Garcia et al., 2015; Kleinhenz et al., 2018; Kleinhenz et al., 2019). To our knowledge, this is the first study that has employed gait analysis to assess pain associated with dehorning in calves. Additional studies are required to gain a better understanding of changes in gait and their association with pain following dehorning.

Mean scores for chute defense behavior differed between the CON group and all other treatment groups suggesting that a local anesthetic alone reduced defensive behaviors at the time of the dehorning procedure. However, the LID group exhibited a higher chute defense score than the SHAM group suggesting that lidocaine alone did not alleviate all pain associated with the dehorning procedure. Drinking was the only activity indicating a treatment difference in the present study, with the BUP group spending more time drinking. Previous findings have suggested that meloxicam may increase quiescence but that was not observed in the present study (Heinrich et al., 2010; Theurer et al., 2012). Calves spent less time eating at 4, 8 and 24 h following dehorning compared to baseline levels, but eating behavior did increase from 4-24 h. Calves may have been in pain and less motivated to eat the day of the dehorning procedure. However, calves were more motivated to eat by the first feeding period at 24 h post-dehorning, but still had not returned to baseline levels. Collecting more data for a longer period of time would be valuable to see when calves returned to baseline levels for eating behavior. Theurer et al. (2012) found that calves only administered meloxicam spent more time eating on days 2 and 6 post-dehorning compared to negative controls. Heinrich et al. (2010) did not find a significant difference in intake between calves administered lidocaine and meloxicam or lidocaine alone before dehorning. Ear flicking (defined as rapidly moves one or both ears to the front and back,

independent of head shaking) results from the current study indicate that the BUP and LID + MEL treatment groups had reduced ear flicking relative to the CON group. However, the CON group mean did not differ from the SHAM group, though the CON mean was numerically larger, the 95% confidence intervals overlapped which may have been influenced by variation between individual animals – a larger sample size may have produced different results. In a meta-analysis of cautery disbudding studies, ear flicks at 1, 6, and 24 h were found to have great heterogeneity, but at 3 or 4 h, less heterogeneity and a protective treatment effect was observed (Winder et al., 2018). Negative mean values were a result of calves being out-of-view of the video camera due to obstructions such as other animals, objects in the pen, and study personnel. Calves averaged being at least partially out-of-view for 24-57% from a single scoring period which likely impacted behavior results. Future studies may implement a more robust system to minimize the risk of calves being out of view of the camera.

Cortisol concentrations increased immediately following dehorning at 0 and 0.5 h but returned to baseline levels from 1 to 2 h following dehorning. Previous studies have characterized the acute cortisol response following dehorning (Heinrich et al., 2009; Stock et al., 2015). Cortisol is reported to be a good indicator of acute pain and distress but showed no evidence of calves still in pain beyond 1 h following dehorning, which did not align with other outcome variables. Cortisol concentrations also did not appear to be sensitive to an analgesic effect. This is not consistent with McMeekan et al. (1998) who administered bupivacaine 20 min prior to dehorning and again a second time 4 h after the procedure and prevented a cortisol response for 8 h; yet quantification methods and limits of detection have progressed since the cited study took place. Cortisol concentrations can be quite variable in response to a stressor, as well as calf age (Stock et al., 2013). In the current study, the cortisol response observed across

treatment groups may be indicative of the stress of restraint despite acclimation. Furthermore, cautery dehorning may have destroyed the nociceptors adjacent to the wound and mitigated the cortisol response specific to pain (Stafford and Mellor, 2005).

The concentration of prostaglandin E_2 metabolites only differed in calves treated with meloxicam which is consistent with previous findings suggesting that NSAIDs reduce prostaglandin E_2 concentrations over the duration of action of the drug (Stock et al., 2016). No differences were observed between CON and SHAM calves which is also consistent with previous findings following dehorning (Stock et al., 2016). The cortisol spike immediately following the dehorning procedure could have potentially inhibited aspects of the inflammatory response and prevented a response in prostaglandin E_2 metabolite concentration across treatment groups (Van Engen and Coetzee, 2018).

Lidocaine is the most widely used local anesthetic in veterinary practice in the U.S. and its widespread use in dehorning procedures is likely attributable to its low cost and long shelf-life (Riviere and Papich, 2018). A multimodal approach of lidocaine and meloxicam has been well-characterized as a more effective option for controlling pain associated with cautery dehorning, with a longer duration of action than lidocaine alone (Heinrich et al., 2010). However, lidocaine use in food animals is no longer allowed in the European Union, demonstrating the need for investigation into alternative analgesic options. Bupivacaine liposome suspension is novel option for pain control during dehorning. Based on the results of the present study, the bupivacaine liposome suspension did not have a delayed onset relative to lidocaine and controlled pain for a duration similar to the combination of lidocaine and meloxicam. No drugs are currently labeled to control pain from dehorning in cattle in the U.S., thus extra-label drug use under the Animal Medicinal Drug Use Clarification Act (AMDUCA) is the only way this use is permitted (FDA,

1994). Producers' ability to implement a change is key for adopting new means of pain control (Jansen et al., 2009) and bupivacaine liposome suspension is not readily available or likely cost-effective for producers to implement at the time of dehorning, currently. However, effective anesthesia for dehorning is becoming increasingly recognized as an industry requirement (Bates et al., 2019; National Milk Producers Federation, 2020), and thus the availability and cost of drugs may evolve with time. While bupivacaine liposome suspension does not seem to provide any extended duration of analgesia relative to a multi-modal approach of lidocaine and meloxicam, it may be more practical for producers to only administer one product. The need for analgesic options that control pain for an extended duration without the need for re-administration and are practical for producers to implement is apparent, and still requires further investigation. In order to improve animal well-being in the future, the use of polled genetics is a viable alternative to causing pain from disbudding and dehorning procedures.

CONCLUSIONS

Evidence provided in the current study indicates that pain from cautery dehorning can last up to 120 h post-dehorning indicated by changes in nociception and gait analysis. These data show that a single administration of bupivacaine liposome suspension as a cornual block at the time of dehorning was as effective at controlling pain, as evidenced by nociceptive threshold and gait distance, as a multi-modal approach of a lidocaine block and systemic meloxicam. A single injection that alleviates both perioperative and postoperative pain would be an attractive option for livestock producers to alleviate pain at the time of dehorning. Further research is needed to determine the repeatability of these results, to quantify pain in different age groups of calves, and to discover effective ways of managing pain for extended durations following painful husbandry procedures.

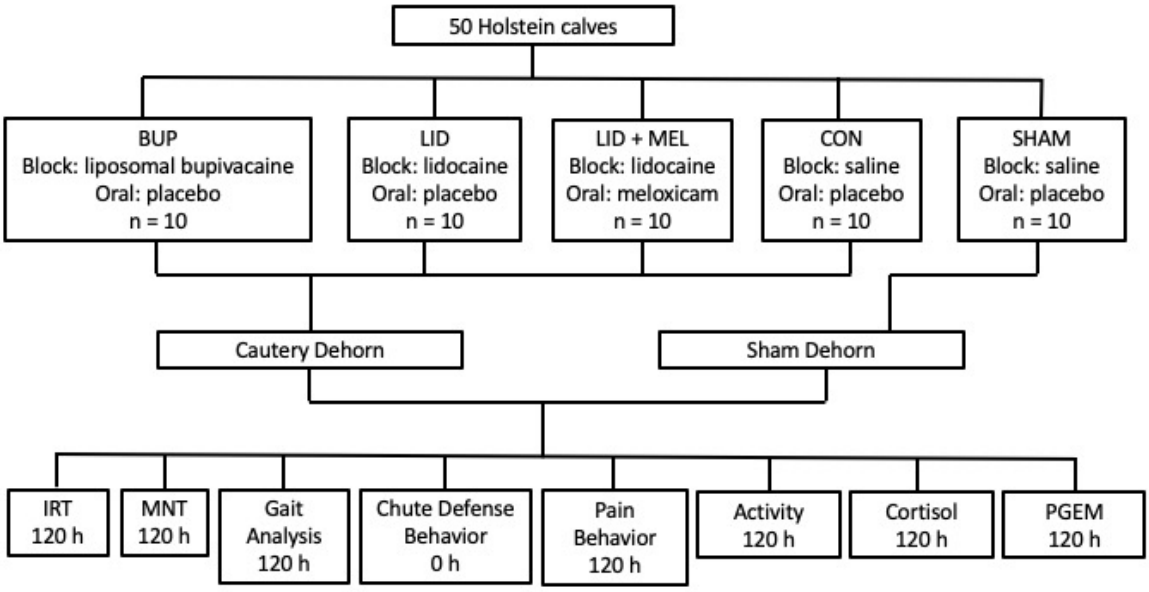

Figure 6.1 Flow chart outlining the timing of study events. Calves were dehorned and outcome variables were collected for the duration of time expressed in h (0-120) below each specific outcome variable.

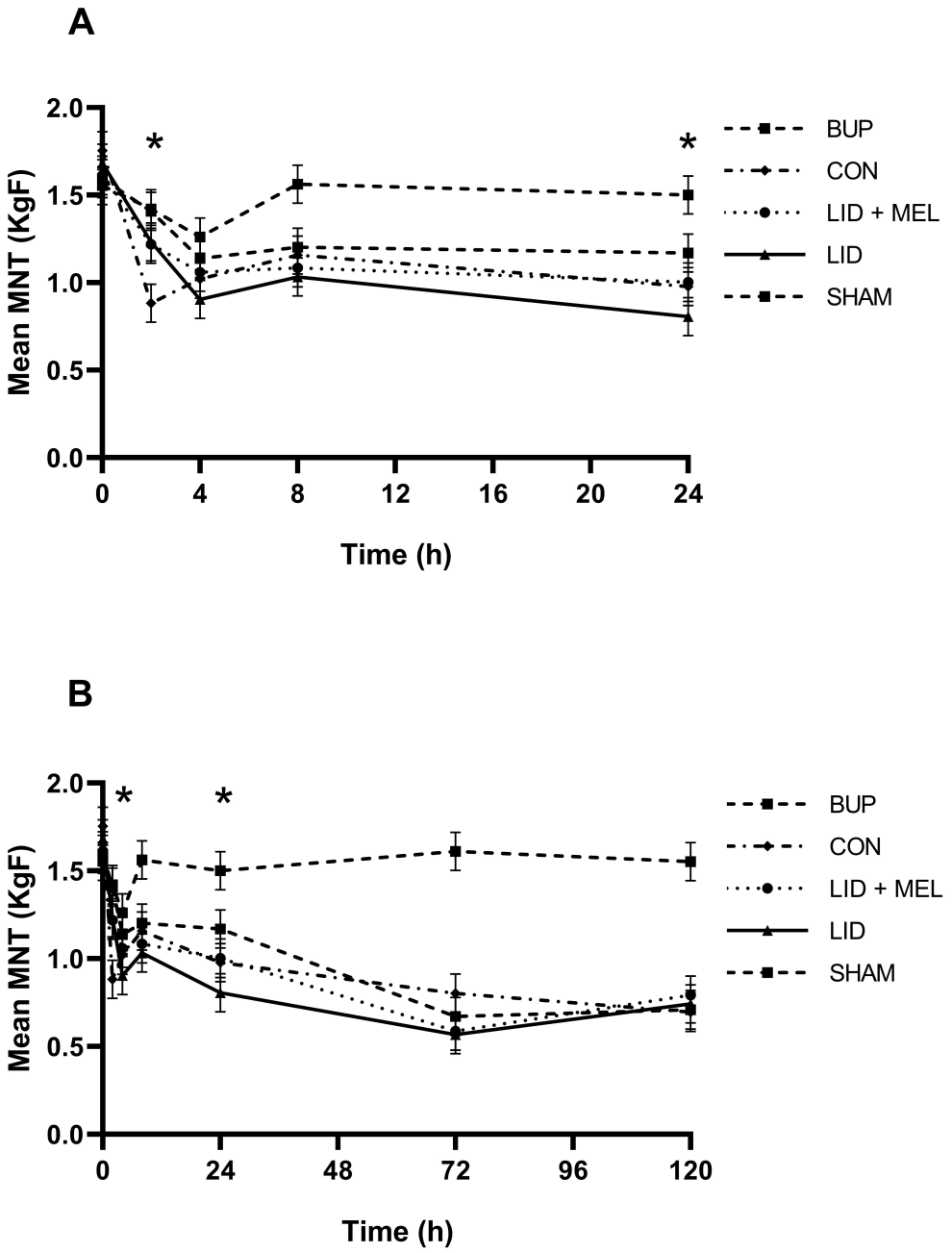

Figure 6.2 Mean mechanical nociceptive threshold values measured in kg of force over the first 24 hours of the study (2.A) and for the study duration (2.B) for each of the five treatment groups.

Error bars indicate SEM.

***Denotes timepoints where a statistically significant difference ($P \leq 0.05$) was observed between at least two treatment groups.**

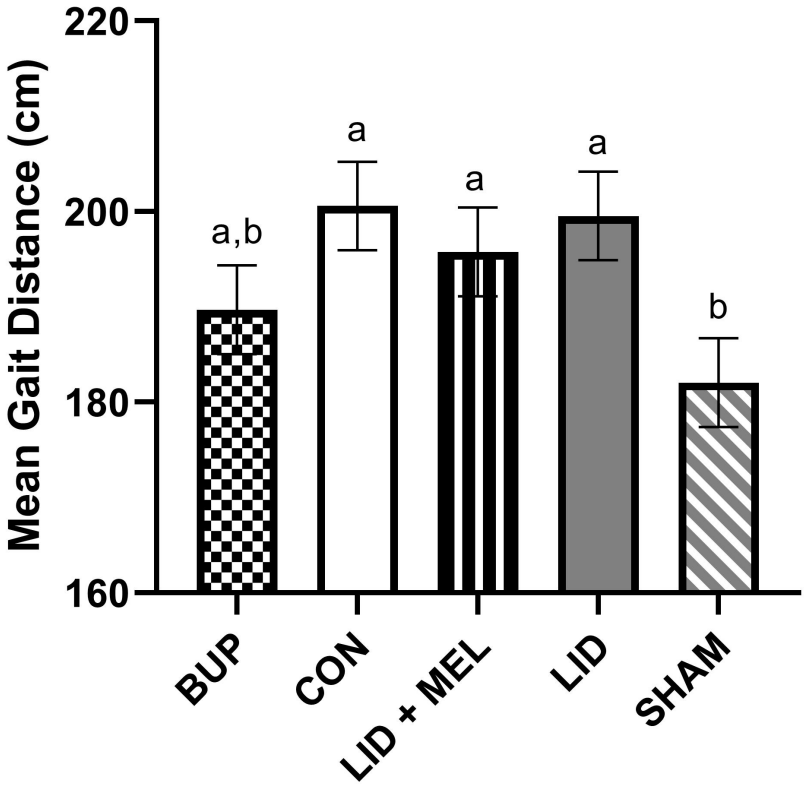

Figure 6.3 Mean gait distance values measured in cm via pressure mat gait analysis for the duration of the study (120 h) for each of the five treatment groups.

Error bars indicate SEM.

[a,b]Different superscripts indicate significant differences between treatment groups ($P \leq 0.05$).

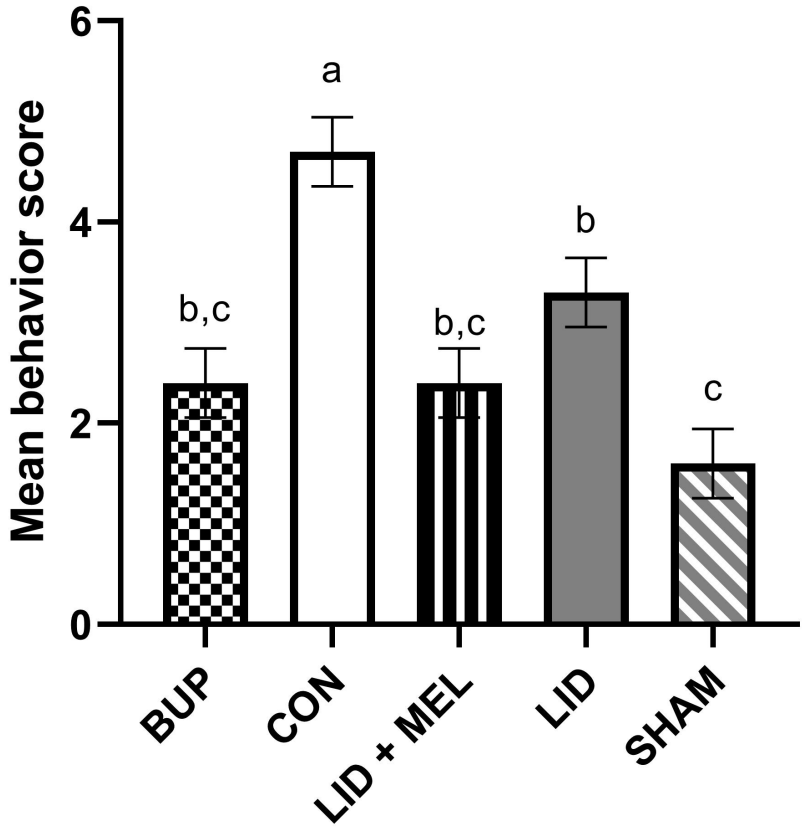

Figure 6.4 Mean chute defense behavior score (0-5), adapted from Grandin (1993); Hoppe et al. (2010) at the time of dehorning for each of the five treatment groups.

Error bars indicate SEM.

[a-c]Different superscripts indicate significant differences between treatment groups ($P \leq 0.05$).

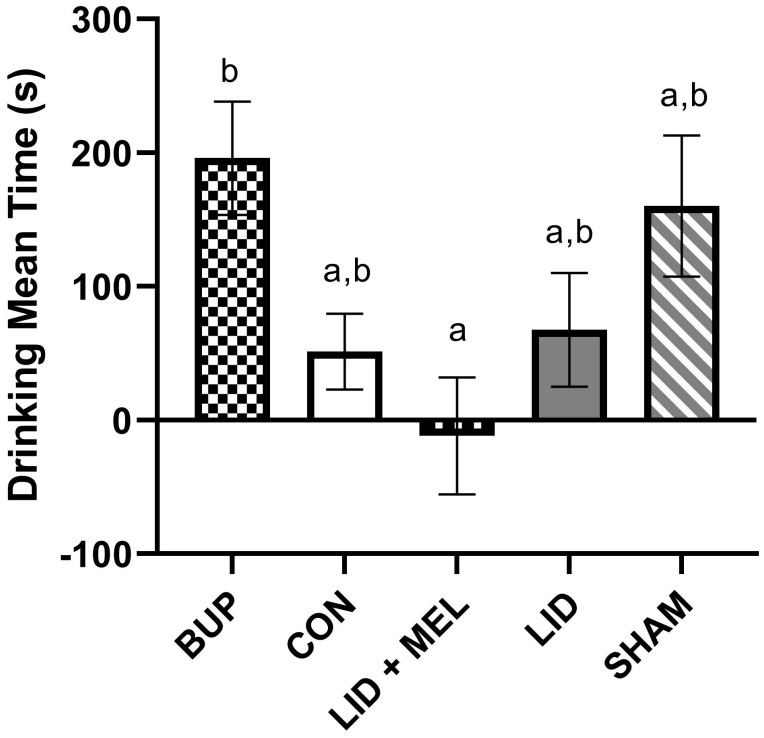

Figure 6.5 Drinking behavior expressed as mean time (s) for the duration of the study (120 h) for each of the five treatment groups.

Error bars indicate SEM.

[a,b]Different superscripts indicate significant differences between treatment groups ($P \leq 0.05$).

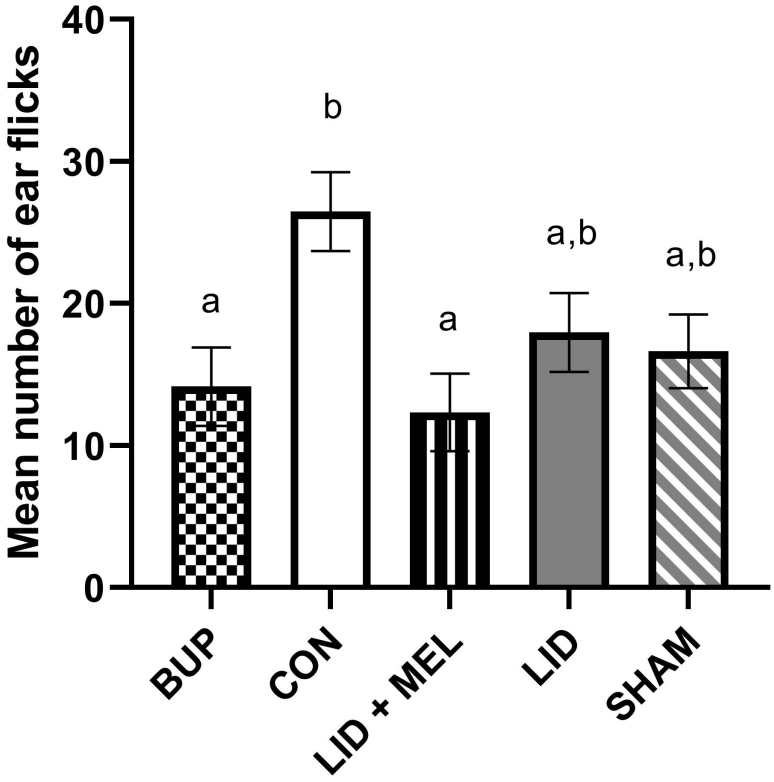

Figure 6.6 Ear flicking behavior expressed as a mean for the duration of the study (120 h) for each of the five treatment groups.

Error bars indicate SEM.

[a,b]Different superscripts indicate significant differences between treatment groups ($P \leq 0.05$).

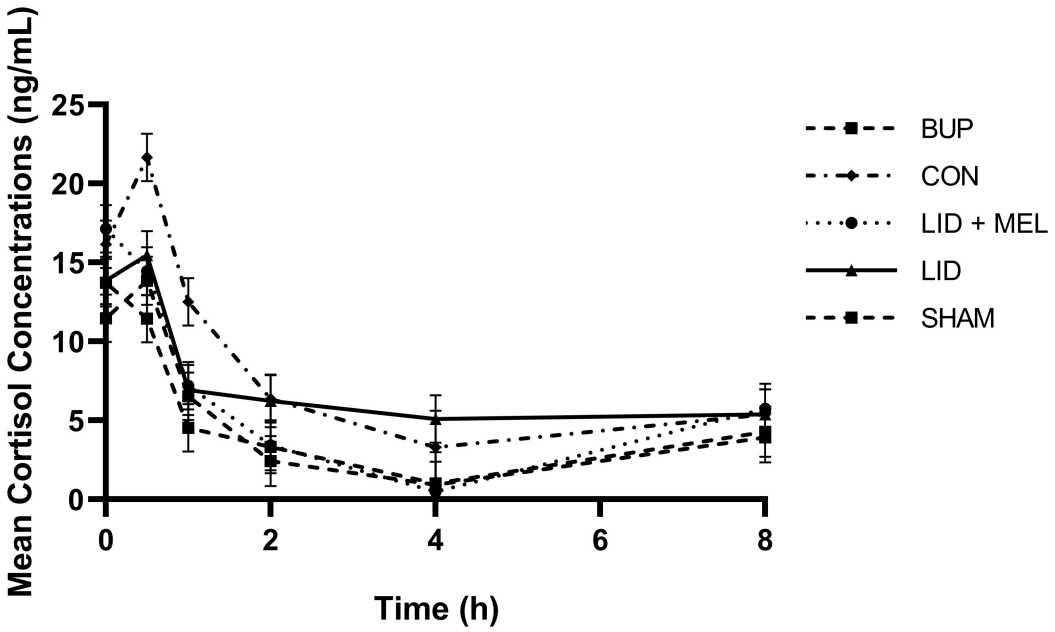

Figure 6.7 Mean cortisol concentrations measured in ng per mL over the first 8 hours of the study for each of the five treatment groups.

No significant treatment differences were observed. Error bars indicate SEM.

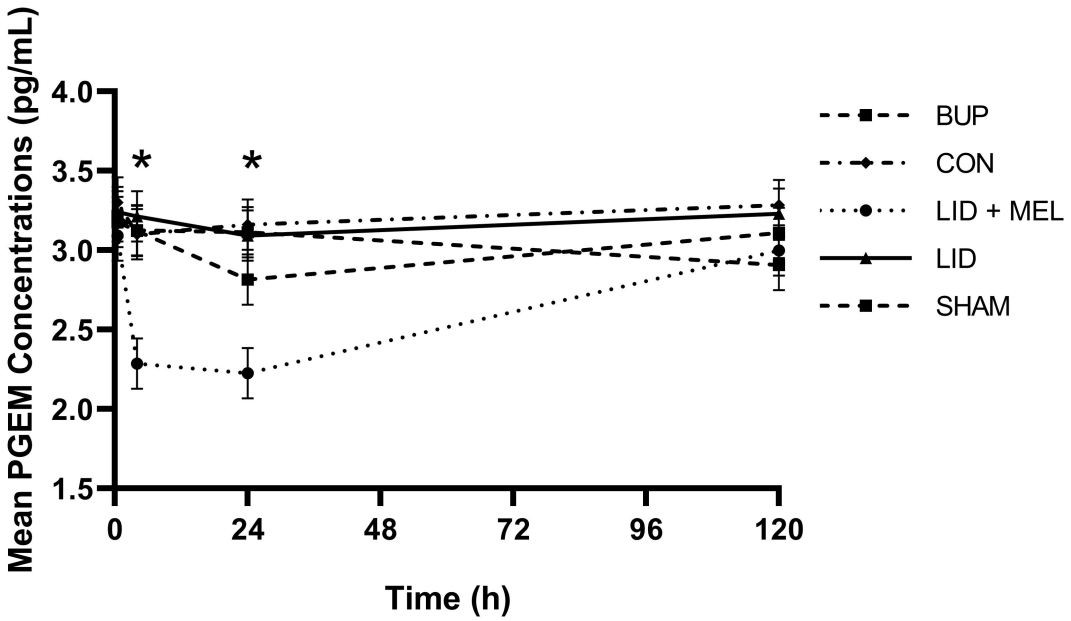

Figure 6.8 Mean Prostaglandin E_2 metabolite concentration measured in pg per mL over the duration of the study for each of the five treatment groups.

Error bars indicate SEM.

***Denotes timepoints where a statistically significant difference ($P \leq 0.05$) was observed between at least two treatment groups.**

Table 6.1 Ethogram used to score calf behavior (adapted from Heinrich et al. (2010) and Sutherland et al. (2013))

Behavior	Description
Eating	Ingesting food provided at feed bunk
Drinking	Consuming water from bucket or waterer
Ruminating	Regurgitating, chewing, and swallowing food
Grooming	Calf moves tongue over body, licking
Walking	Moving forward at a normal pace
Standing	Calf is upright and all four hooves are in contact with the ground
Lying	Calf is recumbent, body is in contact with the ground
Ear flicking	Calf rapidly moves one or both ears to the front and back, independent of head shaking
Head shaking	Calf rapidly shakes head from one side to the other
Head rubbing	Calf lifts hind leg to scratch top of head with foot or rubs head against sides of the pen or gate
Tail flicking	Calf rapidly moves tail from side to side. May include multiple tail movements within one tail-flicking event. A new tail flicking event occurs after the tail moves slowly or is in a resting position
Foot stamping	Calf raises one foot and brings it down again firmly

Table 6.2 Least squares means (upper and lower 95% confidence interval) of outcome variables

Variable	CON	LID	BUP	LID +MEL	SHAM	TRT	TIME	TRT*TIME
MAX IRT (°C)	38.15	38.07	38.17	37.80	38.10	0.7213	< 0.0001	0.5149
CI	37.74 to 38.57	37.66 to 38.49	37.75 to 38.58	37.39 to 38.22	37.68 to 38.51			
Mean MNT (KgF)	1.04[b]	1.00[b]	1.13[b]	1.05[b]	1.49[a]	< 0.0001	< 0.0001	< 0.0001
CI	0.91 to 1.18	0.86 to 1.13	0.99 to 1.26	0.92 to 1.18	1.36 to 1.63			
Gait distance (cm)	200.59[a]	199.54[a]	189.70[a,b]	195.78[a]	182.10[b]	0.0362	0.0423	0.5458
CI	191.17 to 210.02	190.05 to 209.03	180.28 to 199.12	186.35 to 205.20	172.63 to 191.48			
Mean stance time (s)	0.76	0.68	0.75	0.73	0.83	0.1941	< 0.0001	0.0360
CI	0.68 to 0.84	0.60 to 0.77	0.67 to 0.83	0.65 to 0.82	0.75 to 0.91			
Mean stride (cm)	95.79	101.04	98.45	95.58	95.53	0.3064	0.0001	0.0604
CI	91.40 to 100.18	96.64 to 105.44	94.06 to 102.84	91.19 to 99.97	91.12 to 99.93			
Mean force (kg)	28.74	27.30	27.59	27.19	27.76	0.9499	< 0.0001	0.8875
CI	25.78 to 31.71	24.33 to 30.26	24.63 to 30.56	24.23 to 30.16	24.80 to 30.73			
Mean impulse (kg*s)	14.89	13.30	15.21	14.26	16.80	0.3847	< 0.0001	0.6719
CI	12.36 to 17.41	10.77 to 15.83	12.69 to 17.74	11.74 to 16.79	14.28 to 19.33			
Mean pressure (kg/cm^2)	5.87	5.93	5.64	5.67	5.58	0.1800	< 0.0001	0.1199
CI	5.63 to 6.12	5.69 to 6.18	5.39 to 5.88	5.42 to 5.91	5.33 to 5.82			
Average cortisol concentration (ng/mL)	8.36	6.88	5.58	6.89	5.33	0.2123	< 0.0001	0.1489
CI	6.73 to 9.99	5.27 to 8.48	3.96 to 7.20	5.27 to 8.50	3.69 to 6.96			
Average PGE metabolite concentration (pg/mL)	27.72[a]	27.73[a]	23.53[a,b]	19.55[b]	23.76[a,b]	0.0219	< 0.0001	< 0.0001
CI	20.45 to 35.00	20.46 to 35.01	16.25 to 30.81	12.27 to 26.82	16.48 to 31.03			

[a,b] Different superscripts indicate significant differences between treatment groups ($P \leq 0.05$)

IRT – Infrared thermography

MNT – Mechanical nociceptive threshold

Table 6.3 Least squares means (upper and lower 95% confidence interval) of behavior outcome variables

Behavior Variable	CON	LID	BUP	LID +MEL	SHAM	TRT
Chute Defense (1-5)	4.70[c]	3.30[b]	2.40[a,b]	2.40[a,b]	1.60[a]	< 0.0001
CI	4.03 to 5.37	2.63 to 3.97	1.73 to 3.07	1.73 to 3.07	0.93 to 2.27	
Drinking (sec)	51.39[a,b]	67.60a,b	195.96[b]	-11.79[a]	160.20[a,b]	0.0266
CI	-13.90 to 116.68	-30.36 to 165.56	98.00 to 293.93	-112.91 to 89.34	38.28 to 282.12	
Eating (sec)	741.41	530.02	510.49	469.65	512.65	0.5528
CI	454.17 to 1028.66	256.62 to 803.4	233.42 to 787.57	160.11 to 779.19	223.29 to 802.01	
Grooming (sec)	23.4014	19.7771	9.3062	21.4992	12.7262	0.6375
CI	3.43 to 43.37	2.07 to 37.48	-12.19 to 30.80	0.41 to 42.59	-4.63 to 30.08	
Lying (sec)	818.22	828.82	819.79	639.00	747.66	0.5384
CI	568.29 to 1068.16	563.02 to 1094.62	577.01 to 1062.56	393.12 to 884.88	547.93 to 947.40	
Ruminating (sec)	287.71	533.18	494.38	499.59	551.67	0.6577
CI	-39.12 to 614.54	266.17 to 800.19	213.17 to 775.60	192.36 to 806.83	291.63 to 811.71	
Standing (sec)	1212.89	1198.51	1151.30	1104.23	1193.04	0.6553
CI	1092.86 to 1332.93	1083.21 to 1313.80	1035.02 to 1267.58	986.70 to 1221.76	1082.66 to 1303.43	
Walking (sec)	70.4307	50.7651	54.2636	51.6310	45.5675	0.2957
CI	52.66 to 88.20	34.37 to 67.15	37.35 to 71.18	34.69 to 68.57	29.88 to 61.27	
Tail flicking (count)	57.36	51.12	45.90	62.47	61.58	0.1693
CI	45.45 to 69.28	39.73 to 62.52	34.31 to 57.49	50.83 to 74.11	50.76 to 72.40	
Ear flicking (count)	26.4619[b]	17.9725[a,b]	14.1519[a]	12.3280[a]	16.6448[a,b]	0.0023
CI	20.99 to 31.93	12.50 to 23.45	8.72 to 19.58	6.95 to 17.71	11.53 to 21.76	
Foot stamping (count)	11.9292	10.5762	9.7200	9.9367	8.6946	0.6377
CI	8.71 to 15.15	7.59 to 13.56	6.63 to 12.81	6.74 to13.14	5.94 to11.45	
Head rubbing (count)	-0.1286	2.0310	3.2349	3.3418	4.0640	0.1867
CI	-2.92 to 2.66	-0.05 to 4.11	0.82 to 5.65	1.29 to 5.39	1.51 to 6.61	
Head shaking (count)	5.0510	4.3124	2.5104	4.3690	2.9767	0.1018
CI	3.57 to 6.53	2.76 to 5.87	0.94 to 4.08	2.75 to 5.99	1.42 to 4.53	

[a-c]Different superscripts indicate significant differences between treatment groups ($P \leq 0.05$)

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Chapter 7 - Comparison of lidocaine administered alone or in combination with a local nerve block of ethanol or bupivacaine liposome suspension, or oral meloxicam, to extend the duration of analgesia after scoop dehorning in male and female Holstein calves

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ABSTRACT

The AVMA recommends the use of procedures and practices that reduce or eliminate pain and discomfort associated with dehorning. Identification of an effective, long-acting local anesthetic that is practical for producers to implement and reduces pain associated with dehorning would benefit animal welfare. Thirty-two Holstein bulls and heifers were enrolled. The objective of this study was to compare the efficacy and duration of activity of bupivacaine liposome suspension (BUP; n= 8), ethanol (ETH; n = 8), or meloxicam (LID + MEL; n = 8), co-administered with lidocaine; compared to lidocaine only (LID; n = 8), and to quantify their effect on pain

biomarkers and behaviors after scoop dehorning with cauterization in approximately 20-week-old calves. Outcome variables collected included: infrared thermography (IRT), mechanical nociceptive threshold (MNT), visual analog scale (VAS) scoring, and blood sampling for serum cortisol and Prostaglandin E_2 metabolites (PGEM). There was evidence of a sex effect for MNT ($P = 0.04$), with bulls demonstrating a higher threshold (13.74 kg F) compared to heifers (12.12 kg F). There was a treatment by time interaction for cortisol concentrations (ng/mL) ($P = 0.03$). At 2 h, the BUP group had higher cortisol values (17.32 ng/mL) compared to the LID + MEL group (3.10 ng/mL) ($P = 0.03$). Heifers also had had higher mean cortisol values (13.88 ng/mL) compared to bulls (6.96 ng/mL) ($P < 0.01$). There was also evidence of a treatment by time interaction for PGEM concentrations ($P < 0.01$) with calves in the LID + MEL group demonstrating lower PGEM values at 4 and 8 h (10.23 and 9.12 pg/mL) compared to -24, 0, and 0.5 h ($P < 0.03$) (20.38, 27.27, and 22.59 pg/mL, respectively). At 4 h, the LID + MEL group had lower PGEM concentrations (10.23 pg/mL) relative to the ETH group (27.08 pg/mL) ($P = 0.03$). At 8 h, the LID + MEL group had lower PGEM concentrations (9.12 pg/mL) compared to both the ETH and BUP groups (24.80 and 20.52 pg/mL) ($P < 0.03$). These data show that lidocaine + meloxicam reduced cortisol and prostaglandin metabolite concentrations more effectively than ethanol + lidocaine or bupivacaine liposome suspension + lidocaine administered as a local infiltration and cornual block prior to scoop dehorning followed by cauterization. The treatments administered in the present study did not seem to extend the duration of analgesia beyond the currently recommended multi-modal approach, including local anesthesia and systemic analgesia such as lidocaine and meloxicam. Evidence provided in the current study suggests that sex influences certain pain biomarkers such as nociceptive threshold

and cortisol concentration, with males having a higher nociceptive threshold and lower cortisol responses.

Keywords: dehorning, pain, analgesia, NSAID, gender

MAIN BODY

The AVMA recognizes that dehorning of cattle increases safety during handling and transportation, allows cattle to take up less bunk space, and reduces carcass bruising (AVMA, 2014). Because dehorning causes pain and discomfort, the AVMA recommends the use of procedures and practices that reduce or eliminate these effects (AVMA, 2019). Many sources recommend the use of a local anesthetic prior to dehorning, which reduces behavioral and physiological pain responses (Stafford and Mellor, 2005). Additionally, the administration of non-steroidal anti-inflammatory drugs (NSAIDs) has been shown to provide extended postoperative analgesia for calves subjected to cautery dehorning (Allen et al., 2013; Kleinhenz et al., 2017). Anesthesia of the horn bud for calf dehorning is commonly attained by injection of local anesthetic over branches of the cornual nerve. The cornual block method described in Stock et al. (2013) begins with palpation of the temporal ridge, followed by insertion of the needle below the ridge and injection of local anesthetic into the cornual nerve. With experienced staff, this method is 88 to 100% effective (Winder et al., 2018). An alternative method to the cornual block outlined in Bates et al. (2019) is local site infiltration of an anesthetic laterally and caudally to the horn bud, which has been found to result in less of a behavioral response during dehorning than in calves administered local anesthetic via a cornual block. Lidocaine is the most widely used local anesthetic but has a limited duration of activity (Riviere and Papich, 2018).

Ethanol has been shown to result in less pressure sensitivity than lidocaine post-dehorning (Tapper, 2011). Bupivacaine has a longer duration of action but a slower onset than lidocaine (Riviere and Papich, 2018; Martin et al., 2021). Administering lidocaine and bupivacaine has been found to combine the benefits of the rapid onset of lidocaine and the prolonged duration of action of bupivacaine (Best et al., 2015). Finding the most effective local anesthetic that is practical for producers to implement and reduces pain associated with dehorning most effectively, would be beneficial to animal welfare. The null hypothesis of this study was that there would be no difference in efficacy, onset and duration of strategies to extend analgesia following scoop dehorning followed by cauterization. The study objectives were to determine the efficacy, onset, and duration of strategies to extend analgesia for bupivacaine + lidocaine, ethanol + lidocaine, or lidocaine + meloxicam compared to lidocaine only; and to determine which local anesthetic or combination of local anesthetic + NSAID most effectively reduces pain biomarkers and pain behaviors following scoop dehorning.

The Midwest Veterinary Services Institutional Animal Care and Use Committee reviewed and approved the experimental protocol for this project (IACUC# MCL 20055). Calves were group housed in outdoor pens of size exceeding the guidelines for calf housing in the Guide for Care and Use of Agricultural Animals in Research and Teaching (FASS, 2020). Calves were fed a grain diet formulated to meet or exceed the nutritional requirements set by the NRC (NRC, 2016), and were fed once daily per normal procedures at the study site. A total of 32 male and female Holstein calves (9 bulls and 23 heifers) weighing 233 ± 11 kg, approximately 20 weeks of age, all weaned, vaccinated, horned and intact, were randomized using the RAND function in Microsoft Excel (Microsoft Excel® 2016, Microsoft Corporation, Redmond, WA) by horn bud width and then enrolled into 1 of 4 experimental treatment groups: 1) lidocaine (LIDOCAINE

HCl 2% INJECTION, MWI, Boise, ID) cornual block + ethanol (Ethanol 200 Proof, Decon Laboratories, King of Prussia, PA) local infiltration around horn buds + oral placebo (ETH; n = 8; 4 heifers and 4 bulls); (2) lidocaine cornual block + lidocaine local infiltration around horn buds + oral placebo (LID; n = 8; 6 heifers and 2 bulls); (3) lidocaine cornual block + lidocaine local infiltration around horn buds + oral meloxicam (1 mg/kg) (Zydus Pharmaceuticals, Pennington, NJ) (LID + MEL; n = 8; 6 heifers and 2 bulls); (4) lidocaine cornual block + bupivacaine liposome suspension (NOCITA, Elanco, Greenfield, IN) local infiltration around horn buds + oral placebo (BUP; n = 8; 7 heifers and 1 bull).

Calves were administered their treatment 10 minutes prior to the dehorning procedure. The local anesthetic block for dehorning consisted of a conventional cornual nerve block and a local block of the horn buds. For the cornual nerve block, 4-5 mL of local anesthetic was injected half-way between the lateral canthus of the eye and horn just ventral to the frontal crest on each side of the head. For the local block of the horn buds as described in Bates et al. (2019), 1 mL of local anesthetic was injected laterally and caudally to the horn bud (12-14 mL total volume injected). The oral meloxicam tablets (Zydus Pharmaceuticals Inc., Pennington, NJ) were placed in a gelatin capsule (Torpac Inc., Fairfield, NJ) and were administered via a bolus gun at a dosage of 1 mg/kg. The oral placebo was lactose monohydrate powder (Thermo Fisher Scientific, Waltham, MA) which is the binder used in meloxicam tablets and was also placed in a gelatin capsule (Torpac Inc.) and administered via a bolus gun. At 5 minutes prior to dehorning, the local anesthetic block was tested by pricking the skin immediately adjacent to the horn with a hypodermic needle. If the animal responded to the needle stick (i.e. not anesthetized), the local anesthetic block would have been repeated though no calves on study responded. Calves were dehorned using a Barnes dehorning instrument (Stone Manufacturing & Supply Company,

Kansas City, KS). Briefly, the opposing blades of the instrument were aligned with the base of the horns at the skin-horn junction. The handles of the instrument were then closed slowly to ensure proper placement of the instrument. Once optimal positioning was achieved, the handles were spread quickly apart to engage the blades and cut off the horn. Following scoop dehorning, calves were cauterized using a pre-heated electric dehorning iron (Stone Manufacturing & Supply Company) placed on the horn tissue for approximately 15-20 seconds per horn bud. All dehorning procedures were performed by a single experienced veterinarian (CC).

Outcome variables were collected at -24, 0, 0.5, 1, 2, 4, 8, 24, 48, and 72 h post-dehorning, with mechanical nociceptive testing beginning at 2 h and visual analog scale scoring beginning at 8 h following dehorning. Outcome variables collected included: infrared thermography (IRT), mechanical nociceptive threshold (MNT), visual analog scale (VAS) scoring, and blood sampling for serum cortisol and Prostaglandin E_2 metabolites (PGEM). All trained evaluators were masked to treatment for the duration of the study.

Blood samples for serum cortisol and PGEM determination were collected from the jugular vein via venipuncture. The whole blood samples were immediately transferred to tubes (Vacutainer, BD Diagnostics, Franklin Lakes, NJ) containing either no additive for cortisol or EDTA anticoagulant for PGEM determination, respectively. Blood samples were then centrifuged for ten minutes at 3,000 g, collected serum and plasma was placed in cryovials via transfer pipette, and stored at -80 °C.

The IRT images captured the medial canthus of the left eye using a research-grade infrared camera (Fluke TiX580, Fluke Corp, Everett, WA) using methods adapted from Kleinhenz et al. (2017). Infrared images were analyzed using research-specific computer

software (SmartView v. 4.3, Fluke Thermography, Plymouth, MN) to determine maximum and minimum temperatures.

A hand-held pressure algometer (Wagner Instruments, Greenwich, CT) was used for MNT determination using methods adapted from Kleinhenz et al. (2017). A force was applied perpendicularly at a rate of approximately 1 kg of force per second at 5 locations, consisting of 2 points (1 laterally and 1 caudally) around each horn at the hair-horn junction and a point in the center of the forehead. A withdrawal response was indicated by an overt movement away from the applied pressure algometer. The calves were blindfolded to prevent any sudden movements and MNT values were recorded by a second investigator to prevent bias by the investigator performing the MNT collection.

A VAS score was assigned by an evaluator masked to treatment allocations using methods adapted from Martin et al. (2020). The VAS used was a 100 mm (10 cm) line anchored at each end by descriptors of "No Pain" or "Severe Pain". Seven parameters were used to assess pain: depression, tail swishing or flicking, stance, head carriage, spine, movement and ear carriage. No pain was characterized by being alert and quick to show interest, no tail swishing, a normal stance, head carriage above spine level, a straight spine, moving freely around the pen and ears forward. Severe pain was characterized by being dull and showing no interest, more than three tail swishes per minute, legs abducted, head held below spine level, a curved spine, reluctant to move, and ears down. The evaluator marked the line between the 2 descriptors to indicate the pain intensity. A millimeter scale was used to measure the score from the zero anchor point to the evaluator's mark.

Serum cortisol concentrations were determined using a commercially available radioimmunoassay (RIA) kit (MP Biomedicals, Irvine, CA) following manufacturer

specifications with minor modifications. The standard curve was extended to include 1 and 3 ng/mL by diluting the 10 and 30 ng/mL manufacturer-supplied standards 1:10 respectively. The standard curve ranged from 1 to 300 ng/mL. A low (25 ng/mL) and high (150 ng/mL) quality control (QC) were ran at the beginning and end of each set to determine inter-assay variability. Plain 12 x 75 mm polypropylene tubes were used as blank tubes to calculate non-specific binding. Input for standards, QCs, and samples was adjusted to 50 µL. Samples were incubated at room temperature for 30 minutes prior to the addition of I-125. Manufacturer instructions were then followed. Tubes were counted on a gamma counter (Wizard2, PerkinElmer, Waltham, MA) for 1 minute. The raw data file was then uploaded onto MyAssays Desktop software (version 7.0.211.1238, 21 Hampton Place, Brighton, UK) for concentration determination. Standard curves were plotted as a 4-parameter logistic curve. Samples with a coefficient of variation (CV) > 18% were re-analyzed. The project average for serum cortisol intra-assay CV was 14.50% and inter-assay CV was 14.87%.

Prostaglandin E_2 metabolites were analyzed using a commercially available ELISA kit (cat. no. 514531, Cayman Chemical, Ann Arbor, MI) following manufacturer specifications with minor modifications. Sample input was adjusted to 375 µL with 1.5 mL ice-cold acetone added for sample purification. Samples were incubated at -20°C for 30 min., then centrifuged at 3,000 x g for 5 min. Supernatant was transferred to clean 13 x 100mm glass tubes and evaporated using a CentriVap Concentrator (cat. no. 7810014, Labconco, Kansas City, MO) overnight (approx. 18h). Samples were reconstituted with 375 µL of appropriate kit buffer. A 300 µL aliquot of the reconstituted sample was derivatized with proportionally adjusted kit components. Manufacturer protocol was then followed. Samples were diluted 1:2 and ran in duplicate. Absorbance was measured at 405 nm after 60 min of development (SpectraMax i3, Molecular Devices, San Jose,

CA). The standard curve ranged from 0.39 to 50 pg/mL. Sample results were excluded if the raw read exceeded the raw read of the highest standard (Standard 1; 50 pg/mL) or was below the lowest acceptable standard. The lowest acceptable standard was defined for each individual plate and was identified by excluding standards that had a ratio of absorbance of that standard to the maximum binding of any well (%B/B0) of ≥ 80% or ≤ 20%. Any individual sample outside the standard curve, with a %B/B0 outside the 20-80% range, or a CV > 15% were re-analyzed. The project average for PGEM intra-assay CV was 16.40% and inter-assay CV was 13.55%.

Mechanical nociceptive threshold was used to determine the number of calves needed per treatment group, as previously described (Heinrich et al., 2010). The study was designed to have power exceeding 0.80 assuming a difference in effect size (Δ) of 0.51, a standard error (σ) of 0.13, and a statistical inference level (α) of 0.05. Based on this calculation, a sample size of 8 animals per treatment group was determined. Concentrations of serum cortisol and PGEM were log-transformed for normality before statistical analysis. Responses (i.e. IRT, MNT, VAS, serum cortisol, and PGEM) were analyzed using linear regression with repeated measures with calf as the experimental unit. Calves nested in a treatment group were designated as a random effect, with treatment, time, treatment by time interaction, and sex designated as fixed effects. F-tests were utilized for testing significance of main effects and interactions. If significant overall differences were identified, pairwise comparisons were performed using the Tukey honestly significant difference (HSD) test. Statistics were performed using statistical software (JMP Pro 15.1.0, SAS Institute, Inc., Cary, NC). Statistical significance was set a priori at $P \leq 0.05$. Data are presented as least squares means.

Treatment means for all the outcome variables collected are outlined in **Table 1**. No evidence was found of a treatment effect ($P = 0.25$), treatment by time interaction ($P = 0.25$), or

sex effect ($P = 0.15$) for IRT. However, there was a time effect ($P < 0.01$) with 0 and 24 h IRT measurements (33.57 and 33.71 °C; 95% CI: 33.20 to 34.08 °C) being less than readings at -24, 2, 4, 8, and 72 h (>34.73; 95% CI: 34.25 to 37.79 °C) ($P < 0.01$).

No evidence was found of a treatment effect ($P = 0.63$) or a treatment by time interaction ($P = 0.11$) for mean MNT. However, there was a time effect ($P < 0.01$) with thresholds at 24, 48 and 72 h (10.80, 9.78, and 10.18 kg F; 95% CI: 8.68 to 11.91 kg F) being lower than at 4 and 8 h (13.31 and 12.87 kg F; 95% CI: 11.76 to 14.42 kg F) which were all lower than at -24 and 2 h (17.18 and 16.39 kg F; 95% CI: 15.28 to 18.29 kg F) ($P < 0.01$). There was evidence of a sex effect ($P = 0.04$) (**Fig. 1**) with bulls having a higher threshold (13.74 kg F; 95% CI: 12.41 to 15.10 kg F) compared to heifers (12.12 kg F; 95% CI: 11.23 to 13.01 kg F).

There was a trend towards significance ($P \leq 0.10$) for a treatment by time interaction ($P = 0.08$) for percent change from baseline MNT. For the ETH group, thresholds decreased at 24, 48, and 72 h (-36.72, -49.13 and -45.71%, respectively; 95% CI: -60.07 to -22.36%) relative to -24 and 2 h (0 and -0.85%; 95% CI: -15.21 to 14.36%) ($P < 0.01$). For the LID group, thresholds decreased at 48 and 72 h (-40.23 and -39.41%; 95% CI: -63.79 to -15.85%) relative to -24 h (1.61%; 95% CI: -21.94 to 25.17%) ($P < 0.04$). For the LID + MEL group, thresholds decreased at 24, 48 and 72 h (-19.63, -35.27, and -34.45%, respectively; 95% CI: -46.31 to -7.77%) relative to -24 and 2 h (2.61 and 10.70 %; 95% CI: -9.25 to 22.54%) ($P < 0.01$). For the BUP group, thresholds decreased at 8, 24, 48 and 72 h (-29.87, -47.23, -38.89, and -34.64%, respectively; 95% CI: -62.49 to -14.61%) relative to -24 h (3.64%; 95% CI: -11.62 to 18.90%) ($P < 0.01$). There was no evidence of a sex effect for percent change from baseline MNT ($P = 0.16$).

There was a trend towards significance ($P \leq 0.10$) for a treatment by time interaction ($P = 0.09$) for VAS. For the ETH group, VAS scores increased at 8 and 24 h (28 and 25.75 mm; 95%

CI: 18.89 to 34.85 mm) relative to -24, 0 and 72 h (0, 0, and 8.5 mm, respectively; 95% CI: 0 to 15.35 mm) ($P < 0.01$). For the LID group, VAS scores increased at 8 and 24 h (31.33 and 37.67 mm; 95% CI: 20.10 to 48.89 mm) relative to -24, 0 h (0 and 0 mm; 95% CI: 0 to 10.89 mm) ($P < 0.03$). For the LID + MEL group, VAS scores increased at 8 and 24 h (16.84 and 16.99; 95% CI: 11.31 to 22.53 mm) relative to -24, 0, and 72 h (0, 0, and 3.43 mm; 95% CI: 0 to 9.14 mm) ($P < 0.04$). For the BUP group, VAS scores increased at 8 h (27.37 mm; 95% CI: 20.28 to 34.46 mm) relative to -24, 0, 48 and 72 h (0, 0, and 4.37 mm; 95% CI: 0 to 11.46 mm) ($P < 0.01$). There was no evidence of a sex effect for VAS score ($P = 0.41$).

There was evidence of a treatment by time interaction for cortisol concentrations (ng/mL) ($P = 0.03$) (**Fig. 2**). For the LID + MEL group, cortisol values were higher 0 and 0.5 h (20.48 and 20.39 ng/mL; 95% CI: 15.44 to 25.43 ng/mL) relative to 2, 4, 24, and 48 h (3.10, 2.60, 5.80, and 3.82 ng/mL, respectively; 95% CI: 0 to 10.93 ng/mL) ($P < 0.02$). At 2 h, the BUP group had higher cortisol values (17.32 ng/mL; 95% CI: 10.98 to 23.67 ng/mL) relative to the LID + MEL group (3.10 ng/mL; 95% CI: 0 to 8.05 ng/mL) ($P = 0.03$). There was also a sex effect for cortisol concentrations ($P < 0.01$). Heifers had higher mean cortisol values (13.88 ng/mL; 95% CI: 11.54 to 16.22 ng/mL) relative to bulls (6.96 ng/mL; 95% CI: 3.41 to 10.50 ng/mL).

There was a treatment by time interaction ($P < 0.01$) for PGEM concentrations. Calves in the LID + MEL group had lower PGEM values at 4 and 8 h (10.23 and 9.12 pg/mL; 95% CI: 3.01 to 16.34 ng/mL) relative to -24, 0, and 0.5 h ($P < 0.03$) (20.38, 27.27, and 22.59 pg/mL, respectively; 95% CI: 14.26 to 33.39 pg/mL). At 4 h, the LID + MEL group had lower PGEM concentrations (10.23 pg/mL; 95% CI: 4.12 to 16.34 pg/mL) relative to the ETH group (27.08 pg/mL; 95% CI: 19.55 to 34.62 pg/mL) ($P = 0.03$). At 8 h, the LID + MEL group had lower PGEM concentrations (9.12 pg/mL; 95% CI: 3.01 to 15.23 pg/mL) relative to the ETH and BUP

groups (24.80 and 20.52 pg/mL; 95% CI: 12.68 to 32.33) ($P < 0.03$). There was no evidence of a sex effect for PGEM concentrations ($P = 0.27$).

Evidence from the current study indicates that scoop dehorning followed by cauterization caused changes from baseline measures in mechanical nociceptive threshold and visual analog scale scores. In the current study, treatment by time interactions were observed for percent change in mechanical nociceptive threshold, visual analog scale score, cortisol and prostaglandin E_2 metabolite concentrations. The authors chose to investigate the use of a lidocaine cornual block in addition to a local infiltration of either lidocaine, ethanol, or bupivacaine liposome suspension based upon the work of Bates et al. (2019), Tapper (2011), and Martin et al. (2021) investigating the use of the local infiltration method, ethanol as a nerve block for dehorning, and bupivacaine liposome suspension as a nerve block for dehorning, respectively. The investigation of these treatments did not seem to extend the duration of analgesia beyond the currently recommended multi-modal approach (AVMA, 2019) including local anesthesia and systemic analgesia such as lidocaine and meloxicam.

For percent change from baseline MNT values, thresholds decreased from baseline (i.e., increased pain sensitivity around the horn buds) beginning at 8 h for the BUP group, 24 h for the ETH and MEL + LID groups, and not until 48 h for the LID group, but there were not significant differences in MNT values between treatments. Calves in all the treatment groups had not returned to baseline values at 72 h indicating that they were likely still painful. Findings from previous research have shown administration of meloxicam in addition to lidocaine resulted in calves being less sensitive to force (higher nociceptive threshold) than calves only administered lidocaine (Heinrich et al., 2010). A significant difference between the LID and LID + MEL group was not detected in the present study for MNT. Visual analog scale scores were

significantly higher than baseline (i.e., calves exhibited more pain behavior) for the ETH, LID and LID + MEL groups at 8 and 24 h but only at 8 h for the BUP group, with VAS scores declining at 24 h. However, there were not significant differences between treatments.

For cortisol concentrations, the LID + MEL group had lower cortisol concentrations at 2 h relative to the BUP group. In a recent cautery dehorning study, differences in cortisol concentrations between administration of a combination of lidocaine and meloxicam and bupivacaine liposome suspension were not observed (Martin et al., 2021); however, bupivacaine liposome suspension was also administered as a cornual block in that study not just a local infiltration, ultimately resulting in a larger amount of bupivacaine liposome suspension being administered which may have had a more positive effect on reducing cortisol levels than in the present study. In the current study, the investigators were uncomfortable administering ethanol as a cornual block due to a lack of safety data, thus the lidocaine cornual block was chosen across all treatment groups. The highest cortisol concentrations were observed at 0 and 0.5 h which is consistent with past research (Martin et al., 2021). The LID + MEL group had lower PGEM concentrations at 4 and 8 h relative to the ETH group, and ETH and BUP groups, respectively. A decrease in PGEM values in the calves treated with meloxicam was anticipated due to previous findings suggesting that NSAIDs reduce prostaglandin E_2 concentrations over the duration of action of the drug (Stock et al., 2016).

Scoop dehorning followed by cauterization in five-month-old calves likely caused more stress and pain relative to calves that are disbudded at a few days old, which likely influenced some of the outcome parameter values and trends in this study relative to previous studies done in younger calves. Results from the 2017 National Animal Health Monitoring Survey of cow-calf operations showed that only 44% of cow-calf operations dehorned horned calves before they left

the operation, revealing that many horned animals have the potential to be dehorned after weaning (USDA-APHIS-NAHMS, 2020). The need for research into analgesic protocols appropriate to the age of these animals is apparent based on differences observed in the present study compared to (Martin et al., 2021) which was conducted in younger animals, as the pain caused by dehorning may be influenced by age and horn bud width, and effective analgesic protocols for younger animals may not be directly translatable.

Sex effects were observed for mechanical nociceptive threshold and cortisol concentrations, with intact males having a higher nociceptive threshold and lower cortisol concentrations post-dehorning when compared to similarly aged heifers. There were more bull calves in the ETH group and less bull calves in the BUP group relative to the other treatment groups but sex by treatment interaction was not significant for MNT or cortisol concentration outcomes ($P > 0.45$) where a sex effect was observed. Since there were more heifers than bull calves on study, sex effects should be interpreted cautiously. Sex differences are not currently well-characterized in cattle pain research. Results from human research show that male subjects have higher pain thresholds and tolerance, are less discriminative between painful sensations, and the NSAID ibuprofen has been shown to be less effective in women (Walker and Carmody, 1998; Vallerand and Polomano, 2000). The need for further investigation into whether these differences exist in cattle among intact males, castrated males and females is apparent to better quantify and alleviate pain.

These data suggest that lidocaine + meloxicam reduces cortisol and prostaglandin metabolite concentrations at certain timepoints more than ethanol + lidocaine or bupivacaine liposome suspension + lidocaine administered as a local infiltration and cornual block prior to scoop dehorning followed by cauterization. The treatments administered in the present study did

not significantly extend the duration of analgesia beyond the currently recommended multi-modal approach, including local anesthesia and systemic analgesia such as lidocaine and meloxicam. Evidence provided in the current study suggests that sex influences certain pain biomarkers specifically nociceptive threshold and cortisol concentration, with males having a higher nociceptive threshold and lower cortisol responses.

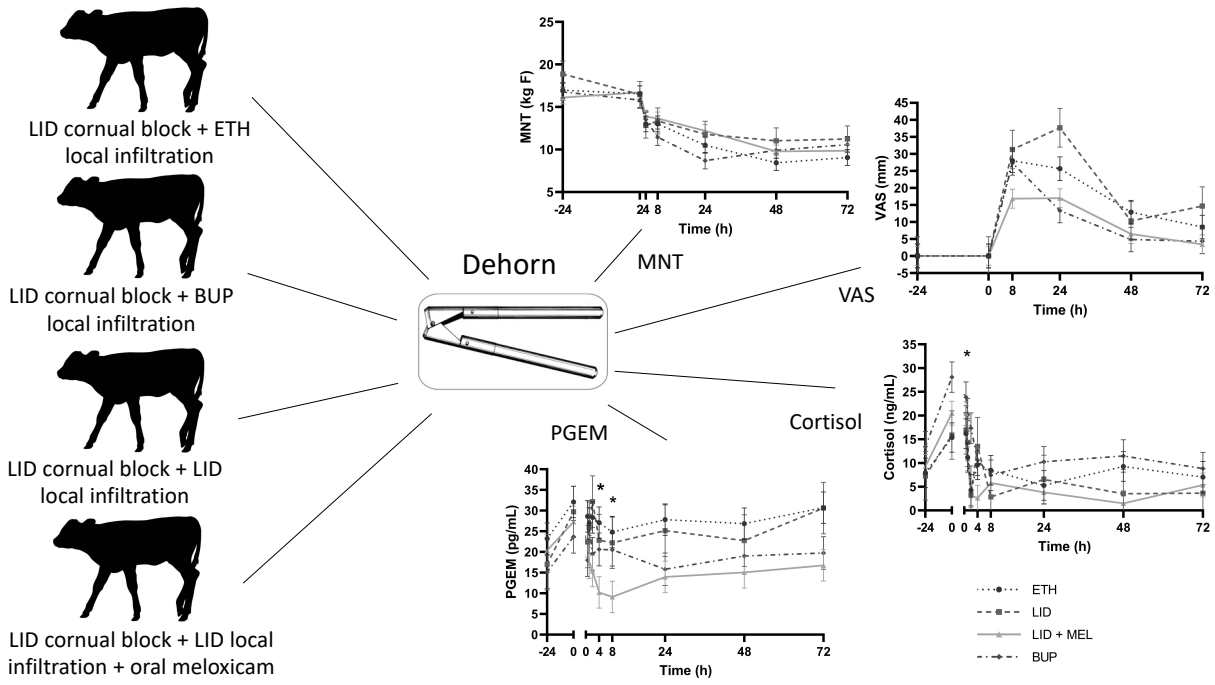

Figure 7.1 Graphical Abstract of treatment groups and outcome variables

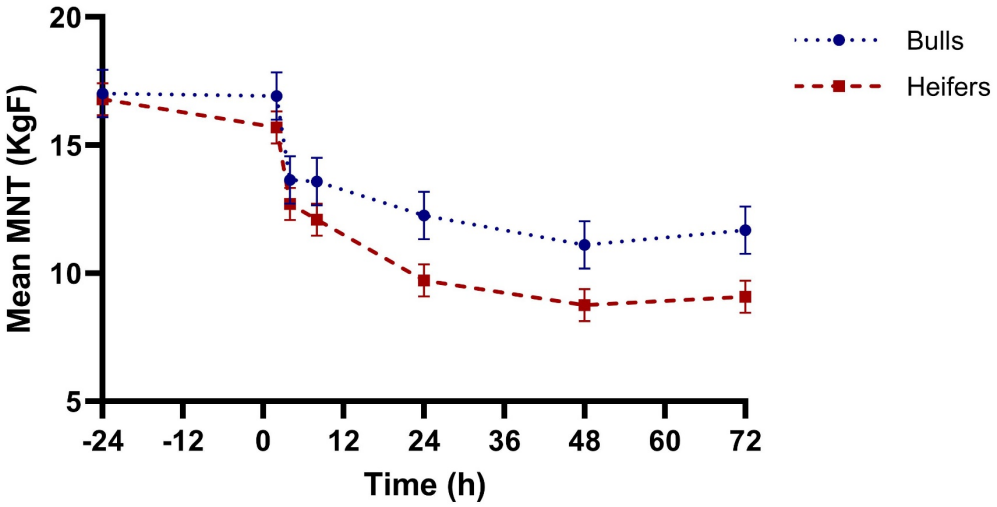

Figure 7.2 Mean mechanical nociceptive threshold values measured in kg of force over the study duration by sex.

Error bars indicate SEM.

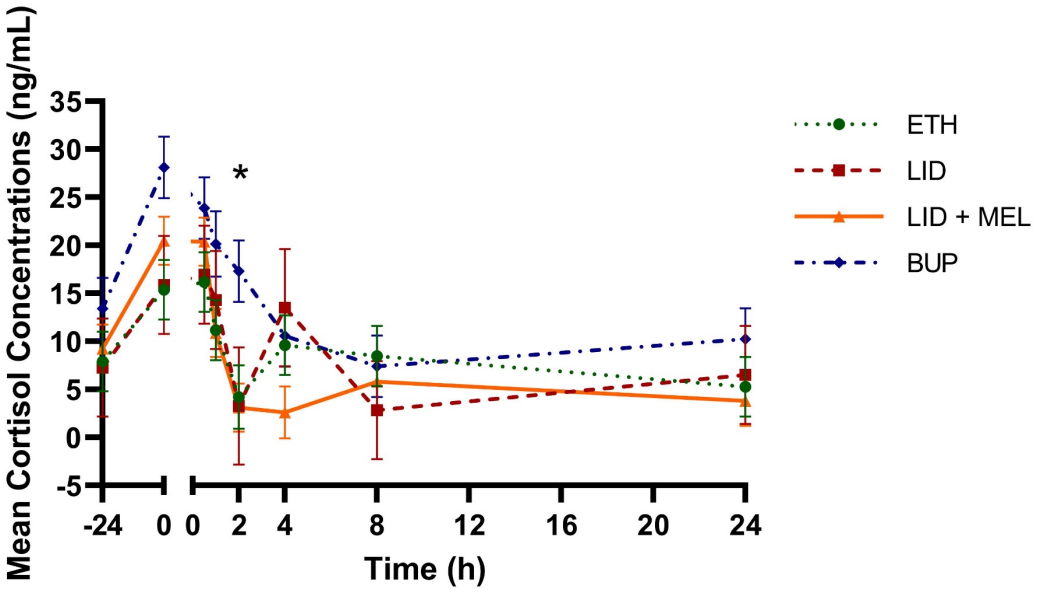

Figure 7.3 Mean cortisol concentrations measured in ng/mL over the first 24 h of the study for each of the four treatment groups.

No significant differences were observed beyond 24 h. Error bars indicate SEM. *Denotes timepoints where a statistically significant difference ($P \leq 0.05$) was observed between at least two treatment groups.

Table 7.1 Least squares means (upper and lower 95% confidence interval) of outcome variables by treatment

Variable	ETH	LID	LID + MEL	BUP	TRT	TIME	TRT*TIME	SEX
Mean IRT (°C)	34.92	34.96	35.15	34.82	0.25	< 0.01	0.25	0.15
CI	34.65 to 35.20	34.51 to 35.41	34.92 to 35.38	34.52 to 35.12				
Mean MNT (Kg F)	12.51	13.66	13.17	12.39	0.63	< 0.01	0.11	0.04
CI	11.18 to 13.84	11.47 to 15.85	12.04 to 14.29	10.93 to 13.84				
% Change MNT (%)	-25.22	-26.27	-13.30	-23.64	0.31	< 0.01	0.09	0.16
CI	-37.09 to -13.34	-45.81 to -6.74	-23.34 to -3.26	-36.61 to -10.67				
Mean VAS (1-100 mm)	12.52	15.55	7.11	8.08	0.08	< 0.01	0.09	0.41
CI	8.29 to 16.75	8.60 to 22.50	3.53 to 10.69	3.47 to 12.69				
Mean cortisol (ng/mL)	9.45	8.77	8.31	15.14	0.06	< 0.01	0.03	< 0.01
CI	5.97 to 12.93	2.99 to 14.55	5.36 to 11.27	11.31 to 18.98				
Mean PGEM (pg/mL)	27.65	25.08	16.81	19.88	< 0.01	< 0.01	< 0.01	0.27
CI	22.57 to 32.72	16.74 to 33.43	12.52 to 21.10	14.33 to 25.42				

IRT – Infrared thermography

MNT – Mechanical nociceptive threshold

VAS – Visual Analog Scale

PGEM – Prostaglandin E_2 metabolite concentration

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Chapter 8 - Effect of bupivacaine liposome suspension administered as a local anesthetic block on indicators of pain and distress during and after surgical castration in dairy calves

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ABSTRACT

Castration is a routine management procedure performed on beef and dairy operations in the United States. All methods of castration have shown to produce behavioral, physiologic, and neuroendocrine changes associated with pain and distress. The American Veterinary Medical Association (AVMA) and the American Association of Bovine Practitioners (AABP) encourage the consideration of administration of local anesthesia, as well as systemic pain relief to minimize pain and stress associated with castration. However, livestock producers are limited in

how to address this challenge, in part due to the lack of approved analgesic drugs. The objective of this study was to evaluate the effectiveness of bupivacaine liposome suspension, a novel, long-acting, local anesthetic formulation administered as a nerve block at castration, as an alternative to current industry standard analgesic approaches using lidocaine nerve blocks alone, or in combination with meloxicam, a non-steroidal anti-inflammatory drug (NSAID). Thirty male Holstein calves, 16-20 weeks of age, were enrolled and randomly assigned to 1 of 4 treatment groups prior to surgical castration: 1) bupivacaine liposome suspension block + oral placebo (BUP); 2) lidocaine block + oral placebo (LID); 3) lidocaine block + oral meloxicam (1 mg/kg) (LID + MEL); and 4) saline block + oral placebo (CON). Biomarkers were collected at baseline (-24 h) and from 0 to 120 hours post-castration and included infrared thermography, pressure mat gait analysis, chute defense and behavior scoring (pain and activity), and blood sampling for serum cortisol and prostaglandin E2 metabolites (PGEM). Responses were analyzed using repeated measures, with calf nested in treatment designated as a random effect, and treatment, time, and their interaction designated as fixed effects. Calves in the CON group had a significantly shorter front stance time from baseline (-8.73%; 95% CI: -24.84 to 7.37%) compared to the BUP and LID + MEL groups (> 5.70%; 95% CI: -22.91 to 23.79%) ($P < 0.03$). The CON group also tended to have an increase in front force from baseline (6.31%; 95% CI: -1.79 to 14.41%) compared to the BUP, LID, and LID + MEL groups (< -5.06%; 95% CI: -14.22 to 0.95%) ($P < 0.04$). Castration had greater impact on posture scores, specifically back position, in the CON group (2.00; 95% CI: 1.68 to 2.32) compared to the LID + MEL group (1.43; 95% CI: 1.13 to 1.72) ($P = 0.05$). Calves in the CON group had higher cortisol concentrations at 24 h (7.70 ng/mL; 95% CI: 1.52 to 13.87 ng/mL) relative to the BUP group (3.11 ng/mL; 95% CI: -2.56 to 8.79 ng/mL) ($P = 0.002$). At 4 and 24 h, the LID + MEL group had lower PGEM

concentrations from baseline (-32.42% and -47.84%; 95% CI: -78.45 to -1.80%) compared with the CON group (27.86% and 47.63%; 95% CI: 7.49 to 82.98%) ($P < 0.02$). These data show that administration of bupivacaine liposome suspension as a local anesthetic block at the time of castration was as effective at controlling pain as a multi-modal approach of lidocaine and meloxicam.

Key words: analgesia, cattle, castration, pain

INTRODUCTION

Castration is routinely performed on beef and dairy operations in the United States. Dairy calves are castrated, on average, at 7.5 weeks, and the majority of beef calves are castrated prior to being sold by the operation they were born on (USDA-APHIS-NAHMS, 2018, 2020). All castration methods have shown to produce behavioral, physiologic and neuroendocrine changes associated with pain and distress (Coetzee, 2011). Wound healing ranges from 35-56 days following surgical castration (Marti et al., 2018).

The American Veterinary Medical Association and the American Association of Bovine Practitioners encourage the consideration of administering anesthesia and analgesia during castration (AVMA, 2014; AABP, 2019). Yet, less than 25% of producers report always using local anesthesia or analgesia for surgical castration in calves < 12 months of age (Johnstone et al., 2021). One factor contributing to the low adoption rate is the lack of FDA-approved analgesics (Robles et al., 2021). However, veterinarians are permitted to prescribe analgesics for extra-label purposes under the Animal Medicinal Drug Use Clarification Act (FDA, 1996).

A liposomal formulation of bupivacaine was approved for dogs in 2016 that provides up to 72 hours of pain control (FDA, 2016). Liposomal bupivacaine has an increased duration of action and a delayed peak plasma concentration when compared to bupivacaine hydrochloride (Tong et al., 2014). Meloxicam is a nonsteroidal anti-inflammatory drug with preferential cyclooxygenase-2 activity (Smith, 2013) that is a practical analgesic option for producers due to its half-life of 27 hours (Coetzee et al., 2009). Meloxicam has shown to reduce pain responses up to 72 hours after castration (Olson et al., 2016). The objective of this study was to evaluate the effectiveness of bupivacaine liposome suspension, a novel, long-acting, local anesthetic formulation administered as a nerve block at castration, as an alternative to current suggested industry analgesic approaches using solely lidocaine or in combination with meloxicam.

MATERIALS AND METHODS

Animals, Housing, and Treatments

The Kansas State University Institutional Animal Care and Use Committee reviewed and approved the experimental protocol for this project (IACUC# 4445). This study was conducted at the Kansas State University College of Veterinary Medicine in Manhattan, KS, in September 2020. All calves were assessed 3 times daily for signs of excessive pain via behavior and inappetence for a 120 h period after castration, in consideration for calves in the control group experiencing pain from castration. The administration of flunixin meglumine (2.2 mg/kg, IV, q 12 h) was established as the rescue analgesic protocol for calves showing excessive lying, reluctance to rise, or inappetence following castration. A total of 30 weaned, vaccinated, and intact male Holstein calves were received from a producer for potential enrollment onto the study in June 2020.

Calves were group housed in outdoor pens with open front run-in sheds of equal size for shelter. Pens had concrete flooring and the pen size exceeded the guidelines for calf housing in the *Guide for Care and Use of Agricultural Animals in Research and Teaching* (FASS, 2020). Calves were fed a grain diet formulated at 3.5% BW twice daily per normal procedures at the study site along with free choice hay. Calves were moved to the study site upon arrival and were given a 2 week acclimation period; throughout the acclimation period calves were trained to be led with a halter and stand haltered for an extended period of time to facilitate biomarker collection.

After the acclimation period, prior to the start of the study, calves were weighed and averaged 155 kg (range: 121-187 kg). Calves (n = 30) were 16-20 weeks of age at the time of enrollment. Calves were castrated in two groups of 15 and were randomly allocated to a castration group, as well as 1 of 4 experimental treatment groups. Randomization was accomplished using the RAND function in Microsoft Excel (Microsoft Excel® 2016, Microsoft Corporation, Redmond, WA). The treatment groups were as follows: 1) bupivacaine liposome suspension block + oral placebo (BUP); 2) lidocaine block + oral placebo (LID); 3) lidocaine block + oral meloxicam (1 mg/kg) (LID + MEL); 4) saline block + oral placebo (CON). The oral meloxicam tablets (Zydus Pharmaceuticals Inc., Pennington, NJ) were placed in a gelatin capsule (Torpac Inc., Fairfield, NJ) and were administered via a bolus gun. As with the oral meloxicam tablets, the oral placebo was also placed into a gelatin capsule (Torpac Inc.) and administered via a bolus gun, except formulated with the binder in meloxicam tablets, lactose monohydrate (Thermo Fisher Scientific, Waltham, MA). Eight calves were assigned each of the BUP, LID, and LID + MEL treatment groups and 6 calves were assigned to the CON treatment group. Each treatment was equally represented in each castration group. Calf was the experimental unit for

the study. The duration of outcome variable collection was 120 h post-castration, with baseline measures collected 24 h prior to calves being castrated. The time of castration was considered the 0 h timepoint.

Treatments were administered 10 min prior to the castration procedure. The scrotum and testicles were anesthetized by injecting 2 mL of lidocaine (MWI, Boise, ID) or bupivacaine liposome suspension (NOCITA, Elanco US Inc., Greenfield, IN) using a 20 gauge needle into the neck of the scrotum (proximal scrotum) and 3-4 mL around each spermatic cord (8-10 mL total lidocaine or bupivacaine liposome suspension). Ten minutes following the local anesthetic block, calves were surgically castrated as described: the scrotum was cleaned and disinfected by applying a mix of water and chlorhexidine and sterile gauze until there was no visible debris, the distal half of the scrotum was surgically excised using a disposable scalpel blade, the testes and spermatic cord were exteriorized by blunt dissection, and the testicles were removed by stripping and twisting the spermatic cord.

Measurements and Sample Collection

Outcome variables were collected at -24, 0, 0.5, 1, 2, 4, 8, 24, 72, and 120 h post-treatment, with chute defense behavior being scored at 0 h (at the time of castration) and pressure mat gait analysis beginning at 4 h. Outcome variables collected included: infrared thermography (IRT), gait analysis using a pressure mat, chute defense and behavior scoring (pain and activity), and blood sampling for serum cortisol and Prostaglandin E_2 metabolites (PGEM) (**Fig. 1**). All trained evaluators were masked to treatment for the duration of the study.

Infrared Thermography

A research-grade infrared camera (Fluke TiX580, Fluke Corp, Everett, WA) was used to capture IRT images of the medial canthus of the left eye applying methods adapted from

(Kleinhenz et al., 2017). Images were obtained 0.5 m from the left eye of the calf. Infrared images were analyzed using research-specific computer software (SmartView v. 4.3, Fluke Thermography, Plymouth, MN) to determine maximum and minimum temperatures.

Pressure Mat Gait Analysis

A commercially available pressure mat gait analysis system (Walkway, Tekscan, Inc., South Boston, MA) was used to record gait and biomechanical parameters. The pressure mat was calibrated using a known mass to ensure accuracy of measurements at each timepoint. Video synchronization was used to ensure consistent gait between and within calves at each timepoint. Using research specific software (Walkway 7.7, Tekscan, Inc.), force, contact pressure, impulse, stance time, stride length, velocity, and gait distance were assessed. A percent change from baseline was calculated for each output and used for statistical analysis.

Behavior

Video cameras (Sony Handycam HDR-CX405, Sony Corporation of America, New York, NY) were placed on tripods outside of the chute area or calf pens, based on the location of calves at each time point.

Chute defense behavior was scored at the time of castration using the scale adapted from (Grandin, 1993) and also cited by Hoppe et al. (2010), summarized as (1) calm, no movement; (2) restless, shifting; (3) squirming, occasionally shaking of the chute; (4) continuous vigorous movement and shaking of the chute; (5) rearing, twisting of the body, or violent struggling.

A pain score was also assigned at the time of castration that was adapted from the pain scale developed by Gleerup et al. (2015) (**Table 1**). The behaviors summed in the pain score were back position, head position, ear position and facial expression along with an additional

variable the authors chose to include—vocalization. Back position, head position and ear flicking were also evaluated as stand-alone pain behaviors.

Calves were video recorded the day prior to the castration procedure for 30 min to collect baseline pain behavior and activity data. Post-castration, calves were video recorded for 30 min at the following time points: 0, 1, 2, 4, 8, and 24 h. The videos were randomized across time point and calf ID using a random number generator (random.org). Three observers blinded to treatment and time point used a detailed ethogram (**Table 2**) and BORIS software (Behavioral Observation Research Interactive Software v 7.7.3, Torino, Italy) to score calf behavior. Focal-animal, continuous sampling was used for behavioral analysis. Pain behaviors (attention to the surgical site, licking the surgical site, tail flicking, and foot stamping) were classified as events and the occurrence of each behavior (i.e., count data) was collected. The rest of the behaviors in the ethogram were classified as states, and total duration (s) of these behaviors across the observation period was collected. A total of 6,300 min (105 h) of behavior recordings were scored and analyzed for this study. The inter-observer reliability between the three individuals scoring behavior was assessed by having all observers score the same calf in three different videos for 30 mins and then calculating the interclass correlation coefficient (ICC). The ICC was ≤ 0.9 between the three individuals scoring behavior across three different samples of video footage, indicating excellent reliability between observers.

Blood Sampling

Blood samples for serum cortisol and PGEM determination were collected from the jugular vein via venipuncture. The whole blood samples were immediately transferred to tubes (Vacutainer, BD Diagnostics, Franklin Lakes, NJ) containing either no additive for cortisol determination or EDTA anticoagulant for PGEM determination. Blood samples were then

centrifuged for ten min at 1,500 g, serum and plasma were placed in cryovials via transfer pipette, and stored at -80 °C.

Cortisol

Serum cortisol concentrations were determined using a commercially available radioimmunoassay (RIA) kit (MP Biomedicals, Irvine, CA) following manufacturer specifications with minor modifications. The standard curve was extended to include 1 and 3 ng/mL by diluting the 10 and 30 ng/mL manufacturer-supplied standards 1:10 respectively. The standard curve ranged from 1 to 300 ng/mL. A low (25 ng/mL) and high (150 ng/mL) quality control (QC) were run at the beginning and end of each set to determine inter-assay variability. Plain 12 x 75 mm polypropylene tubes were used as blank tubes to calculate non-specific binding. Input for standards, QCs, and samples was adjusted to 50 µL. Samples were incubated at room temperature for 30 min prior to the addition of I-125. Manufacturer instructions were then followed. Tubes were counted on a gamma counter (Wizard2, PerkinElmer, Waltham, MA) for 1 min. The raw data file was then uploaded onto MyAssays Desktop software (version 7.0.211.1238, 21 Hampton Place, Brighton, UK) for concentration determination. Standard curves were plotted as a 4-parameter logistic curve. Samples with a coefficient of variation (**CV**) > 18% were re-analyzed. The project average for serum cortisol intra-assay CV was 20.95% and inter-assay CV was 20.81%.

Prostaglandin E_2 Metabolites

Prostaglandin E_2 metabolites were analyzed using a commercially available ELISA kit (cat. no. 514531, Cayman Chemical, Ann Arbor, MI) following manufacturer specifications with minor modifications. Sample input was adjusted to 375 µL with 1.5 mL ice-cold acetone added for sample purification. Samples were incubated at -20°C for 30 min., then centrifuged at 3,000 x

g for 5 min. Supernatant was transferred to clean 13 x 100mm glass tubes and evaporated using a CentriVap Concentrator (cat. no. 7810014, Labconco, Kansas City, MO) overnight (approx. 18 h). Samples were reconstituted with 375 µL of appropriate kit buffer. A 300 µL aliquot of the reconstituted sample was derivatized with proportionally adjusted kit components. Manufacturer protocol was then followed. Samples were diluted 1:2 and ran in duplicate. Absorbance was measured at 405 nm after 60 min of development (SpectraMax i3, Molecular Devices, San Jose, CA). Sample results were excluded if the raw read exceeded the raw read of the highest standard (Standard 1; 50 pg/mL) or was below the lowest acceptable standard. The lowest acceptable standard was defined for each individual plate and was identified by excluding standards that had a ratio of absorbance of that standard to the maximum binding of any well (%B/B_0) of ≥ 80% or ≤ 20%. Any individual sample outside the standard curve, with a %B/B_0 outside the 20-80% range, or a CV > 15% were re-analyzed. The project average for PGEM intra-assay CV was 15.08% and inter-assay CV was 11.40%.

Calculations and Statistical Analysis

Concentrations of serum cortisol and PGEM were log-transformed for normality before statistical analysis. Responses (i.e. IRT, gait analysis, behavior, serum cortisol, and PGEM) were analyzed using repeated measures with calf as the experimental unit. Calves nested in a treatment group were designated as a random effect, with treatment, time, and treatment by time interaction designated as fixed effects. F-tests were utilized for testing significance of main effects and interactions. If significant overall differences were identified, pairwise comparisons were performed using the Tukey honestly significant difference (HSD) test. Statistics were performed using statistical software (JMP Pro 15.1.0 and Statistical Analysis System 9.4, SAS

Institute, Inc., Cary, NC). Statistical significance was set *a priori* at $P \leq 0.05$ with $P \leq 0.10$ considered to be a trend towards significance. Data are presented as least squares means.

RESULTS

None of the calves required rescue analgesia. Least squares means by treatment for each outcome variable are outlined in Table 3. We found no evidence of a treatment effect for IRT maximum ocular temperature (P = 0.80). Treatment groups had similar maximum ocular temperatures. There was a significant time effect (P < 0.01) with the highest ocular temperatures at 72 and 120 h (37.16°C; 95% CI: 34.65 to 39.66 and 37.01°C; 95% CI: 34.51 to 39.52°C, respectively) relative to all other timepoints (< 36.7 °C) (P < 0.05).

The percent change in front stance time (s) differed significantly between treatment groups (P = 0.04) with the LID + MEL group (6.35% : 95% CI: -11.77 to 24.48%) having a more positive percent change from the LID and CON groups (< -5.70%; 95% CI: -24.84 to 11.51%) (P < 0.05) and the CON group (-8.73%: 95% CI: -24.84 to 7.37%) having a more negative percent change from the BUP and LID + MEL groups (> 5.70%; 95% CI: -22.91 to 23.79%) (P < 0.03) (Fig. 2). The percent change in front force (kg) between treatment groups trended towards significance (P = 0.06). The CON group (6.31%: 95% CI: -1.79 to 14.41%) had a more positive percent change in force compared to the BUP, LID, and LID + MEL groups, who showed negative changes (< -5.06%; 95% CI: -14.22 to 0.95%) (P < 0.04). There was evidence of a trend towards significance for a treatment by time interaction for front pressure (kg/cm2) (P = 0.07). At the 2 h timepoint, the LID group had a more positive percent change in front pressure (7.65%; 95% CI: -5.62 to 20.92%) relative to the CON group (-16.30%; 95% CI: -32.37 to -0.24%) (P = 0.02). There was no evidence of a treatment effect for percent change in gait distance (cm) (P = 0.17), velocity (cm/s) (P = 0.46), front stride length (cm) (P = 0.22), front impulse (kg*s) (P =

0.38), rear stance time (s) (P = 0.35), rear stride length (cm) (P = 0.14), rear force (kg) (P = 0.54), rear impulse (kg*s) (P = 0.37), or rear pressure (kg/cm2) (P = 0.99).

Behavior outcome means are outlined by treatment in Table 4 and timepoint in Table 5. There was no evidence of a treatment effect for chute defense behavior (P = 0.58). There was no evidence of a treatment effect for pain score (P = 0.11). However, back position did differ by treatment (P = 0.05) (Fig. 3). The LID + MEL group had a significantly lower back position score (1.43; 95% CI: 1.13 to 1.72) compared to the CON group (CON: 2.00; 95% CI: 1.68 to 2.32) (P = 0.05). There was no evidence of treatment effect for ear position or head position (P = 0.59 and P = 0.49, respectively).

Tail flicking differed by time (P < 0.01) with more tail flicking at 2 and 8 h (93.72 and 92.96 times; 95% CI: 66.7 to 120.73 and 65.94 to 119.97 times, respectively) compared to 0 and 24 h (21.86 and -4.10 times; 95% CI: -5.63 to 49.34 and -44.72 to 36.53 times, respectively) (P < 0.01). Foot stamping differed by time (P = 0.01) with more foot stamping at 0 and 2 h (7.19 and 7.21 times; 95% CI: 4.40 to 9.98 and 4.47 to 9.95 times, respectively) compared to 4 h (0.83 times; 95% CI: -1.91 to 3.57 times) (P < 0.05). There were trends toward significance by treatment for foot stamping (P = 0.10) and attention to the surgical site (P = 0.10) with the CON group stamping the least (1.58 times; 95% CI: -0.96 to 4.13 times) and paying the most attention to the surgical site (2.44 times; 95% CI: 1.52 to 3.36 times). Attention to surgical site differed by time, with the most attention paid to the surgical site at 8 and 24 h (5.01 and 4.89 times; 95% CI: 4.01 to 5.99 and 3.39 to 6.38 times, respectively) relative to all other timepoints (< 0.57 times; 95% CI: -0.97 to 1.87 times) (P < 0.01). Licking the surgical site differed by time at 8 and 24 h (2.26 and 2.08 times; 95% CI: 1.73 to 2.80 and 1.27 to 2.88 times, respectively) compared to all other timepoints (< 0.27 times; 95% CI: -0.53 to 0.90 times) as well (P < 0.02).

Eating differed by time ($P < 0.01$) with less time spent eating at 4 and 8 h (225 and 207 sec; 95% CI: 77.33 to 373.02 and 42.87 to 372.54 sec, respectively) relative to -24 and 24 h (974 and 869 sec; 95% CI: 731.90 to 1217.01 and 644.43 to 1093.94 sec, respectively) ($P < 0.01$). There was a trend towards significance ($P \leq 0.10$) by treatment for ruminating ($P = 0.06$) with calves in the CON group spending the most time ruminating (568 sec; 95% CI: 256.80 to 880.12 sec) ($P = 0.06$). Standing differed by time ($P < 0.01$) with less time spent standing at 4 h (375 sec; 95% CI: 231.25 to 520.56 sec) compared to all other time points (< 1319 sec; 95% CI: 1141.25 to 2000.76 sec) ($P < 0.01$). Walking differed by time ($P < 0.01$) with more time spent walking at -24, 2 and 8 h (127, 127 and 113 sec; 95% CI: 79.94 to 175.26, 101.54 to 153.82, and 86.29 to 141.55 sec, respectively) compared to all other timepoints (< 50 sec; 95% CI: -12.27 to 77.01 sec) ($P < 0.02$). There was no evidence of a treatment or time effect for drinking, grooming, or lying behavior ($P > 0.20$).

There was evidence of a trend towards significance ($P \leq 0.10$) for a treatment by time interaction for average cortisol concentrations (ng/mL) when log transformed ($P = 0.12$) (Fig. 4). Calves in the CON group had lower cortisol concentrations at -24 h (2.41 ng/mL; 95% CI: -4.32 to 9.14 ng/mL) relative to 0, 0.5, 1, 2, 4, 8, and 24 h (≥ 6.95 ng/mL: 95% CI: 0.78 to 29.91 ng/mL) ($P < 0.01$). Calves in the LID group had lower cortisol concentrations at -24 h (5.85 ng/mL; 95% CI: -0.28 to 11.98 ng/mL) relative to 0, 0.5, 1, 2, and 4 h (≥ 14.41 ng/mL: 95% CI: 9.09 to 39.13 ng/mL) ($P < 0.01$). Calves in the LID + MEL group had lower cortisol concentrations at -24 h (4.01 ng/mL; 95% CI: -2.11 to 10.14 ng/mL) relative to 0, 0.5, 1, 2, and 4 h (≥ 9.42 ng/mL: 95% CI: 4.10 to 27.79 ng/mL) ($P < 0.04$). Calves in the BUP group had lower cortisol concentrations at -24 h (2.77 ng/mL; 95% CI: -4.71 to 10.26 ng/mL) relative to 0, 0.5, 1, 2, and 4 h (≥ 11.40 ng/mL: 95% CI: 6.09 to 27.53 ng/mL) ($P < 0.01$). Calves in the CON group

had higher cortisol concentrations at 24 h (7.70 ng/mL; 95% CI: 1.52 to 13.87 ng/mL) relative to the BUP group (3.11 ng/mL; 95% CI: -2.56 to 8.79 ng/mL) (P = 0.02). Calves in the CON group had lower cortisol concentrations at 120 h (1.82 ng/mL; 95% CI: -6.79 to 10.43 ng/mL) relative to the LID + MEL group (8.34 ng/mL; 95% CI: 0.92 to 15.76 ng/mL) (P = 0.01).

There was evidence of a significant treatment by time interaction for percent change from baseline in PGE metabolite concentration (pg/mL) (P = 0.01) (Fig. 5). At 4 h, the LID + MEL group had a negative percent change from baseline (-32.42%: 95% CI: -63.03 to -1.80%) that differed significantly from the positive percent change in the CON group (27.86%: 95% CI: -7.49 to 63.20%) (P = 0.01). At 24 h, the LID + MEL group had a negative percent change from baseline (-47.84%: 95% CI: -78.45 to -17.23%) that differed significantly from the positive percent change in the CON group (47.63%: 95% CI: 12.28 to 82.98%) (P = 0.01).

DISCUSSION

Evidence from the current study indicates that surgical castration resulted in changes from baseline values in ocular temperature, pressure mat gait analysis, attention to and licking the surgical site, time spent standing and walking, and cortisol and PGEM concentrations. Previous research reports changes in ocular temperature (Stewart et al., 2010; Kleinhenz et al., 2018), gait analysis (Kleinhenz et al., 2018), behavior (Laurence et al., 2016; Meléndez et al., 2018a), and cortisol concentrations (Kleinhenz et al., 2018; Meléndez et al., 2018b) following surgical castration. Significant changes from baseline values were still evident 120 h following castration, at the completion of the study, for ocular temperature, gait distance, velocity, front and rear stride length, front and rear force, front pressure, rear stance time, rear impulse, and PGEM concentrations. Previous studies have shown that pain associated with castration may persist up to 35 d after the procedure (Marti et al., 2017) which was well beyond the duration of

the present study. In the present study, a treatment effect was observed for the following outcomes: stance time, front limb force, back position, and PGEM concentrations.

Ocular temperatures increased from baseline values immediately following castration, then decreased 0.5 h afterwards, and then slowly increased for the duration of the study. Environmental factors as well as distance from the animal can be very influential upon IRT readings (Church et al., 2014). The highest maximum ocular temperatures were recorded on days with corresponding high ambient temperatures. The study investigators maintained the same distance from each calf and avoided capturing images in direct sunlight. Stewart et al. (2010) observed an immediate decrease in ocular temperature following castration in calves who did not receive a local anesthetic, followed by an increase in ocular temperature. Additionally, calves administered a local anesthetic exhibited increased ocular temperature immediately following castration (Stewart et al., 2010) which is consistent with findings in the present study. In another study, calves administered an NSAID showed an increase in ocular temperature relative to calves who did not receive analgesia and negative controls (Kleinhenz et al., 2018), however a similar treatment effect was not observed in the current study.

A significant time effect for changes from baseline values was observed for gait distance, velocity, front and rear stride length, front and rear force, front pressure, rear stance time and rear impulse across all treatment groups throughout the study. Gait distance, front and rear stride length, front and rear force, and front pressure all decreased from baseline values over the study duration. Gait velocity, rear stance time, and rear impulse increased from baseline values over the study duration. Surgical castration seems to impact multiple outcomes captured by pressure mat gait analysis for both front and rear limbs. Kleinhenz et al. (2018) found that surgically castrated calves placed more force on their forelimbs compared to non-castrated controls, which

is consistent with our findings. Positive controls (castrated without analgesia) also had a decreased front stance time relative to calves administered an NSAID in the present study. Shortened front stance time may be indicative of calves being painful and warrants further investigation.

No significant treatment effects were observed for chute defense scores or pain scores. Relatively low chute defense scores and relatively high pain scores were observed across all treatment groups. The two scoring systems quantify different behaviors that, given the results of the current study, may not always agree. When individual pain behaviors were analyzed that comprised the pain scale adapted from Gleerup et al. (2015), differences were seen in back position between the calves administered an NSAID and all other treatment groups not administered an NSAID. A previous study evaluating pain in Nellore cattle following castration found that restricted movement, such as an arched back, was more often observed in cattle experiencing pain (de Oliveira et al., 2014). In the present study, foot stamping seemed to increase immediately following castration whereas attention to, and licking of, the surgical site did not increase until the 8 and 24 h timepoints, indicating that different pain behaviors may be more pronounced at different timepoints following surgical castration. Attention to, and licking of, the surgical site may have become more pronounced at 8 and 24 h due to these timepoints being beyond the local anesthetic duration of action (Riviere and Papich, 2018). Due to the diminishing anesthesia effects, the calves may be experiencing more localized pain. Ruminating was higher in the CON group relative to other treatments. Teeth grinding is a pain behavior described in Gleerup et al. (2015) that may have been quantified as ruminating in the ethogram used to score behavior and activity, further contributing to the idea that increased specificity in identifying behaviors may be valuable as opposed to combined pain and activity scoring. Some

activities such as eating, standing, and walking were likely influenced by whether calves were fed during data collection. However, the -24 and 24 h timepoints were likely comparable and time spent walking at 24 h was less than at -24 h, indicating that activity may be reduced following castration. Failing to detect further differences in chute defense, pain and activity behaviors may have been due to a limited sample size and calves being partially or fully out-of-view of the camera on average 60 ± 4% of the time.

Cortisol concentrations were elevated immediately following castration and remained elevated for 24 h in CON calves and 4 h in treated calves, indicating that local anesthesia seemed to have an effect between 4 and 24 h on cortisol levels. At 24 h, calves in the BUP group had lower cortisol levels than the CON group indicating that while the duration of effect for lidocaine had likely ended, the bupivacaine liposome suspension may have still been having an effect. Roberts et al. (2015) did not find evidence of an effect of meloxicam on cortisol concentrations following castration which is similar to our findings. A meta-analysis of castration with and without analgesia found no significant differences in cortisol concentrations between surgically castrated calves not given analgesia and shams, and a tendency for analgesia to decrease cortisol levels after 120 min of intervention (Canozzi et al., 2017). Cortisol concentrations should be interpreted cautiously due to the stress of restraint during castration, as well as blood sample collection.

In the current study, the concentration of prostaglandin E2 metabolites only differed between treatments in which calves were treated with meloxicam relative to negative controls. These findings are consistent with previous findings suggesting that NSAIDs reduce prostaglandin E2 concentrations over the duration of action of the drug (Stock et al., 2016). Concentrations in calves who did not receive an NSAID showed the greatest percent change

from baseline at 4 h when the cortisol spike was beginning to decline and potentially was no longer suppressing aspects of the inflammatory response causing a rise in prostaglandin E2 metabolite concentration (Van Engen and Coetzee, 2018).

Lidocaine is a widely used local anesthetic in veterinary practice which is likely attributable to its low cost and long shelf-life (Riviere and Papich, 2018). A multimodal approach of lidocaine and meloxicam has been well-characterized as a more effective option for controlling pain associated with painful husbandry procedures, due to a longer duration of action than lidocaine alone and lidocaine and meloxicam reducing pain at different time points (Heinrich et al., 2010; Allen et al., 2013; Laurence et al., 2016; Meléndez et al., 2018b). Bupivacaine liposome suspension is a novel option for pain control during castration. Based on the results of the present study, the bupivacaine liposome suspension did not have a delayed onset relative to lidocaine, and it was able to control pain for a duration similar to a multi-modal approach of lidocaine and meloxicam. In a recent study comparing the effect of bupivacaine liposome suspension to a combination of lidocaine and meloxicam on pain biomarkers at the time of dehorning, similar results were observed for bupivacaine liposome suspension, including an onset similar to lidocaine and duration of pain control similar to a combination of lidocaine and meloxicam (Martin et al., 2021).

No drugs are currently labeled to control pain from castration in cattle in the U.S., thus extra-label drug use under the Animal Medicinal Drug Use Clarification Act (AMDUCA) is the only way this use is permitted (FDA, 1994). In a recent survey, both producers and veterinarians selected, “I am not comfortable using an analgesic unless it has been approved by the FDA” as a common reason for not using an analgesic (Robles et al., 2021). Producers’ ability to implement a change is key for adopting new means of pain control (Jansen et al., 2009). Currently,

bupivacaine liposome suspension is not readily available or likely cost-effective for producers to implement. However, a recent survey suggests that analgesic use at the time of castration may be more common than it was ten years ago (Johnstone et al., 2021) and the cost, availability, and approval of analgesics may evolve over time, providing more options for pain control at the time of castration.

CONCLUSIONS

Evidence provided in the current study demonstrates that pain from surgical castration can last up to 120 h post-castration, indicated by changes in ocular temperature, gait analysis, and PGEM concentrations. These data show that administration of bupivacaine liposome suspension as a local anesthetic block at the time of castration was as effective at controlling pain as a multi-modal approach of lidocaine and meloxicam. A single injection that alleviates both perioperative and postoperative pain would be an attractive option for livestock producers to alleviate pain at the time of castration. Further research is needed to discover effective ways of managing pain for extended durations following painful husbandry procedures.

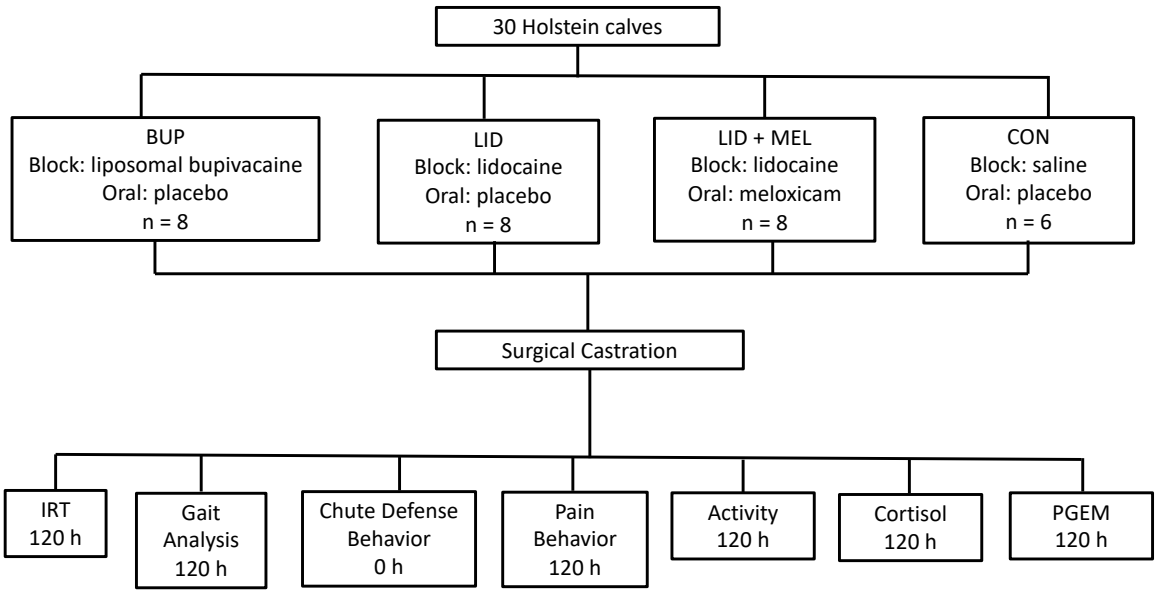

Figure 8.1 Flow chart outlining the timing of study events. Calves were castrated and outcome variables were collected at baseline and for the duration of time expressed in h (0-120) below each specific outcome variable.

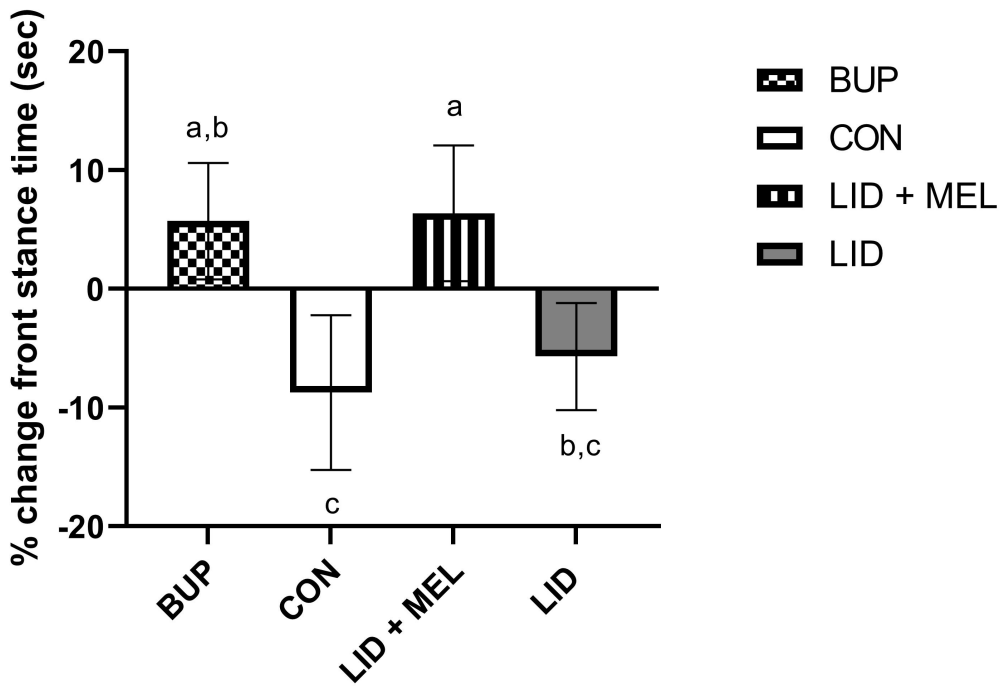

Figure 8.2 Mean percent change from baseline for front stance time (sec) over the duration of the study for each of the four treatment groups.

Error bars indicate SEM. Different superscripts (^{a,b,c}) indicate significant differences between timepoints ($P \leq 0.05$).

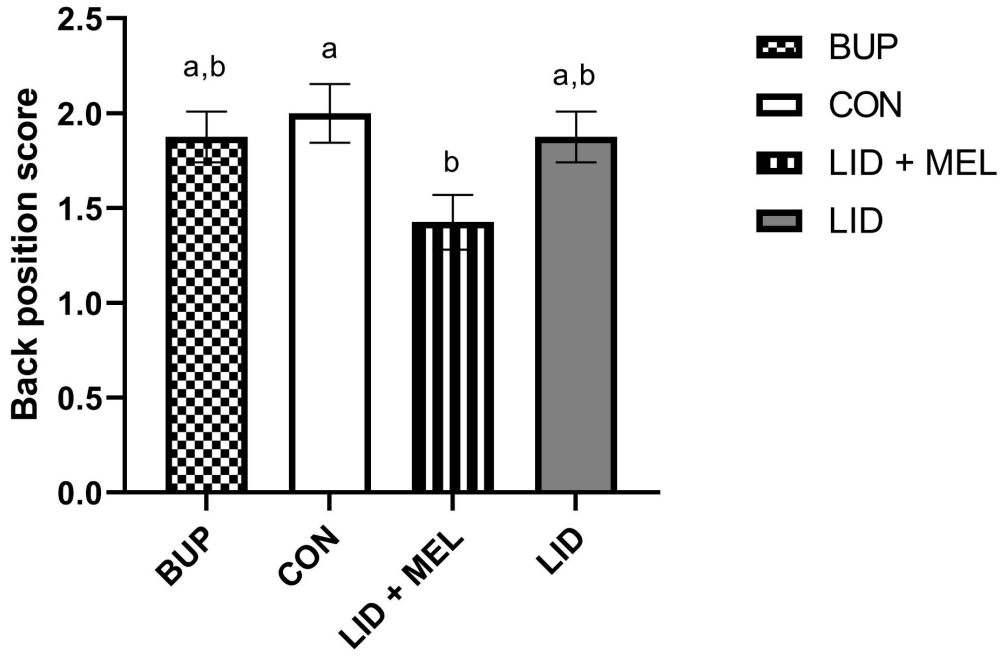

Figure 8.3 Mean back position score (0-2) during castration for each of the four treatment groups.

Error bars indicate SEM. Different superscripts (^{a,b}) indicate significant differences between timepoints ($P \leq 0.05$).

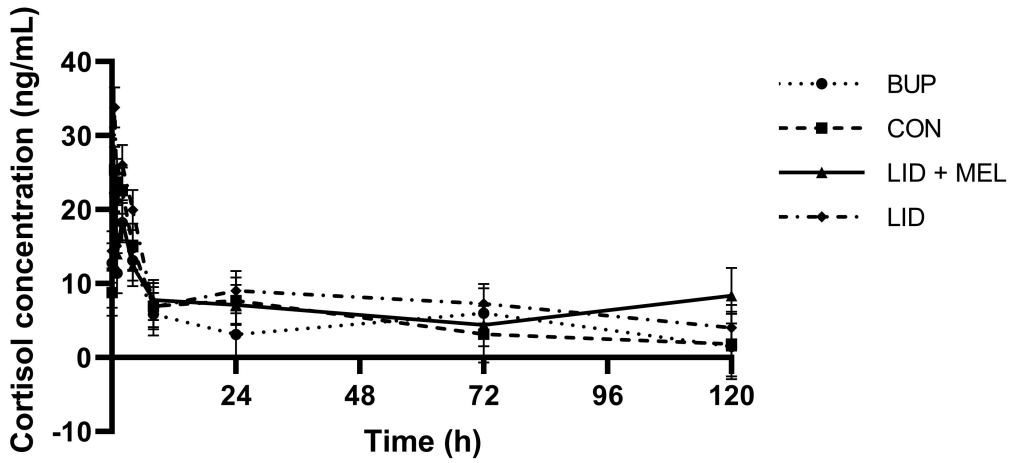

Figure 8.4 Mean percent change from baseline for cortisol concentration (ng/mL) over the duration of the study for each of the four treatment groups.

Error bars indicate SEM.

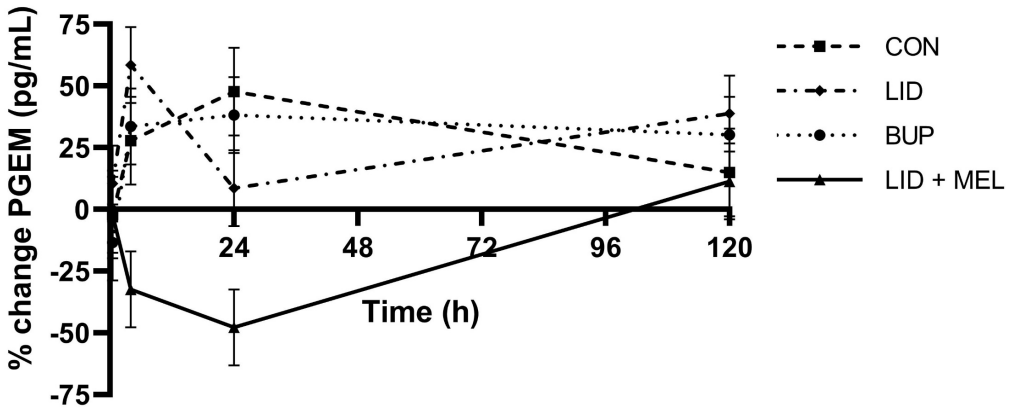

Figure 8.5 Mean percent change from baseline for prostaglandin E_2 metabolite concentration (pg/mL) over the duration of the study for each of the four treatment groups.

Error bars indicate SEM.

Table 8.1 Modified pain scale used to score calf behavior and assign a pain score (adapted from (Gleerup et al., 2015)).

Score	0	1	2
Head position	High/level of withers Animal is active, eating, ruminating or is contact seeking/curious	Level of withers Animal is not active, not eating, ruminating, grooming, or sleeping	Low Animal is not active, not eating, ruminating, grooming, or sleeping; may lie down quickly after getting up
Ear position	Both ears forward or one ear forward or back and the other listening	Ears back/asymmetric ear movements Both ears back or moving in different directions	Both ears to the sides and lower than usual (i.e., lambs' ears); the pinna facing slightly down
Facial expression	Attentive/neutral look Animal is attentive, focused on a task (e.g., eating or ruminating) or sleeping	Tense expression/strained appearance Animal has a worried or strained look, furrows above the eyes and puckers above the nostrils	
Back position	Normal	Slightly arched back	Arched back
Vocalization	No vocalization	Vocalization	

Table 8.2 Ethogram used to score calf behavior and activity (adapted from Heinrich et al. (2010) and Sutherland et al. (2013))

Behavior	Description
Eating	Ingesting food provided at feed bunk
Drinking	Consuming water from bucket or waterer
Ruminating	Regurgitating, chewing, and swallowing food
Grooming	Calf moves tongue over body, licking
Walking	Moving forward at a normal pace
Standing	Calf is upright and all four hooves are in contact with the ground
Lying	Calf is recumbent, body is in contact with the ground
Attention to surgical site	Turning head back towards hind end with attention focused on scrotal area. May involve lifting a hind limb. No attempts are made to lick surgical site
Licking/Attempting to lick surgical site	Lifting a hind limb and licking (or attempting to lick) scrotal area
Tail flicking	Calf rapidly moves tail from side to side. May include multiple tail movements within one tail-flicking event. A new tail flicking event occurs after the tail moves slowly or is in a resting position
Foot stamping	Calf raises one foot and brings it down again firmly

Table 8.3 Least squares means (upper and lower 95% confidence interval) of outcome variables by treatment with associated *P*-values

Variable	CON	LID	BUP	LID +MEL	TRT	TIME	TRT*TIME
Maximum IRT (°C)	36.07	36.23	36.16	36.25	0.80	<0.01	0.99
CI	(33.97 to 38.16)	(33.80 to 38.65)	(33.74 to 38.57)	(33.82 to 38.67)			
% change gait distance (cm)	-15.76	-10.14	-31.87	-32.01	0.17	<0.01	0.27
CI	(-37.13 to 5.62)	(-28.90 to 8.63)	-(49.84 to -14.83)	(-49.69 to -14.05)			
% change velocity (cm/s)	37.98	31.33	64.55	85.93	0.46	<0.01	0.70
CI	(-24.93 to 100.88)	(-18.90 to 81.55)	(21.32 to 107.79)	(42.72 to 129.13)			
% change front stance time (s)	-8.73[c]	-5.70[b,c]	5.70[a,b]	6.35[a]	0.04	0.46	0.12
CI	(-24.84 to 7.37)	(-22.91 to 11.51)	(-12.38 to 23.79)	(-11.77 to 24.48)			
% change front stride length (cm)	-3.84	-4.63	-11.33	-8.98	0.22	<0.01	0.25
CI	(-10.36 to 2.69)	(-10.01 to 0.74)	(-16.19 to -6.47)	(-13.84 to -4.12)			
% change front force (kg)	6.31[a]	-5.64b[b]	-5.06[b]	-8.32[b]	0.06	0.06	0.36
CI	(-1.79 to 14.41)	(-12.24 to 0.95)	(-10.96 to 0.85)	(-14.22 to -2.42)			
% change front impulse (kg*s)	47.11	28.31	234.76	67.37	0.38	0.42	0.49
CI	(-185.17 to 279.40)	(-160.81 to 217.43)	(61.30 to 408.23)	(-104.56 to 239.29)			
% change front pressure (kg/cm^2)	-7.07	1.31	-14.40	-8.23	0.12	0.01	0.07
CI	(-19.73 to 5.59)	(-10.37 to 12.99)	(-25.83 to -2.96)	-(19.67 to 3.2)			
% change rear stance time (s)	55.69	33.05	44.33	95.15	0.35	<0.01	0.51
CI	(-11.46 to 122.84)	(-25.12 to 91.22)	(-10.42 to 99.08)	(40.42 to 149.89)			
% change rear stride length (cm)	-1.49	-6.81	-11.65	-11.17	0.14	<0.01	0.18
CI	(-8.62 to 5.64)	(-12.59 to -1.04)	(-16.76 to -6.53)	(-16.28 to -6.06)			
% change rear force (kg)	-3.15	-6.95	-3.31	-10.60	0.54	0.01	0.94
CI	(-15.64 to 9.34)	(-18.87 to 4.98)	(-15.21 to 8.59)	(-22.50 to 1.30)			
% change rear impulse (kg*s)	44.13	24.21	58.98	73.87	0.37	<0.01	0.64
CI	(-5.64 to 93.91)	(-14.88 to 63.30)	(26.42 to 91.53)	(41.34 to 106.39)			
% change rear pressure (kg/cm^2)	-1.84	-2.83	-1.59	-0.76	0.99	0.01	0.97
CI	(-15.53 to 11.84)	(-13.91 to 8.24)	(-11.44 to 8.26)	(-10.59 to 9.08)			

Average cortisol (ng/mL)	11.74	14.84	9.69	10.85	0.19	<0.01	0.12
CI	(8.64 to 14.85)	(12.22 to 17.46)	(6.93 to 12.45)	(8.21 to 13.48)			
% change PGEM (pg/mL)	17.68	23.25	17.71	-14.22	0.08	0.01	0.01
CI	(-7.59 to 42.94)	(1.38 to 45.13)	(-4.17 to 39.58)	(-36.10 to 7.66)			

[a,b] Different superscripts indicate significant differences between treatment groups ($P \leq 0.05$) or trends toward significance between treatment groups ($P \leq 0.1$)

IRT – Infrared thermography

PGEM – Prostaglandin E_2 metabolite

Table 8.4 Least squares means (upper and lower 95% confidence interval) of behavior outcome variables by treatment with associated *P*-values

Behavior Variable	CON	LID	BUP	LID +MEL	Treatment *P*-value
Chute defense score (1-5)	2.58	1.94	2.63	2.48	0.58
CI	(1.67 to 3.50)	(1.15 to 2.73)	(1.83 to 3.41)	(1.63 to 3.33)	
Pain score (0-8)	6.17	5.75	6.00	5.13	0.11
CI	(5.50 to 6.83)	(5.17 to 6.33)	(5.42 to 6.58)	(4.51 to 5.75)	
Back position (0-2)	2.00[a]	1.88[a,b]	1.88[a,b]	1.43[b]	0.05
CI	(1.68 to 2.32)	(1.50 to 2.15)	(1.60 to 2.15)	(1.13 to 1.72)	
Ear position (0-2)	0.83	1.00	1.13	0.99	0.59
CI	(0.51 to 1.16)	(0.72 to 1.28)	(0.84 to 1.41)	(0.69 to 1.29)	
Head position (0-2)	1.83	1.63	1.75	1.44	0.49
CI	(1.42 to 2.25)	(1.26 to 1.99)	(1.39 to 2.11)	(1.05 to 1.82)	
Tail flicking (count)	42.23	30.28	66.14	37.83	0.15
CI	(17.14 to 67.31)	(7.61 to 52.95)	(41.82 to 90.46)	(16.55 to 59.10)	
Foot stamping (count)	1.58	3.21	5.63	4.71	0.10
CI	(-0.96 to 4.13)	(0.91 to 5.51)	(3.16 to 8.10)	(2.55 to 6.87)	
Attention to surgical site (count)	2.44	1.28	1.99	1.10	0.10
CI	(1.52 to 3.36)	(0.44 to 2.11)	(1.09 to 2.88)	(0.31 to 1.88)	
Licking surgical site (count)	0.79	0.45	0.74	0.64	0.73
CI	(0.29 to 1.28)	(-0.0004 to 0.90)	(0.25 to 1.22)	(0.22 to 1.06)	
Drinking (sec)	9.18	18.42	0.31	31.20	0.74
CI	(-63.83 to 82.20)	(-29.38 to 66.22)	(-72.71 to 73.32)	(-0.09 to 62.49)	
Eating (sec)	515.67	643.60	539.89	577.37	0.78
CI	(313.75 to 717.59)	(456.81 to 830.39)	(351.88 to 727.89)	(418.42 to 736.32)	
Grooming (sec)	10.39	3.52	16.42	17.04	0.22
CI	(-1.27 to 22.04)	(-8.20 to 15.24)	(4.42 to 28.42)	(4.84 to 29.25)	
Lying (sec)	573.78	472.27	248.82	639.03	0.41
CI	(186.48 to 961.08)	(142.51 to 802.03)	(-173.62 to 671.26)	(230.81 to 1047.25)	

Ruminating (sec)	568.46	169.21	219.04	153.67	0.06
CI	(256.80 to 880.12)	(-37.51 to 375.92)	(-16.09 to 454.16)	(-17.30 to 324.65)	
Standing (sec)	1398.87	1354.08	1435.97	1401.10	0.82
CI	(1262.71 to 1535.02)	(1231.55 to 1476.61)	(1305.74 to 1566.21)	(1285.99 to 1516.21)	
Walking (sec)	56.77	90.72	93.48	66.76	0.07
CI	(31.80 to 81.73)	(67.99 to 113.45)	(69.93 to 117.03)	(45.95 to 87.57)	

[a,b] Different superscripts indicate significant differences between treatments ($P \leq 0.05$)

Table 8.5 Least squares means (upper and lower 95% confidence interval) of behavior outcome variables by timepoint with associated *P*-values

Behavior Variable	-24	0	1	2	4	8	24	Time *P*-value
Tail flicking (count)	38.38[a,b]	21.86[a]	46.92[a,b]	93.72[b]	19.10[a]	92.96[b]	-4.10[a]	<0.01
CI	(-10.84 to 87.61)	(-5.63 to 49.34)	(19.91 to 73.94)	(66.70 to 120.73)	(-7.92 to 46.11)	(65.94 to 119.97)	(-44.72 to 36.53)	
Foot stamping (count)	0.54[a,b]	7.19[a]	5.93[a,b]	7.21[a]	0.83[b]	3.41[a,b]	1.36[a,b]	<0.01
CI	(-4.45 to 5.54)	(4.40 to 9.98)	(3.19 to 8.67)	(4.47 to 9.95)	(-1.91 to 3.57)	(0.67 to 6.15)	(-2.76 to 5.48)	
Attention to surgical site (count)	0.06[a]	0.04[a]	0.56[a]	0.80[a]	0.56[a]	5.01[b]	4.89[b]	<0.01
CI	(-1.75 to 1.87)	(-0.97 to 1.05)	(-0.44 to 1.55)	(-0.20 to 1.79)	(-0.43 to 1.55)	(4.01 to 5.99)	(3.39 to 6.38)	
Licking surgical site (count)	-0.08[a]	0.01[a]	0.02[a]	0.02[a]	0.26[a]	2.26[b]	2.08[b]	<0.01
CI	(-1.05 to 0.90)	(-0.53 to 0.56)	(-0.52 to 0.55)	(-0.52 to 0.55)	(-0.27 to 0.80)	(1.72 to 2.80)	(1.27 to 2.88)	
Drinking (sec)	11.73					17.82		0.77
CI	(-33.13 to 56.59)					(-24.55 to 60.19)		
Eating (sec)	974.46[b]				225.18[a]	207.71[a]	869.19[b]	<0.01
CI	(731.90 to 1217.01)				(77.33 to 373.02)	(42.87 to 372.54)	(644.43 to 1093.94)	
Grooming (sec)	23.52		1.55	8.07	10.35	22.53	5.04	0.20
CI	(10.12 to 36.91)		(-25.90 to 29.01)	(-9.80 to 25.94)	(-2.60 to 23.30)	(14.47 to 30.59)	(-10.02 to 20.09)	
Lying (sec)	615.13	104.88	365.55	352.71	423.65	549.70	972.69	0.66
CI	(102.08 to 1128.19)	(-1073.59 to 1283.36)	(-170.38 to 901.47)	(-53.45 to 758.87)	(133.64 to 713.66)	(222.47 to 876.93)	(295.53 to 1649.84)	
Ruminating (sec)	258.56	284.51	295.53	508.44	237.23	278.84	80.055	0.68

CI	(-33.87 to 550.99)	(-110.22 to 679.23)	(38.56 to 552.49)	(229.43 to 787.45)	(-31.74 to 506.20)	(110.94 to 446.74)	(-470.33 to 630.44)	
Standing (sec)	1737.05[a,b]	1584.76[a]	1696.05[a]	1711.57[a]	375.90[c]	1318.21[b]	1358.99[a,b]	<0.01
CI	(1473.34 to 2000.76)	(1437.61 to 1731.92)	(1551.39 to 1840.70)	(1566.91 to 1856.22)	(231.25 to 520.56)	(1165.33 to 1471.09)	(1141.25 to 1576.73)	
Walking (sec)	127.60[b]	42.17[a]	48.87[a]	127.68[b]	49.86[a]	113.92[b]	28.44[a]	<0.01
CI	(79.94 to 175.26)	(15.57 to 68.76)	(22.72 to 75.01)	(101.54 to 153.82)	(22.71 to 77.01)	(86.29 to 141.55)	(-12.27 to 69.15)	

[a,b] Different superscripts indicate significant differences between timepoints ($P \leq 0.05$)

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Chapter 9 - The effect of breed, sex and oral meloxicam administration on pain biomarkers following hot-iron branding in Hereford and Angus calves

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ABSTRACT

Hot-iron branding uses thermal injury to the skin to permanently identify cattle, resulting in tissue damage that is considered painful. The primary objective of this study was to examine the physiological and behavioral effects of oral meloxicam, compared to a control group, administered at the time of hot-iron branding in Angus and Hereford steers and heifers. The secondary objectives were to investigate the effects of breed and sex on pain biomarkers at branding. Seventy yearling calves, consisting of 35 heifers and 35 steers (Angus, Hereford, or Angus x Hereford), were enrolled onto the study. Animals were blocked by sex, randomized

across weight, and assigned to receive oral meloxicam (1 mg/kg) (MEL) or a placebo (CON). Biomarkers were assessed for 48 h after hot iron branding with an electric branding iron, and included infrared thermography (IRT), mechanical nociceptive threshold (MNT), behavioral assessment using accelerometers and a visual analog scale (VAS), and blood sampling for serum cortisol and prostaglandin E_2 metabolites (PGEM). Wound healing was assessed for 12 weeks after branding. Responses were analyzed using repeated measures with calf nested in treatment designated as a random effect, and treatment, time, treatment by time interaction, breed and sex designated as fixed effects. There was evidence of a treatment by time interaction for prostaglandin E_2 metabolite (PGEM) concentrations ($P < 0.01$) with MEL having lower concentrations than CON at 6, 24, and 48 h (MEL: 18.34 ± 3.52, 19.61 ± 3.48, and 22.24 ± 3.48 pg/mL, respectively) (CON: 32.57 ± 3.58, 37.00 ± 3.52, and 33.07 ± 3.48 pg/mL) ($P < 0.01$). Calves in the MEL group had less of a difference in maximum IRT values between the branded (2.27 ± 0.29 ℃) and control site (3.15 ± 0.29 ℃) ($P < 0.01$). Calves in the MEL group took fewer lying bouts at 0 to12 h (4.91 bouts ± 0.56) compared to CON (6.87 bouts ± 0.55) ($P < 0.01$). Compared to Hereford calves, Angus calves had higher plasma cortisol concentrations, higher PGEM concentrations, exhibited more lying bouts, and had lower (less healed) wound healing scores at 3, 4, and 5 wk following branding. Compared to heifers, steers had lower PGEM concentrations, lower branding site and ocular IRT temperatures, and a higher MNT. Furthermore, steers spent more time lying and exhibited more lying bouts, with higher VAS pain and wound scores (healed more quickly) at 5 wk, and had higher average daily gain than heifers. These data suggest that meloxicam administration at hot-iron branding reduced the difference in temperature between the branding site and control site and reduced lying bouts for the first 12 h after branding. However, meloxicam administration alone did not have an effect on the majority

of pain biomarkers. Breed and sex effects were observed across a wide range of biomarkers. These data suggest that long-acting analgesics should be considered to alleviate pain from branding, and that breed and sex may impact pain expression in beef calves.

Key words: cattle, pain, branding, breed, gender, NSAID

INTRODUCTION

Hot-iron branding permanently identifies cattle via thermal injury to the skin and results in tissue damage which is considered painful. In the most recent USDA survey, 75% of large cattle operations of 200 head or more used hot iron brands (USDA, 2019). Branding is generally used for the purposes of identifying imported cattle, disease control, theft prevention, and permanent identification on open range when cattle are not within enclosures. Hot-iron branding results in second or third degree burn injuries described as initial tissue damage causing cell death, inflammation of the tissue leading to a local edema and the invasion of inflammatory cells, and re-epithelialization of the damaged wound (Laycock et al., 2013). Tucker et al. (2014) showed that only 67% of brand wounds were fully healed 10 weeks after hot-iron branding.

The analgesic class of nonsteroidal anti-inflammatory drugs inhibit the cyclo-oxygenase enzymes, reduce inflammation, and decrease prostaglandin production which attenuates the response of the nervous system to noxious stimuli, which reduces the response to pain (Ochroch et al., 2003). Meloxicam when administered at the time of branding had a positive effect on weight gain the five days following branding (Moreno Berggren, 2019). Meloxicam was also shown to reduce indicators of acute pain associated with knife castration along with branding

(Meléndez et al., 2018). However, the effect of meloxicam on pain biomarkers has not been solely investigated at the time of hot-iron branding.

Meloxicam is a practical analgesic option for producers due to its long half-life of 27 hours (Coetzee et al., 2009). Meloxicam alone has not been shown to mitigate signs of acute distress but has the potential to have long term physiological, behavior and performance effects (Coetzee et al., 2012). The primary objective of this study was to examine the physiological and behavioral effects of oral meloxicam administered at the time of hot-iron branding compared to a control group. The secondary objectives were to further characterize the pain associated with branding and investigate the effects of breed and sex on pain biomarkers at branding.

MATERIALS AND METHODS

This experiment was conducted at the Colorado State University Agricultural Research, Development and Education Center and was reviewed and approved by the Colorado State University Institutional Animal Care and Use Committee (#19-9609A). Branding without any form of pain control is a standard procedure at this facility. Calves were monitored daily throughout the study for signs of swelling, bleeding, or lethargy. If any of these signs had been observed, the attending veterinarian would have been contacted immediately.

Animals and Treatments

Seventy yearling calves consisting of 35 heifers (mean ± SE BW = 353 ± 6.32 kg) and 35 steers (mean ± SE BW = 373 ± 6.34 kg) (Angus, Hereford, and Angus x Hereford) were enrolled onto the study (**Table 1**). Animals were blocked by sex, randomized across weight, and assigned using the RAND function in Microsoft Excel (Microsoft Excel® 2016, Microsoft Corporation, Redmond, WA) to one of two treatment groups, control (CON) or meloxicam (MEL; 1 mg per kg BW *per os*). Calves were weighed the day prior to branding and meloxicam dosage was

determined based upon that measured BW. Meloxicam (Zydus Pharmaceuticals Inc., Pennington, NJ) was administered via 1 or 2 gelatin capsules (Torpac Inc., Fairfield, NJ) containing 15 mg tablets. Meloxicam was administered *per os* at a dosage of 1 mg/kg via 15 mg tablets, dosages were rounded to the nearest whole tablet (actual calculated dosage 0.999 mg/kg). Calves in the control group received 1 or 2 gelatin capsules (Torpac Inc.) containing the placebo – lactose monohydrate powder (Thermo Fisher Scientific, Waltham, MA) which is the binder in meloxicam tablets. Treatments were administered via a bolus gun at the time of branding. Calves were weighed again 30 days post-branding and individual weights were recorded.

Branding Procedure

All animals were restrained in a hydraulic chute for approximately 5 minutes during branding. The branding site of approximately 400 cm2 was clipped prior to branding. Animals were branded on the left hip with an electric branding iron by the same trained individual who conducts the branding procedure in calves at this facility annually. The branding iron symbol was the letters CSU. A control site was clipped on the opposite hip for IRT and MNT analysis.

Biomarker Collection

Baseline sample collection was completed 24 hours prior to branding. Biomarkers were then collected 6, 24, and 48 h post-branding.

Blood Sampling

Blood samples were collected at the following timepoints: -24, 6, 24, and 48 h post-branding. Blood samples for serum cortisol and prostaglandin E_2 metabolite (PGEM) determination were collected from the jugular vein via venipuncture. The whole blood samples were immediately transferred to tubes (Vacutainer, BD Diagnostics, Franklin Lakes, NJ) containing either no additive for cortisol determination or EDTA anticoagulant for PGEM

determination. Samples were immediately placed on ice after collection, centrifuged within 30 min of collection for ten minutes at 1,500 g, and serum and plasma were placed in cryovials via transfer pipette and stored at -80 °C.

Serum Cortisol

Serum cortisol concentrations were determined using a commercially available radioimmunoassay (RIA) kit (MP Biomedicals, Irvine, CA) following manufacturer specifications with minor modifications; the standard curve was extended to include 1 and 3 ng/mL by diluting the 10 and 30 ng/mL manufacturer-supplied standards, 1:10 respectively. The standard curve ranged from 1 to 300 ng/mL. A low (25 ng/mL) and high (150 ng/mL) quality control (QC) were ran at the beginning and end of each set to determine inter-assay variability. Plain 12 x 75 mm polypropylene tubes were used as blank tubes to calculate non-specific binding. Input for standards, QCs, and samples was adjusted to 50 µL. Samples were incubated at room temperature for 30 minutes prior to the addition of I-125. Manufacturer instructions were then followed. Tubes were counted on a gamma counter (Wizard2, PerkinElmer, Waltham, MA) for 1 minute. The raw data file was then uploaded onto MyAssays Desktop software (version 7.0.211.1238, 21 Hampton Place, Brighton, UK) for concentration determination. Standard curves were plotted as a 4-parameter logistic curve. Samples with a coefficient of variation (**CV**) > 18% were re-analyzed. The coefficient of variation for the intra-assay variability was 16.7%, and the inter-assay variability was calculated to be 16.0%.

Prostaglandin E_2 metabolites

Prostaglandin E_2 metabolites were analyzed using a commercially available ELISA kit (Cayman Chemical, Ann Arbor, MI) following manufacturer specifications with minor modifications. Sample input was adjusted to 375 µL with 1.5 mL ice-cold acetone added for

sample purification. Samples were incubated at -20°C for 30 min., then centrifuged at 3,000 x g for 5 min. The supernatant was transferred to clean 13 x 100mm glass tubes and evaporated using a CentriVap Concentrator (Labconco, Kansas City, MO) overnight (approx. 18h). Samples were reconstituted with 375 µL of appropriate kit buffer. A 300 µL aliquot of the reconstituted sample was derivatized with proportionally adjusted kit components. Manufacturer protocol was then followed. Samples were diluted at 1:2 and ran in duplicate. Absorbance was measured at 405 nm after 60 min of development (SpectraMax i3, Molecular Devices, San Jose, CA). Sample results were excluded if the raw read exceeded the raw read of the highest standard (Standard 1; 50 pg/mL) or was below the lowest acceptable standard. The lowest acceptable standard was defined for each individual plate and was identified by excluding standards that had a ratio of absorbance of that standard to the maximum binding of any well ($\%B/B_0$) of $\geq 80\%$ or $\leq 20\%$. Any individual sample outside the standard curve, with a $\%B/B_0$ outside the 20-80% range, or a CV > 15% were re-analyzed. The project average for PGEM intra-assay CV was 17.40% and inter-assay CV was 10.89%.

Infrared Thermography

Infrared thermographic (IRT) images of the branding site, a control site on the opposite hip which was also clipped, as well as the left eye capturing the medial canthus were taken at the following timepoints: -24, 6, 24, and 48 h post-branding using a research-grade infrared camera (Fluke TiX580, Fluke Corp, Everett, WA). Images were obtained at a 45° angle, .5 - .75 m from the branding site, control site, and eye. Infrared images were analyzed using research-specific computer software (SmartView v. 4.3, Fluke Thermography, Plymouth, MN) to determine maximum and minimum temperatures. The difference between the temperature of the medial canthus baseline and timepoints following branding were determined and difference between the

temperature of the branding and control site were determined for each time point. These differences were used for statistical analysis.

Mechanical Nociception Threshold

The mechanical nociception threshold (MNT) was determined using a handheld algometer (FPX 100, Wagner Instruments, Greenwich, CT) at the following timepoints: -24, 6, 24, and 48 h post-branding. Animals were restrained in a hydraulic chute and allowed to stand for all MNT measurements. The MNT was measured at four locations surrounding the brand site and one control location on the opposite hip by applying slow, steady pressure until the animal responded. The average of 3 readings for each location at each time point was taken and the mean used for analysis. The difference between the control site and each location around the brand site were determined for each time point. These differences were used for statistical analysis. The investigator determining the MNT was blinded to treatment and the reading of the algometer, to prevent testing bias. A second investigator recorded algometer readings, to prevent testing bias.

Accelerometer Activity

IceTag (IceRobotics Ltd, South Queensferry, Edinburgh, Scotland UK) accelerometers were placed on the left rear leg of a subset of 30 animals, 12 hours prior to branding. Fifteen accelerometers were placed on heifers and were equally and randomly assigned to the two treatment groups. Fifteen accelerometers were placed on steers and were equally and randomly assigned to the two treatment groups. In total, 15 accelerometers were randomly assigned to each of the two treatment groups and 15 accelerometers were assigned to each sex. Animals were chosen using the RAND function in Microsoft Excel (Microsoft Excel® 2016, Microsoft Corporation, Redmond, WA). Accelerometers were removed after the 48-hour post-branding

timepoint and data was downloaded for analysis. Accelerometers recorded motion index, standing and lying time, step count, and lying bouts. One accelerometer did not record data throughout the study.

Behavior Scoring

Behavior scoring was done by two trained evaluators blinded to treatment prior to calves being moved from their home pen into the working facility at each of the following timepoints: -24, 6, 24, and 48 h post-branding. Calves were observed for a minute and then a score was assigned. Animals who were chosen using the RAND function in Microsoft Excel (Microsoft Excel® 2016, Microsoft Corporation, Redmond, WA) to have accelerometers placed on their left rear leg were also used for behavior scoring. A visual analog scale (VAS) behavior scoring scale was used. The VAS used was a 100 mm (10 cm) line anchored at each end by descriptors of "No Pain" or "Severe Pain". Six parameters were used to assess pain; depression, tail swishing or flicking, stance, head carriage, foot stomping or kicking, and wound licking. No pain was characterized by being alert and quick to show interest, no tail swishing, a normal stance, head held above spine level, absence of foot stomping, and absence of wound licking. Severe pain was characterized by being dull and showing no interest, more than three tail swishes per minute, legs abducted, head held below spine level, numerous stomps, and any wound licking. The evaluator marked the line between the 2 descriptors to indicate the pain intensity. A millimeter scale was used to measure the score from the zero anchor point to the evaluator's mark. The mean VAS measures of the two evaluators were combined into one score for statistical analysis.

Wound Scoring

Branding site wounds were assessed weekly for 12 weeks following branding. Animals were walked through the working facility weekly and a photo was taken of the branding site.

Wound healing was then scored by a trained evaluator, blinded to treatment, using a six-point scale adapted from Tucker et al. (2014). A score of 1 represented all of the initial scab being present, 2: a majority of the brand covered by a scab, 3: minority of the initial scab present, 4: initial scab gone and tissue becoming re-pigmented, 5: secondary scabbing present and majority of tissue re-pigmented, a score of 6 represented no presence of scabbing and 100% re-pigmentation.

Statistical Analyses

Concentrations of serum cortisol and PGEM were log-transformed for normality before statistical analysis. Continuous responses (i.e., IRT, MNT, accelerometer activity, VAS, serum cortisol, and PGEM) were analyzed using a mixed effects model with repeated measures with calf as the experimental unit. Calves nested in a treatment group were designated as a random effect, with treatment, time, treatment by time interaction, breed, and sex designated as fixed effects. F-tests were utilized for testing significance of main effects and interactions. If significant overall differences were identified, pairwise comparisons were performed using the Tukey honestly significant difference (HSD) test. Categorical responses (wound healing) were analyzed using contingency tables and Fisher's exact test. Sample sizes of purebred Angus and Hereford calves were large enough to compare breed effects, crossbred Angus x Hereford sample size was not. Statistics were performed using statistical software (JMP Pro 15.1.0, SAS Institute, Inc., Cary, NC). Statistical significance was set a priori at $P \leq 0.05$. Data are presented as least squares means.

RESULTS

Outcome measure means are outlined by treatment in **Table 2**, by breed in **Table 3**, and by sex in **Table 4**.

Serum Cortisol Concentrations

There was no evidence of a significant treatment effect (P = 0.73) or treatment by time interaction (P = 0.86) for cortisol concentrations but there was evidence of a time effect (P < 0.01). Cortisol concentrations were higher at -24 h (18.93 ng/mL; 95% CI: 16.28 to 21.58) compared to 24 and 48 h (12.57 ng/mL; 95% CI: 9.90 to 15.24 ng/mL and 11.97 ng/mL; 95% CI: 9.31 to 14.63 ng/mL, respectively) (P < 0.01). Cortisol concentrations were highest at -24 h (18.93 ng/mL; 95% CI: 16.28 to 21.58 ng/mL) and declined from 6 to 48 h following branding, however there were not significant differences between the 6, 24 and 48 h time points (13.82 ng/mL; 95% CI 11.13 to 16.50 ng/mL, 12.57 ng/mL; 95% CI: 9.90 to 15.24 ng/mL and 11.97 ng/mL; 95% CI: 9.31 to 14.63 ng/mL, respectively) (P > 0.06).

There was a significant breed effect (P < 0.01) with Angus calves having higher cortisol concentrations (14.81 ng/mL; 95% CI: 12.73 to 16.89 ng/mL) compared to Hereford calves (10.69 ng/mL; 95% CI: 8.66 to 12.72 ng/mL) (P = 0.02). There was no evidence of a sex effect on cortisol concentrations (P = 0.87).

Prostaglandin E_2 metabolites

There was evidence of a treatment by time interaction for PGEM concentrations (*P* < 0.01) (**Fig. 1**) with calves in the MEL group having lower concentrations than CON at 6, 24, and 48 h (MEL: 18.34, 19.61, and 22.24 pg/mL, respectively) (CON: 32.57, 37.00, and 33.07 pg/mL, respectively) (*P* < 0.01). The MEL group specifically had lower PGEM concentrations at 6, 24, and 48 h (18.34 pg/mL; 95% CI: 11.37 to 25.31 pg/mL, 19.61 pg/mL; 95% CI: 12.71 to 26.50 pg/mL, and 22.24 pg/mL; 95% CI: 15.34 to 29.14 pg/mL, respectively) relative to -24 h (38.72 pg/ml; 95% CI: 31.82 to 45.61 pg/mL) (*P* < 0.01).

There was a significant breed effect with Angus calves exhibiting higher serum PGEM concentrations (33.52 pg/mL; 95% CI: 28.61 to 38.43 pg/mL) relative to Hereford calves (23.77 pg/mL; 95% CI: 19.01 to 28.53 pg/mL) ($P = 0.02$). There was a significant sex effect with heifers exhibiting higher PGEM concentrations (36.14 pg/mL; 95% CI: 29.77 to 42.52 pg/mL) than steers (23.13 pg/mL; 95% CI: 17.30 to 28.95 pg/mL) ($P < 0.01$).

Infrared Thermography

There was evidence of a treatment effect ($P < 0.01$) and time effect ($P < 0.01$) for maximum IRT values on the left hip (branded side of the calf) minus the right hip (control), but the treatment by time interaction was not significant ($P = 0.39$). Calves in the MEL group had less of a difference in maximum temperature between the branded and control site (2.27℃; 95% CI: 1.70 to 2.85 ℃) relative to CON (3.15 ℃; 95% CI: 2.58 to 3.73 ℃) ($P < 0.01$). Calves showed larger differences at 24 and 48 h (3.87℃; 95% CI: 3.31 to 4.43℃ and 3.30℃; 95% CI: 2.74 to 3.85℃, respectively) compared to -24 and 6 h (1.64℃; 95% CI: 1.08 to 2.19℃ and 2.06; 95% CI: 1.50 to 2.61℃, respectively) ($P < 0.01$). There was no evidence of breed or sex effects for the difference in maximum IRT hip readings ($P > 0.24$).

There was evidence of a time effect for left hip (branded side of the calf) maximum IRT values ($P < 0.01$) with values at -24 and 6 h (35.33℃; 95% CI: 34.91 to 35.76℃ and 35.79℃; 95% CI: 35.37 to 36.22℃, respectively) being higher than at 24 and 48 h (34.25℃; 95% CI: 33.83 to 34.68℃ and 34.25℃; 95% CI: 33.82 to 34.68℃) ($P < 0.01$). There was no evidence of a treatment ($P = 0.53$), treatment by time interaction ($P = 0.71$), or breed effect ($P = 0.13$) for left hip maximum IRT values. There was evidence of sex effect ($P < 0.01$) with heifers having higher left hip maximum IRT values (35.44℃; 95% CI: 35.13 to 35.57℃) compared to steers (34.38℃; 95% CI: 34.09 to 34.66℃).

There was evidence of a time effect for maximum ocular temperatures ($P < 0.01$) with values at -24 and 6 h (37.44℃; 95% CI: 37.12 to 37.76℃ and 37.08℃; 95% CI: 36.77 to 37.40℃, respectively) being higher than at 24 and 48 h (35.74℃; 95% CI: 35.42 to 36.06℃ and 36.01℃; 95% CI: 35.69 to 36.33℃, respectively) ($P < 0.01$). There was no evidence of a treatment ($P = 0.60$), treatment by time interaction ($P = 0.57$), or breed effect ($P = 0.49$) for maximum ocular IRT values. There was evidence of sex effect ($P < 0.01$) with heifers having maximum ocular IRT values (36.85℃; 95% CI: 36.56 to 37.15 ℃) compared to steers (36.28℃; 95% CI: 36.02 to 36.55℃).

Mechanical Nociception Threshold

There was evidence of a time effect for mean brand site MNT values ($P < 0.01$) with thresholds at 6 and 24 h (0.56 kg F; 95% CI: 0.52 to 0.59 kg F and 0.55 kg F; 95% CI: 0.51 to 0.59 kg F, respectively) being lower than at -24 and 48 h (0.80 kg F; 95% CI: 0.76 to 0.83 kg F and 0.71 kg F; 95% CI: 0.68 to 0.75 kg F, respectively) ($P < 0.01$). There was no evidence of a treatment ($P = 0.92$), treatment by time interaction ($P = 0.63$), breed ($P = 0.13$), or sex effect ($P = 0.24$) for mean brand site MNT values.

There was evidence of a time effect for mean control site MNT values ($P < 0.01$) with the threshold at 48 h (1.33 kg F; 95% CI: 1.21 to 1.46 kg F) being higher than at all other timepoints (-24, 6, and 24 h) (1.03 kg F; 95% CI: .91 to 1.1.6, 1.20 kg F; 95% CI: 1.08 to 1.32 kg F, and 1.12 kg F; 95% CI: 1.00 to 1.25 kg F, respectively) ($P < 0.03$). There was no evidence of a treatment ($P = 0.16$), treatment by time interaction ($P = 0.32$) or breed effect ($P = 0.72$) for mean control site MNT values. There was a sex effect ($P = 0.04$) with steers having a higher mean control site threshold (1.23 kg F; 95% CI: 1.13 to 1.33 kg F) compared to heifers (1.11 kg F; 95% CI: 1.01 to 1.22 kg F).

Accelerometer

There was evidence of a time effect for motion index ($P < 0.01$). Motion index at 0 to 12 h (13,063; 95% CI: 12,373 to 13,754) was higher than at all other time points ($P < 0.01$) and at 24 to 36 h (7,692; 95% CI: 7,002 to 8,383) was higher than at baseline (-12 to 0 h), 12 to 24 and 36 to 48 h (2,229; 95% CI: 1,539 to 2,920, 1,489; 95% CI: 799 to 2,180, and 1,578; 95% CI: 888 to 2,269, respectively) ($P < 0.01$). There was no evidence of a treatment ($P = 0.80$), treatment by time interaction ($P = 0.96$), breed ($P = 0.43$), or sex effect ($P = 0.11$) for motion index.

There was evidence of a time effect for standing and lying time ($P < 0.01$). A higher proportion of time was spent lying than standing at 12 to 24 and 36 to 48 h (0.40 and 0.40; 95% CI: 0.39 to 0.42) relative to -12 to 0, 0 to 12, and 24 to 36 h (0.35; 95% CI: 0.34 to 0.37, 0.12; 95% CI: 0.13 to 0.16, and 0.21; 95%: 0.20 to 0.23, respectively) ($P < 0.01$). There was no evidence of a treatment ($P = 0.83$), treatment by time interaction ($P = 0.42$), or breed effect ($P = 0.61$) for standing and lying time. There was a sex effect ($P < 0.01$) with steers spending a greater proportion of time lying (0.32; 95% CI: 0.31 to 0.33) relative to heifers (0.29; 95% CI: 0.28 to 0.30).

There was evidence of a time effect for step count ($P < 0.01$) with calves taking less steps at 12 to 24 and 36 to 48 h (347 steps; 95% CI: 206 to 488 steps and 360 steps; 95% CI: 219 to 500 steps) compared to -12 to 0, 0 to 12, and 24 to 36 h (551 steps; 95% CI: 410 to 691 steps, 2,569; 95% CI: 2,428 to 2,710 steps, and 1,600 steps; 1,459 to 1,741 steps, respectively) ($P < 0.01$). There was no evidence of a treatment ($P = 0.77$), treatment by time interaction ($P = 0.94$), breed ($P = 0.42$), or sex effect ($P = 0.07$) for step count.

There was evidence of a treatment by time interaction for lying bouts ($P = 0.03$) (**Fig. 2**). CON calves took more lying bouts at 0 to 12 h (6.87 bouts; 95% CI: 5.78 to 796 bouts) relative

to -12 to 0 h (4.74 bouts; 95% CI: 3.65 to 5.83 bouts) ($P = 0.03$). Calves in the MEL group took fewer lying bouts at 0 to12 h (4.91 bouts; 95% CI: 3.79 to 6.03 bouts) compared to CON (6.87 bouts; 95% CI: 5.78 to 7.96) ($P < 0.01$). There was a breed effect ($P = 0.04$) with Angus calves taking more lying bouts (6.42 bouts; 95% CI: 5.71 to 7.13 bouts) relative to Hereford calves (5.43 bouts; 95% CI: 4.80 to 6.06 bouts). There was evidence of sex effect ($P < 0.01$) with steers taking more lying bouts (6.22 bouts; 95% CI: 5.43 to 7.01 bouts) compared to heifers (4.70 bouts; 95% CI: 3.87 to 5.49 bouts).

VAS

There was a time effect for VAS score ($P < 0.01$) with pain scores being lowest at -24 h (4.64 mm; 95% CI: 3.97 to 5.32 mm) compared to 6, 24, and 48 h (7.48 mm; 95% CI: 6.80 to 8.17 mm, 11.13 mm; 95% CI: 10.44 to 11.81 mm, and 10.95 mm; 95% CI: 10.27 to 11.64 mm, respectively) ($P < 0.01$). Scores at 6 h (7.48 mm; 95% CI: 6.80 to 8.17 mm) were also lower than at 24 and 48 h (11.13 mm; 95% CI: 10.44 to 11.81 mm and 10.95 mm; 95% CI: 10.27 to 11.64 mm, respectively) ($P < 0.01$). There was no evidence of a treatment ($P = 0.75$), treatment by time interaction ($P = 0.28$), or breed effect ($P = 0.95$) on VAS score. There was a sex effect ($P = 0.03$) with steers having higher mean VAS scores (8.87 mm; 95% CI: 8.36 to 9.37 mm) compared to heifers (8.23 mm; 95% CI: 7.75 to 8.73 mm).

Wound Healing

There was no evidence of a treatment effect for wound scoring (P = 0.69). There was evidence of a breed effect on wound scoring at 3, 4, and 5 wk (P < 0.05) (Fig. 3) with Hereford calves having higher wound scores (more healed) at 3, 4, and 5 wk (3 wk: score 2: 6, score 3: 2; 4 wk: score 2: 16, score 3: 2, score 4: 2; 5 wk: score 2: 22, score 3: 3, score 4: 3, score 5: 2, score 6: 1) compared to Angus calves (3 wk: score 2: 1, score 3: 0; 4 wk: score 2:7, score 3:0, score

4:0; 5 wk: score 2: 20, score 3: 4, score 4: 0, score 5: 0, score 6: 0). There was a sex effect on wound scoring at 5 wk (P = 0.01) (Fig. 4) with steers having higher wound scores (more healed) (score 2: 22, score 3: 7, score 4: 1, score 5: 2, score 6: 1) than heifers (score 2:22, score 3: 0, score 4: 2, score 5: 0, score 6: 0).

Average Daily Gain

There was no evidence of a treatment ($P = 0.61$) or breed effect ($P = 0.72$) for ADG for the 30-d following branding. There was evidence of a sex effect ($P < 0.01$) with steers having a higher ADG (1.46 kg/d; 95% CI: 1.30 to 1.62 kg/d) compared to heifers (0.30 kg/d; 95% CI: 0.13 to 0.48 kg/d).

DISCUSSION

Branding is not mandatory in the majority of U.S. states but is required for cattle imported unless they are for immediate slaughter from Canada; two forms of identification are required for cattle from Mexico, with a brand typically being one form (AVMA, 2011; USDA-APHIS, 2018; USDA-APHIS-VS, 2018). Brand inspections are often required for cattle to be sold or cross state lines and branding is generally required for cattle grazing open range, branding regulations vary by state. Previous studies have quantified pain from hot-iron branding (Lay Jr et al., 1992; Schwartzkopf-Genswein et al., 1997) and have begun to investigate analgesic strategies (Tucker et al., 2014; Meléndez et al., 2018; Moreno Berggren, 2019). Meloxicam has shown promise in reducing physiologic pain biomarkers following a combination of castration and branding procedures (Meléndez et al., 2018), and has been evaluated in cattle branded on the jaw (Moreno Berggren, 2019).

Changes from baseline in IRT, MNT, lying time, step count, VAS pain score and wound scoring from the current study all support that hot-iron branding cattle is painful. The highest

cortisol levels observed were at baseline and 6 h after branding with calves likely becoming more acclimated to moving through the chute at subsequent time points and thus exhibiting a reduced stress response. The first collection time point following branding was at 6 h which did not allow for an acute cortisol response to be captured if it did exist. Previous research has shown a cortisol spike 20 and 40 min following branding (Schwartzkopf-Genswein et al., 1997) which were timepoints not assessed in the current study. Calves who received meloxicam had lower PGEM concentrations than controls at 6, 24, and 48 h which is consistent with previous findings suggesting that NSAIDs reduce prostaglandin E_2 concentrations over the duration of action of the drug (Stock et al., 2016; Martin et al., 2021).

Calves in the MEL group showed less of a difference in infrared thermography readings between the branding and control site on their opposite hip than CON. The branding site IRT readings were higher at -24 and 6 h relative to 24 and 48 h. Previous findings have shown that hot-iron branding caused elevated readings at the branding site 168 h post-branding (Schwartzkopf-Genswein and Stookey, 1997) which is beyond the duration of the present study. Differences in pressure sensitivity of the branding site have been shown to be most pronounced in the days immediately following branding but lasting out to 71 d (Tucker et al., 2014). In the present study, the lowest nociceptive thresholds were observed at 6 and 24 h following branding around the branding site. The control site threshold was highest at the end of the study indicating that calves had not become sensitized to the algometer.

There was evidence of a diurnal effect in accelerometer activity with calves being more active during the 0 to 12 h and 24 to 36 h daytime increments. The -12 to 0, 12 to 24, and 36 to 48 h increments corresponded with the same time of day. Calves spent more time lying and took less steps at 12 to 24 and 36 to 48 h compared to baseline and CON calves took more lying bouts

than MEL from 0 to 12 h indicating that increased lying time and lying bouts may have been due to calves being uncomfortable or painful. Previous literature has shown that calves who received an NSAID (flunixin IV) spent less time lying on the day of branding (Tucker et al., 2014). This was not observed in the present study which may be due to route of administration and thus different pharmacokinetics. Visual analog scale scores based on pain behavior were highest at 24 and 48 h indicating that calves were still painful at the end of the study sampling period.

Steers and heifers were wound scored until 8 weeks following branding, the heifers were then turned out to pasture and the steers continued to be scored out to 12 wk following branding. At 8 wk, wound scores ranged from 2-6 with the majority of calves being scored 3-6. At 11 wk, all the steers scored a 5 or 6 except one calf. At 12 wk, all the steers scored a 5 or 6 with 17 calves scoring a 6, and 2 calves scoring a 5, indicating that at 12 wk nearly all of the branding sites were completely healed. These results show consistencies in healing time and associated scores outlined in Tucker et al. (2014). However, in the previously mentioned study, branding sites were beginning to be fully healed at 8 wk whereas branding sites were identified as fully healed beginning at 6 wk in the current study.

Meloxicam has been shown to have a positive effect on weight gain five days immediately following branding (Moreno Berggren, 2019). A second weight was recorded for animals in the current study 30 d following branding and average daily gain was calculated. No statistical differences were observed between treatments, though the MEL group ADG was numerically larger. Further investigation via a study with power designed to detect performance differences following NSAID administration at branding is warranted.

Stafford and Mellor (2005) make the point that rigorously designed pain studies must make an allowance for animal breed and sex. Breed differences were observed for cortisol,

PGEM, lying bouts, and wound healing scoring in the present study. Angus calves had higher cortisol concentrations, higher PGEM concentrations, took more lying bouts, and had lower (less healed) wound healing scores at 3, 4, and 5 weeks following branding compared to Hereford calves. Breed differences were not observed by Schwartzkopf-Genswein et al. (1997) but a smaller sample size and larger pool of breed types was used in that study.

Sex differences were observed for PGEM, branding site and ocular IRT, MNT, lying time, lying bouts, VAS, wound healing scoring, and ADG. Steers had lower PGEM concentrations, lower branding site and ocular temperatures, a higher nociceptive threshold, spent more time lying and took more lying bouts, had higher VAS scores, had higher wound scores (healed more quickly) at 5 weeks, and had higher ADG than heifers. Human research has shown that male subjects have higher pain thresholds and tolerance, are less discriminative between painful sensations, and the NSAID ibuprofen has been shown to be less effective in women (Walker and Carmody, 1998; Vallerand and Polomano, 2000). The males in this study were castrated which may have influenced sex differences. The need for further investigation into whether these differences exist in cattle among intact males, castrated males, and females is apparent to better characterize and alleviate pain.

These data suggest that meloxicam administration at hot-iron branding reduced the difference in temperature between the branding and control site and reduced lying bouts for the first 12 h following branding. However, meloxicam administration alone did not have an effect on the majority of pain biomarkers collected in the present study. The practicality of administering an NSAID once at the time of branding is attractive. However, a multi-modal approach using a combination of analgesics or longer acting analgesic option warrants further investigation to alleviate pain and discomfort caused by hot-iron branding.

CONCLUSION

These results show that infrared thermography, mechanical nociceptive threshold, lying time, step count, visual analog scale score and wound scoring all support that hot-iron branding cattle is painful and investigation into analgesic strategies is needed. Oral meloxicam administration reduced infrared thermography differences from the branding and control site and reduced lying bouts. Breed and sex effects were observed across a wide range of biomarkers and should be considered in future pain studies. The need for long-acting analgesic options that demonstrate pain alleviation across multiple biomarkers is apparent and would be beneficial to alleviating pain from routine husbandry procedures like branding.

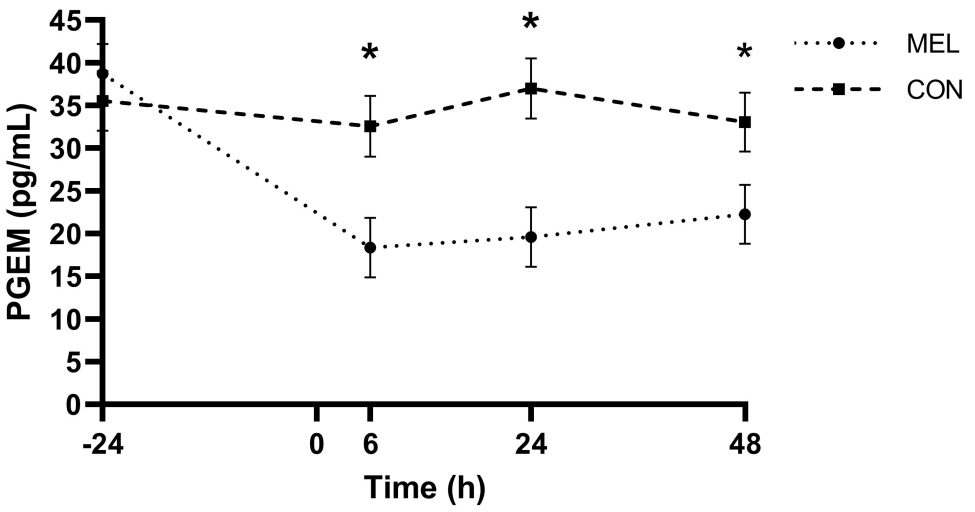

Figure 9.1 Mean Prostaglandin E_2 metabolite (PGEM) concentrations measured in pg/mL over the duration of the study for each of the two treatment groups receiving oral meloxicam at 1 m/kg (MEL; n = 35) or placebo (CON; n = 35) following hot-iron branding.

Error bars indicate SEM.

***Denotes timepoints where a statistically significant difference ($P \leq 0.05$) was observed between treatment groups.**

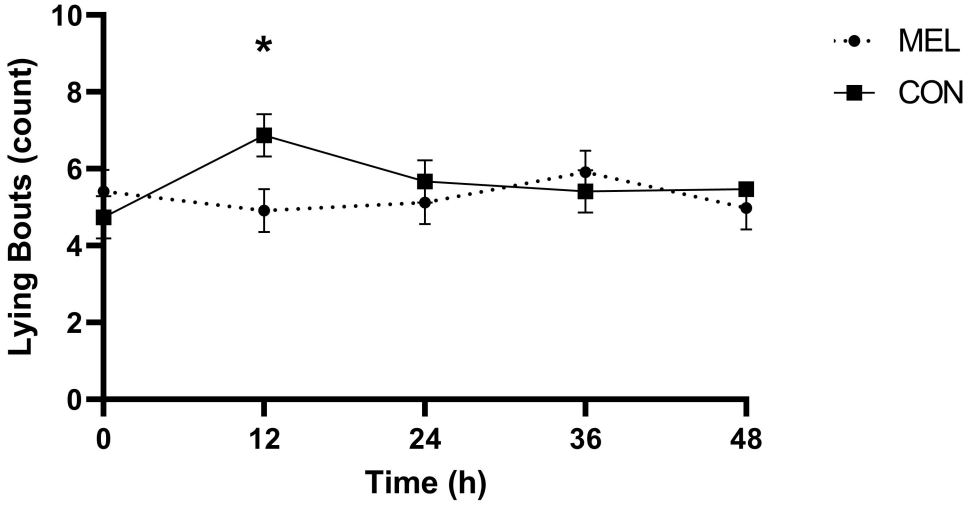

Figure 9.2 Mean lying bouts over the duration of the study for each of the two treatment groups receiving oral meloxicam at 1 m/kg (MEL; n = 35) or placebo (CON; n = 35) following hot-iron branding.

Error bars indicate SEM.

***Denotes timepoints where a statistically significant difference ($P \leq 0.05$) was observed between treatment groups.**

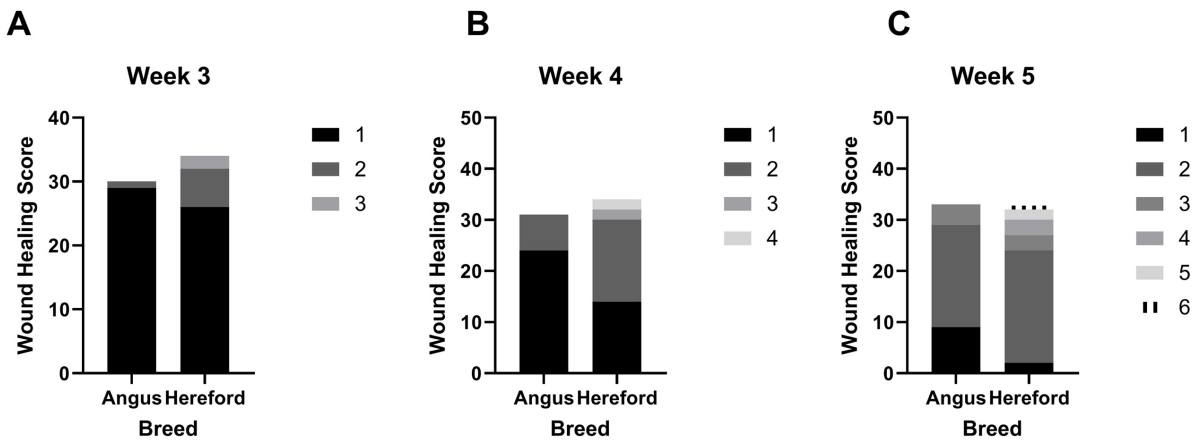

Figure 9.3 Individual wound healing scores for each of the two breed types following hot-iron branding for week 3 (3.A), 4 (3.B) and 5 (3.C) when significant differences ($P < 0.05$) between breeds were observed.

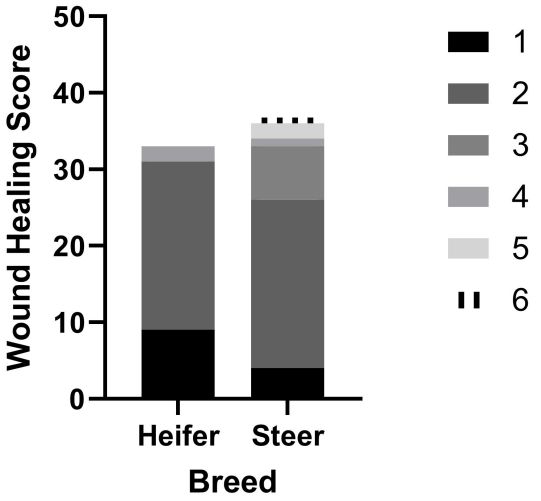

Figure 9.4 Individual wound healing scores by sex following hot-iron branding for week 5 when a significant sex effect (*P* = 0.01) was observed.

Table 9.1 Number of observations by treatment allocation, breed type, and sex calves undergoing hot-iron branding and receiving meloxicam at 1 mg/kg orally (MEL; n = 35) or placebo (CON; n = 35).

Treatment Assignment	Breed Type	Sex	n
MEL	Angus	Heifer	9
MEL	Hereford	Heifer	8
MEL	Angus	Steer	10
MEL	Hereford	Steer	6
MEL	Angus x Hereford	Steer	2
CON	Angus	Heifer	7
CON	Hereford	Heifer	10
CON	Angus x Hereford	Heifer	1
CON	Angus	Steer	6
CON	Hereford	Steer	10
CON	Angus x Hereford	Steer	1

Table 9.2 **Least squares means (upper and lower 95% confidence interval) of outcome variables by treatment for calves undergoing hot-iron branding and receiving meloxicam at 1 mg/kg orally (MEL; n = 35) or placebo (CON; n = 35).**

VARIABLE	MEL	CON	TRT	TIME	TRT*TIME
Mean cortisol (ng/mL)	14.27	14.37	0.73	< 0.01	0.86
CI	11.67 to 16.88	11.77 to 16.97			
Mean PGEM (pg/mL)	24.73	34.54	< 0.01	< 0.01	< 0.01
CI	18.61 to 30.84	28.42 to 40.67			
Mean Max IRT Hip Difference(°C)	2.28[a]	3.15[b]	< 0.01	< 0.01	0.39
CI	1.70 to 2.85	2.58 to 3.73			
Mean Max IRT Left Hip (°C)	34.86	34.96	0.53	< 0.01	0.71
CI	34.56 to 35.15	34.66 to 35.26			
Mean Max Ocular IRT (°C)	36.61	36.53	0.60	< 0.01	0.57
CI	36.33 to 36.89	36.25 to 36.81			
Mean Brand Site MNT (kg F)	0.66	0.65	0.92	< 0.01	0.63
CI	0.62 to 0.69	0.62 to 0.69			
Mean Control Site MNT (kg F)	1.13	1.21	0.16	< 0.01	0.32
CI	1.03 to 1.24	1.11 to 1.31			
Mean Motion Index	5,259	5,161	0.80	< 0.01	0.96
CI	4,557 to 5,962	4,469 to 5,852			
Mean Lying Time (Proportion)	0.30	0.30	0.83	< 0.01	0.42
CI	0.29 to 0.31	0.29 to 0.31			
Mean Step Count	1,097	1,074	0.77	< 0.01	0.94
CI	951 to 1,243	930 to 1,217			
Mean Lying Bouts	5.27	5.63	0.42	0.37	0.03
CI	4.46 to 6.07	4.84 to 6.42			
Mean VAS (1-100 mm)	8.60	8.51	0.75	< 0.01	0.29
CI	8.10 to 9.09	8.00 to 9.01			

Mean ADG (kg/d)	0.91	0.86	0.61
CI	0.74 to 1.07	1.02 to 0.88	

[a,b] Different superscripts indicate significant differences between treatment groups ($P \leq 0.05$)

PGEM – Prostaglandin E_2 metabolite concentration

IRT – Infrared thermography

MNT – Mechanical nociceptive threshold

VAS – Visual Analog Scale

ADG – Average Daily Gain

Table 9.3 **Least squares means (upper and lower 95% confidence interval) of outcome variables by breed (Angus or Hereford) for calves undergoing hot-iron branding and receiving meloxicam at 1 mg/kg orally (MEL; n = 35) or placebo (CON; n = 35).**

VARIABLE	ANGUS	HEREFORD	BREED
Mean cortisol (ng/mL)	14.81[a]	10.69[b]	< 0.01
CI	12.73 to 16.89	8.66 to 12.72	
Mean PGEM (pg/mL)	33.52[a]	23.77[b]	< 0.01
CI	28.61 to 38.43	19.01 to 28.53	
Mean Max IRT Hip Difference(°C)	2.46	2.59	0.63
CI	2.00 to 2.91	2.14 to 3.03	
Mean Max IRT Left Hip (°C)	35.11	34.77	0.13
CI	34.87 to 35.35	34.54 to 35.00	
Mean Max Ocular IRT (°C)	36.44	36.63	0.49
CI	36.22 to 36.67	36.41 to 36.85	
Mean Brand Site MNT (kg F)	0.67	0.69	0.13
CI	0.64 to 0.69	0.66 to 0.71	
Mean Control Site MNT (kg F)	1.16	1.21	0.72
CI	1.08 to 1.24	1.13 to 1.29	
Mean Motion Index	5,381	4,870	0.43
CI	4,763 to 5,999	4,319 to 5,421	
Mean Lying Time (Proportion)	0.31	0.31	0.61
CI	0.30 to 0.31	0.30 to 0.31	
Mean Step Count	1,127	1,017	0.42
CI	999 to 1,256	903 to 1,132	
Mean Lying Bouts	6.42[a]	5.43[b]	0.04
CI	5.71 to 7.13	4.80 to 6.06	
Mean VAS (1-100 mm)	8.54	8.62	0.95
CI	8.09 to 8.98	8.22 to 9.02	
Mean ADG (kg/d)	0.94	0.88	0.72
CI	0.81 to 1.08	0.75 to 1.01	

[a,b] Different superscripts indicate significant differences between treatment groups ($P \leq 0.05$)

PGEM – Prostaglandin E_2 metabolite concentration

IRT – Infrared thermography

MNT – Mechanical nociceptive threshold

VAS – Visual Analog Scale

ADG – Average Daily Gain

Table 9.4 Least squares means (upper and lower 95% confidence interval) of outcome variables by sex (heifer or steer) for calves undergoing hot-iron branding and receiving meloxicam at 1 mg/kg orally (MEL; n = 35) or placebo (CON; n = 35).

Variable	HEIFER	STEER	SEX
Mean cortisol (ng/mL)	14.25	14.40	0.87
CI	11.53 to 16.96	11.92 to 16.87	
Mean PGEM (pg/mL)	36.14[a]	23.13[b]	0.02
CI	29.77 to 42.52	17.30 to 28.95	
Mean Max IRT Hip Difference(°C)	2.53	2.90	0.24
CI	1.93 to 3.13	2.35 to 3.45	
Mean Max IRT Left Hip (°C)	35.44[a]	34.38[b]	< 0.01
CI	35.13 to 35.75	34.09 to 34.66	
Mean Max Ocular IRT (°C)	36.85[a]	36.28[b]	< 0.01
CI	36.56 to 37.15	36.02 to 36.55	
Mean Brand Site MNT (kg F)	0.64	0.66	0.24
CI	0.61 to 0.68	0.63 to 0.70	
Mean Control Site MNT (kg F)	1.11[a]	1.23[b]	0.04
CI	1.01 to 1.22	1.13 to 1.33	
Mean Motion Index	5,536	4,884	0.11
CI	4,829 to 6,243	4,194 to 5,575	
Mean Lying Time (Proportion)	0.29[a]	0.32[b]	< 0.01
CI	0.28 to 0.30	0.31 to 0.33	
Mean Step Count	1,161	1,009	0.07
CI	1,014 to 1,308	865 to 1,153	
Mean Lying Bouts	4.68[a]	6.22[b]	< 0.01
CI	3.87 to 5.49	5.43 to 7.01	
Mean VAS (1-100 mm)	8.24[a]	8.87[b]	0.03
CI	7.75 to 8.73	8.36 to 9.37	
Mean ADG (kg/d)	0.30[a]	1.46[b]	< 0.01
CI	0.13 to 0.48	1.30 to 1.62	

[a,b] Different superscripts indicate significant differences between treatment groups ($P \leq 0.05$)

PGEM – Prostaglandin E_2 metabolite concentration

IRT – Infrared thermography

MNT – Mechanical nociceptive threshold

VAS – Visual Analog Scale

ADG – Average Daily Gain

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Chapter 10 - Comparative pharmacokinetics and tissue concentrations of flunixin meglumine and meloxicam in tilapia (*Oreochromis* spp.)

This chapter has been submitted for publication to Fishes.

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ABSTRACT

Evidence of pain perception in fish is well-established, but analgesic use in aquaculture is limited as little information is available on the properties of analgesic drugs in fish. The objective of this study was to investigate the comparative pharmacokinetics and tissue concentrations of flunixin administered intramuscularly and meloxicam administered intramuscularly or orally in tilapia. Two hundred-seventy fish were enrolled and assigned to 1 of 3 treatment groups 1)

flunixin meglumine administered intramuscularly at a dose of 2.2 mg/kg; 2) meloxicam administered intramuscularly at a dose of 1 mg/kg; or 3) meloxicam administered orally at a dose of 1 mg/kg. Blood and tissue samples were collected from 6 fish per treatment group at 14 timepoints during the 10 days post-drug administration. Plasma and tissue drug concentrations were determined using ultra-high pressure liquid chromatography coupled with mass spectroscopy. The plasma concentration versus time data were analyzed with a non-compartmental approach using a commercially available software (Phoenix®, Version 8.3, Certara, Inc., Princeton, NJ, USA). Flunixin reached a mean maximum concentration (Cmax) of 4826.7 ng/mL at 0.5 h, had a terminal half-life (T1/2) of 7.34 h, and an area under the concentration time curve extrapolated to infinity (AUCINF_obs) of 25,261.62 h*ng/mL. Intramuscular meloxicam had a T1/2 of 9.4 h after reaching a Cmax of 11.3 ng/mL at 2 h, with an AUCINF_obs of 150.31 h*ng/mL. Oral meloxicam had a T1/2 of 1.9 h after reaching a Cmax of 72.2 ng/mL at 2 h, with an AUCINF_obs of 400.83 h*ng/mL. Tissue concentrations of both drugs were undetectable by 9 h post-administration. In conclusion, flunixin, when administered intramuscularly, reached a sufficient plasma concentration to potentially have an analgesic effect, while meloxicam when administered either intramuscularly or orally at the given dosage likely would not reach an analgesic concentration due to the relatively low plasma concentration.

Key words: pharmacokinetics, pain, fish, analgesia

INTRODUCTION

Aquaculture is one of the largest growing sectors of the world food supply. Between 1961 and 2016, the average annual increase in global food fish consumption (3.2%) outpaced

population growth (1.6%) and exceeded that of meat from all terrestrial animals combined (2.8%) (FAO, 2018). In 2013, global aquaculture production accounted for about 50 million of the 125 million metric tons of seafood produced annually for human consumption (Kite-Powell, et al., 2013). Ornamental fish make up another large sector with around 27 million ornamental fish being traded each year (Townsend, 2011). With the continued growth of aquaculture as well as the ornamental fish trade, and increasing scientific discussion over the potential for pain and suffering, research into the welfare of fish is vital (Ashley, 2007).

Evidence of pain perception in fish is supported by a similar sensory system, evidence of adverse behavioral and physiological responses and normal behavior being suspended during a potentially painful event (Sneddon, 2006). Administration of morphine to fish has been shown to significantly reduce pain-related behaviors and opercular beat rate, showing evidence that morphine can act as an analgesic in fish (Sneddon, 2003). The Farm Animal Welfare Committee base their guidelines for farmed fish welfare on the "Five Freedoms", therefore, the use of analgesia in aquaculture could potentially play a role in allowing for freedom from discomfort, pain, injury, and distress (FAWC, 2014). Analgesic use in aquaculture is limited with little information available on the properties of analgesic drugs in most fish species. Interspecies variation has been reported and the current lack of a validated approach to assessing pain in fish limits our ability to evaluate analgesic efficacy (Chatigny, et al., 2018).

Fish pharmacokinetic studies have primarily focused on drugs used to treat infectious disease, with little attention given to analgesic drugs. Studies published on analgesic drugs have focused on clinical efficacy and pain control with less focus on drug pharmacokinetics. The analgesic class of NSAIDs inhibit the cyclo-oxygenase enzymes, reduce inflammation that accompanies tissue injury, and decrease prostaglandin production which attenuates the response

of the peripheral and central components of the nervous system to noxious stimuli, which results in a reduction in the response to pain (Ochroch, et al., 2003). Few NSAIDs have been evaluated with respect to analgesia in fish (Sneddon, 2012). Flunixin has been found to be an ineffective water treatment but has been determined to be effective when injected intra-peritoneally at a dose of 2.2 mg/kg in channel catfish (Brown, et al., 1986). Meloxicam when administered intramuscularly or intravenously at a dose of 1 mg/kg was rapidly eliminated suggesting that clinically relevant concentrations may be difficult to maintain in Nile tilapia (Fredholm, et al., 2016). Neither study examined tissue residue depletion of flunixin or meloxicam in fish.

Off-label usage of dosing regimens are often extrapolated from other species and in fish, extrapolation is not recommended because of excessive pharmacokinetic variability between species, route of administration, and drug formulation (Rigos, Smith, 2015). Aquaculture pharmacokinetics are also uniquely affected by environmental factors such as temperature, pH, and water salinity in which the fish are raised. Thus, the purpose of this study was to investigate the comparative pharmacokinetics of flunixin administered intramuscularly and meloxicam administered intramuscularly or orally in tilapia under a controlled environmental setting.

MATERIALS AND METHODS

2.1. Animals

Three hundred juvenile tilapia (*Oreochromis* spp.) were obtained from a commercial aquaculture facility (Blue Ridge Aquaculture, Martinsville, VA, USA) and maintained at the Aquaculture Lab of the Department of Food Science and Technology at Virginia Tech. The pharmacokinetic trial was performed using an indoor freshwater recirculating aquaculture system equipped with twelve one-meter diameter polyethylene tanks (~ 340 liters each), bubble bead filter for mechanical-solids filtration, fluidized-bed bioreactors for biological treatment to nitrify

ammonia and nitrite to nitrate, UV disinfection units, heating element, and distributed diffuse aeration. Sodium bicarbonate was supplemented to the system to maintain sufficient alkalinity levels for efficient nitrification.

Water quality was rigorously monitored during the fish trial. All water quality parameters were analyzed using methods approved and adapted from Baird (2017). Dissolved oxygen and pH were measured using a YSI ProODO (Yellow Spring Instruments, Yellow Springs, OH) and an Accumet AB15 (Accumet Instruments Pte Ltd., Singapore) meters, respectively. Temperature was monitored every minute using an Onset HOBO 64K Pendant® (Onset, Cape Cod, MA) temperature data logger. Total ammonia nitrogen-N, nitrite-N, and nitrate-N were determined using a HACH DR/2400 spectrophotometer (HACH Company, Loveland, CO). Alkalinity as calcium carbonate was measured using a HACH 16900 manual-digital titrator HACH Company, Loveland, CO).

Fish were fed a standard pelleted tilapia maintenance diet containing approximately 35% protein and 6% fat (Zeigler Brothers, Inc., Gardners, PA). Total fish acquired were 300 (84 × 3 groups + 18 controls = 270 + 30 extra = 300) where the additional 30 fish were included to account for any transportation mortality and/or unacceptable small sized fish. All fish were handled and humanely euthanized using methods approved by Virginia Tech's Institutional Animal Care and Use Committee (VT-IACUC #19-155) under the auspices of Virginia Tech's Animal Welfare Assurance Program (#A-3208-01).

2.2. Treatment groups

After a two-week acclimation period, fish were arbitrarily divided into three treatment groups consisting of 100 fish each and were placed into six separate 200-250 gallon tanks (50 fish/tank) labeled by treatment. Six fish from each treatment group were used as controls and

were blood sampled and euthanized prior to treatment administration. Following treatment administration, 84 fish from each treatment group were sampled at various time points leaving approximately 10 fish remaining to account for unacceptable small sized fish.

2.3. Dosing and sampling

Flunixin meglumine (Banamine injectable solution; 50 mg/ml; Merck Animal Health, Madison, NJ) was diluted with 0.9% NaCl to a ratio of 1:10 (1 part flunixin to 9 parts 0.9% NaCl) in a sterile container to reach a concentration of 5 mg/ml. The flunixin at a concentration of 5 mg/ml was then administered to fish by intramuscular injection at a dosage of 2.2 mg/kg.

Meloxicam (Metacam injectable; 5mg/ml; Boehringer Ingelheim Vetmedica Inc., St. Joseph, MO) was diluted with 0.9% NaCl to a ratio of 1:5 (1 part Meloxicam to 4 parts 0.9% NaCl) in a sterile container to reach a concentration of 1 mg/mL. The meloxicam at a concentration of 1 mg/mL was then administered to fish by intramuscular injection at a dosage of 1 mg/kg.

Meloxicam (Metacam oral; 1.5 mg/mL; Boehringer Ingelheim Vetmedica Inc.) was diluted with 0.9% NaCl to a ratio of 1:1.5 (1 part Meloxicam to 0.5 parts 0.9% NaCl) in a sterile container to reach a concentration of 1 mg/mL. The meloxicam at a concentration of 1 mg/mL was then administered to fish by oral gavage at a dosage of 1 mg/kg after a 24 h fasting period prior to dosing. Oral meloxicam was administered using a curved stainless steel 20-gauge 3″ gavage tube (Popper and Sons, Inc., New Hyde Park, NY) attached to a 100 µL Hamilton syringe. Gavage tube placement in the posterior portion of the stomach was confirmed manually.

At the start of the study (0 time), 6 fish from each group were netted, sedated with sodium bicarbonate buffered (1:1) tricaine methanesulfonate (150 µg/L, MS-222, Sigma Scientific, St. Louis, MO) and bled. Blood was collected from the caudal tail vessels using a 23-

gauge needle and syringe, and after sample collection fish were euthanized with buffered MS-222 (250 μg/L) followed by cervical separation to ensure death. Following flunixin or meloxicam administration, blood samples (~2 mL) were collected at 14 time points: 0.25, 0.5, 1, 2, 4, 6, 9, 12, 24 h, and 2, 4, 6, 8, and 10 days. At each sampling time, six fish from each group were netted, sedated with buffered MS-222, bled and then euthanized. Blood samples were immediately placed in individual plasma separator tubes containing lithium heparin (BD Microtainer, Becton, Dickinson and Company, Franklin Lakes, NJ), mixed by inversion several times and kept on ice until centrifugation at 3000 × g for 10 min at 12 °C. Plasma samples were separated and placed in individual micro-centrifuge tubes and stored at –80 °C until analyzed for flunixin or meloxicam concentration. Following euthanasia, a sample of muscle tissue approximately 1.0-1.5 g was harvested from each fish. Skeletal muscle ventral to the dorsal fin was harvested opposite the side of the intramuscular injection to avoid sampling the injection site. All samples were places in micro-centrifuge tubes and stored at –80 °C until analyzed for flunixin or meloxicam concentration.

2.4. Flunixin and Meloxicam determination

Plasma meloxicam and flunixin analysis

Plasma extraction and clean-up. All solvents used such as methanol, acetonitrile and ammonium formate, formic acid were LC-MS grade and purchased from Fisher Scientific (Hampton, NH). Ultrapure 18Ω water was obtained in-house with a Millipore Synergy UV-R system. Concentrated phosphoric acid (~85%) was also purchase from Fisher Scientific. Meloxicam (MEL) and piroxicam (PIR, internal standard) were purchased from Cayman Chemicals (Ann Arbor, MI). 5'-Hydroxymethyl 5'-desmethyl meloxicam (HDM), a meloxicam metabolite, flunixin, 5-hydroflunixin and flunixin-d_3 standards were purchased from Toronto

research Chemicals (Toronto, ON, Canada).Solid phase extraction μElution plates Oasis PRIME HLB, 2 mg sorbent were purchased from Waters Co (Milford, MD). Stock solutions of MEL and HDM at 100 μg/mL were prepared in methanol and stored at -20 °C. A stock solution of the internal standard at 10 μg/mL was prepared in methanol and kept at -20 °C. For the calibration standards, working solutions of a mixture of HDM and MEL were prepared daily in aqueous ammonium formate 10 mM with 2 % formic acid at the following concentrations: 1, 2.5, 5, 10, 25, 50, 100, 250, 500 and 1,000 ng/mL. Quality controls plasma samples were prepared by spiking untreated fish plasma with HDM and MEL at the following concentrations: 4, 10 and 40 and 400 ng/mL and with 5-hydroxyflunixin and flunixin to at the following concentrations: 1.5, 15 and 150 ng/mL.A working solution of piroxicam at 100 ng/mL in aqueous ammonium formate 10 mM with 2 % formic acid was prepared as well. All the samples were stored at -80 °C and thawed at room temperature (x °C) for 20 min prior to extraction. The negative control plasma for meloxicam was prepared by adding 100 μL of untreated fish plasma, and 200 μL of aqueous ammonium formate 10 mM with 2 % formic. The negative control plasma for flunixin was prepared by adding 100 μL of untreated fish plasma, and 200 μL of phosphoric acid 4%. For the calibration standards, 100 μL of untreated fish plasma was mixed with 100 μL of working standards and 100 μL of internal standard mixture at 100 ng/mL in at 100 ng/mL aqueous ammonium formate 10 mM with 2 % formic for meloxicam or aqueous phosphoric acid 4% for flunixin. For the meloxicam samples and QCs, to 100 μL of fish plasma was added 100 μL of piroxicam working solution at 100 ng/mL and 100 μL of aqueous ammonium formate 10 mM with 2% formic and for the flunixin, 100 μL of fish plasma was added to 100 μL of flunixin-d_3 and 100 μL of aqueous phosphoric acid 4%. The samples were mixed and cleaned-up by solid phase extraction. The negative control, standards, quality controls and samples were loaded on

the SPE plate using positive pressure with nitrogen. Each well was washed twice with 0.25 mL of a mixture of methanol-water (25:75). The compounds were eluted with 2 x 25 µL aliquots of acetonitrile-methanol (90:10) and diluted with 50 µL of water with 0.1% formic acid before analysis.

Plasma Meloxicam LC-MS/MS analysis. Samples were analyzed using a QExactive instrument (Thermo Fisher, Waltham, MA) connected to a Vanquish UPLC system. The chromatographic separation was performed using a column Agilent Technologies Eclipse Plus C18 2.1 x 100 mm, 1.8 µ with a gradient of aqueous formic acid 0.1% (A) and acetonitrile (B) as follows: from 0-0.5 min 30% B, at 4 min 80% B, from 4.010-4.50 min wash with 100% B followed by and a re-equilibration from 4.51-6.5 min with 30% B. The total run time was 6.5 min, the flow rate 0.4 mL/min, the column temperature was kept at 55 °C and the autosampler temperature at 10 °C. In these conditions, meloxicam, 5-hydroxymethyl-5'-desmethyl meloxicam and piroxicam retention times were 2.91 min, 2.17 min and 3.56 min respectively. The analysis was performed in positive mode using Parallel Reaction Monitoring (PRM). The system was controlled through QExactive Tune 2.11 and TraceFinder software 4.1. The Heated Electrospray Ionization (HESI) ion source parameters were set as follows: sheath gas flow rate at 70, auxiliary gas flow rate at 20, sweep gas flow rate at 5, spray voltage at 2.5 kV, the capillary temperature at 280 °C and the auxiliary gas heater temperature at 400 °C. The PRM method combined two scan events starting with a full scan event followed by targeted MS/MS for the single charged precursor ions scheduled in an inclusion list. The full scan event employed a *m/z* 300–800 mass selection, an Orbitrap resolution of 35,000 at m/z 200, a target automatic gain control (AGC) value of 2×10^5, and maximum fill times (IT) of 50 ms. The targeted MS/MS was run at an Orbitrap resolution of 17,500 at *m/z* 200, a target AGC value of 1×10^5, and an IT time

of 50 ms and an isolation window of 1.2 *m/z* unit window. MS/MS fragmentation was performed using the high energy collision dissociation (HCD) mode, with normalized collision energy (NCE) of 50 eV for both meloxicam and its metabolite and 40 eV for the piroxicam (IS). Meloxicam, 5-hydroxymethyl-5'-desmethyl meloxicam and piroxicam were quantified using the products ions at *m/z* 115.032, 131.027 and 95.060, respectively. Identity of meloxicam and 5-hydroxymethyl-5'-desmethyl meloxicam were confirmed with the qualifier ions at *m/z* 141.012 and 157.007, respectively. The calibration curve was linear from 1 ng/mL to 1,000 ng/mL for meloxicam and 5-hydroxymethyl-5'-desmethyl meloxicam with a minimum R^2 of 0.99 and a weighing factor of 1/x. Quality controls were used at 4 ng/mL, 40 ng/mL and 400 ng/mL with accuracies comprised between 80-120% and precision <15%.

Plasma Flunixin LC-MS/MS analysis. The analysis was performed with a system from Waters Corporation (Milford, MA) including an Acquity H UPLC and a TQ-S triple quadrupole mass spectrometer. MassLynx and TargetLynx software 4.2 from Waters Co. (Milford, MA), were used for the data acquisition and data analysis, respectively. The chromatographic separation was done with a column Kinetex from Phenomenex (Torrance, CA) 50 x 2.1 mm, 1.6 µ, heated at 55 °C. The flow rate was set at 0.4 mL/min, the mobile phase consisted of a gradient of acetonitrile (B) and water containing 0.1% formic acid (A) as follow: 0 min: 30% B, 2.0 min: 70% B, 2.01 min: 100% B, 3.00 min: 30% B until 4 min. The total run time was 4 minutes. The injection volume was 5 µL and the temperature of the autosampler was maintained at 8 °C. The data acquisition was performed by Electrospray Ionization (ES) in positive (ES+) mode using multiple reaction monitoring (MRM). The operating parameters for the mass spectrometer were as follows: the capillary voltage was set at 3.5 kV, the source and nitrogen desolvation temperatures were 150 °C and 600 °C, respectively. The desolvation nitrogen flow was set at

1,000 L/hr and the cone nitrogen flow at 150 L/hour. The cone energy was set at 38 V for 5-Hydroxyflunixin, 10 V for flunixin, and 42 V for flunixin-d_3. Helium was used as the collision gas. Desolvation and cone gas flow (nitrogen) were 1,000 and 150 L/h, respectively and the collision gas flow (helium) was 0.15 mL/min. The data acquisition was done by Electrospray Ionization in positive mode (ESI+) using multiple reaction monitoring (MRM). From each precursor ion, two transition product ions were recorded, including one quantifier ion (Q) and one qualifier ion (q). The collision energy (CE) was expressed in volts (V). The dwell time was set automatically in order to get 20 points per peak. For flunixin, MRM transitions were *m/z* 297.144 > 279.1 (Q, CE=20 V), *m/z* 297.1 > 259.1 (q; CE=28 V). For 5-Hydroxyflunixin, MRM transitions were *m/z* 313.1 > 295.1 (Q, CE=22 v) and 313.1 > 197.0 (q; CE=46 V). For flunixin-d_3, MRM transition was *m/z* 300.1 > 282.1 (Q, CE=22 V). The calibration curve was linear from 1.0 ng/mL to 100 ng/mL for both flunixin and 5-hydroxyflunixin with a minimum R^2 of 0.99 and a weighing factor of 1/x. Quality controls were used at 1.5 ng/mL and 15 ng/mL, 150 ng/mL and 1,000 ng/mL with accuracies comprised between 70-120% and precision <15%.

Tissue meloxicam and flunixin analysis

Meloxicam sample extraction. Sample extraction for meloxicam followed the procedure described previously. Fish muscle tissue was weighted (1.0 g±0.1 g) in a 15-mL polypropylene tube and 50 µL of piroxicam (IS) 100 ng/mL in methanol was added. After mixing, 2 mL of 0.2 M sodium acetate buffer pH 4.75 was added. The mixture was homogenized and 25 µL of ß-glucuronidase (Sigma Aldrich, Catalog #G0876) was added and after mixing the tube was placed in an oscillating water bath at 37 °C for 1 h. After letting the tube cool down to room temperature, 5 mL of acetonitrile was added. The tube was shaken for 15 min and centrifuged at 2,900 x g for 7 min. The supernatant was transferred to a clean tube and the extraction repeated

with 5 mL of acetonitrile, and the second extract being combined to the previous one. The final extract was centrifuged at 4,500 x g for 10 min before the clean-up steps. A 10-mL syringe was connected on top of an Alumina N column (Waters Co., P/N WAT020510). The extract was added to the syringe, the vacuum was turned on and the extract was passed through the Alumina column and collected in a clean 15-mL polypropylene tube. The acetonitrile was evaporated at 40 °C under vacuum concentrator until about a volume of 2 mL remained. The extract was centrifuged at 4,500 x g for 10 min and then cleaned-up on a Nexus C18 column 60 mg (Agilent Technologies, P/N 12103101). After conditioning the column with 2 mL of methanol followed by 2 mL of water, the aqueous extract was loaded on the column. The column was washed with 2 mL of water, let dry and then washed with 2 mL of *n*-hexane and let dry. Meloxicam and metabolite were eluted with 4 mL of hexane-ethyl acetate 50:50. The extract was evaporated at 40 °C under vacuum and reconstituted in 200 µL of acetonitrile-0.1% formic acid (30:70) following by centrifugation at 13,000 x g for 10 min before being transferred to a HPLC vial.

Flunixin sample extraction. Sample extraction for flunixin and 5-hydroxyflunixin followed the procedure described below. All the solvents were LC-MS grade. Flunixin, 5-hydroxyflunixin and flunixin-d3 were purchased from Toronto Research Chemicals (Toronto, ON, Canada). Working standards of flunixin and 5-hydroxyflunixin were prepared in a mixture of acetonitrile-water (4:1). Fish muscle tissues were weighted (1.0 g±0.1 g) in a 15-mL polypropylene tube. Additional drug free tilapia tissue from a commercially available source was homogenized and used as a negative control. For calibrators or QCs, 0.5 mL of the following working standards were added to 1 .0 g of NEG CTRL homogenized tissue: 1, 5, 10, 25, 50, 100, 250, 500 and 1,000 ng/mL. For quality controls (QCs), 0.5 mL of the following working standards were added: 2, 20, 200 and 800 ng/mL. then 0.5 mL of flunixin-d_3 at 100 ng/mL was

added to each calibration, QC and sample. After mixing, 4 mL acetonitrile-water (4:1) were added and the tubes were shaken for 5 minutes followed by a centrifugation step at 4,500 x g for 5 minutes. The supernatant was transferred to a 15-mL polypropylene tube containing 0.25 g of C18 sorbent (Agilent, P/N5982-1188). Tubes were shaken for 30 seconds and centrifuged at 4,500 x g for 5 minutes and 0.1 mL of supernatant was transferred to a HPLC vial.

LC-MS/MS analysis. Samples were analyzed using a QExactive instrument (Thermo Fisher) connected to a Vanquish UPLC system. The chromatographic separation was performed using a column Agilent Technologies Eclipse Plus C18 2.1 x 100 mm, 1.8 μ with a gradient of aqueous formic acid 0.1% (A) and acetonitrile (B) as follows: from 0-0.5 min 30% B, at 4 min 80% B, from 4.010-4.50 min wash with 100% B followed by and a re-equilibration from 4.51-6.5 min with 30% B. The total run time was 6.5 min, the flow rate 0.4 mL/min, the column temperature was kept at 55 °C and the autosampler temperature at 10 °C. In these conditions, meloxicam, 5-hydroxymethyl-5'-desmethyl meloxicam and piroxicam retention times were 2.91 min, 2.17 min and 3.56 min, respectively, and 5-hydroxyflunixin, flunixin and flunixin-d_3 retention times were 1.87, 2.02 min and 2.02 min, respectively. The analysis was performed in positive mode using Parallel Reaction Monitoring (PRM). The system was controlled through QExactive Tune and TraceFinder software. The Heated Electrospray Ionization (HESI) ion source parameters were set as follows for meloxicam: sheath gas flow rate at 70, auxiliary gas flow rate at 20, sweep gas flow rate at 5, spray voltage at 2.5 kV, the capillary temperature at 280 °C and the auxiliary gas heater temperature at 400 °C and as follows for flunixin: sheath gas flow rate at 70, auxiliary gas flow rate at 15, sweep gas flow rate at 5, spray voltage at 3.0 kV, the capillary temperature at 370 °C and the auxiliary gas heater temperature at 400 °C. The approximate room temperature of the laboratory was 21ºC. The PRM method combined two scan

events starting with a full scan event followed by targeted MS/MS for the single charged precursor ions scheduled in an inclusion list.

For meloxicam, the full scan event employed a *m/z* 300–800 mass selection, an Orbitrap resolution of 35,000 at m/z 200, a target automatic gain control (AGC) value of 2×10^5, and maximum fill times (IT) of 50 ms. The targeted MS/MS was run at an Orbitrap resolution of 17,500 at *m/z* 200, a target AGC value of 1×10^5, and an IT time of 50 ms and an isolation window of 1.2 *m/z* unit window. MS/MS fragmentation was performed using the high energy collision dissociation (HCD) mode, with normalized collision energy (NCE) of 50 eV for both meloxicam and its metabolite and 40 eV for the piroxicam (IS). Meloxicam, 5-hydroxymethyl-5'-desmethyl meloxicam and piroxicam were quantified using the products ions at *m/z* 115.032, 131.027 and 95.060, respectively. Identity of meloxicam and 5-hydroxymethyl-5'-desmethyl meloxicam were confirmed with the qualifier ions at *m/z* 141.012 and 157.007 respectively. The calibration curve was linear from 1.25 ng/mL to 50 ng/mL for meloxicam and from 5 to 50 ng/mL for 5-hydroxymethyl-5'-desmethyl meloxicam with a minimum R^2 of 0.99 and a weighing factor of 1/x. Quality controls were used at 2 ng/g and 20 ng/g with accuracies comprised between 80-120% and precision <15%.

For flunixin, the full scan event employed a *m/z* 80-500 mass selection, an Orbitrap resolution of 17,500 at m/z 200, a target automatic gain control (AGC) value of 1×10^6, and maximum fill times (IT) of 50 ms. 5-Hydroxyflunixin, flunixin, and flunixin-d_3 precursor ions were *m/z* 313.079, 298.085, 300.103, respectively. The targeted MS/MS was run with an Orbitrap resolution of 17,500 at *m/z* 200, a target AGC value of 1×10^5, and an IT time of 50 ms and an isolation window of 1.2 *m/z* unit window. MS/MS fragmentation was performed using the high energy collision dissociation (HCD) mode, with normalized collision energy (NCE) of 40

eV for 5-hydroxyflunixin and a combination of 35 and 65 eV for flunixin. 5-Hydroxyflunixin, flunixin, and flunixin-d_3 were quantified using the products ions at *m/z* 280.045, 279.073 and 282.092, respectively. Identity of 5-hydroxyflunixin and flunixin were confirmed with the qualifier ions at *m/z* 295.0684 and 109.045 respectively. The calibration curve was linear from 2.5 ng/mL to 500 ng/mL for both flunixin and 5-hydroxyflunixin with a minimum R^2 of 0.99 and a weighing factor of 1/x. Quality controls were used at 10 ng/g and 100 ng/g and 400 ng/g with accuracies comprised between 80-120% and precision <15%.

2.5. Pharmacokinetic analysis

The plasma concentration versus time data were analyzed with a non-compartmental approach using a commercially available software (Phoenix®, Version 8.3, Certara, Inc., Princeton, NJ, USA). Mean concentration versus time profile for each treatment group was generated by taking the mean concentration value of the data from six fish for each time point. Only concentrations above the limit of quantification (LOQ) for all six fish each time point were included in the analysis. PK parameters were calculated based on the mean concentration versus time profile for each treatment group. The following PK parameters were calculated, including; slope of the terminal phase (λ_z), terminal half-life (T ½), maximum plasma concentration (C_{max}); time to achieve peak concentration (T_{max}), time of last measurable (positive) concentrations in all six fish (T_{last}), area under the curve from the time of dosing ($Dosing_{time}$) to the last measurable (positive) concentration ($AUC_{0\text{-}last}$), AUC from $Dosing_{time}$ extrapolated to infinity, based on the last observed concentration (obs) ($AUC_{0\text{-}\infty}$), percentage of $AUCINF_{obs}$ due to extrapolation from T_{last} to infinity ($AUC_{\%Extrap}$), volume of distribution based on the terminal phase per fraction of dose absorbed (Vz_F), total body clearance per fraction of dose absorbed (Cl_F), area under the moment curve from the time of dosing ($Dosing_{time}$) to the last measurable (positive)

concentration ($AUMC_{0\text{-}last}$), area under the first moment curve (AUMC) extrapolated to infinity, based on the last observed concentration ($AUMC_{0\text{-}\infty}$), percent of $AUMC_{0\text{-}\infty}$ that is extrapolated ($AUMC_{\%Extrap}$), mean residence time from the time of dosing ($Dosing_{time}$) to the time of the last measurable concentration (MRT_{last}), and mean residence time (MRT) extrapolated to infinity ($MRT_{0\text{-}\infty}$). The λ_z was calculated using linear regression of the terminal part of the log plasma concentration versus time curve and a linear trapezoidal linear interpolation method was used to determine $AUC_{0\text{-}last}$. The AUMC was calculated by combining the trapezoid calculation of $AUMC_{0\text{-}last}$ and extrapolated area. The MRT was calculated as MRT=AUMC/AUC and CL/F was calculated as CL=dose/AUC. The C_{max} represented the observed peak plasma concentration, and the T_{max} was the time to reach C_{max}.

RESULTS

Fish and Environmental Parameters

Mean weights of the FLU, MEL-IM, and MEL-PO treatment groups at the time of drug administration were 297.0 ± 6.21 g, 296.0 0 ± 6.60 g, and 287.5 ± 6.80 g, respectively (**Table 1**). Overall mean ± SE of water quality parameters: temperature 25.5 ± 0.1 °C, dissolved oxygen 6.13 ± 0.07 mg/L, pH 7.95 ± 0.04, total ammonia-N 0.15 ± 0.03 mg/L, nitrite-N 0.16 ± 0.03 mg/L, nitrate-N 13.5 ± 5.4 mg/L, and alkalinity as CaCO3 79.3 ± 2.0 ppm.

Plasma Concentrations

The mean ± SEM plasma concentrations of drug at time intervals between 0.25 h-240 h are given in **Table 2**. The timepoints included are those at which at least 1 of the 6 fish sampled contained a drug concentration above the LOQ. The comparative log drug concentration versus time curves are shown in **Figure 1** and **2**. The mean profiles of the two meloxicam treatments showed a similar trend with oral administration reaching a higher peak concentration and both

drug concentrations becoming non-detectable at 12 h (**Fig. 1**). 5'Hydroxy-desmethyl-meloxicam was detectable out to 6 d post-drug administration. Flunixin drug concentrations reached a much higher peak concentration and indicated much slower excretion of the drug from the body, with drug concentrations detectable out to 96 h post-administration (**Fig. 2**). 5-hydroxyflunixin was detectable out to 24 h post-drug administration.

The PK parameters derived from plasma concentration versus time data of the three treatment groups are shown in **Table 3**. Drug absorption appeared to be quick for all three treatment groups with peak concentrations at 0.5 h for FLU and 2 h for both MEL-IM and MEL-PO. The C_{max} of the three treatments were 4826.72 ng/mL, 11.29 ng/mL, and 72.22 ng/mL in the FLU, MEL-IM and MEL-PO groups, respectively and corresponding T_{max} values were 0.5 h, 2 h, and 2 h. Plasma concentrations for the two meloxicam treatments were much lower than flunixin concentrations at all timepoints when concentrations were detectable. The $AUC_{0-\infty}$ (25261.62 h*ng/mL) from the FLU group was approximately 60 times higher than that obtained from the MEL-PO group (400.83 h*ng/mL) and 160 times higher than the $AUC_{0-\infty}$ obtained from the MEL-IM group (150.31 h*ng/mL). The Cl_F of the FLU, MEL-IM, and MEL-PO groups were 87.09 mL/h/kg, 6653.13 mL/h/kg, and 2494.81mL/h/kg, respectively, and the corresponding T ½ were 7.34 h, 9.4 h, and 1.91 h.

Tissue Concentrations

The mean ± SEM tissue concentrations of drug at time intervals between 0.25 h-240 h are given in **Table 4**. The timepoints included are those at which at least 1 of the 6 fish sampled contained a drug concentration above the LOQ. Data was unable to be collected from the flunixin tissue samples for the 0.25 and 0.5 h timepoints. The comparative tissue drug concentration versus time curves are shown in **Figure 3** and **4** for each of the samples above the

LOQ. The mean tissue profiles of the two meloxicam treatments show a similar trend to the plasma profiles with oral administration reaching a higher peak concentration and both drug concentrations becoming non-detectable at 9 h (**Fig. 3**). 5'Hydroxy-desmethyl-meloxicam was not detected throughout the study following both oral and intramuscular administration. Flunixin tissue concentrations reached a higher peak concentration than meloxicam with tissue drug concentrations detectable out to 24 h post-administration (**Fig. 4**). 5-hydroxyflunixin was detectable out to 9 h post-drug administration.

DISCUSSION

There were no mortalities in any of the groups of fish, and no negative effects of drug treatment were observed during the study. Water quality variables during the study were within typical ranges for tilapia aquaculture (Rakocy, 1990). A recent study found that MS-222 had no effect on florfenicol pharmacokinetics in Nile tilapia (Rairat, et al., 2021). The use of MS-222 along with an NSAID is probable due to the widespread use of MS-222 as a sedative; there is no current data available on the effect of MS-222 on NSAID pharmacokinetics in Nile tilapia.

Tilapia belong to a class of fish known as cichlids from the family Cichlidae (Dunz, Schliewen, 2013) which encompasses numerous species that are a part of aquaculture as well as the ornamental fish trade. Analgesics are an integral part of routine pain management in mammals, yet their use in aquaculture as well as the ornamental fish trade is still limited (Chatigny, et al., 2018). Research into post-operative analgesia investigating the use of an opioid or NSAID in ornamental fish (*Cyprinus carpio)* showed promise but has not been expanded to different species (Harms, et al., 2005). Fish that are subject to tissue damaging, invasive procedures, traumatic injury or aggression may require that pain and discomfort be reduced by the use of an analgesic such as an NSAID; yet, validation of analgesic protocols is very limited

and a great deal of species variation exists making extrapolation difficult (Sneddon, 2012). Meloxicam has been administered intravenously and intramuscularly to Nile tilapia and reached relatively high plasma concentrations, but was eliminated faster than what is reported in the literature for other mammals (Fredholm, et al., 2016). Flunixin has been found to be an ineffective water treatment but has shown promise to reach a clinically effective concentration when administered intraperitoneally in catfish (Brown, et al., 1986). This study was the first to report on the pharmacokinetics of flunixin administered intramuscularly and meloxicam administered intramuscularly or orally at the given drug concentration and dosage in Nile tilapia.

Meloxicam when administered intramuscularly or orally at a concentration of 1 mg/mL and a dosage of 1 mg/kg likely did not reach clinically effective plasma concentrations and was quickly eliminated. The quick elimination is consistent with the findings of Fredholm, et al. (2016) when meloxicam was administered intravenously or intramuscularly at the same dose of 1 mg/kg, yet higher plasma concentrations were achieved compared to the present study. Meloxicam administered orally resulted in a larger AUC and longer terminal half-life than when administered intramuscularly, though both plasma concentrations were relatively low. 5'Hydroxy-desmethyl-meloxicam was quantified in the current study and was detectable in plasma out to 6 d post drug administration for both intramuscular and oral routes indicative of a possible drug depot. The findings of the current study are consistent with previous findings suggesting that multiple daily administrations would be necessary to maintain a plasma concentration that could effectively control pain. Unfortunately, multiple dosing is impractical and stressful for large populations of fish maintained in aquaculture settings.

When flunixin was administered intramuscularly at a concentration of 50 mg/mL and a dosage of 2.2 mg/kg, relatively high plasma concentrations were achieved. Flunixin

concentrations were detectable out to 96 h post administration and 5-hydroxyflunixin was detectable out to 24 h. These findings are consistent with Brown, et al. (1986) who injected flunixin intra-peritoneally and reached a high plasma concentration deemed to be effective in catfish without signs of toxicity at a dosage of 2.2 mg/kg. Evaluation of clinical efficacy was outside the scope of this pharmacokinetic study, thus recommendations for effective dosing in Nile tilapia cannot be made. However, when administered intramuscularly, flunixin achieved a high C_{max} (4826.72 ng/mL) and long enough terminal half-life (7.34 h) to potentially maintain clinically relevant plasma concentrations. Immediately following flunixin administration, an area of hyperpigmentation appeared around the injection site indicative of possible drug irritation which has also been reported in mammals upon intramuscular administration (Pyörälä, et al., 1999). This localized darkening of the skin disappeared within 30 minutes from all fish involved in this study group.

Meloxicam concentrations were detectable in plasma for longer periods of time than in tissue for both oral and intramuscular administration. Meloxicam levels were below the LOQ of 2.5 ng/g at 8 h following drug administration and remained nondetectable. Flunixin peak concentrations in tissue were higher and detectable in tissue for a longer period of time than meloxicam tissue concentrations. Flunixin levels were below the LOQ of 2.5 ng/g at 48 h following drug administration and remained nondetectable. These results show a similar pattern of plasma concentrations for flunixin and meloxicam. However, these data suggest that meloxicam and flunixin reach nondetectable levels more quickly in tissue relative to plasma given the study conditions. The disparities between tissue and plasma data suggest that drug pharmacokinetics do differ between plasma and tissue. The meloxicam and flunixin metabolites quantified in this study (5'Hydroxy-desmethyl-meloxicam and 5-hydroxyflunixin) have been

shown to be active metabolites in cattle pharmacokinetic trials, however, (Kissell, et al., 2012; Mosher, et al., 2012), whether they are the most appropriate metabolites to quantify in fish has not been established.

CONCLUSION

In conclusion, flunixin administered intramuscularly reached a sufficient plasma concentration to potentially have an analgesic effect, while meloxicam administered either intramuscularly or orally at the given dosage likely would not reach an analgesic concentration due to the relatively low plasma concentration. The feasibility of dosing individual fish is limited in commercial operations but may be relevant to settings where individual fish are more valuable and are handled on occasion. Development of an effective granular formulation of an NSAID would be more likely to be integrated into commercial operations. Further studies investigating different drug concentrations and dosage regimens of meloxicam, as well as clinical efficacy of flunixin and meloxicam in Nile tilapia are warranted to provide effective options for pain control in fish.

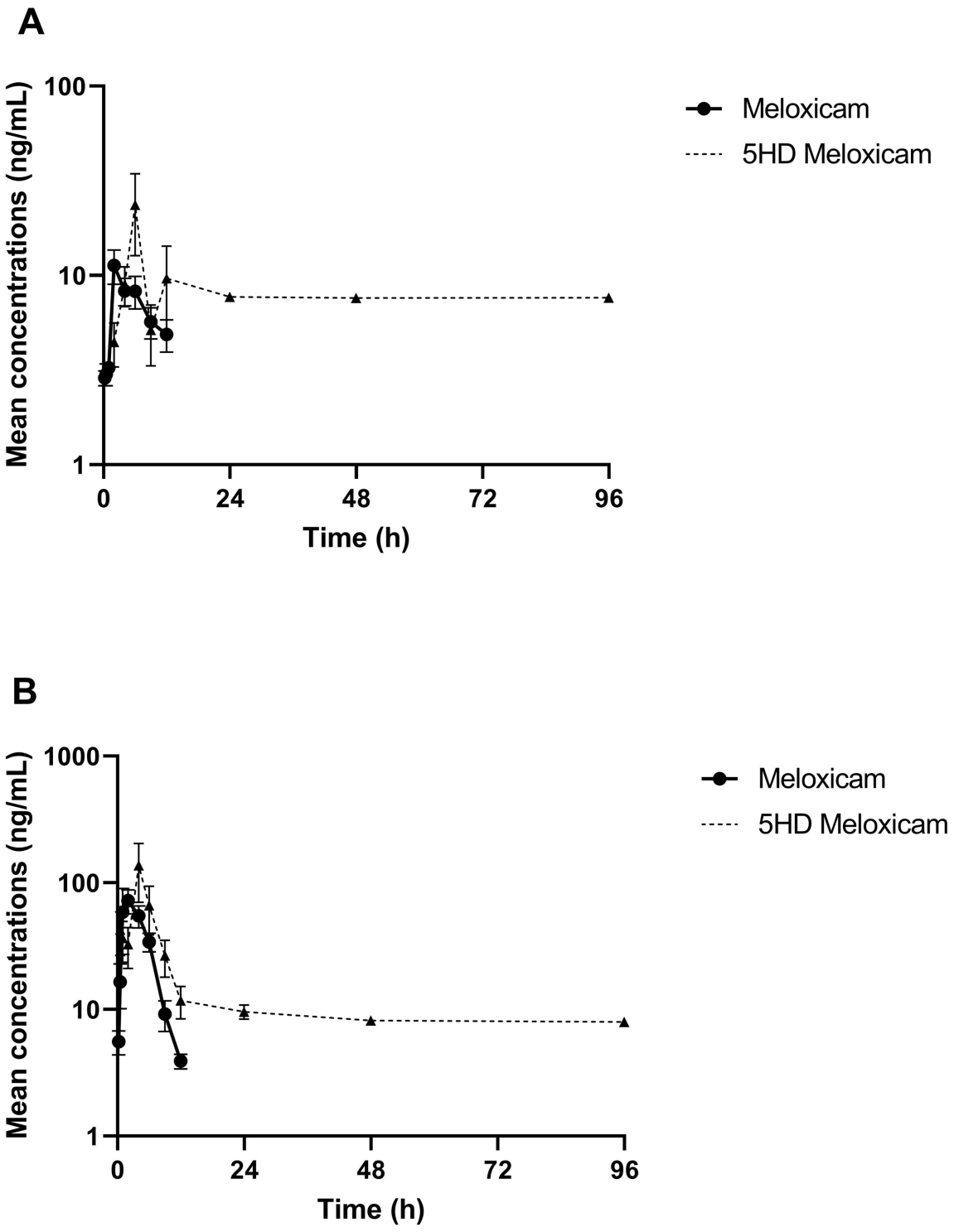

Figure 10.1 Mean ± SEM parent drug and metabolite concentration in plasma after administration of a single dose of meloxicam (1 mg/kg) orally (Fig. 1.A) or intramuscularly (Fig. 1.B) (n = 6) from 0-96 h post-administration in tilapia

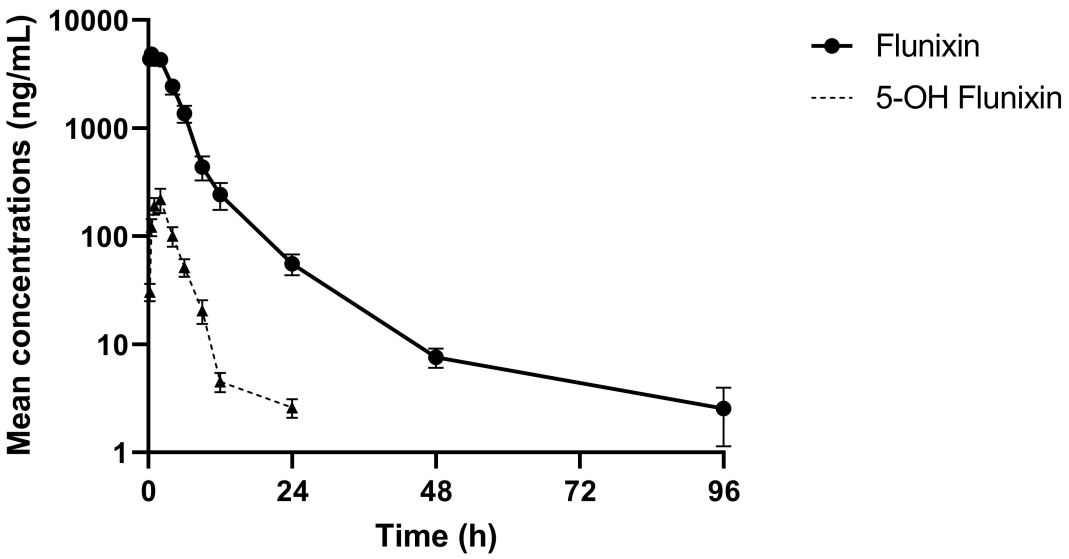

Figure 10.2 Mean ± SEM parent drug and metabolite concentration in plasma after administration of a single dose of flunixin (2.2 mg/kg) intramuscularly (n = 6) from 0-96 h post-administration in tilapia

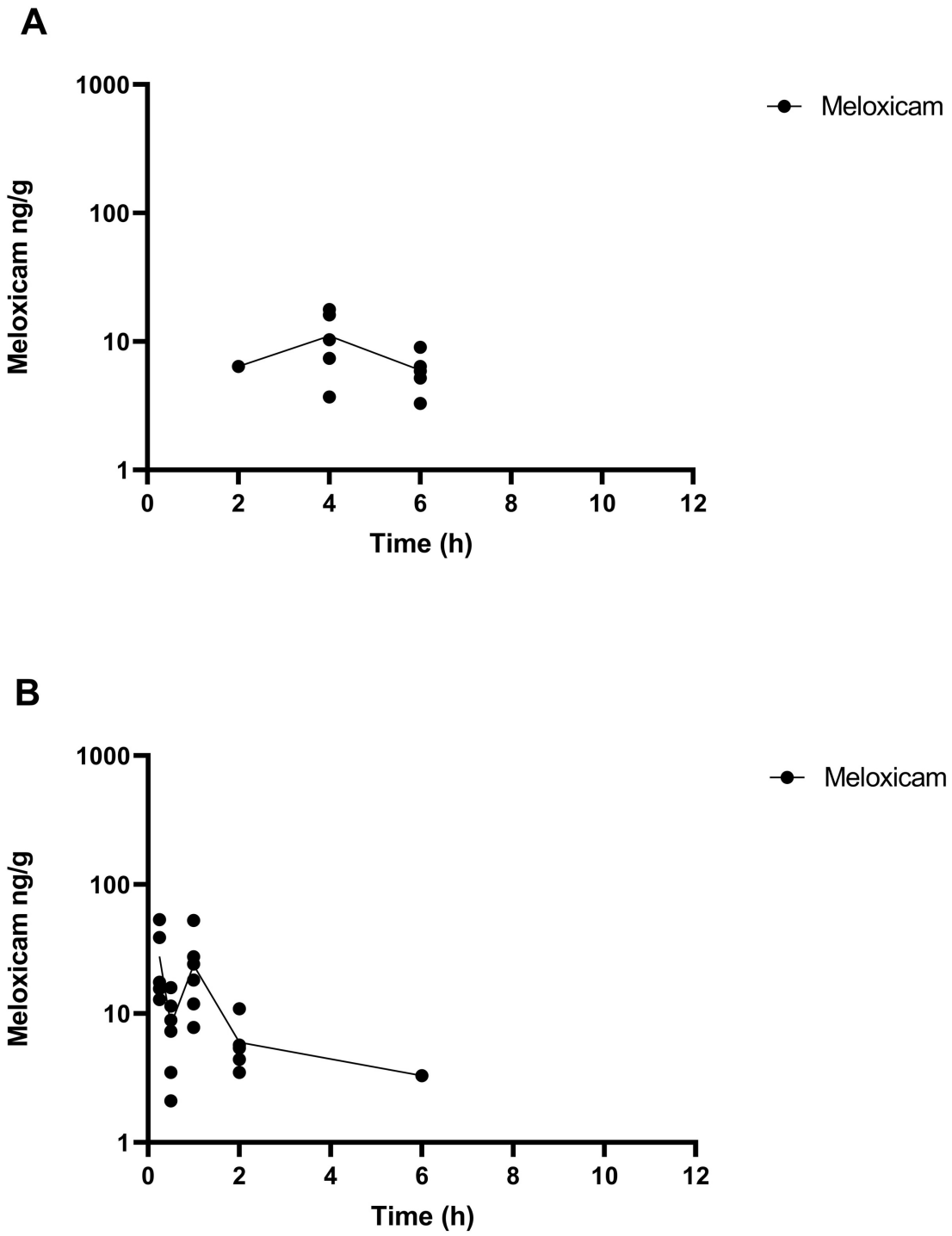

Figure 10.3 Mean ± SEM parent drug concentration in tissue after administration of a single dose of meloxicam (1 mg/kg) orally (Fig. 2.A) or intramuscularly (Fig. 2.B) (n = 6) from 0-12 h post-administration in tilapia

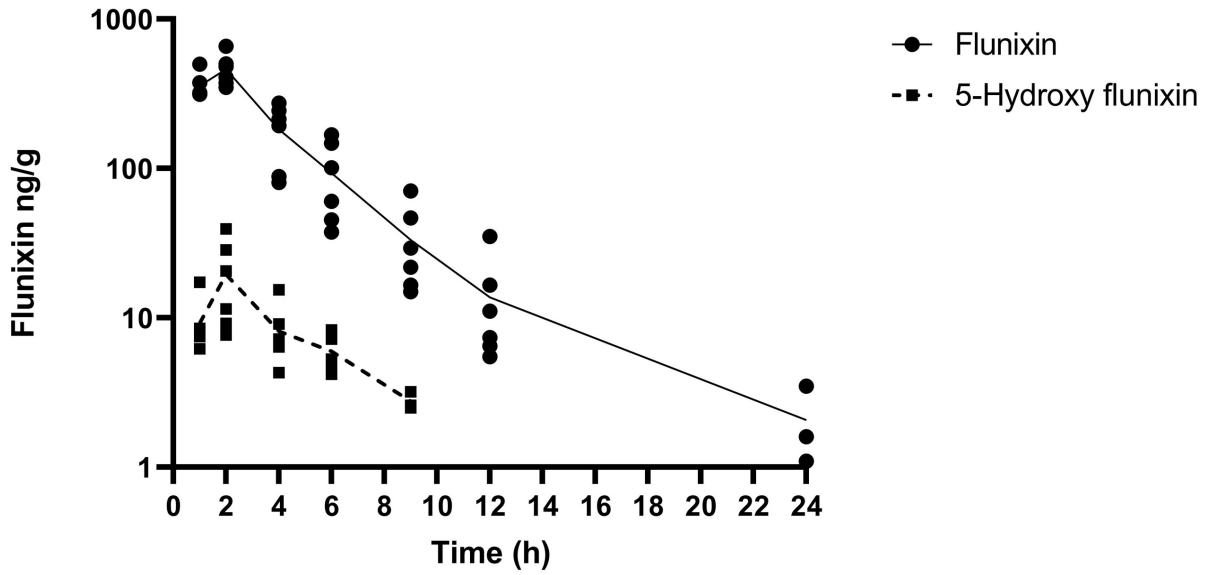

Figure 10.4 Mean ± SEM parent drug and metabolite concentration in tissue after administration of a single dose of flunixin (2.2 mg/kg) intramuscularly (n = 6) from 0-24 h post-administration in tilapia

Table 10.1 Experimental design and mean weights of the FLU, MEL-IM, and MEL-PO groups at the time of drug administration in tilapia.

	Treatment groups		
	FLU	MEL-IM	MEL-PO
Number of tilapia per group (n)	84	84	84
Number of sample times collected	14	14	14
Number of fish sampled at each timepoint	6	6	6
Weight of tilapia (g)	Mean = 297.0 ± 6.21 Min =194 Max =440	Mean = 296.0 ± 6.60 Min =189 Max =513	Mean = 287.5 ± 6.80 Min =185 Max =467
Dose (mg/kg)	2.2	1.0	1.0
Mortality during experiment	0	0	0

Table 10.2 Mean ± SEM parent drug and metabolite concentration in plasma after administration of a single dose of flunixin (2.2 mg/kg) intramuscularly or meloxicam (1 mg/kg) intramuscularly or orally (n = 6) in tilapia.

Time (h)	Concentration (ng/mL)																
	FLU			FLU-OH			MEL-IM			MEL-IM-5HD			MEL-PO			MEL-PO-5HD	
	n	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n	Mean	SEM
0.25	6	4375.79	171.40	6	30.71	5.53	3	2.87	0.26	6	< LOQ		4	5.57	1.18	6	< LOQ	
0.5	6	4826.72	402.71	6	122.38	21.83	2	3.01	0.40	6	< LOQ		5	16.49	6.38	3	42.71	16.03
1	6	4372.20	237.12	6	192.19	33.17	1	3.25		6	< LOQ		6	58.70	31.49	6	36.55	13.04
2	6	4303.00	172.82	6	219.42	55.10	6	11.29	2.30	3	4.45	1.17	5	72.22	15.40	5	32.71	11.62
4	6	2439.61	387.80	6	100.83	20.52	6	8.27	1.37	4	8.97	2.13	6	54.70	10.82	6	137.00	67.08
6	6	1368.40	245.71	6	51.66	9.61	6	8.26	1.62	3	23.61	10.89	6	34.13	5.72	6	66.25	27.56
9	6	439.50	110.87	6	20.54	5.10	6	5.68	1.07	3	5.16	1.82	6	9.19	2.51	5	26.58	8.57
12	6	243.49	67.55	6	4.53	0.92	6	4.88	0.94	4	9.65	4.63	3	3.91	0.51	4	11.77	3.37
24	6	55.95	12.26	4	2.60	0.50	6	< LOQ		6	7.70	0.08	6	< LOQ		6	9.58	1.22
48	6	7.61	1.52	6	< LOQ		6	< LOQ		5	7.61	0.01	6	< LOQ		5	8.16	0.19
96	4	2.56	1.41	6	< LOQ		6	< LOQ		5	7.61	0.01	6	< LOQ		5	7.94	0.20
144	6	< LOQ		6	< LOQ		6	< LOQ		5	7.62	0.01	6	< LOQ		5	7.72	0.05
192	6	< LOQ		6	< LOQ		6	< LOQ			< LOQ		6	< LOQ		6	< LOQ	
240	6	< LOQ		6	< LOQ		6	< LOQ			< LOQ		6	< LOQ		6	< LOQ	

Where n < 6 the remaining sample concentrations were < LOQ and the mean concentration is only based on sample concentrations >LOQ

Table 10.3 Pharmacokinetic parameters in plasma after administration of a single dose of flunixin (2.2 mg/kg) intramuscularly or meloxicam (1 mg/kg) intramuscularly or orally (n = 6) in tilapia.

Parameter	Units	FLU	MEL-IM	MEL-PO
λ_z	1/h	0.09	0.07	0.36
T ½	(h)	7.34	9.4	1.91
T_{max}	(h)	0.5	2	2
C_{max}	(ng/mL)	4826.72	11.29	72.22
T_{last}	(h)	48	12	9
$AUC_{0\text{-}last}$	(h*ng/mL)	25180.93	84.13	375.54
$AUC_{0\text{-}\infty}$	(h*ng/mL)	25261.62	150.31	400.83
$AUC_{\%Extrap}$	(%)	0.32	44.03	6.31
Vz_F	(mL/kg)	922.84	90245.53	6868.6
Cl_F	(mL/h/kg)	87.09	6653.13	2494.81
$AUMC_{0\text{-}last}$	(h*h*ng/mL)	119745.13	476.38	1348.97
$AUMC_{0\text{-}\infty}$	(h*h*ng/mL)	124473.36	2168.13	1646.19
$AUMC_{\%Extrap}$	(%)	3.8	78.03	18.06
MRT_{last}	(h)	4.76	5.66	3.59
$MRTI_{0\text{-}\infty}$	(h)	4.93	14.42	4.11

Table 10.4 Mean ± SEM parent drug and metabolite concentration in tissue after administration of a single dose of flunixin (2.2 mg/kg) intramuscularly or meloxicam (1 mg/kg) intramuscularly or orally (n = 6) in tilapia.

Time (h)	Concentration (ng/g)																	
	FLU			FLU-OH			MEL-IM			MEL-IM-5HD			MEL-PO			MEL-PO-5HD		
	n	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n	Mean	SEM
0.25							6	<LOQ		6	<LOQ		5	27.62	7.92	6	<LOQ	
0.5							6	<LOQ		6	<LOQ		6	8.18	2.08	6	<LOQ	
1	6	357.4	30.20	6	9.23	1.64	6	<LOQ		6	<LOQ		6	23.73	6.53	6	<LOQ	
2	6	461.65	46.18	6	19.5	5.14	1	6.4		6	<LOQ		4	6.125	1.64	6	<LOQ	
4	6	182.42	32.93	6	8.13	1.57	5	11.04	2.62	6	<LOQ		6	<LOQ		6	<LOQ	
6	6	93.37	22.44	5	5.98	0.78	5	5.96	0.93	6	<LOQ		1	3.3		6	<LOQ	
9	6	33.34	8.81	3	2.77	0.23	6	<LOQ		6	<LOQ		6	<LOQ		6	<LOQ	
12	6	13.7	4.59	6	<LOQ		6	<LOQ		6	<LOQ		6	<LOQ		6	<LOQ	
24	3	2.07	0.74	6	<LOQ		6	<LOQ		6	<LOQ		6	<LOQ		6	<LOQ	
48	6	<LOQ		6	<LOQ		6	<LOQ		6	<LOQ		6	<LOQ		6	<LOQ	
96	6	<LOQ		6	<LOQ		6	<LOQ		6	<LOQ		6	<LOQ		6	<LOQ	
144	6	<LOQ		6	<LOQ		6	<LOQ		6	<LOQ		6	<LOQ		6	<LOQ	
192	6	<LOQ		6	<LOQ		6	<LOQ		6	<LOQ		6	<LOQ		6	<LOQ	
240	6	<LOQ		6	<LOQ		6	<LOQ		6	<LOQ		6	<LOQ		6	<LOQ	

Where n < 6 the remaining sample concentrations were < LOQ and the mean concentration is only based on sample concentrations >LOQ

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