ASSESSMENT OF RESPONSE VARIABLES IN BOVINE MODELS OF PAIN AND STRESS, WITH AND WITHOUT MELOXICAM

by

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BS, Kansas State University, 1984
DVM, Kansas State University, 1986
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AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

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Department of Diagnostic Medicine and Pathobiology
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Abstract

The absence of pain management for common husbandry procedures, such as dehorning and castration of cattle, is considered to be an important animal welfare consideration, but there are currently no drugs approved by the FDA for the purpose of providing pain relief in cattle. The FDA's Center for Veterinary Medicine (CVM) recognizes the need for the availability of pain-relieving drugs and has encouraged research into the development of behavioral and physiologic measures which can reliably demonstrate the effectiveness in that species. The USDA has also recognized this need by providing grants for research into investigating pain models for cattle that can be used for the development of pain mitigation methods. The studies reported in this dissertation were funded by the USDA and the American Association of Bovine Practitioners. They add to the body of knowledge from which a pain model in cattle may eventually be validated for use in the drug approval process and also contribute to knowledge base for a candidate non-steroidal anti-inflammatory drug for convenient use in cattle.

The study reported in Chapter 2 was conducted to support research of a candidate pain-assessment variable, substance P. This study provides future researchers with recommended sample handling procedures for obtaining reliable and repeatable results, which is important if substance P is to be validated as pain biomarker in cattle. The study in Chapter 3 investigated the use of several variables for use in a pain model. The results provided researchers, veterinarians and policy-makers with evidence to support the common practice of castrating and dehorning calves at the same time rather than as individual procedures separated by a healing interim. The study in Chapter 4 investigated the pharmacokinetics of oral meloxicam when administered to juvenile ruminant and pre-ruminant calves. This study added to the growing knowledge base of the pharmacokinetics of oral meloxicam in cattle and also provided practitioners with practical information concerning the administration of the drug in milk replacer. Chapter 5 investigated the use of oral meloxicam in a production setting and indicated that meloxicam administration prior to surgical castration may reduce the incidence of respiratory disease in the post-surgical period.

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Chapter 1 - Literature Review

Introduction

The absence of pain management for common surgical procedures, such as dehorning and castration of cattle, is considered to be an important animal welfare concern and has been under consideration in the development of international trade agreements (Phillips, 2008; Thiermann & Babcock, 2005). There are currently no drugs approved by the U.S. Food and Drug Administration (FDA) for the express purpose of providing pain relief in cattle. Lidocaine, is approved for the administration of short-acting local anesthesia. Flunixin meglumine, a non-steroidal anti-inflammatory drug, is approved for controlling pyrexia and inflammation in cattle with intravenous administration. The FDA's Center for Veterinary Medicine (CVM) recognizes the need for the availability of pain-relieving drugs, and with the aim of improving the availability of drugs to control pain in cattle, the CVM is currently encouraging research into the development of behavioral and physiologic measures which can reliably demonstrate the effectiveness in that species (Smith, 2013).

The research for this dissertation was conducted to investigate measures which may be used for the development of pain-mitigation strategies, when performing castration and dehorning of cattle. Furthermore, this research was conducted to further the understanding of the pharmacokinetics of an analgesic drug, meloxicam, in cattle and to investigate its use as a candidate for providing analgesia in cattle when subjected to castration.

Overview of animal husbandry procedures in modern livestock production settings

The castration and dehorning of cattle are common husbandry procedures which, in modern society and production agriculture, convey benefits to producers, consumers, and to the animals themselves. Although the procedures inflict pain and are associated with a post-procedure period of reduced feed efficiency and reduced growth rate, the benefits are considered to outweigh the negative aspects. Castration of bulls has long been practiced to produce steers which are less aggressive to man and to each other. Additionally, castration prevents unwanted matings with co-mingled females and produces carcass traits which are desirable to consumers

(AVMA, 2011a). Horns on cattle increase the risk of injury to man and animals alike. Cattle with horns may injure each other while eating at the feed bunk, during aggressive displays of dominance, and during close confinement and transportation. The carcasses of dehorned cattle tend to have less wastage due to bruising (AVMA, 2011b).

Cattle destined to be dehorned and/or castrated are derived from two distinct cattle production systems: beef and dairy. The different handling processes and the different genetics within those industries affect when the dehorning and castration procedures occur. In the dairy industry, calves are usually separated from the dam within 24 h after parturition and raised by producers with the provision of milk-replacer and a target weaning date of approximately 6 to 8 weeks of age. Whether calves are retained on the farm, sent to a heifer-raising facility, or sold to another producer for entry into the veal or finished beef channels, the animals are handled from birth and there is opportunity to perform husbandry procedures at a young age. Most dairy calves require dehorning since the horned Holstein breed comprises 90% of all dairy cows in the U.S. (USDA, 2007a). In a USDA survey of dairy farm practices, the majority of heifer calves were disbudded by hot iron (68%) or caustic paste (12%) before 8 weeks of age which is approximately when the horn bud begins to attach to the frontal bone. Tube, spoon, or gouge tools were used at a mean age of 17 weeks in 12% of heifer calves, and saws, wires, or Barnes dehorners were used on the remaining 8% of heifers at an average age of 24 weeks. Analgesics or anesthesia were used in 18% of operations. A survey of north central and northeastern dairies reported a similar method and age structure for dehorning calves, but reported lower percentages of operations using anesthetics (12.4%) and analgesics (1.8%) (Fulwider et al., 2008). When retained on the dairy farm, most calves were castrated at an average age of 9 weeks with either band (61%), knife (27%), or burdizzo (12%). Analgesics or anesthesia were used routinely for castration in 3.2% of operations. (USDA, 2007a). Information from producers concerning the timing of castration and dehorning of bull calves not retained on a dairy farm is not readily available, but in a survey of veterinarians in bovine practice, more respondents reported castrating greater numbers of dairy calves weighing between 90 – 270 kg, than those weighing more than 270 kg (Coetzee, et al., 2010).

In the beef industry, calves generally remain with the dam in a pasture environment until weaning at approximately 6 to 10 months of age. Although some beef calves may be dehorned and castrated prior to weaning, many calves are not handled until removed from the dam. In

some operations, prior to marketing, calves undergo a post-weaning backgrounding period, in which they are vaccinated, castrated, dehorned, and acclimated to feeding from a bunk. In a USDA survey of beef cow-calf operations, approximately 80% of bull calves were castrated prior to sale in 2007-08 (USDA. 2008a). The remaining 20% of calves would not be castrated until after going through the stresses of the marketing process and the transported to a stocker operation or feeding facility. Due to a higher percentage of polled genetics in beef breeds, fewer calves require dehorning than dairy breeds. In a USDA survey of beef cow-calf operations, the percent of beef calves born with horns decreased from 28% in 1997 to 12% in 2007. Horned calves were dehorned at an average age of 120 days, with 43% of calves being dehorned by 3 months of age, but with 25% not being dehorned until after 6 months of age (USDA, 2008a).

The USDA study did not report beef producers' use of anesthetics or analgesics in castration and dehorning procedures. Two recent surveys of bovine practitioners, however, allow some insight into the provision of pain relief when a veterinarian is called upon to perform routine husbandry procedures. Coetzee, et al (2010) reported that most producers castrated calves themselves in the perinatal period when animals weighed under 90 kg, but hired a veterinarian to perform castrations of calves over 270 kg, which is the approximate weaning weight of beef breeds. Between 90 and 270 kg, producers and veterinarians performed the castrations in nearly equal proportions. The survey does not indicate whether veterinarians used surgical or non-surgical methods more in one weight group than another, but does indicate that a scalpel method is used most often in surgical castration and that elastic bands or rings are most commonly used in non-surgical castration. The survey also reported that 20% of veterinarians used analgesia or anesthesia at the time of castration, and when dehorning was necessary, 90% of veterinarians performed dehorning and castration concurrently. Another survey (Fajt et al., 2011) of bovine practitioners reported that 30% of veterinarians provided anesthesia or analgesia to calves under 6 months of age when castrating, but 45% provided local anesthesia and/or an alpha-2 adrenergic agonist to calves 6 months of age or older. When dehorning calves at either < 6 mo-old or > 6 mo-old, a smaller percentage of veterinarians provided anesthesia or analgesia to beef calves (49% and 64%) than to dairy calves (63% and 73%). Although Fajt et al. (2011) did not speculate on the reason for the lower percentages associated with beef calves, one reason may be that the administration of medication slows the rate of processing. Commonly cited reasons why analgesia is not provided to cattle when undergoing castration and dehorning

include practical and economic factors, scarcity of analgesic agents licensed for use in cattle, difficult in administration and concern of drug residues in food tissues. (Vinuela-Fernandez et al., 2007).

It is important to note that veterinarians in the U.S. are hampered in the provision of analgesia to food animals because no drugs have been approved by the FDA for the treatment of pain in those species. The only analgesic drug licensed for use in food animals in the U.S. is flunixin meglumine, and its labeled use is for the control of pyrexia associated with bovine respiratory disease and for the control of inflammation and pyrexia associated with endotoxemia (FDA, 2003). However, due in part to increased societal concern over animal welfare issues in the popular press, there has been increased public scrutiny of the use (or non-use) of analgesia and anesthesia in routine husbandry procedures of cattle and other farm livestock (Martin, 2008; McKinley, J. 2010). In the U.S., the performance of these routine surgeries in livestock, without the benefit of pain-reducing techniques, has been exempt from state cruelty laws because it has been a widespread practice. There is indication, however, that this may change. The New Jersey Supreme court has declared that castration without alleviation of pain cannot be considered humane just because the practice is widespread. Pressure to change the status quo concerning the lack of pain-mitigating standards in the U.S. comes not only from internal sources, but external as well. The absence of pain management for common surgical procedures is considered to be an important animal welfare concern and is under consideration in the development of international trade agreements (Phillips, 2008; Thiermann & Babcock, 2005). It is understandable that countries which regulate how and when animal husbandry procedures are performed within their own borders feel that trading partners should follow similar standards, both for economic and ethical reasons. Whether from domestic or foreign pressure, officials in the U.S. have been, or are being, charged with revising legislation governing the humane treatment of food-producing animals (Cowan, 2011).

In order for these new standards to actually increase the well-being of animals, it is necessary that they be based upon solid scientific basis. To assist the formulation of regulations concerning castration and dehorning of cattle, the scientific literature must contain reproducible studies which investigate the effects of animal age and procedure method upon the animals' response. Measuring pain in animals difficult because they cannot describe the subjective amount of pain they feel in terms which humans can readily understand and quantify. Therefore,

we attempt to measure the amount of pain which an animal experiences due to a specific procedure through changes which occur in the animals' behavior and physiology. We gauge the effectiveness of a specific pain-mitigating strategy by the absence, or lessening, of those behavioral and physiological changes when the procedure is performed with the provision of that strategy.

Response variables in bovine pain research

The potential response variables with which pain may be estimated are derived from the animal's responses which can be measured. Physiologic variables which have been measured in association with pain in cattle include heart rate (Schwartzkopf-Genswein et al.,2005; Stewart et al., 2008, 2010), heart rate variability (Stewart et al., 2008, 2010), external temperature of skin or membranes (Stewart et al., 2008, 2010); brain waves (Bergamasco et al., 2011), electrodermal activity (Kotschwar et al., 2009; Baldridge et al., 2011) and circulating concentrations of biological molecules such as cortisol (Stafford and Mellor, 2005), epinephrine and norepinephrine (Stewart et al., 2010), substance-P (Coetzee et al., 2008), interferon-γ, and acute phase proteins (Early and Crowe, 2002). Changes in behavior have been used as summary measures of the animal's overall response as it attempts to maintain homeostasis in response to external threats and internal changes. Variables associated with behavior changes which have been utilized include vocalization during the procedure (Schwartzkopf-Gentzwein et al., 2005), velocity with which the animal leaves the area where the procedure occurred (Baldridge et al., 2011), behavioral count measures such as the number of kicks, ear flicks, tail flicks, scratching, or turning to look at the area, and behavior duration measures such as the amount of time spent standing, lying, eating, drinking, and specific postures (Millman, 2013). Performance variables such as weight gain, dry matter intake, and the incidence of succumbing to infectious disease have an underlying basis in physiology and behavior, are useful as summary indicators of an animal's overall wellbeing, and are important to producers (Fisher et al., 1996; Baldridge et al., 2011; Massey et al., 2011).

The physiological variables measured in pain studies are not necessarily specific to nociception, but may change in response to the stress of a perceived threat as well or due to inflammation. Perceived or actual threat to the body is met with activation of two intertwined

stress-axes: the sympatho-adrenal (SA) and the hypothalamic-pituitary-adrenal (HPA) axes. The signal of an actual threat to the body is transmitted to the brain by nociceptive neural impulses from the site of tissue stimulation or damage.

The adrenal glands play a crucial role in both SA and HPA axes, with the secreted catecholamines and glucocorticoids forming an immediate "fight-or-flight" reaction and a slower adaptive response which functions to return the body to normal homeostatic conditions. In the SA axis, neural transmission causes a response from the adrenal medulla to occur within milliseconds, whereas activation of the adrenals via the HPA axis is slower, requiring a chain of events to occur via the bloodstream. In the HPA axis, following stimulation, the hypothalamus releases corticotrophin-releasing hormone (CRH), which then stimulates the anterior pituitary to release adreno-corticotropic hormone (ACTH), which in turn stimulates the cells in the adrenal cortex to release glucocorticoids, such as cortisol, into the bloodstream. The activity of the HPA axis is excited by catecholamines and suppressed by cortisol in a negative-feedback mechanism which limits its own production. Cortisol also functions to limit the biosynthesis and release of epinephrine from the adrenal gland to dampen the state of excitation. (Axelrod & Reisine, 1994; Ehrhart-Bornstein, 2998)

Catecholamines are quickly cleaved enzymatically, with a plasma half-life of 1 to 2 minutes (Hjemdahl, 1993), which presents logistical difficulty in collecting, preserving, and assaying samples that are representative of the biological state at the time of a procedure. Therefore, alternative methods have been investigated to approximate the level of arousal of the SA axis. The effects of catecholamine release in the body are supportive of the rapid fight-or-flight response by promoting energy mobilization, diverting blood supply from skin and internal organs toward the muscles, and increasing respiration, as well as cardiac output. Thus, response variables which are intended to approximate the level of SA axis activation include respiratory rate, electrodermal conductivity, heart rate, and heart rate variability. Cortisol has been utilized extensively in the literature as a measure of the stress associated with a procedure, with the notion that more painful procedures are more stressful to the animal. The value of cortisol as a biomarker for the approximation of pain is limited because its circulating concentration may be influenced by perceived threats as well as actual threats. Furthermore, cortisol levels are regulated by a negative feedback mechanism which creates a ceiling effect whereby increased

pain may not result in increased cortisol concentration within the body (Axelrod and Reisine, 1984).

Numerous studies have investigated the various abovementioned response variables in the single procedure of either castration (Stafford and Mellor, 2005) or dehorning (Stafford and Mellor, 2004). However, studies are scarce which have compared the relative noxiousness of castration to dehorning or to a procedure in which castration and dehorning are performed concurrently, yet in a survey of U.S. veterinarians in bovine practice, 90% of respondents indicated that they dehorn calves at the same time as castration (Coetzee et al., 2010). One study has investigated separate castration and dehorning procedures in the same calves, but the procedures were performed in series, with castration always following dehorning, so comparisons between procedures were confounded with order of application (Schwartzkopf-Genswein, et al., 2005). Another study has investigated castration, dehorning, and the concurrent procedure, in parallel but not in series (Ballou et al., 2013; Sutherland et al., 2013.) Therefore, Chapter 3 of this dissertation investigates the utility of a number of these variables in a study involving castration, dehorning, and concurrent dehorning and castration in sequence and in parallel.

Substance P has recently come under investigation as a biomarker which may be more specific to the pain response than substances produced in the SA or HPA axes (Coetzee et al., 2008, 2012; Sutherland et al., 2012; Glynn et al., 2013; Dockweiler et al., 2013, Allen et al. 2013.). A member of the tachykinin family, substance P is known classically as a neuropeptide which is produced and released at both the central and peripheral terminals of bi-polar peptidergic C fibers which, in concert with the primary neurotransmitter glutamate, transmits the message of nociception (Otsuka and Yoshioka, 1993). Substance P is also produced in adrenal chromaffin cells, and co-stored with other neuropeptides and adrenomedullary catecholamines (Ehrhart-Bornstein et al., 1998). Substance P production and activity is not, however, specific to neurologic tissue. Substance P also plays a role in the inflammatory process by causing vasodilation, microvascular leakage, and the attraction and activation of cells with immunological function. The molecule has been shown to be produced by non-neural mammalian cells including vascular endothelium, monocytes, macrophages, eosinophils and tissues associated with the respiratory, gastrointestinal and genito-urinary tracts (Linnik and Moskowitz, 1989; Ho et al.,1997; Lecci and Maggi, 2001; Pinto et al., 2004). Since the use of

Substance P is relatively novel as a biomarker of pain in cattle, more research is necessary to validate its use, and to provide researchers with methodological information such as best practices for collecting and handling samples destined for analysis of SP. Chapter 2 of this dissertation investigates the effects on SP in bovine blood of various protease inhibitors, the length of time samples were held prior to the harvesting of plasma, and the temperature at which samples were held.

NSAIDs in dehorning and castration research

Non-steroidal anti-inflammatory drugs which have been investigated in either dehorning or castration studies include flunixin meglumine (Stillwell, 2008; Gonzalez et al., 2010; Webster, 2010), ketoprofen (Earley & Crowe, 2002; Ting et al., 2003a, 2003b), salicylic acid (Coetzee et al., 2007; Baldridge et al., 2011), carprofen (Pang et al., 2006; Stillwell, 2008), and recently, oral meloxicam (Glynn et al., 2013).

The rationale for the use of NSAIDs, and meloxicam in particular, is discussed in depth in Chapters 4 and 5.

NSAIDs in bovine respiratory disease research

In the beef cattle industry at weaning time, calves generally undergo a period of extreme stress in which they are weaned from their dams, transported for various distances, commingled with other calves at either the livestock market or feedlot facility, and undergo a change in feeding practice. The immune system is negatively affected by such stressors, which makes the animal more susceptible to contracting infectious agents and succumbing to disease. Bovine respiratory disease (BRD), with multiple causative bacterial and viral organisms, is the most common cause of illness and death in feedlot calves, with the highest incidence of disease occurring within 28 days of arrival at the feedyard (Buhman et al., 2000; Duff and Galyean, 2007).

Bull calves may be castrated before weaning, but many are not castrated until reaching the feedlot, further stressing the animal. The surgical castration of cattle is generally followed by a period of decreased animal performance as evidenced by reduced average daily gain (ADG), daily feed intake (DFI), and gain-to-feed ratio (ADG:DFI), (Faulkner et al., 1992; Bretschneider,

2005; Stafford and Mellor, 2005; AVMA, 2011; Massey et al., 2011). Furthermore, surgical castration is associated with increased levels of cortisol and acute phase proteins such as haptoglobin, with ensuing immune suppression which may increase the likelihood of the animal succumbing to disease. (Fisher et al., 1997; Earley and Crowe, 2002).

In a study in which an inflammatory response was induced in calves by intravenously injecting the endotoxin of *Escherichia coli*, LPS, a single administration of meloxicam was shown to stimulate synthesis of leukotriene B_4 compared to controls. (Bednarek & Kondracki, 2006). This stimulation indicates a positive immune reaction because leukotriene B_4 is an immune cell chemoattractant and is thought to activate macrophages for enhanced phagocytosis and to activate neutrophils for enhanced killing of pathogens (Bednarek & Kondracki, 2006; Yoo et al., 1995.) In a later study, in calves suffering from BRD, the NSAID flunixin meglumine was administered as an adjunctive therapy to antimicrobial treatment. Calves treated with the NSAID showed a faster clinical recovery from BRD and blood samples showed increased numbers of lymphocytes and their subpopulations (CD2(+), CD4(+), CD8(+), WC4(+) cells) than calves not receiving the NSAID (Bednarek et al., 2013).

In a model of induced bovine respiratory disease, carprofen has also been shown to reduce the clinical disease scores and the extent of pulmonary lesions in cattle (Wallemacq et al., 2007).

In the study reported in Chapter 6, the results of administering meloxicam to newly-received calves prior to castration at the feedlot are reported.

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Chapter 2 - Effects of sample handling methods on substance P concentrations and substance P immunoreactivity in bovine blood[1]

Introduction

Due to societal concern for the welfare of farm animals, numerous organizations have implemented or are considering the implementation of regulations concerning painful husbandry procedures such as dehorning and castration.[1] In order to provide proper scientific evidence for regulatory decisions and for the development of pain-mitigating options, there is need for studies which investigate pain-relieving techniques in food animal species. A major impediment in drawing conclusions from these studies is the absence of an objective measure of pain in animals. Cortisol has been used as a response variable to approximate the relative noxiousness of a procedure in numerous studies, but its limitations have also been recognized.[2] Substance P has been investigated as a response variable which may be more specific to pain experienced by an animal.[3-10] In support of research which may utilize SP as a response variable, this study investigated the effects of various sample handling techniques on the recovery of SP in blood samples when analyzed by ELISA or LC-MS/MS methodology.

Substance P is a biologically active peptide, long known to play a role in the neural transmission of nociceptive signals from sites of peripheral insult to the central nervous system.[11] Substance P is released by peptidergic peripheral sensory nerve fibers at both, the central synaptic junctions in the dorsal horn of the spinal cord and at the peripheral terminals, where the peptide plays a signaling role in the transmission of pain as well as the induction of inflammation and central sensitization.[12]

Substance P is a member of the tachykinin family and is synthesized in cell bodies from transcripts of the gene, Preprotachykinin-A. The parent SP peptide is composed of 11 amino

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acids with an amidated carboxyl terminus (Arg_1-Pro_2-Lys_3-Pro_4-Gln_5-Gln_6-Phe_7-Phe_8-Gly_9-Leu_{10}-Met_{11}-NH_2) and will be specifically identified from here on as SP_{1-11}. The various enzymes which may cleave SP_{1-11} include members of the serine- and metallo-protease families: angiotensin I converting enzyme, aminopeptidase, neutral endopeptidase, dipeptidyl peptidase IV, and post-proline cleaving enzyme.[13,14] Enzymes of the different classes require inhibitors with specific affinity for their catalytic site, thus the protection of SP_{1-11} from enzymatic degradation may require the addition of at least two inhibitor types. Various degradation products are derived from SP_{1-11}, and are named according to the fragment of amino acids contained. For example, the cleavage of SP_{1-11} between the Pro_2 and Lys_3 results in the formation of two fragments, SP_{1-2}, and SP_{3-11}. Fragments containing at least the five C-terminal amino acids, i.e. fragments from SP_{1-11} to SP_{7-11}, elicit similar biological effects as the parent SP peptide, though potency decreases with fewer amino acids.[15] Opposite effects have been demonstrated between fragments containing either the hydrophobic C-terminal or the hydrophilic N-terminal, with a recent study suggesting that the antinociceptive effects of morphine were enhanced when combined with SP_{1-7}.[16] Knowledge of the fragmentation profile of substance P may therefore increase our understanding of pain processing and perception in animals.

Concentrations of SP can be measured by ELISA or LC-MS/MS methodology. Whereas LC-MS/MS has the ability to measure concentrations of SP_{1-11} specifically, the ELISA assay provides a summary measure of SP immunoreactivity which includes not only SP_{1-11}, but C-terminal fragments and other related immunoreactive peptides as well. One commercially available ELISA test[a] indicates cross reactivities between the assay and metabolites as: SP_{1-11} (100%), SP_{3-11} (87.9%), SP_{4-11} (11.7%), and SP_{7-11} (5.9%). From here on this summary measure will be identified as SP_{ELISA}.

In studies involving livestock, sample-handling conditions are not always ideal. With barns often being distant from laboratory facilities, refrigeration or ice may not be available for chilling samples. Furthermore samples may be held in batches for transport to the laboratory where delayed processing occurs. Therefore, in support of research which utilizes the assay of SP as a response variable, we wanted to compare the effects of certain handling procedures upon the concentrations of SP_{1-11}, SP_{3-11}, and SP_{7-11} measured by LC-MS/MS and upon levels of SP_{ELISA} immunoreactivity as measured by ELISA. In this exploratory study, we investigated the effects of delayed blood sample processing (up to 24 h) and the temperature (ice bath or ambient)

at which the sample was held during that period. With respect to the ability to protect SP_{1-11} and SP_{ELISA} from degradation, we compared the enzyme inhibitor aprotinin, 500 KIU/ml in EDTA, as recommended by the ELISA kit[a] used in the study, with a lower concentration of 50 KIU/ml. We also evaluated whether a commercially-marketed complete protease inhibitor cocktail may be more effective at preventing the decay of SP than aprotinin, and whether those treatments are more effective than simply collecting blood in readily available EDTA or heparin tubes.

Materials and Methods

All experimental procedures in this study were approved by the Kansas State University Institutional Animal Care and Use Committee under the supervision of the University Veterinarian. An overview of the study is provided in Figure 1.

Collection Tube Preparation

Enzyme inhibitor treatments were prepared and added to blood collection tubes approximately eight hours prior to the study commencement. Aprotinin powder was dissolved and serially diluted with deionized water to create two stock solutions that would provide final concentrations of 50 KIU/ml and 500 KIU/ml when diluted in the collected blood sample. A commercially available protease inhibitor cocktail tablet[b] was dissolved and serially diluted in deionized water according to manufacturer's recommendations to provide solutions of single-strength and double-strength when diluted in the blood sample. To prepare tubes containing one of the levels of aprotinin or complete protease inhibitor cocktail, 100 µL of a concentrated stock solution was added to an uncapped 4 mL K_3EDTA blood collection tube. All tubes were recapped and refrigerated overnight.

Approximately 1 h prior to the blood collection, a spiking solution of SP_{1-11} was prepared by adding 1 mL of 0.1% formic acid to a vial containing 425,000 ng SP_{1-11}. From this solution, serial dilutions were made with 0.1% formic acid to obtain a solution containing 50 ng/mL SP_{1-11}. Fifty microliters of the SP_{1-11} solution were added to each of the prepared enzyme inhibitor tubes, as well as tubes containing lithium heparin or only K_3EDTA, to provide a final concentration of 625 pg/mL when mixed with 4 mL of whole blood.

The blood tubes were arranged in 3 replicate arrays for each holding period. The arrays destined for holding periods of 1, 3, 6, 12, and 24 h, each contained two sets (one for each temperature treatment) of the following 6 enzyme inhibitor treatments spiked with 625 pg/ml

SP_{1-11}: 1) heparin, 15 USP/ml (HEP); 2) EDTA, 1.8 mg/mL (EDTA); 3) aprotinin, 50 KIU/ml in EDTA (AP50); 4) aprotinin 500 KIU/ml in EDTA (AP500); 5) single-strength protease inhibitor tablet in EDTA (TAB1X); and 6) double-strength complete protease inhibitor tablet in EDTA (TAB2X). Arrays destined for immediate processing contained only one set of the 6 inhibitor treatments because there was no temperature treatment. In addition to the treatment tubes, each array also contained one non-spiked TAB2X tube that would be processed immediately to detect levels of endogenous SP. In collecting the non-spiked samples in the TAB2X inhibitor, it was assumed *a priori*, that this inhibitor cocktail might provide the fullest extent of protection against the different types of enzymes which degrade SP. All tubes were then held in a coolers filled with crushed ice for transport to the barn facility where the blood collection procedure occurred.

Blood Collection Procedure

Whole blood was obtained from a healthy 6-mo-old Holstein calf. Approximately 18 hours prior to the study, the calf was restrained in a chute while an indwelling jugular catheter was surgically placed using local anesthesia. To maintain patency, the catheter was flushed with a heparin solution.

On the morning of the study, the calf was again restrained in the chute while the tubes in three arrays were uncapped in preparation for filling from the first blood collection series. The heparin solution was cleared from the catheter by attaching a syringe, and in three repetitions, drawing 5 ml blood into the syringe, then depressing the plunger to return the blood to the calf. A collection series consisted of three syringes of blood, collected as follows. A 60 mL syringe was attached to the uncapped catheter and filled over a period of 30-60 seconds, after which 4 mL blood was rapidly transferred to each of the 13 tubes in the first array. As soon as each tube was filled, it was recapped and rapidly inverted 10 times to ensure thorough mixing of the contents. During this time, another 60 ml syringe was attached to the catheter and the next volume of blood obtained. Once each array was completed, in approximately one minute, the tubes were immediately placed in their holding environment and the start time and temperature were recorded. In this manner, the process was repeated to quickly obtain three complete replicate treatment arrays for each holding period. To facilitate the processing of samples, the order of draw for the separate holding periods was: 12, 6, 3, 24, 0, and then 1 h. Collecting and processing the samples for the three arrays in each holding period was achieved in 5 min (SD =

2 min). Approximately 5 min were needed to assure readiness for the next series of blood draws, resulting in a spacing of 11 min (SD = 2 min) between the first syringe draws of each collection series.

Ambient temperature (AMB) samples were placed in a test tube rack for holding, whereas chilled samples were immediately plunged into a bath of crushed ice maintained in an insulated portable cooler (ICE). With respect to the chilled samples, care was taken to assure that only the cap and small amount of the tube remained above the ice surface. Ambient temperature was recorded by a digital thermometer placed on the bench top near the samples. Temperature in the cooler at the ice bath/air interface was recorded from a similar thermometer protected in a plastic bag. Ambient temperatures ranged from 20.3° to 21.2°C during the 1 hour sample collection period at the barn, then ranged from 23.9 to 25.4°C during the remaining 24 h holding period in the laboratory. The ice/air interface in the cooler ranged from 4.5 to 7.3°C during the 1 hour period while samples were being collected and ranged from 0.1 to 2.2°C, during the remaining holding period in the laboratory; however, the samples themselves were held below the ice surface where the presumed temperature at the ice/water interface was 0°C.

Samples were either processed within 5 minutes after collection (0 h) or held 1, 3, 6, 12 or 24 h prior to processing, which consisted of centrifuging the samples in a refrigerated unit at 10,000 x G for 15 min. Tubes were then removed from the centrifuge and held in an ice bath while plasma was harvested and divided among paired cryovials (aliquots #1 and #2) for holding at -70°C until analysis. The 0 h spiked samples and the non-spiked reference samples were processed in the barn using the same refrigerated centrifuge utilized for the other samples. For holding periods processed in the barn, the cryovials of harvested plasma were immediately placed in a cooler on icepacks (which had cooled overnight at -70°C), then were transported to the laboratory, where they were observed to have frozen during the < 10 minute transit, then placed in a -70°C freezer for storage. Samples from all other holding periods were processed in the laboratory, with care taken to assure similar handling as those processed in the barn.

Analysis of SP_{1-11} and metabolites by LC-MS/MS

Only samples held 0, 1, 3 and 6 h were analyzed by LC-MS/MS. Analysis of aliquot #1 samples occurred within 6 weeks after collection and storage at -70°C.

An LC-MS/MS method was utilized for the analysis of SP_{1-11}, SP_{3-11}, and SP_{7-11} in bovine plasma. A structurally related peptide, substance P [Tyr8] was used as the internal standard for all analytes. The analytes and internal standard were isolated from 0.5 mL of bovine plasma by solid phase extraction using HLB cartridges. Extracts were evaporated to dryness and re-constituted in starting mobile phase. Electrospray ionization and MS/MS analysis was carried out using an HPLC system coupled with an API 4000 Triple Quadrupole Mass Spectrometer[c]. Chromatographic separation of all analytes and internal standard was achieved using an XBridge Shield RP18 analytical column and a gradient elution from 100% Mobile Phase A (0.2% Acetic Acid in water) to 80% Mobile Phase B (0.2% Acetic Acid in Acetonitrile) and re-equilibration over a 15.0 minute runtime. The method was proven to be accurate and precise across a linear dynamic range from the LLOQ of 50 pg/mL to 1,000 pg/mL.

Analysis of SP_{1-11} and metabolites by ELISA

Approximately 18 months following collection and storage at -70°C, aliquot #2 samples for all holding periods from 0 to 24 h were analyzed by ELISA. Samples were analyzed for SP_{ELISA} immunoreactivity using a validated analytical method as previously described (Coetzee et al., 2008). Briefly, analytes were extracted from plasma by acidifying with acetic acid and fractionating with reverse-phase solid-phase extraction columns. The peptide was eluted from the column using an organic-aqueous solvent mixture and concentrated by drying under nitrogen. The dried extract was reconstituted and analyzed in duplicate according to the manufacturer's instructions in the Substance P ELISA kit.[a] Assay performance was monitored using 5 replicates of bovine plasma samples fortified with 0, 200 or 800 pg/mL of substance P purified standard. The method was linear across the 5 replicates at each concentration (R-squared > 0.99) and the coefficient of variation (CV%) at each concentration within a run was < 15%. The CV% between the 5 ELISA assay runs in the study was 42% for 0 pg/mL samples, 20% for 200 pg/mL samples and 35% for 800 pg/mL samples.

Statistical Analysis

Since the three syringes of blood in each collection series were not independent, and because holding period was confounded with collection series, we refrained from investigating rates of decay and from making comparisons across holding periods. Instead we confined our analysis to comparisons of the three replicates of time-by-temperature combinations within each

holding period. Furthermore, because the analyses of samples by LC MS/MS and ELISA did not occur after the same freezer storage time and because the ELISA method cross reacts with SP metabolites ,we also refrained from statistical comparison of levels of SP_{ELISA} immunoreactivity with a summary measure of SP_{1-11}, SP_{3-11}, and SP_{7-11} analyzed by LC MS/MS.

The response variables SP_{1-11}, SP_{3-11}, and SP_{7-11} and SP_{ELISA} were transformed (natural log) and analyzed using analysis of variance models. For graphical presentation, the means and 95% confidence intervals were transformed back to the original units. Fixed effects included temperature (ICE, AMB) and enzyme inhibitor (AP50, AP500, TAB1X, TAB2X, EDTA, and HEP). For analyses of SP_{ELISA} data, ELISA run was used as a random effect. Differences among treatment groups were assessed using Tukey's pairwise t-tests by time. The data analyses were performed using statistical software.[d] Statistical significance was designated a-priori as $P \leq 0.05$.

Results

Non-spiked samples. In the non-spiked blood samples which were processed immediately following collection, there were no detectable levels of endogenous SP_{1-11}, SP_{3-11}, or SP_{7-11} analyzed by LC-MS/MS. In samples analyzed by ELISA, the level of immunoreactivity was in the same range as reference samples with 0 pg/mL.

Spiked Samples: SP_{1-11} SP_{3-11} and SP_{7-11} peptide analysis by LC-MS/MS.

With respect to the *ex vivo* metabolism of SP_{1-11}, SP_{3-11} and SP_{7-11}, the main effects of holding temperature and enzyme inhibitor were significant ($P < 0.0001$) although there was marginal ($P = 0.06$ for SP_{1-11}) and significant ($P < 0.0001$ for SP_{3-11} and SP_{7-11}) evidence of an interaction between holding temperature and enzyme inhibitor. Back-transformed mean concentrations and lettered differences are presented to show the nature of these interactions for SP_{1-11} (Figure 2), SP_{3-11} (Figure 3), and SP_{7-11} (Figure 4).

When samples were processed at 0 h, immediately following collection, there were no significant differences ($P > 0.12$) between the mean concentrations of SP_{1-11}, SP_{3-11}, or SP_{7-11} associated with any of the enzyme inhibitor treatments.

Comparing between temperatures in samples held 1, 3, or 6 h prior to processing; the SP_{1-11} concentrations of all ICE samples within a holding period were significantly greater than all AMB samples ($P < 0.005$).

In ICE samples held for 1 h prior to processing, the only significant differences in SP_{1-11} concentrations were between either AP500 or AP50 and the other enzyme inhibitors. In those samples, the SP_{1-11} means of AP500 samples were greater than those of HEP, TAB2X, and TAB1X ($P < 0.018$); and the SP_{1-11} concentrations of AP50 samples were greater than HEP and TAB2X ($P < 0.04$). Within the ICE samples held for 1 h, there were no significant differences in SP_{1-11} concentrations between the two levels of aprotinin, AP50 and AP500, nor between the two levels of protease inhibitor tablet, TAB1X and TAB2X ($P > 0.99$).

Spiked samples: ELISA.

Samples in all holding periods, from 0 - 24 h, were analyzed for substance P by ELISA (Figure 5). The interaction term between holding temperature and enzyme inhibitor was significant ($P < 0.0001$).

When samples were processed immediately following collection, there were no significant differences detected between the mean SP_{ELISA} immunoreactivity levels associated with any of the enzyme inhibitor treatments ($P > 0.93$).

Within the 1 and 3 h holding periods, there were no significant differences between the mean SP_{ELISA} immunoreactivity levels of AP50, AP500, TAB1X, TAB2X, and EDTA samples whether held in ICE or AMB conditions ($P > 0.92$). Within the 1 h holding period, the mean SP_{ELISA} immunoreactivity level of HEP samples was lower than that of all other enzyme inhibitors when held at AMB temperature ($P < 0.0001$) and lower than AP50 and TAB2X in samples held at ICE temperature ($P < 0.04$).

Although not statistically comparable, levels of SP_{ELISA} immunoreactivity appeared to increase in samples held longer than 3 h prior to processing, reaching similar numerical levels at 6 and 24 h as at 0 h. Within each enzyme inhibitor group, when held at ICE temperature, the levels of SP_{ELISA} appeared to be lower at 12 h than at 6 and 24 h.

Discussion

This study examined the effects of enzyme inhibitor type and holding temperature during the period of time elapsing from the point of blood collection to the start of sample processing to harvest and freeze plasma destined for analysis of SP by LC-MS/MS and ELISA methodology. Because animals vary with regard to stress response and to the chemical, enzymatic and cellular

components of the blood, and because it was not possible to collect and pool whole blood from several individuals, only one animal was used in this study to control for this inherent source of variability which might otherwise have obscured differences between the in vitro treatments. The individual was a healthy calf, selected at random, so might be considered representative of its class and therefore, the use of blood from only one calf is not thought to have affected inferences made in this study. Furthermore, because blood components may vary over time within an individual and because each holding period was associated with blood from a distinct point in time, we refrained from making inferences between holding periods where differences might have been caused by intra-individual variation.

As suggested by the approximately equal concentrations of SP1-11 and SP3-11 in the 0 h samples analyzed by LC-MS/MS, there appeared to be rapid early cleavage of parent SP1-11 to SP3-11 which was observed in all enzyme treatments. Immediately after the blood was added to the individual test tubes, there was a short period of time, during the tube inversion process before the inhibitors were homogenously mixed throughout the blood sample that the enzymes in the blood were able to act relatively freely upon the SP1-11 peptide. This source of SP1-11 degradation might have been minimized by spiking the tubes with exogenous SP1-11 after, rather than before, the addition of whole blood, but the intent of the study was to follow degradation due to handling procedures as if the exogenous SP1-11 had been present in the bloodstream.

When samples were held at ambient temperature, enzymatic processes appeared to proceed more rapidly than when held in the ice bath. Enzymes are generally known to be more active at physiologic temperatures than at cooler temperatures, so these results were not unexpected. In samples held for 1 h, concentrations of SP_{1-11} analyzed by LC-MS/MS were approximately 50% less in ambient samples than in ice bath samples. Even when samples were held in an ice bath, SP_{1-11} concentrations were near the LLOQ when held 3 h. Therefore, for analysis of SP_{1-11} by LC-MS/MS, the results of our study indicated that samples should either be processed immediately or held in an ice bath for processing within one hour of collection. When samples were processed immediately, the type of enzyme inhibitor did not significantly affect concentrations of SP_{1-11} or SP_{3-11}. When samples were held 1 h in an ice bath, aprotinin appeared to be the superior enzyme inhibitor for protecting SP_{1-11} from enzymatic degradation, and there was not a significant difference detected between the protection provided by either the

50 or 500 KIU/mL concentrations. Because the composition of the complete protease inhibitor cocktail tablet is proprietary information, it is not known if aprotinin was included in the formulation, but in 1 h ice bath samples, neither the 1X nor 2X concentration of the tablet appeared to provide more protection of the SP_{1-11} peptide than EDTA alone.

Similar to the analysis of SP1-11 and SP3-11 by LC-MS/MS, when samples processed immediately were analyzed by ELISA, the type of enzyme inhibitor did not significantly affect levels of SP_{ELISA} immunoreactivity. But unlike the results for LC-MS/MS, when samples were held for 1 or 3 h, there was no effect of temperature detected on the samples analyzed by ELISA. This is likely because the ELISA test measures not only the parent SP1-11 molecule, but also includes, to lesser extents, the family of degradation products with the SP1-11 carboxyl terminus. It must also be pointed out that that this study may not have had enough power to detect some differences between treatments with the ELISA test, given the method's inter-assay coefficient of variation of 20 – 35% in the range from 200 – 800 pg/mL. Although the ELISA test appeared to be relatively forgiving of holding temperature, especially when processed within the first hour, it is important to recognize that only the SP1-11 molecule has 100% cross reactivity with the ELISA test, and therefore the goal of sample handling should be to preserve the SP1-11 molecule to obtain the highest and most accurate concentrations. In animal studies where a difference in SP between groups is the alternate hypothesis, true differences between groups might be missed if SP1-11 is unnecessarily allowed to degrade to SP3-11 and other metabolites which are measured to a lesser degree by the ELISA test. Pronounced intra- and inter-individual variability of plasma SP immunoreactivity has been reported in control and in castrated calves.[3] Therefore, in animal studies it is important to control for as much extraneous procedure-related variability and to measure the highest concentrations of SP1-11 as possible in order to not miss true differences between treatment groups due to artificially low SP immunoreactivity means paired with inherently high error.

With respect to both SP_{1-11} and SP_{ELISA}, we could not test if the differences between the 1 h and 0 h samples were significant, though they were often numerically large. This observation suggests that a strict processing order should be adhered to which follows the collection order, without large discrepancies in the amount of time elapsing between the times of collection and processing. Otherwise, significant differences could occur due to sample-handling alone.

Alternately, the variability among samples may be great enough to obscure the significance of differences due to treatment.

The lower concentrations of SP_{1-11} generally observed in all samples after the 3 h holding period support the recommendation that samples destined for analysis of SP should be processed within 1 h following collection. Beyond 3 h from the time of collection, another factor besides enzymatic degradation appears to affect SP concentrations in whole blood samples in that in the ELISA analysis, levels of SP_{ELISA} immunoreactivity tended to rise. Notably in the samples held in the ice bath for 24 h, the levels of SP_{ELISA} immunoreactivity were in a similar range as the samples processed immediately following collection, regardless of the enzyme inhibitor present. Levels of SP_{ELISA} immunoreactivity at 6 h and 12 h were variably in the same range as the 0 h and 24 h samples, depending upon the enzyme inhibitor. Similarly, concentrations of SP_{1-11} in ice bath samples appeared to increase from 3 h to 6 h.

The higher concentrations of SP in the samples held for longer than 3 h introduce the possibility that SP (or other molecules with cross reactivity to the ELISA test), was being produced by cells in the whole blood samples. If SP was being produced *in vitro*, this leads to the question of why SP_{ELISA} concentrations at 12 h were usually less than at 6 h rather than *vice-versa*. This counter-intuitive finding might be explained by the order in which the individual holding periods were collected. Notably, the samples in the 12 h holding period were from the first group of blood draws collected from the calf. Although the calf was well-acclimated to the facility and to most of the study personnel, there was more activity than usual on the day of the study and the animal was unfamiliar with the person drawing blood from the catheter. It is likely therefore that a certain amount of stress was involved with the procedure. In an *in vitro* study of normal human bone marrow aspirate, it was demonstrated that the addition of ACTH (100 ng/ml) to bone marrow stromal cells induced peak concentrations of SP at 48h post-stimulation.[17] In our study, it is possible that the samples collected first (and held 12 h prior to processing) contained lower levels of ACTH than those in subsequent collection series. That might explain the apparent lower concentrations of SP_{ELISA} observed in samples held 12 h than those held 6 or 24 h, though it would have been necessary to assay levels of ACTH in the samples to confirm this hypothesis.

Conclusion

This study indicated that various biological processes may affect the concentration of SP following blood sample collection. Thus, to obtain the best snapshot of SP concentrations present in the blood at any certain time, samples should be processed as soon as possible and handled according to a strict protocol which ensures that all samples are immediately chilled to the temperature of an ice bath following sample collection. Furthermore, it is essential that plasma is harvested from samples in the same order in which they were collected, with similar amounts of time elapsing between the point of collection and the point of plasma harvest. Otherwise, significant differences may occur, or be obscured, due to sample-handling technique alone. This procedure is recommended when samples are to be analyzed by either LC-MS/MS or by ELISA. Although the ELISA test appeared to be relatively forgiving of the holding temperature during the first hour after collection, the goal of sample handling should be to preserve the SP1-11 molecule, for which the test has the greatest cross reactivity.

For samples destined for analysis by either ELISA or LC-MS/MS, if samples are chilled and processed within 5 minutes of collection, a standard blood tube containing heparin or EDTA appears to be as effective in preserving SP as the combination of EDTA with aprotinin or with an inhibitor cocktail.

Footnotes

a. Assay Designs, now Enzo Life Sciences, Farmingdale, NY

b. Complete protease inhibitor tablet, Santa Cruz Biotechnology Corp, Santa Cruz, CA

c. AB SCIEX, Framinghan, MA

d. SAS,Version 9.2, SAS Institute Inc., Cary, NC

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Figures

Figure 2.1. Study Diagram.

Blood was collected through a jugular catheter from one calf in a series of 6 separate collections. Each collection was comprised of 3 consecutive, closely-spaced syringe draws. One syringeful of blood filled one complete array of treatment tubes (6 enzyme inhibitors x 2 holding temperatures) spiked with SP_{1-11}, 625 pg/mL, plus 1 non-spiked reference tube. The enzyme inhibitors were: 1) heparin, 15 USP/ml (HEP); 2) EDTA, 1.8 mg/mL (EDTA); 3) aprotinin, 50 KIU/ml in EDTA (AP50); 4) aprotinin 500 KIU/ml in EDTA (AP500); 5) single-strength protease inhibitor tablet in EDTA (TAB1X); and 6) double-strength complete protease inhibitor tablet in EDTA (TAB2X). Samples from one collection series were held in an ice bath or at ambient temperature (20.3 - 25.4°C) for 0, 1, 3, 6, 12, or 24 h prior to centrifugation. Plasma was divided into matched samples for analysis by LC-MS/MS and by ELISA.

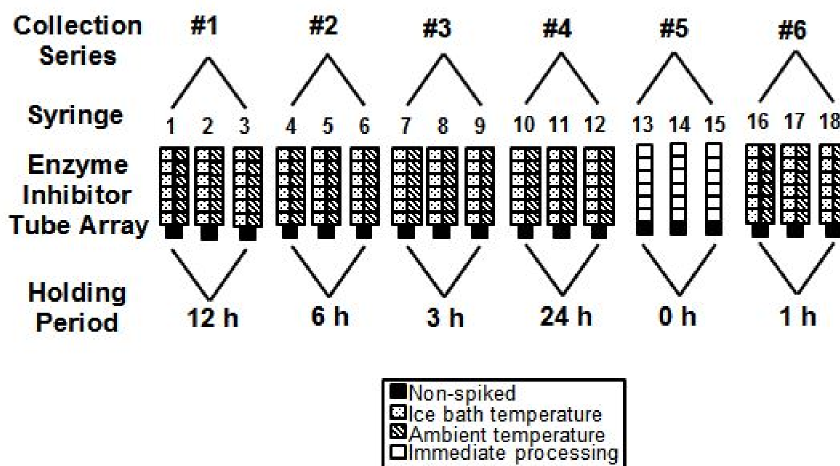
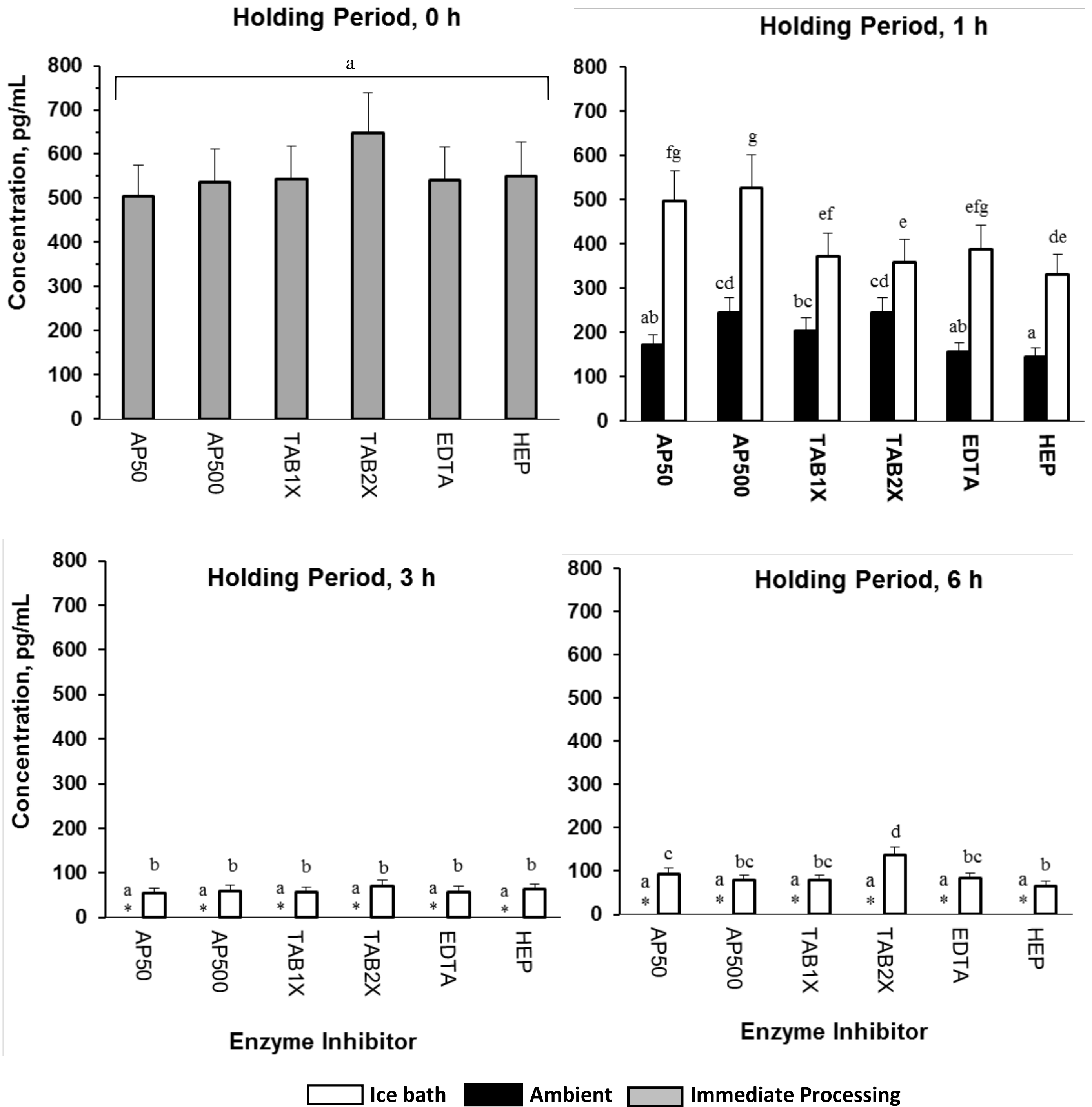


Figure 2.2. Back-transformed least squares median concentration estimates (and upper 95% confidence limits) of substance P (SP_{1-11}) analyzed by LC-MS/MS.

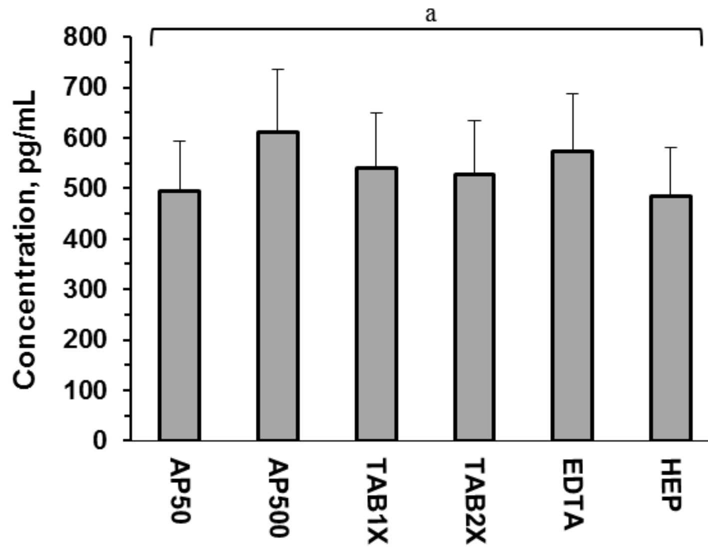
Whole bovine blood samples, spiked with 625 pg/mL SP_{1-11}, were either processed immediately following collection (0 h, represented by gray bars), held at ambient temperature (23.9 – 25.4°C) or in an ice bath for 1, 3, or 6 h prior to centrifugation and harvesting of plasma. Sample tubes contained one of the following enzyme inhibitors: 1) heparin, 15 USP/ml (HEP); 2) EDTA, 1.8 mg/mL (EDTA); 3) aprotinin, 50 KIU/ml in EDTA (AP50); 4) aprotinin 500 KIU/ml in EDTA (AP500); 5) single-strength protease inhibitor tablet in EDTA (TAB1X); and 6) double-strength complete protease inhibitor tablet in EDTA (TAB2X). An asterisk near the x-axis represents a sample having either no detected analyte peak or a peak below the lower limit of quantitation. Within each holding period, means without a common letter are significantly different ($P < 0.05$).

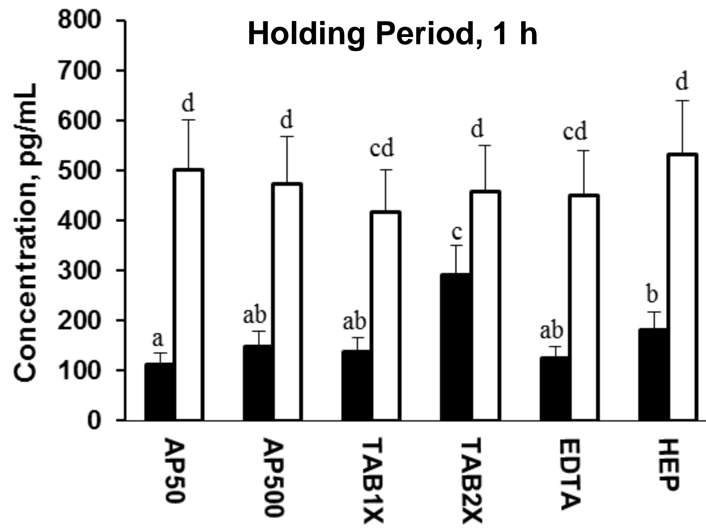


Holding Period, 0 h
Holding Period, 1 h
Holding Period, 3 h
Holding Period, 6 h
Concentration, pg/mL
Enzyme Inhibitor
AP50
AP500
TAB1X
TAB2X
EDTA
HEP
Ice bath
Ambient
Immediate Processing

Figure 2.3. Back-transformed least squares median concentration estimates (and upper 95% confidence limits) of the substance P metabolite, $SP_{3\text{-}11}$, analyzed by LC-MS/MS.

Whole bovine blood samples, spiked with 625 pg/mL $SP_{1\text{-}11}$, were either processed immediately following collection (0 h, represented by gray bars), held at ambient temperature (23.9 – 25.4°C) or in an ice bath for 1, 3, or 6 h prior to centrifugation and harvesting of plasma. Sample tubes contained one of the following enzyme inhibitors: 1) heparin, 15 USP/ml (HEP); 2) EDTA, 1.8 mg/mL (EDTA); 3) aprotinin, 50 KIU/ml in EDTA (AP50); 4) aprotinin 500 KIU/ml in EDTA (AP500); 5) single-strength protease inhibitor tablet in EDTA (TAB1X); and 6) double-strength complete protease inhibitor tablet in EDTA (TAB2X). An asterisk near the x-axis represents a sample having either no detected analyte peak or a peak below the lower limit of quantitation. Within each holding period, means without a common letter are significantly different ($P < 0.05$).


Holding Period, 0 h
a
Concentration, pg/mL
0
100
200
300
400
500
600
700
800
AP50
AP500
TAB1X
TAB2X
EDTA
HEP


Holding Period, 1 h
Concentration, pg/mL
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100
200
300
400
500
600
700
800
a
d
ab
d
ab
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b
d
AP50
AP500
TAB1X
TAB2X
EDTA
HEP

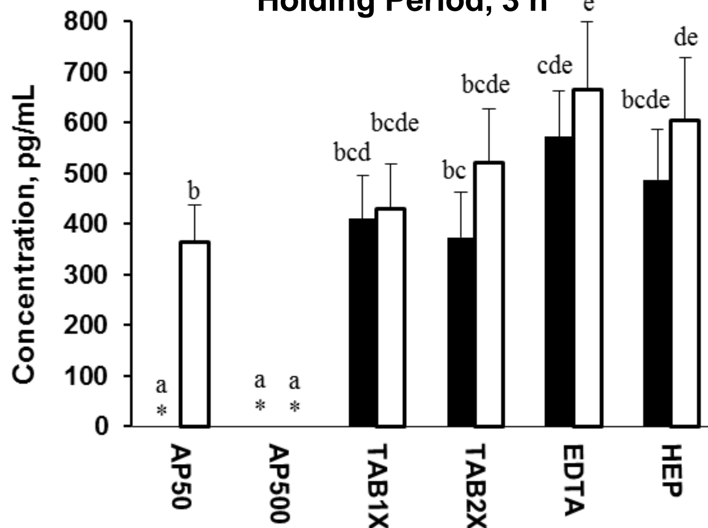

Holding Period, 3 h
Concentration, pg/mL
0
100
200
300
400
500
600
700
800
a
*
b
a
*
a
*
bcd
bcde
bc
bcde
cde
e
bcde
de
AP50
AP500
TAB1X
TAB2X
EDTA
HEP
Enzyme Inhibitor
Ice bath
Ambient
Immediate Processing

Figure 2.4. Back-transformed least squares median concentration estimates (and upper 95% confidence limits) of the substance P metabolite, $SP7_{-11}$, analyzed by LC-MS/MS.

Whole bovine blood samples, spiked with 625 pg/mL SP_{1-11}, were either processed immediately following collection (0 h, represented by gray bars), held at ambient temperature (23.9 – 25.4°C) or in an ice bath for 1, 3, or 6 h prior to centrifugation and harvesting of plasma. Sample tubes contained one of the following enzyme inhibitors: 1) heparin, 15 USP/ml (HEP); 2) EDTA, 1.8 mg/mL (EDTA); 3) aprotinin, 50 KIU/ml in EDTA (AP50); 4) aprotinin 500 KIU/ml in EDTA (AP500); 5) single-strength protease inhibitor tablet in EDTA (TAB1X); and 6) double-strength complete protease inhibitor tablet in EDTA (TAB2X). An asterisk near the x-axis represents a sample having either no detected analyte peak or a peak below the lower limit of quantitation. Within each holding period, means without a common letter are significantly different ($P < 0.05$).

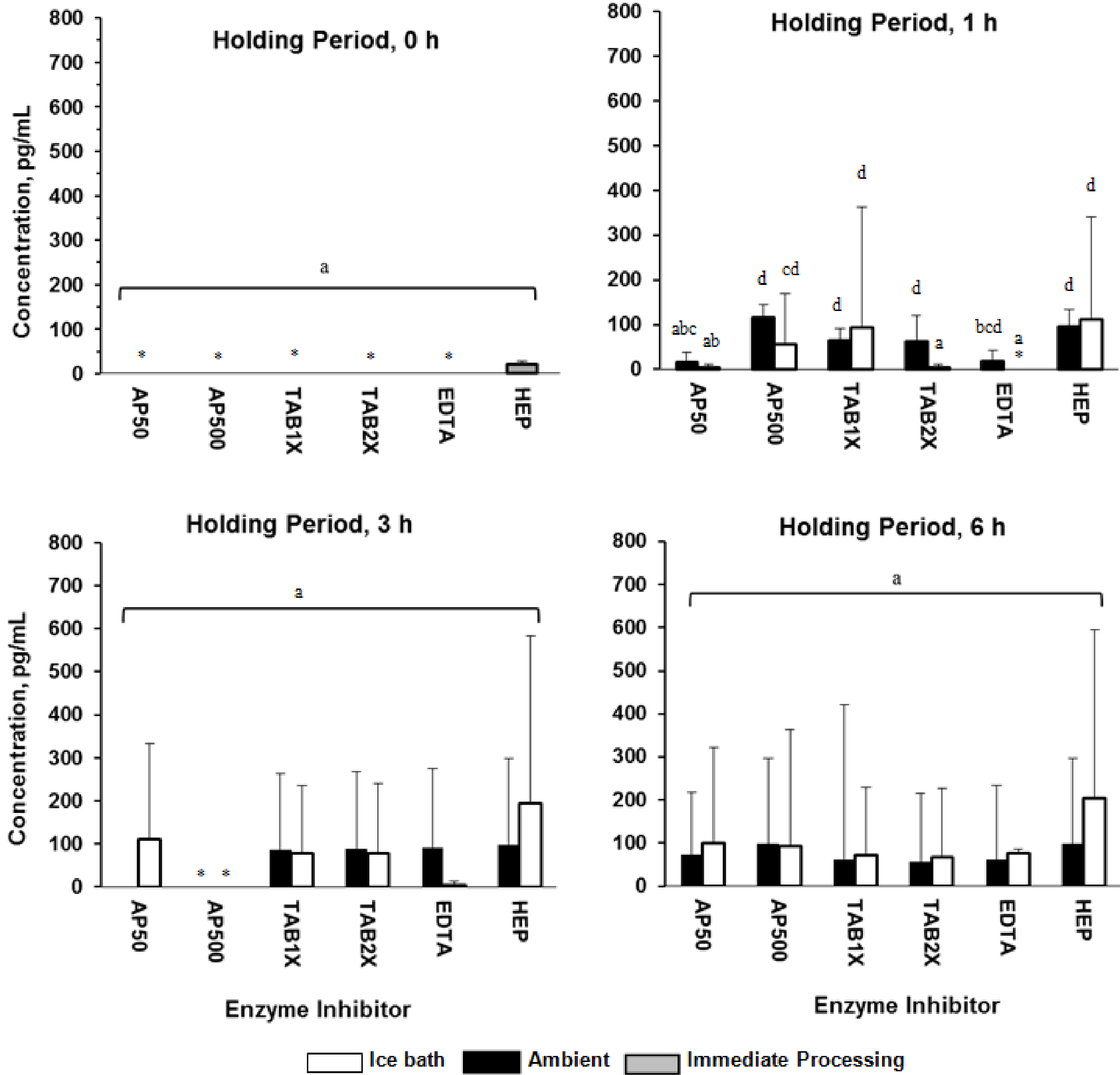
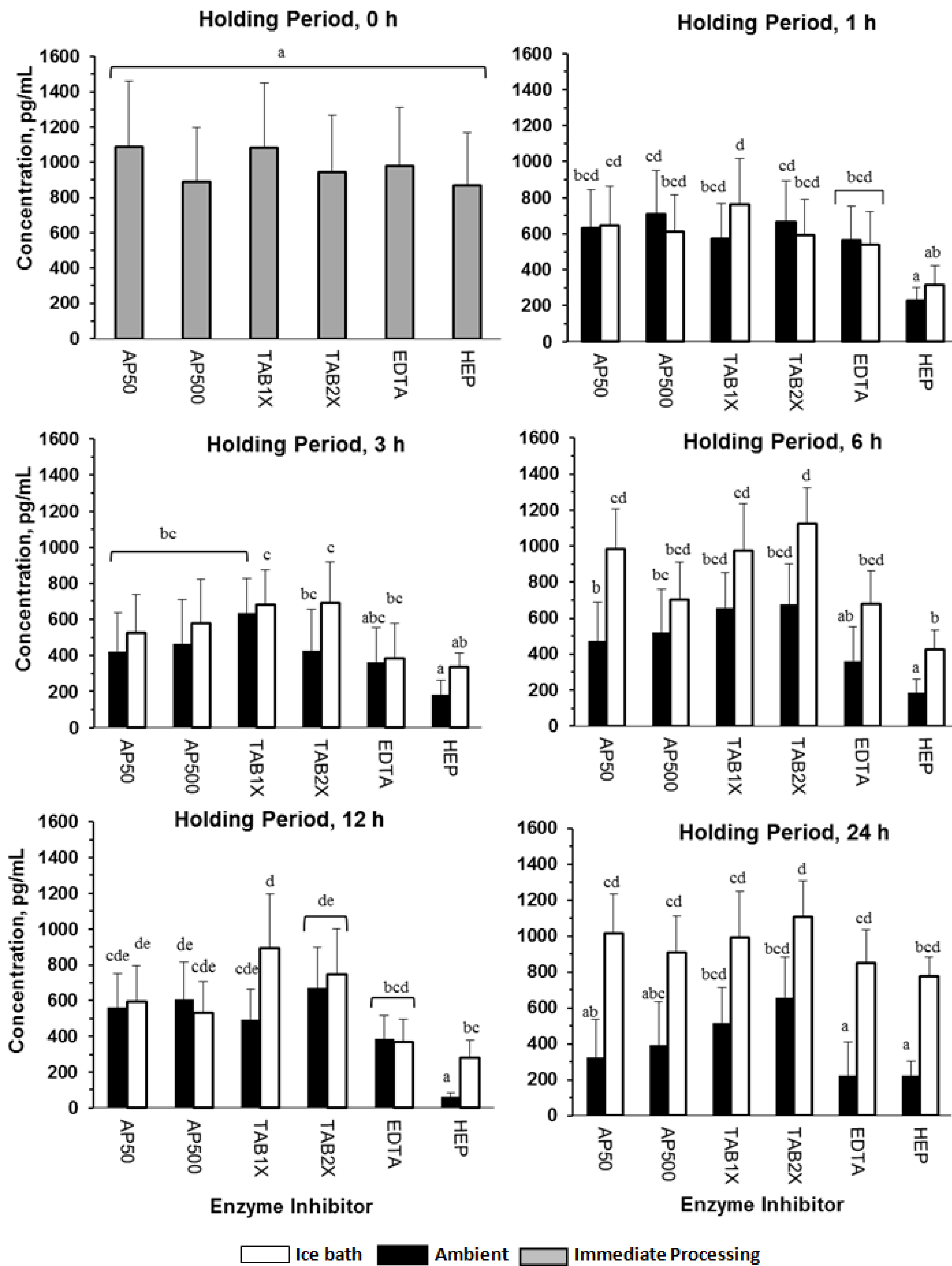

Holding Period, 0 h
Holding Period, 1 h
Holding Period, 3 h
Holding Period, 6 h
Concentration, pg/mL
AP50
AP500
TAB1X
TAB2X
EDTA
HEP
Enzyme Inhibitor
Ice bath
Ambient
Immediate Processing

Figure 2.5. Back-transformed least squares median estimates (and upper 95% confidence limits) of SP immunoreactivity levels analyzed by ELISA (SP_{ELISA}).

Whole bovine blood samples, spiked with 625 pg/mL SP_{1-11}, were either processed immediately following collection (0 h, represented by gray bars), held at ambient temperature (23.9 – 25.4°C) or in an ice bath for 1, 3, 6, 12 and 24 h prior to centrifugation and harvesting of plasma. Sample tubes contained one of the following enzyme inhibitors: 1) heparin, 15 USP/ml (HEP); 2) EDTA, 1.8 mg/mL (EDTA); 3) aprotinin, 50 KIU/ml in EDTA (AP50); 4) aprotinin 500 KIU/ml in EDTA (AP500); 5) single-strength protease inhibitor tablet in EDTA (TAB1X); and 6) double-strength complete protease inhibitor tablet in EDTA (TAB2X). Within a holding period, means without a common letter are significantly different ($P < 0.05$).



Holding Period, 0 h
Holding Period, 1 h
Holding Period, 3 h
Holding Period, 6 h
Holding Period, 12 h
Holding Period, 24 h
Concentration, pg/mL
AP50
AP500
TAB1X
TAB2X
EDTA
HEP
Enzyme Inhibitor
Ice bath
Ambient
Immediate Processing

Chapter 3 - Comparative effects of castration and dehorning in series, or concurrent castration and dehorning procedures on stress responses and production in Holstein calves[2]

Introduction

Research assessing the physiological, behavioral and production effect of dehorning and castration has focused on the consequences of performing these procedures individually, with and without pain relief (Stafford and Mellor, 2004 and 2005). A comparison between the individual procedures and the possible additive effects of their concurrent implementation, however, has received little attention as noted by at least one regulatory body (NAWAC, 2005). Yet in a survey of U.S. veterinarians in bovine practice, 90% of respondents indicated that they dehorn calves at the same time as castration (Coetzee et al., 2010).

One study has investigated separate castration and dehorning procedures in the same calves, but the procedures were performed in series, with castration always following dehorning, so comparisons between procedures were confounded with order of application (Schwartzkopf-Genswein, et al., 2005). Another study has investigated castration, dehorning, and the concurrent procedure, in parallel but not in series (Ballou et al., 2013; Sutherland et al., 2013.)

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[2] Reprinted with permission from "Comparative effects of castration and dehorning in series, or concurrent castration and dehorning procedures on stress responses and production in Holstein calves" by R. A. Mosher, C. Wang, P. S. Allen, J. F. Coetzee, 2013. Journal of Animal Science, 91:4133-4145. American Society of Animal Science.

The objective of the study was to compare the acute stress variables of serum cortisol and chute exit velocity, along with ADG as an indicator of performance and well-being following castration, dehorning, or concurrent castration/dehorning of calves when performed in parallel and in series. The null hypothesis was that there would be no difference between treatments whether performed in parallel or in series.

The methods of surgical castration and amputation dehorning, without local anesthesia or analgesia, were selected for this study because they were reported as the most common methods employed in light weight calves in the survey of U.S. veterinarians in bovine practice (Coetzee et al., 2010). The results of this study are intended to provide scientific support for the development of best practices in the field concerning castration and dehorning of calves.

Materials and Methods

This study was approved by the Institutional Animal Care and Use Committee at Kansas State University (Protocol # 2649). Calves were assessed hourly for behavioral signs of excessive pain over a period of 10 hours after surgery, then twice daily for 7 days. Calves exhibiting postural changes, prolonged recumbency, anorexia or depression were scheduled to receive rescue analgesia with flunixin meglumine at 2.2 mg/kg IV, every 12 h. No calves were deemed to require rescue analgesia during the study.

Animals and housing

Forty intact male horned Holstein calves, 3 to 4 months of age, were obtained from commercial dairy herds located in Central Kansas and allowed to acclimate for one week prior to study commencement in June 2009. Upon arrival, all calves were identified with numeric ear tags, weighed and measured for scrotal circumference, horn length and horn base diameter (Table 1). These measurements were used to assess the balance of the treatment allocation in the

randomization process and for possible inclusion as covariates in statistical modeling. Calves were then vaccinated for bovine infectious rhinotracheitis, viral diarrhea, parainfluenza3, and syncytial virus (Bovishield Gold, Pfizer Animal Health, New York, NY). Calves were administered oxytetracycline (Noromycin 300 LA, Norbrook Labs, Newry, Northern Ireland), IM, 3ml/100 lbs bodyweight, and doramectin (Dectomax Pour-on, Pfizer Animal Health, New York, NY), topically at 500 µg/kg bodyweight. Amprolium (Corid, Duluth, GA) was added to the drinking water to provide 10 mg/kg PO for 5 days. During the acclimation period, calves were trained on a daily basis to stand restrained by halter and lead rope tied to posts in the study barn while study personnel circulated among them, talking and touching the calves as would occur on procedure days.

Calves were blocked by body weight and assigned to 5 pens of 8 calves each, so that each pen contained 2 calves from each treatment sequence. Pens were comprised of a linear row of outdoor concrete pads (9.75 m x 18.29 m), each with a partial roof over straw bedding. The diet consisted of water and grass hay *ad libitum* with a ration composed of cracked corn, oats, soybean meal, molasses, vitamins, and minerals delivered at 3 to 4 kg per calf per day, divided and offered twice daily in open bunks. Due to the nature of the diet and the housing arrangement, it was not possible to measure individual feed intake.

Experimental design

A diagram of the two-period study is shown in Figure 1. After an initial sham handling procedure, calves underwent two surgical treatments in sequence separated by approximately 2-3 wk. The purpose of the sham procedure was to collect baseline stress-related data for each calf in a non-painful situation to compare to the data from the painful situation encountered in the surgical periods. Thus, the sham procedure allowed each calf to act as its own historical control.

A period began and ended with the collection of body weight and encompassed the surgical treatment and the ensuing 7 days. The two periods were separated by a balanced interim of 14 to 21 d with a mean ± SD of 17 ± 2 d.

The treatments within a period were: surgical castration (CAST), amputation dehorning with thermocautery (DH), concurrent surgical castration and amputation dehorning with thermocautery (CD), and control chute experience (CONT). With subscripts indicating either sham handling (S) or the period (1 or 2) in which the treatment was performed, the sequences were: sham castration, castration, then dehorning ($CAST_S$--$CAST_1$--DH_2); sham dehorning, dehorning, then castration (DH_S--DH_1--$CAST_2$); and sham dehorning and castration, concurrent dehorning and castration, then non-surgical control (CD_S--CD_1--$CONT_2$); and non-surgical control for Period 1 ($CONT_S$—$CONT_1$). Calves in the $CONT_1$ group participated in Period 1, and then were removed from the pens and from the study.

Calves were initially blocked by receiving weight in groups of 4 and randomly assigned to a treatment sequence. In order of weight, blocks were combined in pairs to form one pen. In Period 1, calves were processed on five separate days over 2.5 weeks, with one pen processed per day. In Period 2, calves were processed on three separate days over 1.5 weeks with either one or two pens processed per day, which resulted in a variable number of days comprising the interval between treatments for different pens; however, the differences were balanced among treatments.

Two days prior to the beginning of each period, calves were restrained in the chute by tying the head to one side of the head gate with a rope halter, then a jugular catheter was surgically placed using local anesthesia.

On the day prior to Period 1 onset, calves were subjected to sham handling in which the animal underwent all manipulations as if surgery were to be performed, but without making an incision. Restraint of calves was conducted in the same fashion as for the jugular catheterization. After the first horn procedure was completed on calves in the DH and CD groups, the head was repositioned and tied to the other side of the chute briefly while the procedure on the other horn was conducted, then the head was returned to the starting position. Sham manipulations were as follows: 1) sham castration ($CAST_S$): the scrotum was washed and manipulated for approximately 30 seconds; 2) sham dehorning (DH_S): the hair was clipped around the horns with electric clippers then each horn was grasped between the surgeon's thumb and forefinger and manipulated for approximately 2-3 seconds; 3) sham castration combined with dehorning(CD_S): manipulations were carried out as for calves in the $CAST_S$ and DH_S groups, always in that order; and 4) sham control group ($CONT_S$): calves stood restrained by the head-catch in the chute. Calves were processed at intervals of 8 minutes, being released approximately 5 minutes following the procedure. The surgical treatments (and sham) were applied to all calves within a span of time approximately 1.5 h or less beginning at the same time each day.

On D0, twenty-four hours after the sham procedure, the control animals were handled in a manner similar to the previous day whereas the surgically-treated calves underwent actual castration and dehorning. Following cleansing of the scrotum, castration was performed by removing the lower one-third of the scrotum with a sterile scalpel blade; the testicles were exteriorized and removed by twisting and manual traction until the spermatic cord separated. Dehorning was achieved by placing the blades of a Barnes-type scoop dehorner around the base of the horn and then quickly forcing the blades together to remove the horn; bleeding was controlled by cauterizing the cornual vessels with a heated iron.

The environmental temperature was measured with a digital thermometer (Model 91551, Springfield, Las Cruces, NM) at 6 am (range, 16 to 27°C) and 2 pm (range, 23 to 39°C) on the actual procedure day for possible inclusion as a covariate in statistical modeling.

Chute exit velocity. Following the chute restraint period, the velocity at which the calf exited the chute was recorded when the calf passed between two points, one meter apart, of a wireless infrared timing system (Polaris Multi-Event Timer, FarmTek, Wylie, TX), with the first device being placed 1.5 m in front of the chute headgate. The chute exit velocity (m/s) was calculated by dividing the distance between the two points (one m) by the time (s) taken to travel the distance.

Blood sample collection for cortisol. Blood samples were collected through the catheter port during both the sham and surgical periods at the following time points after the procedure initiation: 5, 10, 20, 30, 40, 50, 60, 120, 240, 360, 480, 600, and 720 minutes. A baseline sample was taken approximately 15 min prior to the procedure, when calves were brought into the holding area of the barn. The 5-min sample was taken while the calf was in the chute, then the calf was released. Following exit from the chute, calves were restrained with a rope halter and a lead long enough to allow the calf to lie down in a common holding area in the working facility in order to obtain blood samples from 10 to 240 min, after which time calves were released to their pen. The remaining samples were obtained following restraint of the calves by rope halter in the pen or in the working facility. Following collection, blood samples were immediately transferred to tubes containing a clot activator (BD Diagnostics, Franklin Lakes, NJ) and allowed to clot for 30 - 60 min prior to centrifugation for 10 min at 1,500 x g. Serum was pipetted to cryovials and frozen at -70°C until analysis.

Serum cortisol determination and calculation of summary variables. Serum cortisol concentrations were analyzed in the Kansas State University Clinical Sciences Laboratory using a solid-phase competitive chemiluminescent enzyme immunoassay kit with an automated analyzer system (Immulite 1000 Cortisol, Siemens Medical Solutions Diagnostics, Los Angeles, CA) which has been validated for bovine plasma and serum. The assay calibration range was 28 to 1,380 nmol/L with a lower limit of quantification of 5.5 nmol/L. Over 21 assay runs, the intra- and inter-assay coefficients of variation of quality control samples with low, mid-range, and high concentrations were < 8.0%. Peak cortisol concentration (C_{max}), and the time at which peak cortisol concentration occurred (T_{max}) were calculated directly from the data. As a summary variable, the integrated cortisol response from baseline to 720 min, (i.e., the observed area under the curve, $AUC_{0\text{-}720}$) was calculated using the linear trapezoidal method (Gabrielsson & Weiner, 2007).

Weight. Calves were weighed in a chute equipped with an electronic scale (EziWeigh, Tru-Test Corp, Aukland, New Zealand) determined to be accurate within ± 1 kg in the range from 100 – 250 kg. Calves were weighed approximately 30 minutes prior to commencing the surgical treatments in Period 1 ($Weight_a$) and Period 2 ($Weight_c$), then again at the same hour seven days following the treatment in Period 1 ($Weight_b$) and Period 2 ($Weight_d$). Calves were weighed immediately after rousing from rest in the morning prior to ration feeding. Hay and water were freely available overnight. Calves were also weighed on the day of the sham procedure for duplication of handling procedures only.

ADG was calculated as follows for the 7 days in Period 1 and Period 2, and for the 7 to 14 days in the interim between periods:

$$Period\ 1\ ADG = \frac{Weight_a - Weight_b}{7}$$

$$Interim\ ADG = \frac{Weight_b - Weight_c}{days\ in\ interim}$$

$$Period\ 2\ ADG = \frac{Weight_c - Weight_d}{7}$$

***Data analysis and statistics*.**

Group means for variables measured at receiving were compared using analysis of variance (JMP, SAS Institute, Cary, NC).

Linear mixed models were fitted to response variables. Models were fitted to data in the original scale (namely cortisol concentration, cortisol C_{max}, cortisol, $AUC_{0\text{-}720}$, chute exit velocity, and maximum eye temperature), or the log-transformed scale (namely, T_{max}), as needed to stabilize variances and meet model assumptions. The following explanatory covariates were evaluated for each model and included in the linear predictor if there was a significant contribution to model fit; namely horn diameter, horn length, scrotal circumference, arrival weight, and environmental temperatures at 6 am and at 2 pm on the treatment day. The linear predictors for all statistical models except ADG included the fixed effects of treatment (CONT, DH, CAST or CD), period (sham, Period 1, Period 2) and their 2-way interaction. Specification of random effects was tailored to each model depending on convergence and estimability of variance components.

The model for chute exit velocity included the random effect of the block-by-treatment combination to recognize the calf as the experimental unit for treatment and the blocking factor for period. In addition, the random effects of day, day-by-block and day-by-block-by-treatment were evaluated but were excluded from the final model due to their corresponding variance components converging to zero. Horn base diameter was included in the final model as a

significant explanatory covariate (P = 0.003). Comparisons were confined within Period because of increasing calf size.

The models for C_{max}, T_{max}, and $AUC_{0\text{-}720}$ included the random effect of day-by-block-by-treatment to recognize the appropriate experimental unit for treatment. Random components for day and day-by-block combination had their variance components converge to zero and thus were effectively removed from the model. The residual variance covariance structure was modeled using a spatial power correlation structure to account for repeated measures over uneven time intervals.

In addition to the fixed effects included in all models, the statistical model for cortisol also included the fixed effect of time (baseline, 5, 10, 20, 30, 40, 50, 60, 120, 240, 360, 480, 600, 720 min) and all 2- and 3-way interactions with treatment and period. Random effects included in the linear predictor were day-by-block-by-treatment combination and day-by-block-by-treatment-by-period combination in order to recognize the appropriate blocking factors and experimental units for the fixed effects of interest. The random blocking factors of day and day-by block combination converged to zero and thus were effectively removed from the model. The residual variance covariance structure was modeled using a spatial power correlation structure to account for repeated measures over uneven time intervals.

Average daily gain was analyzed using a general linear mixed model. The linear predictor in the statistical model included the fixed effects of treatment and ADG segment (Period 1, Interim and Period 2) and their two-way interactions. The combinations of day-by-block and day-by-block-by-treatment were fitted to recognize the random blocking factor for treatment and its experimental unit, respectively, but both variance components converged to zero and were thus removed from the model. Comparisons were confined within ADG segment.

For all response variables, Satterthwaite's method was used to estimate degrees of freedom and Kenward Roger's procedure was used for the corresponding adjustments in estimated standard errors. Models were fitted using the GLIMMIX procedure of SAS (Version 9.2, SAS Institute, Cary, NC) implemented using Newton-Raphson with ridging as the optimization technique. Model assumptions were considered to be appropriately met based on diagnostics conducted on studentized residuals. Estimated least square means and corresponding standard errors, or 95% confidence intervals, are presented. Relevant pairwise comparisons were conducted using Tukey-Kramer or Bonferroni adjustments, as appropriate in each case, to avoid inflation of Type I error rate due to multiple comparisons.

A significant difference was considered to exist when $P \leq 0.05$, and a marginal difference was considered to exist if $0.05 < P \leq 0.10$.

Results

No significant differences were detected between treatment groups with respect to weight, horn base diameter, horn length, or scrotal circumference measured at receiving (Table 1).

Cortisol. Mean cortisol concentration-time profiles for treatments within period are shown in Figure 2. There was a significant 3-way interaction ($P < 0.0001$) between treatment, period, and time point for serum cortisol concentrations. The *P*-values for selected simple effect comparisons are shown in Table 2; C_{max} and T_{max} are shown in Table 3; and $AUC_{0\text{-}720}$ is shown in Figure 3. For all differences between cortisol means reported below, the SED = 10 nmol/L.

Comparisons within Period.

Within the sham handling procedure, cortisol concentrations were significantly increased for CD_S calves compared to $CONT_S$ and DH_S calves from 5 to 30 minutes after initiation of the

procedure (with a difference range of 26-39 nmol/L, $P \leq 0.05$), but there were no differences in the integrated cortisol response (AUC_{0-720}) between the $CAST_S$, CD_S, DH_S, or $CONT_S$ groups. The previous comparisons also included C_{max} because mean maximum concentrations were reached in all groups within 20 min.

Within Period 1 after the first surgical treatment, relative to the $CONT_1$ group, cortisol concentrations were significantly higher from 5 to 60 min in the DH_1 group (27-73 nmol/L, $P < 0.04$) and from 10 to 240 min (and at 720 min) in both, the $CAST_1$ (28-70 nmol/L, $P < 0.04$) and CD_1 groups (37-95 nmol/L, $P < 0.01$). The previous comparisons also included C_{max} because mean maximum concentrations were reached in all groups within 40 min; T_{max} was significantly shorter in the $CONT_1$ group than DH_1, $CAST_1$, and CD_1 groups ($P \leq 0.01$). In contrast, there was no evidence for significant differences between the 3 surgically-treated groups with respect to T_{max}, C_{max}, or any cortisol concentrations during the first 40 min post-procedure. After that time, cortisol concentrations in DH_1 calves were significantly lower than in $CAST_1$ calves at 120 min post procedure (35 nmol/L, $P < 0.001$) and in CD_1 calves from 50 to 240 min (31-46 nmol/L, $P < 0.02$). Within Period 1, cortisol concentrations were not significantly different between the $CAST_1$ and CD_1 groups at any time point, though there was marginal evidence that concentrations in the CD_1 group were higher than those in the $CAST_1$ group at 60 min (26 nmol/L, $P > 0.06$). The AUC_{0-720} was significantly lower in the $CONT_1$ group than the $CAST_1$ ($P = 0.002$) and CD_1 ($P = 0.006$) groups, with marginal evidence of being lower than the DH_1 group ($P = 0.07$). The AUC_{0-720} of the CD_1 group was significantly higher than the DH_1 ($P = 0.006$) group, but was not significantly different than the $CAST_1$ group.

Within Period 2 after the second surgical treatment, relative to the $CONT_2$ group, cortisol concentrations were significantly higher from 20 to 360 min in the DH_2 group (35-63 nmol/L, $P < 0.01$) and from 20 to 240 min in the $CAST_2$ group (32-57 nmol/L, $P < 0.001$). Relative to the $CONT_2$ group, C_{max} was significantly higher in the DH_2 group (P = 0.003) with marginal evidence of being higher in the $CAST_2$ group (P = 0.08); and T_{max} was significantly longer in both DH_2 and $CAST_2$ groups ($P \leq 0.01$). There were no significant differences between DH_2 and $CAST_2$ calves with respect to C_{max} and T_{max}, however, cortisol concentrations of DH_2 calves were significantly lower than $CAST_2$ calves at 120 min (33 nmol/L, $P = 0.005$), then were higher from 360 to 480 min (27 to 29 nmol/L, $P < 0.002$). The $AUC_{0\text{-}720}$ of the $CONT_2$ group was significantly lower than the $CAST_2$ and DH_2 groups ($P < 0.001$), but there was no significant difference detected between the $CAST_2$ and DH_2 groups.

Comparisons within treatment sequence.

Within CD_1-$CONT_2$ calves, there were no significant differences detected between the sham handling and $CONT_2$ with respect to T_{max}, C_{max}, $AUC_{0\text{-}720}$ or cortisol concentrations at any time point. Within the same calves, cortisol concentrations were higher in CD_1 than in both sham and $CONT_2$ from approximately 20 to 360 min and at 720 min (23-76 nmol/L, $P < 0.05$ except 360 min in $CONT_2$ for which $P = 0.06$). The $AUC_{0\text{-}720}$ was significantly higher in CD_1 than $CONT_2$ ($P < 0.0001$).

Within DH_1-$CAST_2$ calves, compared to sham handling, cortisol concentrations were higher from 10 to 60 min (33-74 nmol/L, $P < 0.01$) and from 5 to 240 min in $CAST_2$ (24-77 nmol/L, $P < 0.03$); and within the same calves, cortisol concentrations were higher in $CAST_2$ than DH_1 from 120 to 240 min (30-51 nmol/L, $P < 0.001$). The $AUC_{0\text{-}720}$ of the sham

handling was significantly lower than in DH_1 ($P = 0.009$) and $CAST_2$ ($P < 0.0001$). The AUC_{0-720} in $CAST_2$ was greater than in DH_1 ($P = 0.04$)

Within $CAST_1$-DH_2 calves, compared to sham handling, cortisol concentrations were higher from 20 to 240 min (37-78 nmol/L, $P < 0.01$) and from 10 to 480 min (31-84 nmol/L, $P < 0.01$, excluding the 120 min time point for which $P = 0.08$); within the same calves, cortisol concentrations were higher in DH_1 than $CAST_1$ from 360 to 480 min (30-32 nmol/L, $P < 0.01$). The AUC_{0-720} of the sham handling was significantly lower than $CAST_1$ ($P < 0.0001$). There was marginal evidence that the AUC_{0-720} of DH_2 was higher than $CAST_1$.

Comparisons within surgical treatment. Between $CAST_1$ and $CAST_2$, there was no significant difference between the AUC_{0-720}, nor were there significant differences in cortisol concentrations between time points except at 720 min, where $CAST_1$ was higher than $CAST_2$ (25 nmol/L, $P = 0.02$). Between DH_1 and DH_2, cortisol concentrations were significantly higher in DH_2 than DH_1 at 20 min and from 240 to 480 minutes (20-26 nmol/L, $P < 0.05$) and the AUC_{0-720} was significantly higher in DH_2 than DH_1 (P = 0.004).

Chute exit velocity. The results for chute exit velocity are shown in Figure 4. There was evidence of an interaction between period and treatment ($P = 0.008$) on chute exit velocity. There were no significant differences between groups when sham handled. In Periods 1 and 2, calves that were castrated ($CAST_1$ and $CAST_2$) exited the chute significantly slower than calves that were dehorned (DH_1 and DH_2) within the respective period ($P < 0.05$).

Average daily gain. Figure 5 shows the segmental ADG of calves through the entire study. The mean ADG outcomes that were significantly different than zero were the $CONT_1$ group in Period 1(1.5 ± 0.4 kg/day), the $CAST_1$ group in the interim between periods (0.9 ± 0.4 kg/day), and the DH_2 group in Period 2 (-1.2 ± 0.4 kg/day). In Period 1, there were no

significant differences between the ADG of $CAST_1$, DH_1, or CD_1 calves. In Period 1, the ADG of the $CONT_1$ group was significantly higher than that of CD_1 (difference ± SED, 1.5 ± 0.6 kg/day, $P = 0.02$), but not $CAST_1$ nor DH_1 groups. In the interim between periods, the ADG of calves in the $CAST_1$ group was significantly higher than that of the CD_1 group (1.4 ± 0.6) kg/day, $P = 0.03$). In Period 2, the ADG of the DH_2 calves was significantly lower than both, $CAST_2$ (-1.5 ± 0.6 kg/day, $P = 0.02$) and $CONT_2$ groups (-1.8 ± 0.6 kg/day, $P = 0.005$).

Discussion

With the aim of evaluating the relative stress and production effects of common husbandry procedures in the U.S. cattle industry, this study investigated the responses of surgical castration, amputation dehorning, and concurrent castration and dehorning of 3- to 4-month-old, acclimated Holstein calves. The immediate indicators of distress included serum cortisol concentration and chute exit velocity. ADG was evaluated as a longer-term indication of production and overall well-being.

Cortisol concentrations in Period 1 appeared to be most influenced by castration, whether performed as a solo procedure or concurrently with dehorning. This was indicated by the mean cortisol concentrations of castrated calves ($CAST_1$ and CD_1) being higher than controls for nearly 4 h, whereas cortisol concentrations of DH_1 calves were higher than controls for approximately 1 h. Furthermore, the overall cortisol responses of $CAST_1$ and CD_1 calves were not significantly different, suggesting the possibility that near-ceiling cortisol concentrations resulted from the castration procedure, leaving little room for an additive increase due to dehorning (Coetzee, 2011, Stock et al., 2013). It is not known why cortisol concentrations of castrated calves were elevated above control concentrations at 720 min post-procedure in Period 1; that response was not observed in Period 2.

Important to the inferences made between groups in Period 2, the cortisol profile of $CONT_2$ calves was not different than when the same calves underwent sham handling, despite an intervening concurrent castration and dehorning procedure. This finding indicates the lack of a memory effect on cortisol concentrations in those calves in Period 2 and suggests the validity of their use as controls in that period. Because all calves were were of similar breed, background, and acclimation, it may be assumed that the painful experience in Period 1 had a similar lack of memory effect on the cortisol response in Period 2, thus suggesting the validity of comparisons made between periods within a treatment or treatment sequence.

Our results support and extend the findings reported by Schwartzkopf-Genswein et al. (2005), in which 1- to 2-mo-old Holstein bull calves were disbudded by hot iron cautery, then approximately 21 d later, were surgically castrated. In that study, as in the present report, cortisol concentrations reached maximum peaks at approximately 30 minutes for both procedure groups, but then a more sustained response in the castrated calves resulted in significant differences between groups in cortisol concentrations at the 120 and 240 min time points, similar to what we observed in calves undergoing the DH_1--$CAST_2$ sequence in our study. Schwartzkopf-Genswein et al. (2005) proposed that memory of a previous negative experience may have caused cortisol concentrations to be higher when calves were castrated than when they were disbudded because, in that study castration always occurred following disbudding. Although the previous negative experience may have contributed to the differences seen in that study, the apparent lack of a memory effect in $CONT_2$ calves in our study, suggests the possibility of a minimum contribution. Furthermore, when we compared cortisol response to castration and dehorning performed in parallel in Period 1 of our study, the results were similar,

with cortisol concentrations of $CAST_1$ calves being significantly (and marginally) elevated above those of DH_1 calves at 120 (and 240) min.

In Period 2, with the exception of the elevated concentrations at 720 min, the cortisol profiles of castrated ($CAST_2$) calves were not different than those which were castrated in Period 1 ($CAST_1$). This indicates that the later castration was not more stressful than the procedure performed earlier. The later dehorning procedure, however, appeared to be more stressful than the earlier procedure. This is indicated by the significantly higher cortisol concentrations in the DH_2 than the DH_1 group soon after the procedure at 20 min, and later at 240 to 480 min. Furthermore, after the dehorning procedure, cortisol concentrations of DH_2 calves did not drop to the level of the contemporaneous control group ($CONT_2$) until 360 to 480 min post-procedure, which is in contrast to the profile noted for DH_1 calves noted above. The higher concentrations of cortisol in the calves dehorned in Period 2 might be attributed to the more advanced stage of horn development in which the horn buds had attached to the skull, and the frontal sinus was possibly invaded in a higher percentage of calves than in Period 1. Horn buds are free floating in the skin until approximately 2 mo of age, when they gradually attach to the skull and to the frontal sinus (AVMA, 2011b; Stock et al., 2013).

Regarding the concurrent castration and dehorning procedure, the cortisol concentrations of the CD_1 group were not significantly different from those of $CAST_1$ at any time point, but compared to DH_1, the concurrent procedure resulted in higher cortisol concentrations for approximately 3 hours resulting in a higher integrated cortisol response as shown by the AUC_{0-720}. In Period 2, when castrated and dehorned calves served as controls ($CONT_2$), their cortisol was lower than that of calves which underwent castration ($CAST_2$) and dehorning (DH_2) for 3.5 h and 5.5 h, respectively. Thus, although our results indicate that castration followed by

dehorning is more stressful to the animal than the concurrent procedure, our results do not suggest a difference between the concurrent procedure and dehorning followed by castration. It should be noted, however, that calves undergoing the single procedure in the field would still need to undergo another stressful procedure, but those undergoing the concurrent procedure would not require another handling such as even the chute experience of the $CONT_2$ calves in our study.

The pattern of our cortisol results observed in Period 2 were similar to the results of a study (reported both in Sutherland et al. (2013) and in Ballou et al. (2013)), in which calves were either surgically castrated or amputation dehorned as a single or combined procedure. As reported in Sutherland et al. (2013), the mean cortisol concentration of dehorned calves was elevated above that of sham-treated controls for at least 6 h post-procedure whereas the mean cortisol concentration of castrated-only calves was elevated from baseline to 4h post-procedure. The cortisol results, as reported in Ballou et al. (2013), indicated that the cortisol concentration of dehorned-only calves was significantly elevated above that of castrated-only calves from 1.5 to 6 h post-procedure. Combining behavior with cortisol data reported both in Sutherland et al. (2013) and Ballou et al. (2013), in Ballou et al. (2013), the authors concluded that dehorning was more painful than castration in 3-mo-old calves. The results of our two-period study based upon cortisol results suggest that it cannot be definitively concluded from a single snapshot in time that one procedure is inherently more painful than another, but that the age of the animal at the time of application is an important determinant of pain and stress perception.

Baldridge et al. (2011) investigated the possibility that the relative noxiousness of a procedure might be associated with the calf's desire to escape the situation, and that this might be measured by the velocity with which the calf left the chute. Baldridge et al. (2011), observed

no difference in chute exit velocity of non-sedated Holstein calves following a sham or an actual surgical castration and dehorning procedure. We observed similar results of no difference between the chute exit velocity of control calves and those which were concurrently castrated and dehorned in Period 1 of our study. Our study extended those results to the single procedures of castration and dehorning, where we observed significantly slower chute exit velocities in CAST than DH calves within each surgical period. It is unknown, however if one procedure was more painful than the other. Given that the chute exit velocity of DH calves was not significantly different (i.e. not faster) than CONT calves in any period, the difference between the CAST and DH calves was possibly that the CAST procedure made the calves more reluctant to move due to a combination of visceral pain and pain caused by moving the rear legs. Furthermore, given the lack of difference between the CD_1 group and either $CAST_1$ or DH_1 group it is possible that the dehorning procedure increased the desire for CD_1 calves to move away from the chute despite any visceral pain or pain caused by movement of the rear legs. Our investigation of chute exit velocity suggests that, in acclimated Holstein calves, the measure may be indicative of a difference in type or area of pain perception, but did not indicate that a painful procedure increased the desire or ability to move away from the chute faster than control calves. Although as reported in Baldridge et al. (2011), chute exit velocity is not useful in sedated calves, the measure might be a useful adjunct in assessing non-sedating methods of pain relief due to castration, but not dehorning.

Surgical castration is generally followed by a period of reduced weight gain, the severity of which is usually related to the age of the animal at the time of the procedure. (AVMA, 2011a; Bretschneider, 2005; Stafford and Mellor, 2005). Losses in ADG following surgical castration tend to be greatest during the first 7 d following the procedure, though cumulative ADG may be

reduced through 27 to 35 days (Cohen et al., 1991; Fisher et al., 1996; Coetzee., 2013). The results of our study followed that pattern, with no significant weight change detected over the 7 d following the single castration procedure in either Period 1 ($CAST_1$) or Period 2 ($CAST_2$), but with a significant weight gain of the $CAST_1$ group during the interim between procedures. However, given that the ADG of both $CAST_1$ and $CAST_2$ calves did not change significantly during the respective period, the CAST procedure did not appear to affect the ADG more adversely when performed as a first or second procedure .

Amputation dehorning is also generally associated with reduced weight gain following the procedure (AVMA, 2011b; Stock et al., 2013). Reductions in ADG following dehorning have been associated with increasing age at the time of the procedure with the associated increasing size of the opening created in the frontal sinus (Winks et al., 1977; Brickell et al., 2009). The results of our study followed that pattern, with no significant weight change of the DH_1 calves in either Period 1 or in the interim between periods. Furthermore, when calves were dehorned at an older age in Period 2 (DH_2) a significant weight loss was observed in the following 7 days, which indicates that dehorning at an older age affects the ADG more adversely than at a younger age. A possible reason why we observed this difference with the DH procedeure, but not CAST, is the rapidly changing horn anatomy occuring in this age class of calves. When a disbudding procedure is performed prior to the attachment of the horn bud to the frontal bone at approximately 2 months of age, the frontal sinus is not invaded, and the healing process may occur more quickly than when an amputation procedure must be performed in older calves. As the horn grows, the horn base diameter enlarges and the likelihood of invading the frontal sinus increases when dehorning. Wounds opening into the frontal sinus may take approximately 4 weeks to heal in calves up to one year of age (Loxton et al., 1982). Sinusitis,

with associated clinical signs such as poor growth and unthriftiness, is a common sequella to dehorning procedures in which the frontal sinus is open and exposed to the environment (Mullville and Curran, 1992).

Few reports describe weight changes following castration and dehorning performed concurrently. In a study of 2- to 4-mo-old Holstein calves, Baldridge et al (2011) reported no net ADG over a period of 13 days following a concurrent castration and dehorning procedure. This result is similar to that observed following the same procedure (CD_1) in the current study.

CONCLUSION

Based on cortisol results following the first surgical treatment, castration appeared to elicit ceiling concentrations because the addition of the dehorning procedure did not significantly increase concentrations. Whereas the cortisol response in Period 1 of DH_1 calves was lower than $CAST_1$ and CD_1 calves for approximately 3 h during post-surgical monitoring, the chronic effect of dehorning appeared to be more detrimental to longer term well-being of calves than castration. This was shown by a significant weight gain by $CAST_1$ but not DH_1 or CD_1 calves over the interim between periods, and by the significant weight loss in Period 2 by DH_2 calves but not $CAST_2$ calves. Our investigation of chute exit velocity suggests that, in acclimated Holstein calves, the measure may be indicative of a difference in type or area of pain perception, but did not indicate that a painful procedure increased the desire or ability to move away from the chute faster than control calves.

Our results indicate that the order in which procedures are performed is important if there must be a time separation between them. Cortisol and ADG results indicate that it is no more stressful or detrimental to longer term well-being to castrate first, to castrate second, or to

concurrently castrate and dehorn first, but that dehorning second is more stressful and detrimental to well-being than dehorning first.

Thus our findings support either, the common practice of concurrent castration and dehorning, or the sequence of dehorning followed by castration after a healing period. In both instances, however, the procedures should be initiated as early during horn development as possible.

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Figures and Tables

Figure 3.1. Study Diagram.

Calves underwent a sham procedure followed by two sequential surgical treatments (one per period) separated by 2 – 3 weeks. A period began and ended with collection of body weight data and was of 7 days duration. With subscripts indicating indicating either sham handling (S) or the period in which the treatment was performed, the sequences were: sham castration, castration, then dehorning ($CAST_S$--$CAST_1$--DH_2); sham dehorning, dehorning, then castration (DH_S--DH_1--$CAST_2$); and sham dehorning and castration, concurrent dehorning and castration, then non-surgical control (CD_S--CD_1--$CONT_2$); and non-surgical control for Period 1 ($CONT_S$--$CONT_1$).

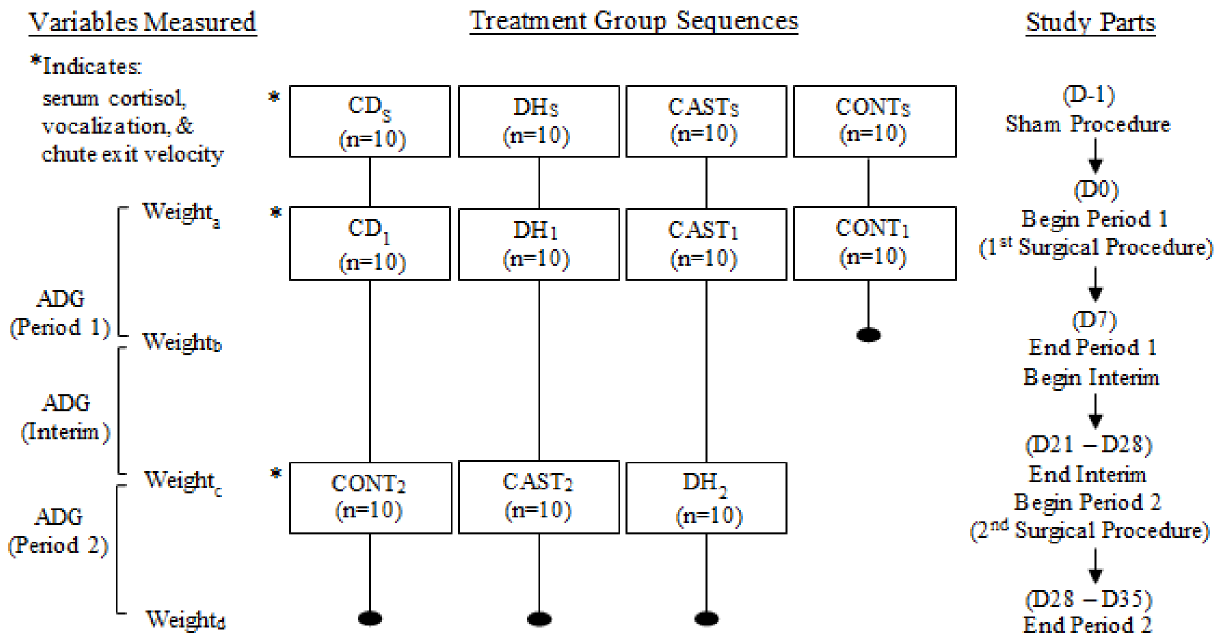
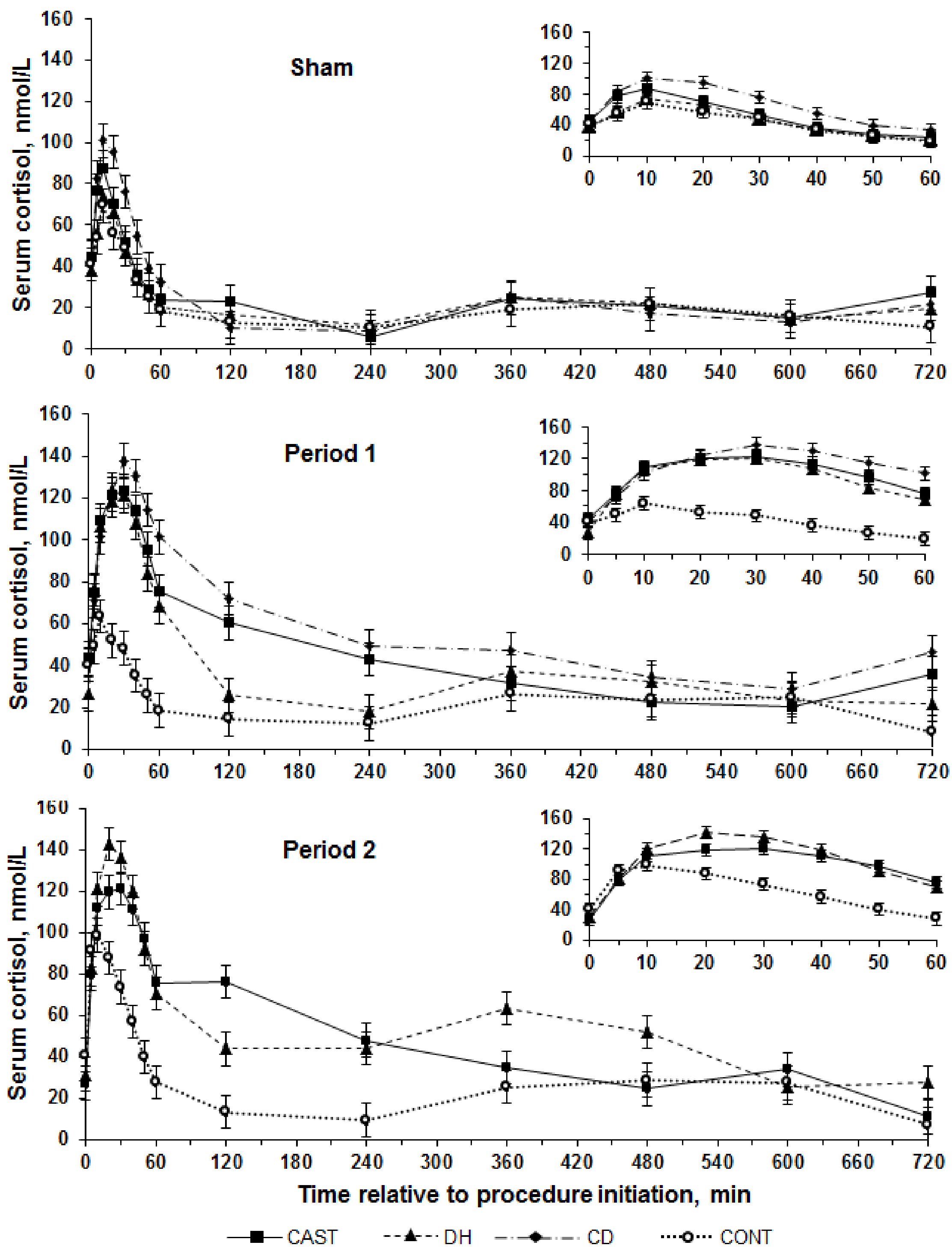


Figure 3.2. Plasma cortisol least squares mean estimates (± SE of the estimate) for calves undergoing sham treatment or surgical treatments in Periods 1 and 2.

With subscripts indicating either sham handling (S) or the period in which the treatment was performed, the sequences were: sham castration, castration, then dehorning ($CAST_S$--$CAST_1$--DH_2); sham dehorning, dehorning, then castration (DH_S--DH_1--$CAST_2$); and sham dehorning and castration, concurrent dehorning and castration, then non-surgical control (CD_S--CD_1--$CONT_2$); and non-surgical control for Period 1 ($CONT_S$--$CONT_1$). Inserts show the cortisol concentration-time profile for the first 60 min after the treatment initiation. For graphical purposes, baseline samples are shown at 0 min. Data are represented as treatment within period (and sham).



Sham
Period 1
Period 2
Serum cortisol, nmol/L
Time relative to procedure initiation, min
CAST
DH
CD
CONT

Figure 3.3. Least squares mean estimates (± SE of the estimate) of the integrated cortisol response from baseline to 720 min post-procedure ($AUC_{0\text{-}720}$) for calves undergoing sham treatment or surgical treatments in Periods 1 and 2.

With subscripts indicating either sham handling (S) or the period in which the treatment was performed, the sequences were: sham castration, castration, then dehorning ($CAST_S$--$CAST_1$--DH_2); sham dehorning, dehorning, then castration (DH_S--DH_1--$CAST_2$); and sham dehorning and castration, concurrent dehorning and castration, then non-surgical control (CD_S--CD_1--$CONT_2$); and non-surgical control for Period 1 ($CONT_S$--$CONT_1$).

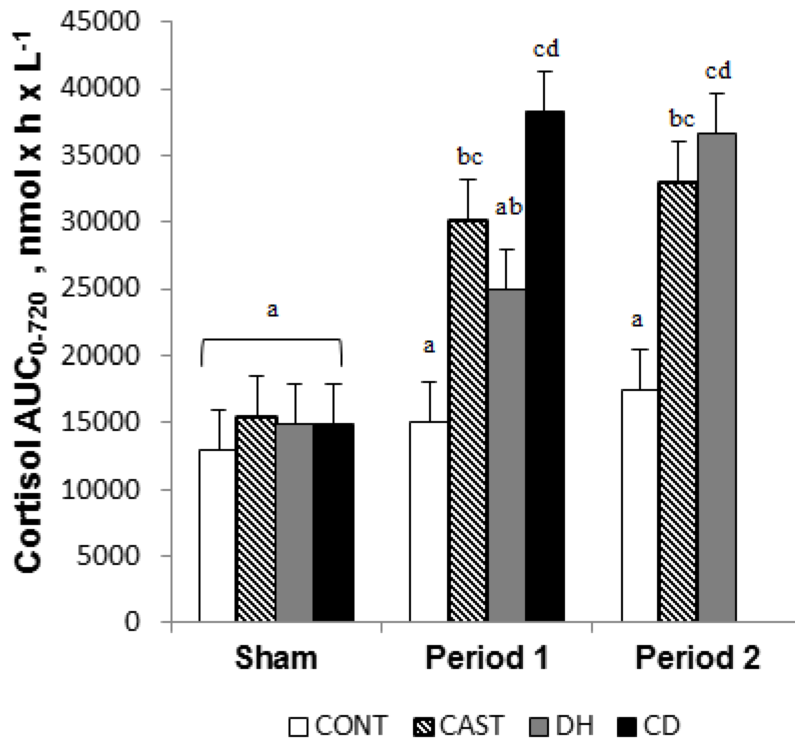


Figure 3.4. Least squares means (± SE of the estimate) for chute exit velocity, which was measured following the sham procedure and the surgical procedures in Period 1 and 2 of the study.

With subscripts indicating either sham handling (S) or the period in which the treatment was performed, the sequences were: sham castration, castration, then dehorning ($CAST_S$--$CAST_1$--DH_2); sham dehorning, dehorning, then castration (DH_S--DH_1--$CAST_2$); and sham dehorning and castration, concurrent dehorning and castration, then non-surgical control (CD_S--CD_1--$CONT_2$); and non-surgical control for Period 1 ($CONT_S$--$CONT_1$).

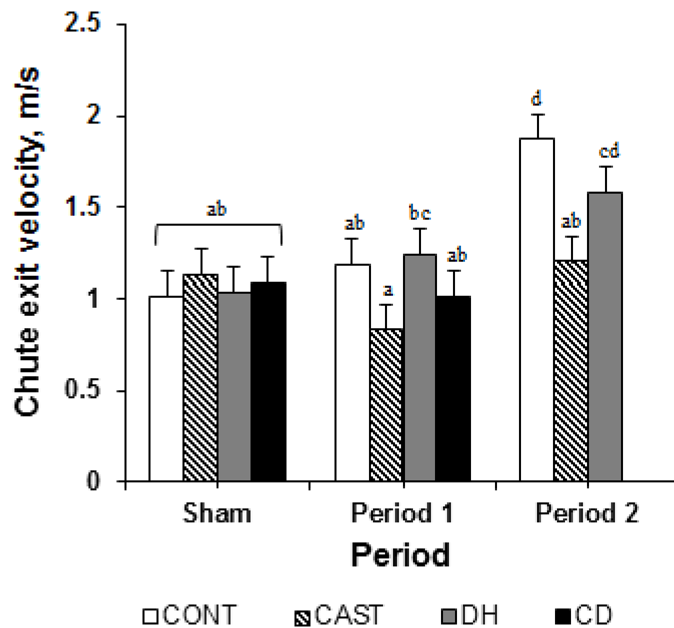


Figure 3.5. Least squares mean estimates (± SE of the estimate), of ADG for calves undergoing sequential surgical treatments separated by 2 – 3 weeks.

With subscripts indicating either sham handling (S) or the period in which the treatment was performed, the sequences were: sham castration, castration, then dehorning ($CAST_S$--$CAST_1$--DH_2); sham dehorning, dehorning, then castration (DH_S--DH_1--$CAST_2$); and sham dehorning and castration, concurrent dehorning and castration, then non-surgical control (CD_S--CD_1--$CONT_2$); and non-surgical control for Period 1 ($CONT_S$--$CONT_1$). Average daily gain was computed from weights obtained on the morning of each surgical treatment and 7 days later. The resulting ADG intervals and time span were: Period 1 (7 d), Interim (7 to 14 d), and Period 2 (7 d). Means within an ADG interval not connected by a common letter differ ($P \leq 0.05$).

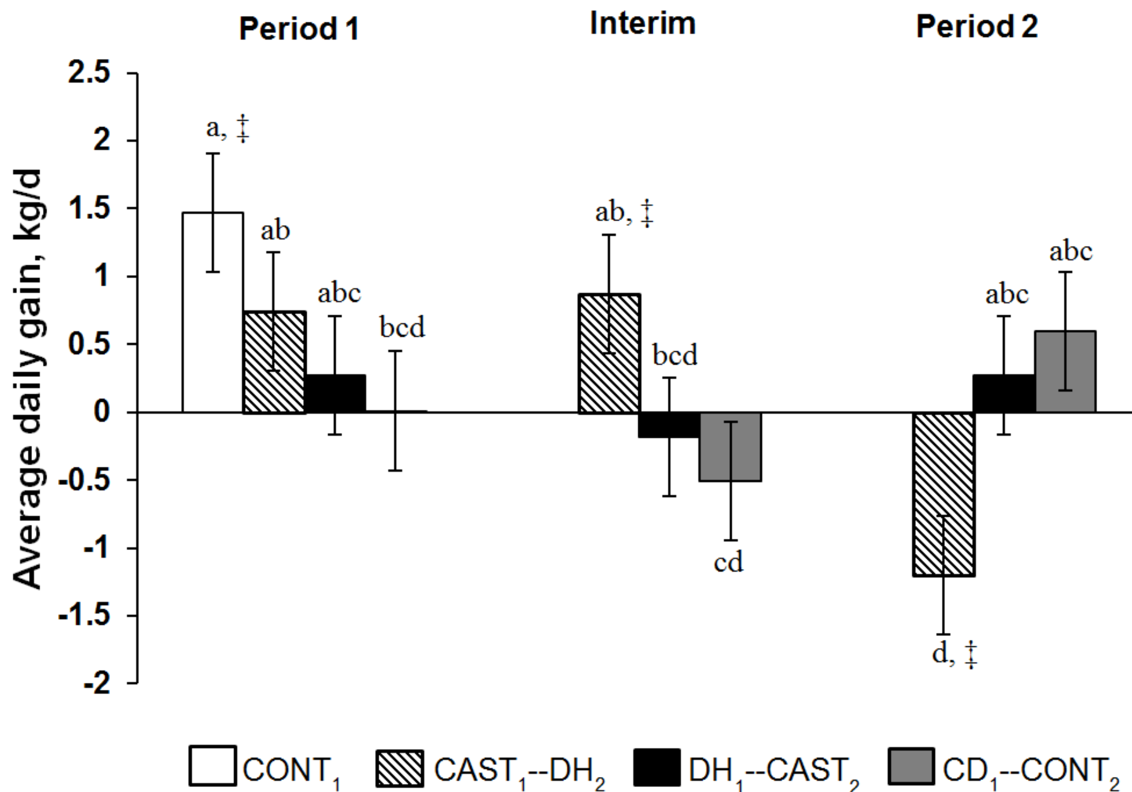


Table 3.1. Treatment sequence group means, (and SEM) of receiving data.

With subscripts indicating either sham handling (S) or the period in which the treatment was performed, the sequences were: sham castration, castration, then dehorning ($CAST_S$--$CAST_1$--DH_2); sham dehorning, dehorning, then castration (DH_S--DH_1--$CAST_2$); and sham dehorning and castration, concurrent dehorning and castration, then non-surgical control (CD_S--CD_1--$CONT_2$); and non-surgical control for Period 1 ($CONT_S$--$CONT_1$). *P*-values from analysis of variance are shown.

	Surgical Treatment Sequence				
Receiving measurement	**$CAST_S$-$CAST_1$-DH_2**	**CD_S-CD_1-$CONT_2$**	**$CONT_S$-$CONT_1$**	**DH_S-DH_1-$CAST_2$**	***P*-Value**
Weight, kg	144 (5)	144 (5)	145 (6)	142 (7)	0.99
Right horn base diameter, mm	32 (3)	33 (1)	32 (2)	32 (4)	0.99
Left horn base diameter, mm	33 (2)	33 (3)	34 (1)	34 (2)	0.98
Right horn length, mm	35 (3)	37 (3)	35 (3)	34 (5)	0.96
Left horn length, mm	36 (2)	35 (4)	35 (3)	34 (3)	0.97
Scrotal circumference, cm	18 (1)	18 (1)	17 (1)	18 (1)	0.76

Table 3.2. *P*-values for selected simple effect comparisons of cortisol concentrations of calves undergoing non-painful handling in the sham procedure, or surgical treatments in Periods 1 and 2.

With subscripts indicating either sham handling (S) or the period in which the treatment was performed, the sequences were: sham castration, castration, then dehorning ($CAST_S$--$CAST_1$--DH_2); sham dehorning, dehorning, then castration (DH_S--DH_1--$CAST_2$); and sham dehorning and castration, concurrent dehorning and castration, then non-surgical control (CD_S--CD_1--$CONT_2$); and non-surgical control for Period 1 ($CONT_S$--$CONT_1$).

Comparison	*P*-value													
	Time, min													
	Baseline	5	10	20	30	40	50	60	120	240	360	480	600	720
Within Sham														
$CAST_S$ v. DH_S	0.9	0.2	0.5	1	1	1	1	1	0.9	0.9	1	1	1	0.9
$CAST_S$ v. CD_S	1	0.9	0.6	0.08	0.09	0.3	0.8	0.8	0.6	1	1	1	1	1
$CAST_S$ v. $CONT_S$	1	0.1	0.3	0.5	1	1	1	1	0.8	1	1	1	1	0.4
CD_S v. DH_S	0.9	**0.05**	**0.05**	**0.02**	**0.03**	0.2	0.6	0.6	0.9	1	1	0.9	1	1
CD_S v. $CONT_S$	1	**0.03**	**0.01**	**	**0.04**	0.2	0.6	0.5	1	1	1	1	1	0.7
DH_S v. $CONT_S$	1	1	1	0.8	1	1	1	1	1	1	0.9	1	1	0.8
Within Period 1														
$CAST_1$ v. DH_1	0.4	1	1	1	1	1	0.7	0.9	**	0.07	0.9	0.8	1	0.5
$CAST_1$ v. CD_1	1	1	0.9	1	0.5	0.4	0.3	0.06	0.7	0.9	0.4	0.6	0.9	0.8
$CAST_1$ v. $CONT_1$	1	0.06	***	***	***	***	***	***	***	**0.02**	1	1	1	**0.04**
CD_1 v. DH_1	0.5	1	0.9	1	0.4	0.1	**0.02**	*	***	**0.01**	0.8	1	1	0.07
CD_1 v. $CONT_1$	1	0.1	*	***	***	***	***	***	***	*	0.2	0.7	1	*
DH_1 v. $CONT_1$	0.5	**0.04**	**	***	***	***	***	***	0.7	1	0.7	0.8	1	0.6
Within Period 2														
$CAST_2$ v. DH_2	1	1	0.6	**0.06**	0.3	0.7	0.9	0.9	*	0.9	**0.02**	**0.02**	0.7	0.2
$CAST_2$ v. $CONT_2$	0.4	0.5	0.4	**	***	***	***	***	***	**	0.6	0.9	0.8	0.9
DH_2 v. $CONT_2$	0.6	0.7	0.08	***	***	***	***	**	*	*	**	0.06	1	0.1
Within DH_1-$CAST_2$ sequence														
DH_S v. DH_1	0.4	0.09	*	***	***	***	***	***	0.6	0.8	0.4	0.6	0.6	1
DH_S v. $CAST_2$	0.5	**0.03**	**	***	***	***	***	***	***	**	0.5	1	0.1	0.7
DH_1 v. $CAST_2$	1	0.9	0.9	1	1	1	0.4	0.7	***	**	1	0.7	0.5	0.5
Within $CAST_1$-DH_2 sequence														
$CAST_S$ v. $CAST_1$	1	1	0.06	***	***	***	***	***	**	**	0.7	1	0.8	0.6
$CAST_S$ v. DH_2	0.3	0.8	*	***	***	***	***	***	**0.08**	**	**	*	0.6	1
$CAST_1$ v. DH_2	0.4	0.7	0.4	0.07	0.4	0.8	0.9	0.9	0.2	1	*	*	0.9	0.7
Within CD_1-$CONT_2$ sequence														
CD_S v. CD_1	0.9	0.5	1	*	***	***	***	***	***	***	**0.05**	0.2	0.2	**0.03**
CD_S v. $CONT_2$	0.9	0.6	1	0.7	1	1	1	0.9	0.9	1	1	0.4	0.3	0.3
CD_1 v. $CONT_2$	1	0.09	1	**	***	***	***	***	***	**	0.06	0.8	1	**
Within $CONT_1$ sequence														
$CONT_S$ v. $CONT_1$	0.9	0.6	0.5	0.7	1	0.8	0.9	1	0.9	0.8	0.4	0.8	0.4	0.8
Within CAST or within DH treatment														
$CAST_1$ v. $CAST_2$	0.1	0.6	0.8	0.8	0.8	0.8	0.9	1	0.1	0.6	0.7	0.8	0.2	**0.02**
DH_1 v. DH_2	0.6	0.6	0.2	**0.02**	0.2	0.3	0.4	0.8	0.08	**0.01**	**0.01**	**0.05**	0.9	0.6

Table 3.3. Least squares means (and estimated 95% confidence intervals) for C_{max} and the time at which C_{max} occurred, namely T_{max}, for each treatment.

Log transformed means for T_{max} were back-transformed to the original units for presentation. Serum cortisol was measured following sham handling and following surgical treatment in Periods 1 and 2 of the study. With subscripts indicating either sham handling (S) or the period in which the treatment was performed, the sequences were: sham castration, castration, then dehorning ($CAST_S$--$CAST_1$--DH_2); sham dehorning, dehorning, then castration (DH_S--DH_1--$CAST_2$); and sham dehorning and castration, concurrent dehorning and castration, then non-surgical control (CD_S--CD_1--$CONT_2$); and non-surgical control for Period 1 ($CONT_S$--$CONT_1$).

Treatment	C_{max}, nmol/L	T_{max}, min
Sham		
$CONT_S$	73.3 (54.6 - 92.0)^{a}	14.1 (9.8 - 21.1)^{ab}
DH_S	77.0 (58.3 - 95.6)^{a}	14.5 (10.1 - 20.6)^{abc}
$CAST_S$	90.2 (71.5 - 108.9)^{ab}	14.8 (10.4 - 21.1)^{abc}
CD_S	107.0 (88.4 - 125.7)^{bc}	17.3 (12.1 - 24.7)^{abcd}
Period 1		
$CONT_1$	83.4 (64.8 - 102.1)^{ab}	13.7 (9.4 - 19.8)^{ab}
DH_1	128.3 (109.7 - 147.0)^{cd}	30.2 (21.1 - 43.0)^{e}
$CAST_1$	136.1 (117.4 - 154.8)^{d}	26.8 (18.8 - 38.3)^{de}
CD_1	143.9 (125.2 - 162.6)^{d}	34.1 (23.9 - 48.6)^{e}
Period 2		
$CONT_2$	105.8 (87.2 - 124.5)^{bc}	11.3 (7.9 - 16.1)^{a}
DH_2	145.9 (127.2 - 164.6)^{d}	23.2 (16.3 - 33.2)^{cde}
$CAST_2$	129.3 (110.7 - 148.0)^{cd}	21.9 (15.4 - 31.3)^{bcde}

a-e. Means within entire columns (including sham, Period 1 and Period 2) not connected by the same letter are significantly ($P < 0.05$) different.

Chapter 4 - Pharmacokinetics of oral meloxicam in ruminant and pre-ruminant calves[3]

Introduction

The absence of pain management for common surgical procedures, such as dehorning and castration of cattle, is considered to be an important animal welfare concern and is under consideration in the development of international trade agreements (Phillips, 2008; Thiermann & Babcock, 2005).

Meloxicam is a non-steroidal anti-inflammatory drug (NSAID) that is a potential candidate for use in providing pain relief to cattle. As a class, NSAIDs exert anti-inflammatory action through variable inhibition of the cyclo-oxygenase (COX) isoenzymes which are pivotal catalysts in the prostaglandin production pathway. While the expression of both COX-1 and COX-2 is constitutive and inducible, COX-2 is the isoform which is greatly upregulated in the presence of inflammatory stimuli and is therefore considered to be the desired target of NSAID activity (Lees, 2009). Although the literature is deficient with respect to cattle, meloxicam is considered to preferentially inhibit the inflammatory effects of COX-2 while tending to spare the homeostatic effects of COX-1 in humans (Warner et al., 1999), dogs (Streppa et al., 2002), cats (Giraudel, et al., 2005), and horses (Beretta, et al., 2005). However, the relative inhibition of the COX isoenzymes by a drug is known to vary between species, therefore COX preference in one species does not guarantee similar preference in another (Lees, 2009).

Although meloxicam is not currently approved for use in cattle in the United States, the drug has been approved for that use in in the European Union as well as in countries such as Canada, New Zealand, and Australia, which are major suppliers of beef products to the U.S (USDA, 2009). Depending upon the country, approved indications variously include the use of meloxicam as ancillary treatment of respiratory disease, diarrhea, mastitis and/or pain due to dehorning or disbudding. Based on a dose of 0.5 mg/kg bodyweight delivered intravenously or

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[3] Reprinted with permission from "Pharmacokinetics of oral meloxicam in ruminant and preruminant calves" by R.A. Mosher, J.F. Coetzee, C.A. Cull, R Gehring, B KuKanich. 2011. Journal of Veterinary Pharmacology and Therapeutics. 35(4):373-8. John Wiley and Sons.

subcutaneously, labeled withdrawal times range from 8 to 20 days for meat, and from 84 hours to 6 days for milk (EMEA, 1999; APVMA, 2010; NZFAZ, 2010; Health Canada, 2009).

In the U.S., although flunixin meglumine is approved for the control of pyrexia associated with bovine respiratory disease and for the control of inflammation and pyrexia associated with endotoxemia (FDA, 2003), there is currently no approved NSAID with indications for alleviating pain in cattle. Provisions in the Animal Medicinal Drug Use Clarification Act of 1994 may therefore allow the extra-label use of meloxicam under specific conditions as discussed previously (Coetzee et al., 2009). Candidate products for extra-label use in cattle include oral and injectable small animal formulations, and oral human formulations.

As determined by a study investigating the pharmacokinetics of generic human-label meloxicam when dosed orally to ruminant calves at 1mg/kg BW, the drug was observed to be well absorbed, with a mean bioavailability of 100% (Coetzee et al., 2009). The pharmacokinetic properties of oral meloxicam have not been reported for pre-ruminant calves. Due to differences in diet as well as differences in gastrointestinal, hepatic, and renal function, meloxicam may exhibit a different pharmacokinetic profile in pre-ruminant vs. ruminant calves.

The current study was conducted in two parts. Experiment #1 was conducted to directly compare pharmacokinetic differences between ruminant and pre-ruminant calves when meloxicam was delivered into the rumen via gavage at a dose of 0.5mg/kg body weight. Experiment #2 was conducted to determine the pharmacokinetic profile of oral meloxicam in pre-ruminant calves when the dose was suckled in milk replacer, and thus would likely bypass the rumen.

Materials and Methods

This study was approved by the Institutional Animal Care and Use Committee at Kansas State University.

Animals and housing

Experiment #1: Weaned and non-weaned male Holstein calves were obtained from Kansas dairy herds and acclimated for 4 weeks prior to study initiation. All calves were black and white Holsteins except one pre-ruminant (#31), which was a red and white Holstein. Six

weaned calves, 4-7 months old, and weighing 95-168 kg at time of study were classified as full ruminants and were group-housed on an outdoor concrete pad (9.8 m x 18.3 m) with a partial roof over straw bedding. Six unweaned calves, 6-8 weeks old and weighing 62-89 kg at time of study were classified as pre-ruminants and were similarly housed nearby in individual contiguous enclosures (1.6 m x 5.3 m) constructed with wire panels. Ruminant calves were maintained on water and grass hay *ad libitum* and supplemented with a typical receiving diet composed of cracked corn, oats, soybean meal, molasses and a protein/vitamin/mineral supplement at 6-8 kg/ head/ day. Pre-ruminant calves were primarily maintained on a bucketfed milk replacer diet, fortified with vitamins and minerals, and containing a minimum of 20% crude fat and 25% crude protein derived from milk (first 5 ingredients) and soy protein (Maxicare, Land O'Lakes, MNWater (in a bucket) and calf starter ration (Herd Maker Supreme B90, Land O'Lakes, MN) were offered *ad libitum* throughout the acclimation period and during the study. Upon arrival, study animals were identified with numeric ear tags, vaccinated (Bovishield Gold, Pfizer Inc., NY, NY), and administered oxytetracycline (Noromycin 300 LA, Norbrook Laboratories, County Down, Northern Ireland) IM, 9 mg/kg bodyweight. All calves were surgically castrated 1-2 weeks after arrival and allowed to heal for a minimum of 10 days prior to the study.

At study initiation, the mean (± standard deviation) weights of the ruminant and pre-ruminant groups were 129.2 ± 32.3 kg and 75.5 ± 9.3 kg respectively. Weights for dose calculation were determined by weighing the calves 24 hours prior to treatment administration. Calves were re-weighed on the morning of the study commencement for determination of the administered dose.

Experiment #2: Six Holstein bull calves, 18 – 28 days of age, with mean (± standard deviation) weight of 46.4 ± 10.5 kg, born at the KSU Dairy Unit, were maintained in their accustomed individual housing units (1.2m x 4m) comprised of a covered hutch with attached outside exercise area. Diet consisted primarily of bottle-and-nipple-fed milk replacer, fortified with vitamins and minerals, and containing a minimum of 18% crude fat and 28% crude protein derived from milk products (Mother's Pride, Ridley Inc, Mankato, MN). Water (in a bucket) and starter ration (Super Krunch 22% Calf Starter, Ridley Inc, Mankato, MN), were offered ad libitum during the study.

Experimental design

Experiment #1 was a parallel design with rumen development classification as the explanatory variable, and with pharmacokinetic parameters as the response variables. All calves received meloxicam, PO, at a target dose of 0.5 mg/kg.

Approximately 24 hours prior to study commencement, calves were restrained with a head gate and halter for intravenous catheter placement. The area over the jugular vein was clipped and surgically prepared with alternating scrubs of 70% isopropyl alcohol and povidone iodine. The catheter site was infiltrated with 2% lidocaine injection, 1 mL s.c., (Hospira Inc, Lake Forest, IL) prior to making a stab incision with a #22 scalpel blade. Using sterile technique, a 14 G x 130 mm extended use catheter (MILACATH®, MILA International, Florence, KY) with injection plug (SURFLOW®, Terumo, Somerset, NY) was inserted into the right jugular vein and sutured to the skin using #3 nylon suture (Braunamid®, Braun, Bethlehem, PA). Catheter patency was maintained by flushing with 3 mL of a heparin saline solution containing 3 USP units heparin sodium/mL saline (Heparin Sodium Injection, Baxter Healthcare, Deerfield, IL).

Neither food nor water was withheld at any time during the study; pre-ruminants were bucket-fed their usual quantity of milk replacer 30 minutes prior to dosing.

Meloxicam was administered orally at 0.5 mg/kg (Meloxicam tablets 15 mg (NDC 60505-2554-1), Apotex Corp, Weston, FL; Lot # JD9485). The dose was rounded to the nearest whole tablet and was based upon body weight obtained 24 hours prior to study. Tablets were crushed and mixed in 50 mL of tap water within 5 minutes of administration. After passing a stomach tube with the aid of a Frick speculum, one operator blew air into the tube while another listened through a stethoscope placed over the rumen to assure placement within the gastrointestinal (GI) tract. The drug suspension was delivered through the stomach tube and then chased with 300 mL of water. Air was then blown through the tube to empty all fluid contents into the GI tract prior to removal.

Approximately 6 mL of blood was collected through the catheter port at 0 and 30 minutes and at 1, 2, 3, 4, 5, 6, 8, 10, 12, 18, 24, 36, 48, 60, 72, 96 and 120 hours after administration. Prior to blood sampling, calves were temporarily restrained with a rope halter and the catheter cap cleaned with 70% isopropyl alcohol. The heparin lock solution was flushed from the catheter before each collection by twice drawing 5 mL of blood into the syringe and then

returning it to the calf through the catheter. Blood drawn into the syringe on the third pull was immediately transferred to a lithium heparin vacutainer tube (BD Diagnostics, Franklin Lakes, NJ). Samples were stored on ice prior to centrifugation for 10 minutes at 1,500 x g within 30 minutes of collection. Plasma was then pipetted into cryovials and frozen at -70°C until analysis.

Experiment #2. With the exception of the dose delivery method, the second experiment was carried out as described above. The crushed tablets were suspended in the morning ration of rehydrated milk replacer and offered to the calves in nipple bottles as they were accustomed to feeding. Following consumption of the contents, the bottle was rinsed with 100 mL of water and reoffered to the calves. Dose and rinse water were readily consumed by all calves within 5 minutes of feeding initiation.

Plasma drug analysis

Plasma concentrations of meloxicam (m/z 352.09→114.90) were determined with high-pressure liquid chromatography (Shimadzu Prominence, Shimadzu Scientific Instruments, Columbia, MD, USA) and mass spectrometry (API 2000, Applied Biosystems, Foster City, CA, USA). Plasma samples or standards (100 µL) were added to 100 µL of internal standard (piroxicam 0.5 µg/mL in methanol, m/z 332.12→95.10) and 300 µL of methanol with 0.1% formic acid to precipitate the proteins. The samples were vortexed for 5 seconds and centrifuged for 10 minutes at 10,000 x g. The supernatant, 200 µL, was transferred to an injection vial with the injection volume set to 10 µL. The mobile phase consisted of A: acetonitrile and B: 0.1% formic acid at a flow rate of 0.4 mL/min. The mobile phase consisted of 85% B from 0 – 0.5 minutes with a linear gradient to 50% B at 2.5 minutes which was maintained until 3 minutes, followed by a linear gradient to 85% B at 4 minutes with a total run time of 5 minutes. Separation was achieved with a C8 column (Supelco Discovery C8, 50 mm x 2.1 mm x 5 µm, Sigma-Aldrich, St. Louis, MO, USA) maintained at 40° C. With a limit of quantification of 0.025 µg/mL, the standard curve was linear from 0.025 µg/mL to 10 µg/mL and was accepted if the correlation coefficient exceeded 0.99 and predicted values were within 15% of the actual values. The accuracy of the assay was 103 ± 7% of the actual value and the coefficient of variation was 7% determined on replicates of 5 each at 0.025, 0.5, and 5 µg/mL.

Pharmacokinetic and statistical analysis

Noncompartmental (NCA) and compartmental (CA) pharmacokinetic analyses were performed with computer software (WinNonlin 5.2, Pharsight Corporation, Mountain View, CA, USA). The pharmacokinetic parameters were estimated based upon the actual dose administered to individual calves. Maximum serum meloxicam concentration (C_{max}) and time to maximum serum concentration (T_{max}) were obtained directly from the data. The parameters calculated with NCA included the plasma clearance per fraction of dose absorbed (Cl/F); the first order elimination rate constant (λ_z); and terminal half-life ($t_{½ \lambda z;}$). The observed area under the curve extrapolated to infinity (AUC_{inf}) was calculated using the trapezoidal rule to calculate AUC to the last observed concentration (C_{last}), then adding the extrapolated portion beyond that point as follows:

$$AUC_{INF} = AUC_{last} + \frac{C_{last}}{\lambda_z}$$

The apparent volume of distribution per fraction of the dose absorbed based on the terminal phase (V_z/F) was calculated by the following equation:

$$V_z/_F = \frac{Dose}{\lambda_z \times AUC_{INF}}$$

The fit of compartmental models was compared by inspection of residuals, the Akaike Information Criterion, and the Schwarz Bayesian Criterion (Gabrielsson & Weiner, 2009). A one-compartment model with no lag time, with weighting of 1/(predicted-C)^2 was determined to best fit the data. The parameters estimated through compartmental modeling of mean data included the absorption rate constant (K_{01}); the elimination rate constant (K_{10}); and apparent volume of distribution per fraction of the dose absorbed (V/F). The data were fit to the following one-compartment model with no lag time and with weighting of 1/(predicted C)^2 :

$$C_t = \left[\frac{DFK_{01}}{V(K_{01} - K_{10})}\right] [e^{-K_{10}t} - e^{-K_{01}t}]$$

where C_t is plasma concentration at time, t; and D is administered dose.

Statistical analyses were performed using JMP (SAS Institute Inc, Cary, NC); P-values of ≤ 0.05, were considered statistically significant. The normality assumption was tested for each variable set with the Shapiro-Wilk W test. Group means from the normally distributed variables, AUC,

T_{max}, λ_z, and dose were compared by one-way analysis of variance. When significant differences were detected, pairwise comparisons were performed using the Tukey-Kramer HSD method to protect the experiment-wide level of significance. Means of the non-normally distributed variables, Cl/F, C_{max}, $t_{½}$, V_z/F, K_{01}, K_{10} were compared using a Kruskal-Wallis nonparametric one-way analysis of variance. When significant differences were detected between means within the group, pairwise comparisons were performed with the Wilcoxon two-level nonparametric test to determine which pairs were different.

Upon initial inspection of the data, the pharmacokinetics of meloxicam in Calf #31 in the PRG group appeared to be different from the rest of the PRG group. For that reason all statistical analyses were performed both with, and without, Calf #31. Although the PK parameters, Cl/F, λ_z , AUC_{inf}, C_{max}, MRT and $t_{½\lambda z}$ for Calf #31 were group extremes which biased the mean, the exclusion of that calf's data did not result in a change in the outcome of significance tests, so data were not excluded.

Plasma chemistry analysis

Experiment #1. To investigate the post hoc hypothesis that hypoproteinemia might have affected the pharmacokinetics of meloxicam in Calf #31 in the PRG group, archived plasma samples obtained immediately prior to dosing calves in the first experiment were submitted to the Kansas State University Diagnostic Laboratory for analysis of albumin, globulin, and total protein. Analysis was performed using an automated, software-controlled system (Cobas 6000 analyzer, Roche Diagnostics, Switzerland).

Results

All calves in the first experiment were determined to have normal levels of plasma proteins.

There was not a significant difference between the dose (mean ± SD; range) of meloxicam administered to calves within the groups: PRF (0.530 ± 0.131, 0.350-0.650 mg/kg), PRG (0.509 ± 0.007; 0.497 – 0.518 mg/kg), and RG (0.515 ± 0.018; 0.5 – 0.545mg/kg). There were quantifiable levels of meloxicam in the plasma of all calves at the first timepoint, 30 minutes after dosing. The model-predicted time-concentration curve with observed mean ± SD

concentration for each group is shown in Figure 1. The figure highlights the greater variation in plasma concentration observed among the PRG calves than in the other groups. In Calf #31, of the PRG group, the plasma concentration of meloxicam was below the limit of quantification by 96 h. For comparison, the predicted curves are overlayed without SD bars in Figure 2.

Summary estimates of pharmacokinetic parameters obtained through NCA are presented in Table 1. The only significant difference revealed by NCA occurred between the two pre-ruminant groups. Cmax was lower (P=0.03) in PRF (1.27 ± 0.430 μg/mL) than PRG calves (2.20 ± 0.467 μg/mL), while Cmax of RG calves (1.95 ± 0.955 μg/mL) was not different from other groups. There was a difference (P = 0.055) in V_z/F of the PRF calves (337 ± 78 mL/kg) and the PRG calves (211 ± 75 mL/kg). Noncompartmental pharmacokinetic parameters associated with Calf #31 in the PRG group represented the group minima for AUC_{inf}, Cmax, MRT and $t_{1/2\lambda z}$ and the group maxima for Cl/F and λ_z.

Summary estimates of the pharmacokinetic parameters obtained by fitting the data to a one-compartment model are presented in Table 2. Estimates of V/F were significantly different between the PRF group (365 ± 57 mL/kg) and both the PRG (177 ± 63 mL/kg, $P < 0.01$) and the RG (232 ± 83 mL/kg, P = 0.01) groups. Whereas compartmental modeling revealed a significant difference ($P < 0.01$) between the rate of absorption in the PRF (0.237 ± 0.0478 h^{-1}) and RG (0.0815 ± 0.0188 h^{-1}) groups, there was no difference between the K_{01} estimates for either of those groups and that of the PRG group (0.153 ± 0.128 h^{-1}). Compartmental pharmacokinetic parameters associated with Calf #31 in the PRG group represented the group maxima for Cl/F and K_{10} and the minimum for AUC_{inf}.

Discussion

Between the two experiments described in this study, we examined the pharmacokinetics of meloxicam in 1) ruminant calves dosed orally via gavage, 2) pre-ruminant calves dosed orally via gavage, and 3) pre-ruminant calves dosed orally by mixing the drug in the usual ration of milk-replacer. The pharmacokinetic parameters of oral meloxicam in the ruminant calves in the current study were similar to those reported earlier (Coetzee et al., 2009).

Upon analyzing the results of the first experiment in this study, there were no significant differences apparent between the PRG and RG groups. It was noted, however, that the $t_{½ \lambda z}$ of meloxicam was much shorter in Calf #31 (9.73 h) in the PRG group than the group mean (40 h).

Since meloxicam is highly bound to plasma albumin, and thus its availability for renal excretion is limited, it was initially considered that the relatively short $t_{½ \lambda z}$ of meloxicam in Calf #31 might be due to low levels of albumin in the bloodstream. To test this hypothesis, archived samples of plasma obtained immediately prior to dosing were analyzed, but revealed no evidence of hypoalbuminemia. It was also considered that the shorter $t_{½ \lambda z}$ in that particular calf may have been due to the calf reflexively closing the reticular groove during dosing and causing the drug to bypass the rumen. To test this hypothesis, the second experiment was conducted to evaluate the pharmacokinetics of meloxicam in calves when the dose was delivered in a manner likely to be delivered directly to the abomasum. The minimum $t_{½ \lambda z}$ value observed in the PRF group however, was 27.3 h, which was similar to the minimum observed in the RG group of 22.8 h, so it was considered unlikely that the short $t_{½ \lambda z}$ observed in Calf #31was due to the drug simply bypassing the rumen. The reason for the shorter $t_{½ \lambda z}$ in that calf was not definitively determined, though a possible explanation could be increased metabolic enzyme activity in that individual. This supposition is supported by Calf #31 exhibiting the group minimum Cmax, the group maximum Cl/F, yet the Vz/F was not an extreme value.

Of note, Calf #31 was the only Holstein of red color in the study. The red color in the Holstein breed is a recessive trait, with black being the dominant color. Thus a red Holstein carries two copies of the gene for red color, whereas a black Holstein may carry two copies of the gene for black color, or may carry one for each color (Specht, 2009). It is not known if there was a genetic association between the coat color of Calf #31 and what appeared to be an increased rate of drug metabolism. As shown in Table 3, the pharmacokinetics of meloxicam in black Holstein calves in an IV/PO randomized crossover study (Coetzee et al. 2009) were of similar range to those in the current study. When meloxicam was given orally to 6 beef calves of mixed breed origins, the ranges for Cl/F, Vz/F, and $t_{1/2\lambda z}$ were similar to those seen with black Holstein calves, with the exception of a minimum $t_{1/2\lambda z}$ value of 8.4 h which is similar to that of the red Holstein (Calf #31, 9.7 h) in the current study. Therefore, Calf #31, while initially appearing to be a possible outlier, may represent a population of calves with increased metabolism.

In cattle, meloxicam is at least 96.5% bound to plasma proteins (EMEA, 1999) and must undergo extensive hepatic biotransormation to be eliminated from the body (Busch et al., 1998). In humans, meloxicam is metabolized by peroxidase and by two enzymes in the cytochrome

P450 monooxygenase superfamily, CYP2C9 and CYP3A4 (Boehringer-Ingleheim, 2010). Both CYP2C9-like and CYP3A4 catalytic activity is expressed in the bovine liver (Ioannides, 2006). Meloxicam metabolites detected in cattle are pharmacologically inactive and include those found in other species (5'-hydroxymethyl-, 5'-carboxy-, and oxalyl- metabolites) as well as an additional polar metabolite (EMEA, 1999). CYP450 genes are highly polymorphic and recent studies of select CYP450 genes in cattle indicate differences in transcription between sexes and between the breeds studied (Ashwell et al., 2011; Giantin et al., 2008). Such differences may underlie the relatively short $t_{½ \lambda z}$ of the red Holstein (Calf #31) in the current study and of the beef calf in Coetzee et al., 2009.

PK data from the PRF group were statistically compared to that of the PRG and RG groups, although the experiments were conducted in trials approximately one month apart in separate facilities. And although the PRF and PRG groups both had immature rumen development and were consuming regular feedings of milk replacer, the PRF calves were 18 to 28 days of age whereas the PRG calves were 6-8 weeks of age. Furthermore, the milk replacer fed to the pre-ruminant groups was of two different brands. Due to these uncontrolled factors, statistical comparisons should be interpreted with caution, but they do allow us to make certain interpretations and conjectures which may be useful clinically and for future controlled studies.

In the second experiment, when the dose was delivered to pre-ruminant calves through suckling a suspension of drug in milk replacer, and thus directly entering the abomasum, Cmax was lower and V/F was higher than in the gavaged pre-ruminants. In comparison with the gavaged ruminant calves, the PRF group estimate of V/F was higher and K_{01} was faster. These differences between the suckled group and the gavaged groups were likely due largely to differences in bioavailability created by the delivery of drug into different stomach compartments and by a probable food-drug interaction with milk-replacer in the PRF calves.

There was greater variability in pharmacokinetic parameter estimates between individuals within each of the pre-ruminant groups than between individuals within the ruminant group. This increased variability within the pre-ruminant groups may have been due to a combination of factors such as amount of starter ration and/or milk replacer consumed on the day of dosing, the presence of undiagnosed enteric pathology, differences in maturation of the hepatic metabolic enzyme system, differences in rumen maturity, and closure kinetics of the reticular groove while

consuming the dose in milk replacer. (De Backer & Debackere, 1979; Marriner, 1979; Nouws,1992).

The V/F of PRF calves was higher than that of both PRG and RG calves. Unless combined with an IV study, the fraction of the dose absorbed cannot be estimated with extra-vascular PK models, and so cannot be entered into calculations of clearance and volume of distribution. Therefore, those parameters are reported relative to the fraction of the dose absorbed. Thus, the difference in V/F noted between groups may be a result of differences in volume of distribution and/or bioavailability. It is unlikely, especially when comparing the pre-ruminant groups, that the volumes of distribution are greatly different, making the observed V/F differences more likely due to bioavailability issues. Differences in hepatic maturation could explain how significant differences in V/F could occur due to bioavailability, yet Cl/F could be similar between all groups. If Cl and F were both reduced in the PRF group, the ratio of Cl/F could remain similar to groups with higher Cl and F. The lower Cmax observed in the PRF group with respect especially to the PRG group supports the premise of a lower bioavailability in the former group.

In addition to anatomic differences, pre-ruminant and ruminant calves differ in the constancy of abomasal pH, which may affect the drug absorption process. Meloxicam is a weak acid which is nearly insoluble in water and has a peak liquid-aqueous partition coefficient of 2.68 at pH 3 (Luger et al., 1996). Thus absorption of the drug is favored in relatively acidic areas of the ruminant gastrointestinal tract. Although pH in the rumen may range from approximately 5.5 – 7.0, it is likely that little absorption of meloxicam occurs in that compartment, with the rumen serving mostly as a reservoir for extended release of drug into the remaining gastrointestinal tract. In studies of acetaminophen, which is unionized at physiological pH, an insignificant amount of the drug was absorbed from the rumen (Schaer et al., 2005). In the non-fasting ruminant calf, the constant flow of ingesta from the forestomach compartments results in a relatively stable pH value of about 2.1 - 2.2 in the abomasum. In the pre-ruminant calf maintained primarily on a diet of milk or milk-replacer, the abomasal pH is much more variable depending upon the time relative to feeding. Immediately following suckling, the abomasal pH quickly rises to approximately 6, depending upon the pH of the ingested milk or milk-replacer, after which the pH remains relatively constant for up to 2 h, then as the milk curd is digested, gradually declines over 7-9 h to reach pre-prandial values in the pH range of 1-2 (Church, 1993;

Constable et al., 2006). In the current study, although both pre-ruminant groups ingested milk replacer in the peri-dosing period, and would have likely experienced a similar rise and fall of abomasal pH, the time differential in dosing relative to feeding may have resulted in a great enough offset of the pattern that the pH environment in the GI tract was less conducive to absorption in the PRF group than either of the gavaged groups.

Although the pre-ruminant groups consumed different brands of milk replacer, another difference was in the timing of consumption relative to administration of the meloxicam dose. While the PRF calves consumed milk replacer at the same time as the drug, PRG calves consumed their ration approximately 30 minutes prior to dosing. There is considerable variation in extent of curd formation between and within brands of milk replacer. (Okada et al., 2009; Heinrichs et al., 1995). We might, however, assume there was at least some curd formation in the PRF and PRG calves in this study, since the primary ingredients of both milk replacers were dried milk products (Heinrichs et al., 1995). In an *in vitro* coagulation study, after acetaminophen was mixed with milk and allowed to clot, it was found that the dose was divided into approximately equal halves between the curd and whey portions (Herrli-Gygi et al., 2008). Likewise, in the PRF calves of the current study, a portion of the meloxicam dose would likely have been trapped within the curds as they formed, whereas the remaining portion of the dose was available to pass into the small intestine for early absorption with the whey fraction. The meloxicam which was bound within the curd would have been released for absorption as the curd was slowly digested. With the PRG calves however, most of the curd was likely already formed by the time the dose of meloxicam was administered. Thus, as the drug entered the abomasum from the rumen of the PRG calves, most of the dose likely flowed past the curd and was available for absorption in the small intestine.

In calves, past studies with ampicillin, penicillin, tetracycline, chloramphenicol, trimethoprim, and acetominophen showed lower bioavailability when fed suspended in milk replacer compared to water (Nouws, 1992; Schaer et al., 2005). Proposed mechanisms included chelation with calcium and/or binding to milk proteins. The literature is deficient in studies investigating the bioavailability of meloxicam administered in electrolyte or milk replacer formulations, but inferences might be drawn from the current study. Considering that a fraction of the meloxicam dose may have been bound in curd in the PRF calves, this could explain the

apparent lower bioavailability with respect to the PRG calves which also received milk in the peri-dosing period.

Although the rates of absorption in the pre-ruminant calves were both numerically greater than the K_{01} of the ruminant group, only the PRF and RG groups were significantly different. This is likely due to the delivery of meloxicam into the rumen of calves in the PRG and RG groups, whereas in the PRF group, most of the dose likely bypassed the rumen and was delivered into the abomasum. Therefore, different rates of oroduodenal passage would have occurred, with the greatest portion of the difference likely occurring in the passage of drug from the rumen to the abomasum. A radiographical imaging study showed that outflow of fluids delivered to the rumen of pre-ruminant calves occurred mostly within 3 hours (Lateur-Rowet & Breukink, 1983). With respect to adult ruminant cattle, one study indicated ruminal turnover rates for liquid, grain, and hay as 8.1, 4.4, and 3.9 percent per hour (Hartnell & Satter, 1979). Thus, although meloxicam was detected in the plasma of all groups at 30 minutes post-dosing, the rate of passage from the rumen into the abomasum and small intestine was likely the limiting factor which resulted in the lower rate of absorption seen in the RG group vs. the PRF group.

Conclusion

Oral delivery of meloxicam may be useful in administering analgesia prior to surgical procedures such as dehorning and castration of calves. Meloxicam can be successfully administered to pre-ruminant calves through feeding a suspension of drug powder in the usual ration of milk-replacer, though peak plasma concentrations may be reduced due to possible interaction with curd-forming proteins. This may possibly reduce therapeutic response. Also, Part 530 of Title 21 in the Code of Federal Regulations, which implements the AMDUCA, prohibits extra-label use of a drug in or on an animal feed (FDA, 21CFR.530). For both of those reasons, it may be preferable to administer oral meloxicam to pre-ruminant calves in a manner which causes the drug to enter the abomasum after milk proteins have clotted. This could be achieved, by delivering the dose directly into the rumen following feeding, as in the current study, or by waiting 1-2 hours after feeding before offering the dose in a non-clotting carrier to be suckled. A commonly suggested conservative withdrawal period for an extra-label drug used in a food animal, is 10 terminal half-lives (Riviere & Sundlof, 2009). Based upon the largest

mean terminal half-life of 40 h observed in this study, a withdrawal period of 20 days might be recommended following the oral dosing of meloxicam to pre-ruminant calves.

Although plasma concentration profiles are more variable in pre-ruminants, the total drug exposure, as indicated by the AUC, is similar to that of older ruminant calves. Controlled studies are necessary, however, to determine if differences in relative bioavailability exist between dosing oral meloxicam at various times, before, during, and after feeding milk replacer. Further study is also needed to determine if any resulting differences are clinically significant.

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Figures and Tables

Figure 4.1. Plasma meloxicam concentration (mean + SD) following single 0.5 mg/kg PO administration to ruminant calves via gavage (RG), pre-ruminant calves via gavage (PRG) and pre-ruminant calves via feeding in milk replacer (PRF).

Solid lines represent group mean concentration fit to a one compartment model, with weighting of 1/(predicted C)^2. In the PRG group, plasma meloxicam concentration was below the limit of quantitation for Calf #31 at 96 and 120 h.

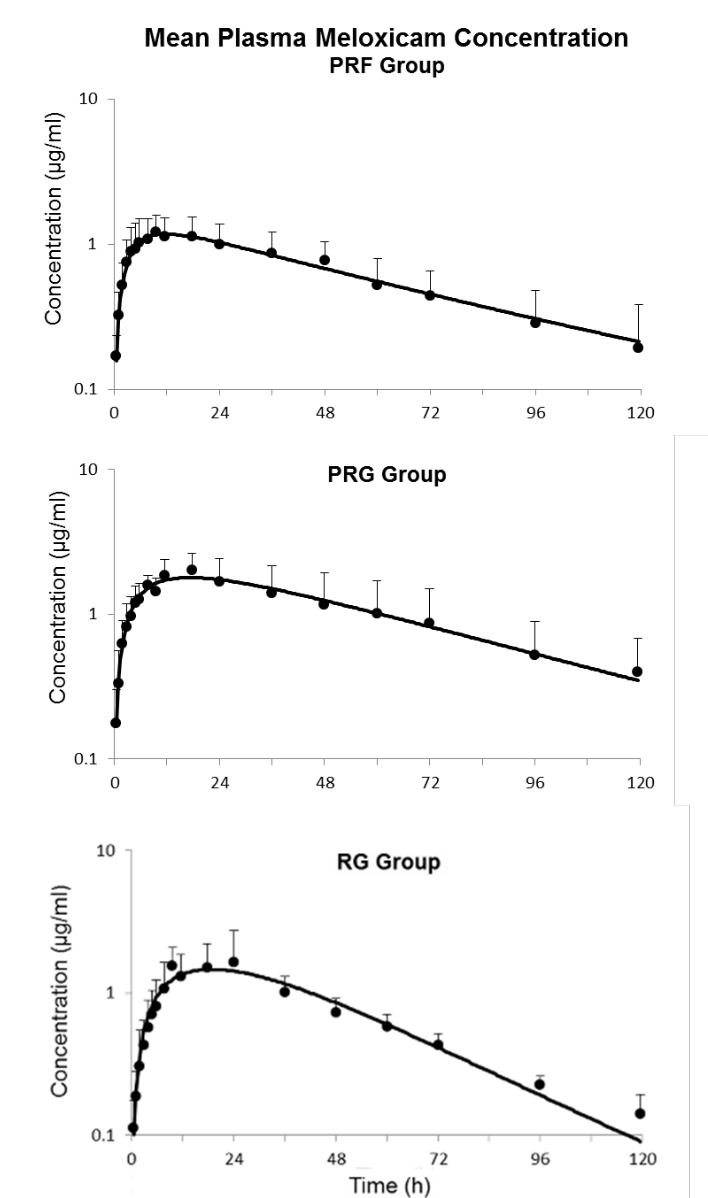

Figure 4.2. Group mean concentration fit to a one compartment model, with weighting of 1/(predicted C)2 of plasma meloxicam concentration following a single 0.5 mg/kg PO administration to ruminant calves via gavage (RG), pre-ruminant calves via gavage (PRG) and pre-ruminant calves via feeding in milk replacer (PRF).

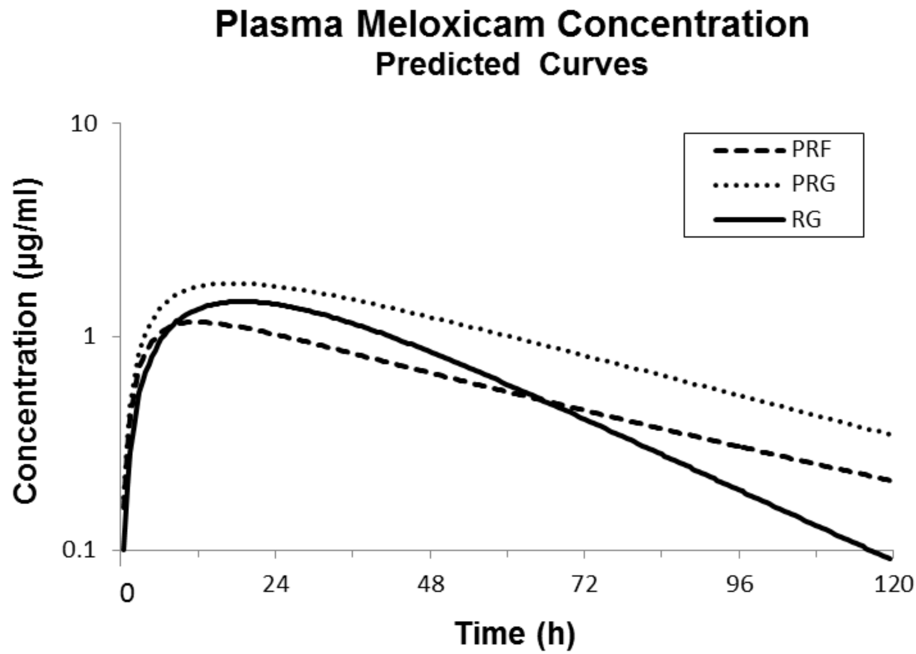

Table 4.1. Pharmacokinetic parameters obtained from non-compartmental analysis of meloxicam after single 0.5 mg/kg PO administration in pre-ruminant calves dosed via ingested milk (PRF) or via gavage (PRG) and ruminant calves via gavage (RG).

Within a column, superscripts not containing the same letter denote a significant difference between associated parameter means (P < 0.05). Similarly, symbols denote a nearly significant difference (P=0.055). †Pharmacokinetic values for Calf #31 in the PRG group which are extremes.

Parameter	Group	Mean	SD	Minimum	Median	Maximum
AUCinf	PRF	85.4	46.6	49.6	69.7	179.0
(h*µg/mL)	PRG	151.0	80.2	39.6†	193.0	218.0
	RG	86.7	28.9	54.2	83.0	137.0
Cl/F	PRF	6.92	2.10	3.66	7.26	9.74
(mL/h/kg)	PRG	5.28	4.52	2.34	2.63	13.10†
	RG	6.45	1.92	3.80	6.45	9.28
C_{max}	PRF	1.27 [a]	0.430	0.726	1.29	1.79
(µg/mL)	PRG	2.20 [b]	0.467	1.630†	2.37	2.65
	RG	1.95 [ab]	0.955	1.020	1.85	3.74
λ_z	PRF	0.0206	0.0051	0.0118	0.0209	0.0254
(h^{-1})	PRG	0.0256	0.0229	0.0106	0.0169	0.0713†
	RG	0.0238	0.0045	0.0188	0.0238	0.0304
MRT	PRF	58.4	17.5	42.4	52.9	91.4
(h)	PRG	63.5	29.4	20.5†	70.5	98.8
	RG	50.3	8.3	41.1	49.5	59.8
$t_{½ \lambda z}$	PRF	36.0	11.7	27.3	33.3	58.5
(h)	PRG	40.0	19.8	9.7†	41.2	65.3
	RG	29.9	5.6	22.8	29.1	37.0
T_{max}	PRF	14.3	6.7	6.0	14.0	24.0
(h)	PRG	17.0	7.0	6.0	18.0	24.0
	RG	17.3	6.3	10.0	18.0	24.0
V_z/F	PRF	337 ▲	78	289	313	495
(mL/kg)	PRG	211 •	75	140	185	340
	RG	281•▲	103	156	304	407

Table 4.2. Pharmacokinetic parameters obtained from fitting a one-compartmental model, with no lag time, and with weighting of 1/(predicted C)^2 to data obtained from administering oral meloxicam, 0.5 mg/kg, to pre-ruminant calves dosed via ingested milk (PRF) or via gavage (PRG) and ruminant calves via gavage (RG).

Within a column, superscripts not containing the same letter denote a significant difference between associated parameter means ($P < 0.05$). †Pharmacokinetic values for Calf #31 in the PRG group which are extremes.

Parameter	Group	Mean	SD	Minimum	Median	Maximum
AUC	PRF	87.0	46.2	49.0	74.2	178
(h*µg/mL)	PRG	149.0	79.0	38.8†	186.0	220
	RG	86.2	26.4	53.6	85.4	129
Cl/F	PRF	6.80	2.16	3.65	7.29	9.74
(mL/h/kg)	PRG	5.34	4.56	2.31	2.74	13.40†
	RG	6.44	1.89	4.01	6.30	9.37
K_{01}	PRF	0.2370[a]	0.0478	0.1770	0.2370	0.294
(h^{-1})	PRG	0.1530[ab]	0.1280	0.0449	0.0898	0.341
	RG	0.0815[b]	0.0188	0.0525	0.0792	0.104
K_{10}	PRF	0.0188	0.0059	0.0112	0.0211	0.0253
(h^{-1})	PRG	0.0310	0.0275	0.0119	0.0219	0.0858†
	RG	0.0289	0.0064	0.0213	0.0285	0.0373
V/F	PRF	365[a]	57	295	353	438
(mL/kg)	PRG	177[b]	63	106	174	283
	RG	232[b]	83	130	235	322

Table 4.3. Selected pharmacokinetic parameters from two published studies in which meloxicam was administered orally to cattle.

In Study 1, calves were 4-6 month old black and white Holstein calves which received meloxicam IV and PO (gavage) in randomized order. In Study 2, calves were 6-8 month old calves of mixed breed origin which were dosed orally via gavage concomitantly with 15mg/kg, PO gabapentin.

Study	Parameter (Units)	Dose (mg/kg)	Route	Mean	SD	Minimum	Median	Maximum
1	Cl (mL/h/kg)	0.5	IV	6.1	0.4	4.8	6.0	7.2
1	Cl/F (mL/h/kg)	1.0	PO	6.4	0.6	3.6	6.6	7.8
2	Cl/F (mL/h/kg)	0.5	PO	6.0	1.2	4.2	6.0	7.1
1	$t_{½\ \lambda z}$ (h)	0.5	IV	20.4	0.7	17.8	20.6	22.8
1	$t_{½\ \lambda z}$ (h)	1.0	PO	28.6	3.6	20	26.3	43.3
2	$t_{½\ \lambda z}$ (h)	0.5	PO	20.5	9.2	8.4	17.6	33.2
1	V_z (mL/kg)	0.5	IV	102	6	80	100	120
1	V_z/F (mL/kg)	1.0	PO	246	20	202	234	321
2	V_z/F (mL/kg)	0.5	PO	160	40	87	156	218

1. Coetzee et al., 2009.
2. Coetzee et al., 2010.

Chapter 5 - Effect of oral meloxicam on performance and health of stocker calves after castration[4]

Introduction

In the beef cattle industry, at weaning time, calves generally undergo a period of extreme stress in which they are weaned from their dams, transported for various distances, commingled with other calves at either the livestock market or feedlot facility, and undergo a change in feeding practice. The immune system is negatively affected by such stressors, which makes the animal more susceptible to contracting infectious agents and succumbing to disease at a time when commingled with calves of different backgrounds. Bovine respiratory disease (BRD), with multiple causative bacterial and viral organisms, is the most common cause of illness and death in feedlot calves, with the highest incidence of disease occurring within 28 days of arrival at the feedyard (Buhman et al., 2000; Duff and Galyean, 2007). The disease is costly in terms of animal welfare, feedlot performance, carcass quality, medicines, time, and manpower. In comparison to calves not treated for BRD, it has been reported that the profit (carcass value - total feedlot costs, in year 2001 dollars) is $40.64 less for calves treated once for BRD, $58.35 less for calves treated twice, and $291.93 less for calves requiring three treatments (Fulton et al., 2002).

Bull calves may be castrated before weaning, but many are not castrated until reaching the feedlot, further stressing the animal. The surgical castration of cattle is generally followed by a period of decreased animal performance as evidenced by reduced average daily gain (ADG), daily feed intake (DFI), and gain-to-feed ratio (ADG:DFI), (Faulkner et al., 1992; Bretschneider, 2005; Stafford and Mellor, 2005; AVMA, 2011; Massey et al., 2011). Furthermore, surgical castration is associated with increased levels of cortisol and acute phase proteins such as

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[4]Data republished with permission from "Effect of oral meloxicam on health and performance of beef steers relative to bulls castrated on arrival at the feedlot" by J.F. Coetzee, L.N. Edwards, R.A. Mosher, N.M. Bello, A.M., O'Connor, B. Wang, B. KuKanich, D. A. Blasi. 2012. Journal of Animal Science. 90:1026-1039. American Society of Animal Science.

haptoglobin, with ensuing immune suppression which may increase the likelihood of the animal succumbing to disease. (Fisher et al., 1997; Earley and Crowe, 2002).

Studies have reported beneficial health and performance effects from administering a non-steroidal anti-inflammatory drug (NSAID) in the peri-castration period (Fisher et al., 1997; Earley and Crowe, 2002; Baldridge et al., 2011). In those studies, the drugs were delivered in either the drinking water (sodium salicylate) or by intravenous injection (ketoprofen). Recent studies indicate that orally-dosed generic meloxicam, with a plasma half-life of approximately 24 h, may be a relatively inexpensive and easily-administered alternative for delivering NSAID medication in a feedlot setting (Coetzee et al. 2009, 2010). The objective of this study was to investigate the effect of oral meloxicam on performance and health of bull calves following surgical castration.

Materials and Methods

This study was approved by the Institutional Animal Care and Use Committee at Kansas State University (KSU).

The study was a stratified parallel design, with calves placed in one of two strata depending upon the time of castration. Calves in the STR strata were castrated prior to arrival to at the study facility, with no evidence of scrotal inflammation or scab. Calves in the XBL strata were received as bulls and then castrated approximately 24h following arrival. The drug treatment factor consisted of two levels: 1) meloxicam, 1 mg/kg bodyweight administered orally on arrival (MEL) and 2) a lactose powder placebo (CONT) administered similarly. Thus, the two strata and treatment combinations were: steers to be sham castrated and receive a placebo (STR-CONT); steers to be sham castrated and receive meloxicam (STR-MEL); bulls to be surgically castrated and receive a placebo (XBL-CONT); and bulls to be surgically castrated and receive meloxicam (XBL-MEL).

Animals and housing

Over a 2 week period in March 2010, two hundred fifty-eight crossbred beef calves, (145 bulls and 113 steers) were obtained from Tennessee livestock commission barns and, in three loads of 83 to 88 calves each, transported approximately 1100 km to the KSU Beef Stocker Unit where the study was conducted. One truckload (Lot) of calves was processed per day, with at

least three days intervening between each load. The calves were approximately 6 to 8 months old, with a mean weight ± SD and range of, 246 ±16 kg, 194 – 285 kg.

Upon arrival, calves were weighed and identified with numeric ear tags. An ear notch tissue sample was collected for bovine viral diarrhea (BVD) analysis by antigen capture ELISA, and all calves were subsequently confirmed negative for BVD virus. Scrotal palpation was performed to determine if calves were to be placed in either the XBL or STR stratum. Only steers with well-healed scrota and no scrotal inflammation were enrolled in the study. Using a previously-prepared assignment sheet (one for each strata) constructed by assigning random numbers to each block of two animals of the same gender status, calves were assigned to either the MEL or CONT group and dosed immediately thereafter. To calves in the MEL group, meloxicam tablets (Meloxicam Tablets USP 15 mg (NDC 29300-125-01), Unichem Pharmaceuticals (USA) Inc. Rochelle Park, NJ; Lot # GMMH09021) were administered orally with a target dose of 1 mg meloxicam/kg bodyweight. The dose was rounded down to the nearest whole tablet, pulverized with mortar and pestle, suspended with tap water in a 60 ml dosing syringe, then administered into the oropharynx of the calf. Calves in the CONT group were similarly dosed, substituting in place of meloxicam, an equivalent volume of D(+) lactose monohydrate powder (Fluka Analytical, Buchs, Germany) which is a pharmacologically inert carrier used in the manufacture of meloxicam tablets. KSU Stocker Unit personnel were blinded to the MEL and CONT treatments.

After processing and dosing, the truckload of calves was housed overnight in 6 holding pens where they were given prairie hay and water. On d 1, approximately 24 h after the dosing, calves were returned to the chute area for further processing and the administration of the castration treatment. Blood samples were collected from the jugular vein of all calves into heparin tubes for determination of plasma meloxicam levels. Calves were vaccinated with a commercial modified-live, viral respiratory vaccine containing infectious bovine rhinotracheitis virus, parinfluenzavirus-3, bovine viral diarrhea virus, bovine respiratory syncytial virus and *Mannheimia haemolytica* (Pyramid 5+ Presponse SQ, Fort Dodge Animal Health, Wyeth, Madison, NJ), a multivalent clostridial vaccine (Calvary 9, Intervet/Schering-Plough Animal Health, Boxmeer, Netherlands), injectable ivermectin (Ivomec, Merial Limited, Duluth, GA) and a metaphylactic antimicrobial, ceftiofur crystalline free acid (Excede, Pfizer Animal Health, New York, NY).

Castration was performed by the same experienced person using an open surgical technique. Briefly, the scrotum of each calf was cleaned with dilute chlorohexidine disinfectant and split twice vertically with a Newberry knife (Jorgensen Lab, Loveland, CO). The testes and spermatic cords were exteriorized by blunt dissection and manual traction. The spermatic cords were cut using a White's Double Crush emasculator (Jorgensen Lab, Loveland, CO) applied for approximately 30 s. The scrota of steers were cleaned and handled, but no incision was made.

Calves were then diverted into their assigned pens, which had been determined by creating two blocks per truckload based upon the previous day's receiving weight. Through random number assignment, calves in each block were assigned to one of two pens per treatment. In the feedlot, pens were blocked in groups of four, with treatments assigned sequentially in the following order: XBL-CONT, STR-CONT, XBL-MEL, and STR-MEL. After all 3 truckloads were received and processed, there were 6 pens of each treatment. Groups of 14 or fewer calves, all treated alike, were housed in open, dirt pens measuring 192 m^2 with 12 m of bunk space and one water source. The stocking density (mean ± SD and range) of XBL calves was 12 ± 1 (10 to 14), and of STR calves was 9 ± 1 (8 to 11); and was balanced between MEL and CONT treatments within the strata.

On d 14, calves were reweighed and revaccinated with the viral respiratory vaccine (Pyramid 5+ Presponse SQ, Fort Dodge Animal Health, Wyeth, Madison, NJ) and given an application of pour-on eprinomectin (Eprinex, Merial Limited, Duluth, GA). On d 28, calves were weighed for the final time at the end of the study.

Feeding program

On arrival, calves were provided a diet consisting of prairie hay containing 7.0% crude protein and 0.44 mcal/kg NEg and ad libitum water. Beginning 2 days after arrival, in the percentages outlined on Table 1, the calves were fed a total mixed ration (TMR) consisting of prairie hay, alfalfa hay, dry rolled corn, wet corn gluten feed, and a commercial premix pellet (Cargill Animal Nutrition, Minneapolis, MN). This ration was formulated to contain 15.2% crude protein and 1.09 mcal/kg NE_g. Beginning 8 days after arrival and continuing through day 18, calves were fed a TMR incorporating the same ingredients as above, but containing 15.2% crude protein and 1.14 mcal/kg NE_g. On day 19 and continuing through the study endpoint, calves were fed a TMR utilizing the same ingredients formulated to contain 14.4% crude protein

and 1.20 mcal/kg NE_g. Daily feed allowances to each pen were recorded along with the weight of unconsumed feed to arrive at daily pen intake.

Health program

Twice daily, personnel at the KSU Beef Stocker Unit evaluated the general appearance and attitude of the cattle. If a calf appeared unthrifty as gauged by posture and behavior, it was brought to the processing area to complete the health evaluation and obtain a rectal temperature and to determine if the animal met the criteria for inclusion in the study as a BRD event. For diagnosis as a BRD event, cattle must have registered a rectal temperature of >103.6ºF (39.8°C) and met a minimum visual depression score of 2 as described on Table 2, without symptoms attributable to bodily injury or disease unrelated to the respiratory system.
Calves with visual signs of BRD and a temperature of <103.6ºF were not treated initially, but if signs were present for two consecutive days, calves were treated with an antimicrobial regardless of temperature. All calves diagnosed with BRD were treated with a single dose of 12.5 mg/kg SC enrofloxacin (Baytril, Bayer Animal Health, Shawnee Mission, KS). After 72 h post-treatment, if the calf's temperature was >103.6ºF (39.78°C), it was treated secondarily with 40 mg/kg florfenicol SC (Nuflor, Intervet/Schering-Plough Animal Health, Boxmeer, Netherlands). If the temperature was calf >103.6°F (39.78°C), after another 72 h, it was treated with 22 mg/kg oxytetracycline (Biomycin 200, Boehringer Ingelheim Vetmedica, Inc, St Joseph, Mo.). After treatments, cattle were returned to their home pen. Animal health data recorded for each calf identified as sick included the pull date, the individual animal identification number, body temperature, clinical score, a presumptive diagnosis, bodyweight and treatment.

Other health conditions that were monitored and included in calculation of overall morbidity included lameness, scrotal infection, and coccidiosis. Diagnosis of lameness was based on signs of persistent limping and reduced weight bearing on one or more limb during standing and walking. A diagnosis of scrotal infection was based on signs of depression, fever and the presence of swelling and purulent discharge from the castration site. A diagnosis of coccidiosis was based on signs of diarrhea, anorexia and depression and the presence of coccidia oocysts on microscopic examination of the feces.

Animals were removed from the study only if a significant illness or injury that compromised the welfare of the animal occurred. All calves that became severely injured or

moribund were humanely euthanized. Animals that died or were euthanized during the study were transferred to the KSU Veterinary Diagnostic Laboratory for necropsy and disposal.

Calculation of response variables

Average daily gain (ADG), days on feed (DOF), and daily dry matter intake (DMI) were calculated for all animals that completed the 28-day study. All calculations, based upon (Hannon et al., 2009) were as follows:

$$ADG = \frac{Weight\ (d\ 28) -\ Arrival\,Weight\ (d\ 0)}{DOF}$$

$$DMI = \frac{Daily\ Pen\ Feed\ allocation -\ Feed\ remaining\ at\ next\ feeding}{Number\ of\ head\ in\ the\ pen}$$

$$Gain\ to\ feed\ ratio\ = \frac{Pen\ level\ ADG}{Pen\ level\ DMI}$$

$$Pull\ Rate\ (\%) = \frac{No.of\ calves\ pulled\ for\ treatment}{No.of\ calves\ on\ study} \times 100\%$$

$$First\ Re - pull\ Rate\ (\%) = \frac{No.of\ calves\ pulled\ a\ second\ time}{No.of\ calves\ pulled\ once} \times 100\%$$

$$Second\ Re - pull\ Rate\ (\%) = \frac{No.of\ calves\ pulled\ a\ third\ time}{No.of\ calves\ pulled\ a\ second\ time} \times 100\%$$

$$Overall\ Morbidity\ rate\ (\%) = \frac{No.of\ calves\ treated\ for\ disease}{No.of\ calves\ on\ study} \times 100\%$$

$$BRD\ morbidity\ rate\ (\%) = \frac{No.of\ calves\ with\ case\ definition\ for\ BRD}{No.of\ calves\ on\ study} \times 100\%$$

$$First\ BRD\ Relapse\ rate\ (\%) = \frac{No.of\ First\ BRD\ Relapses}{No.of\ animals\ treated\ for\ BRD} \times 100\%$$

$$Second\ BRD\ Relapse\ rate\ (\%) = \frac{No.of\ Second\ BRD\ Relapses}{No.of\ first\ BRD\ Relapses} \times 100\%$$

Plasma meloxicam analysis

Although blood samples were obtained from all calves for consistency of treatment, only samples from calves in the MEL group were assayed for meloxicam concentration. Blood samples collected on d 1 were centrifuged for 10 min at 1,500 *g,* plasma was then harvested, then placed in cryovials, and held at -70° C until analysis. All samples were analyzed within 60 days after sample collection.

Plasma concentrations of meloxicam (mass:charge ratio [*m/z*] 352.09→114.90) were determined with high-pressure liquid chromatography (Shimadzu Prominence, Shimadzu Scientific Instruments, Columbia, MD) and mass spectrometry (API 2000, Applied Biosystems, Foster City, CA). Plasma samples or standards (100 µl) were added to 100 µl of internal standard (piroxicam 0.5 µg/ml in methanol, *m/z* 332.12→95.10) and 300 µl of methanol with 0.1% formic acid to precipitate the proteins. The samples were vortexed for 5 seconds and centrifuged for 10 minutes at 10,000 ×*g*. The supernatant, 200 µl, was transferred to an injection vial with the injection volume set to 10 µl. The mobile phase consisted of A: acetonitrile and B: 0.1% formic acid at a flow rate of 0.4 ml/min. The mobile phase consisted of 85% B from 0 to 0.5 minutes with a linear gradient to 50% B at 2.5 minutes, which was maintained until 3 minutes, followed by a linear gradient to 85% B at 4 minutes, with a total run time of 5 minutes. Separation was achieved with a C8 column (Supelco Discovery C8, 50 mm × 2.1 mm × 5 µm; Sigma-Aldrich, St. Louis, MO) maintained at 40°C. The standard curve was linear from 0.01 to 10 µg/ml and was accepted if the correlation coefficient exceeded 0.99 and predicted values were within 15% of the actual values. The accuracy of the assay was 103% ± 7% of the actual value, and the coefficient of variation was 7%, determined on replicates of 5 each at 0.025, 0.5, and 5 µg/ml.

Statistics

When all calves were received, enrolled, and randomized, the treatment groups and number of animals in each were: XBL-CONT (n=74), XBL-MEL (n=71), STR-CONT (n=55), and STR-MEL (n=58).

Health, performance, and plasma meloxicam data were analyzed using generalized linear mixed models fitted with the GLIMMIX procedure of SAS Version 9.2 (SAS Institute, Cary, NC). Performance outcomes (DMI, ADG, G:F ratio) and meloxicam concentrations were modeled as Gaussian using an identity link function. The frequency of health events (Pull, morbidity, BRD) in a given pen were modeled using a binomial distribution whereby the number

of binomial trials was defined by number of animals in a pen. The logit link function was used to model health events. Pen served as the experimental unit for all outcomes. Least square mean estimates for each treatment group and the corresponding estimated SE are reported. Pairwise comparisons were conducted using Bonferroni's method to adjust for multiple comparisons and avoid inflation of Type I error rate. Statistical significance for these multiple comparisons was designated a priori as a P-value ≤ 0.05.

For the statistical models on performance outcomes, the linear predictor included the fixed effects of stratum (XBL or STR), drug treatment (MEL or CONT), time (d 14 and d 28), and all 2- and 3-way interactions. Included in the model were the random blocking effect of Lot and the random effect of Pen to recognize the experimental unit for treatment groups. Repeated observations within a pen were modeled using a compound symmetry residual variance-covariance structure. For DMI, the residual variance-covariance was expanded to accommodate heterogeneous variances at each time period. In turn, for meloxicam concentrations measured 24 h after drug administration, the linear predictor included the fixed effect of stratum (XBL, STR) and the random blocking factor of Lot. For all models fitted on Gaussian responses, Satterthwaite method was used to estimate degrees of freedom and Kenward Rogers was used for bias correction in standard error estimation. Newton-Raphson with ridging was the estimation algorithm implemented. Model assumptions were evaluated using externally studentized residuals and were considered to be appropriately met. For health events at each period of observation, the linear predictor for the statistical model included the fixed effects of stratum (XBL or STR), drug treatment (MEL or CONT), and their 2-way interaction. Entry BW did not enhance model fit and thus, was excluded from the final model. The random effect of Lot was also fit in the linear predictor as a blocking factor to account for variability in health events between lots. In addition, Pen nested within stratum and drug treatment was incorporated in the linear predictor to recognize Pen as the experimental unit for these factors. For binomial responses, inference was conducted after checking for absence of overdispersion based on the Pearson Chi-Square/degrees of freedom fit statistic. Model parameters were estimated using Laplace integral approximation to maximum likelihood.

Least squares means (SEM) and mean differences (SED) are reported unless specifically identified as a mean ± SD for descriptive statistics such as receiving weight of calves.

Results

Eight calves were removed from the study prior to completion as shown on Table 3. One calf was found dead in the pen due to diffuse necrotizing phlebitis of the external iliac and femoral veins with associated thrombo-embolic pneumonia. Including only the calves which completed the study, the mean receiving weight ± SD of the treatment groups was: STR-CONT (245 ± 15 kg), STR-MEL (245 ± 16 kg), XBL-CONT (248 ± 16 kg), XBL-MEL (247 ± 17).

Meloxicam concentrations

Due to handling error, plasma samples were not available for meloxicam assay for 3 XBL-MEL and 2 STR-MEL calves. Dividing the actual dose administered by the receiving weight of the calf, the dose of meloxicam rounded down to the nearest whole tablet ranged from 0.89 – 1.00 mg/kg. There was no difference ($P = 0.70$) in mean ± SEM plasma meloxicam concentrations at the time of castration, between XBL (6.01 ± 0.07 µg/mL) and STR (5.97 ± 0.7 µg/mL) groups.

Performance

Meloxicam administration did not affect the ADG, DMI, or the ADG:DMI ratio of calves: there was no evidence ($P > 0.3$) of a main effect nor any interaction with strata and/or days on feed (Table 4).

Dry matter intake was significantly affected by strata ($P = 0.016$) and DOF ($P < 0.001$), but there was no interaction between those fixed effects as shown on Figure 2a. Over the course of the 28-day study, DMI of calves in the STR stratum was 0.37 ± 0.13 kg/d higher ($P = 0.02$) than those in the XBL stratum. For all calves, dry matter intake was 2.19 kg/d higher in the 15-28 d period than in the 1 – 14 d period ($P < 0.001$).

With regard to ADG and the ADG:DMI ratio, as shown on Figure 2b and c, there was an interaction between strata and DOF ($P \leq 0.002$), with the STR group having a higher ADG (difference, 0.75 ± 0.14 kg/d; $P < 0.0001$) and ADG:DMI ratio (difference, 0.13 ± 0.02; $P < 0.0001$) than the XBL group from 1 to 14 DOF, but no difference between strata from 15 to 28 DOF ($P \leq 0.31$).

Health

The various pull rates, overall morbidity, and the incidence of BRD are listed in Table 5a and 5b and depicted in Figure 3. The rate at which XBL calves were pulled for further health evaluation was significantly higher than STR calves (P = 0.01). Within the STR stratum, there was no difference (P = 0.78) between the pull rate of calves which received meloxicam (17%) and those which did not (20%). Within the XBL stratum, however, the pull rate of calves which received meloxicam (26%) was significantly lower (P = 0.04) than those which did not (45%). Within the placebo treatment, the pull rate of XBL calves (46%) was higher (P = 0.007) than STR calves (20%); but within the meloxicam treatment, there was no difference (P = 0.43) between XBL (26%) and STR (17%) calves.

The highest daily detection of BRD cases peaked within the first 14 days of the study (Figure 4). Over the entire 28-day study, with respect to the incidence of BRD, there was no evidence (P = 0.31) to indicate a main effect of drug treatment, but there was marginal evidence (P = 0.06) of an effect of stratum. Comparing within stratum, there was a significantly (P = 0.03) higher incidence of BRD in castrated calves which received the placebo (34%) than those which received meloxicam (17%), whereas within the STR stratum, there was no difference (P = 0.67) in BRD incidence between calves receiving either drug treatment.

As shown in Table 5a and 5b, there were no differences between strata or treatment groups for the first and second re-pull rates and their associated rates of BRD relapse.

Discussion

This study investigated the effect of administering oral meloxicam at a dose of 1 mg/kg to transportation-stressed beef calves immediately upon arrival to the feedlot, followed by castration of bulls 24 h later. Steer calves, with a sham castration, were used as controls for the castration procedure.

The mean plasma concentration of meloxicam in this study was approximately 6 µg/mL 24 h after administration. In pharmacokinetic studies with calves, when meloxicam was administered orally at doses of 1 mg/kg (Coetzee et al., 2009) and 0.5 mg/kg (Coetzee, et al. 2010) the mean peak plasma concentrations were reached in approximately 12 h with values of 3.1 µg/mL (dose, 1 mg/kg) and 2.1 µg/mL (dose, 0.05 mg/kg). Although it is not possible to calculate the maximum plasma concentration from the one sample collected in the current study,

it is likely that peak concentrations ranged between 2 and 3 times that observed in Coetzee, et al. 2009 with a similar dose. The reason for the higher concentrations observed in the current study is not known. But in previous studies, food and water were not withheld prior to dosing, whereas in the current study, calves did not have access to food and water for at least 12 h prior to dosing. A lower volume of rumen contents may have resulted in less opportunity for the drug to bind to ingesta, and when the calves drank water following release into their pens, a greater portion of the dose would likely have been free to wash out of rumen and travel to the small intestine for absorption than if the rumen had been full. To test this hypothesis, it would be necessary to perform a pharmacokinetic study with oral meloxicam being administered to calves when food and water are withheld for approximately 12 h prior to dosing.

Castration significantly reduced the ADG, DMI, and ADG:DMI of calves during the first two weeks following the procedure. Reduced gains and feed efficiency in castrated calves was expected since surgical castration is generally followed by a period of reduced weight gain, the severity of which is usually directly related to the age of the animal at the time of the procedure. (Faulkner et al., 1992; Bretschneider, 2005; Stafford and Mellor, 2005; AVMA, 2011; Massey et al., 2011). Even though gains and feed intake in the ensuing weeks may be similar between castrated and non-castrated calves, as observed in our study, cumulative ADG may be reduced through 27 to 35 days. (Cohen et al., 1991; Fisher et al., 1996). ADG values were higher in this study than in some others; this is likely the result of using the artificially low receiving weight as the beginning weight, whereas the ending weights were obtained without restriction of food and water.

The administration of meloxicam did not improve the ADG, DMI or ADG:DMI of castrated calves in this study. Performance effects of NSAID administration upon castration have been mixed. Administration of the NSAID ketoprofen at various dosage regimens in the peri-surgical period did not prevent the reduced ADG in 11-mo-old castrated calves in the 1-35 day period. (Ting et al. 2003). Earley and Crowe (2002), however, reported that 5.5-month-old-calves receiving ketoprofen combined with local anesthesia prior to surgical castration had a higher ADG during the 35 days after castration compared with untreated calves. Baldridge et al. (2010) found that 2- to 4-month-old calves receiving the NSAID sodium salicylate in drinking water beginning 3 d prior to concurrent surgical castration and dehorning and continuing for 2 d after surgery had a higher ADG over 13 days after the procedure than untreated calves. It is

possible that NSAID administration is more effective in maintaining ADG in younger calves, though more evidence is needed to support this hypothesis.

The most noteworthy finding of the current study was that the administration of meloxicam significantly reduced the percentage of castrated calves which were diagnosed with BRD. Within the XBL strata, calves castrated after administration of the placebo were twice as likely to be treated for BRD as those castrated after administration of meloxicam. The incidence of BRD in the castrated calves which received meloxicam was reduced to that of the steers. Within the STR strata, the administration of meloxicam had no effect on the rate of BRD.

The finding of increased risk of developing BRD in castrated calves was expected. In a multi-year study of 3,380 stocker calves (65% bulls, 35% steers) similarly shipped from sources in Tennessee and Kentucky to the KSU Stocker Unit, Massey et al (2011) reported that calves which were surgically castrated 24 h post-arrival were treated for BRD significantly more often than calves which were received as steers. Similar observations have been reported by others (Daniels et al., 2000; Pinchak et al., 2004). Additionally, Pinchak et al. (2004) reported marginal evidence of decreased ADG in calves with BRD.

The mechanism by which castrated calves may be more susceptible to developing BRD is thought to be due to immunosuppression resulting from stress and surgery. Surgical castration is associated with increased plasma concentrations of cortisol and acute phase proteins such as haptoglobin (Fisher et al., 1997; Earley and Crowe, 2002). Acute phase proteins have been shown to suppress lymphocyte function in cattle and may act as a negative feedback mechanism to inhibit further inflammation (Murata, 2003). Ting et al., (2003) and Earley and Crowe (2002) demonstrated that administration of the NSAID, ketoprofen, to surgically castrated calves decreased the concentrations of circulating cortisol and haptoglobin, and also prevented suppression of the gamma-interferon response. Although concentrations of cortisol, haptoglobin, or gamma-interferon were not measured in the current study, the findings of Ting et al (2003) and Early and Crowe (2002) suggest the mechanism whereby meloxicam administration may have may limited the immunosuppression usually associated with surgical castration, thus fewer XBL-MEL calves succumbed to BRD than XBL-CONT calves.

Meloxicam is a non-steroidal anti-inflammatory drug (NSAID) that is a potential candidate for use in providing pain relief to cattle. As a class, NSAIDs exert anti-inflammatory action through variable inhibition of the cyclo-oxygenase (COX) isoenzymes which are pivotal

catalysts in the prostaglandin production pathway. While the expression of both COX-1 and COX-2 is constitutive and inducible, COX-2 is the isoform which is greatly upregulated in the presence of inflammatory stimuli and is therefore considered to be the desired target of NSAID activity (Lees, 2009). Although the literature is deficient with respect to cattle, meloxicam is considered to preferentially inhibit the inflammatory effects of COX-2 while tending to spare the homeostatic effects of COX-1 in humans (Warner et al., 1999), dogs (Streppa et al., 2002), cats (Giraudel, et al., 2005), and horses (Beretta, et al., 2005). However, the relative inhibition of the COX isoenzymes by a drug is known to vary between species, therefore COX preference in one species does not guarantee similar preference in another (Lees, 2009).

Although meloxicam is not currently approved for use in cattle in the United States, the drug has been approved for that use in in the European Union as well as in countries such as Canada, New Zealand, and Australia, which are major suppliers of beef products to the U.S (USDA, 2009). Depending upon the country, approved indications variously include the use of meloxicam as ancillary treatment of respiratory disease, diarrhea, mastitis and/or pain due to dehorning or disbudding. Based on a dose of 0.5 mg/kg bodyweight delivered intravenously or subcutaneously, labeled withdrawal times range from 8 to 20 days for meat, and from 84 hours to 6 days for milk (EMEA, 1999; APVMA, 2010; NZFAZ, 2010; Health Canada, 2009).
In the U.S., although flunixin meglumine is approved for the control of pyrexia associated with bovine respiratory disease and for the control of inflammation and pyrexia associated with endotoxemia (FDA, 2003), there is currently no approved NSAID with indications for alleviating pain in cattle. Provisions in the Animal Medicinal Drug Use Clarification Act of 1994 may therefore allow the extra-label use of meloxicam under specific conditions as discussed previously (Coetzee et al., 2009). Candidate products for extra-label use in cattle include oral and injectable small animal formulations, and oral human formulations.

As determined by a study investigating the pharmacokinetics of generic human-label meloxicam when dosed orally to ruminant calves at 1mg/kg BW, the drug was observed to be well absorbed, with a mean bioavailability of 100% and a plasma elimination half-life of 27 h (Coetzee et al., 2009). With the long half-life and the relative ease of oral dosing, meloxicam may provide producers with an affordable and effective route for providing pain relief and to possibly enhance animal well-being in the weeks following transport and subsequent castration in a feedlot receiving environment. The generic human product used in the current study was

available commercially to veterinarians at a price of $0.0033/mg, which translates to an approximate base cost of $0.83 per 250 kg calf. Even after adding a margin for profit, the cost compares favorably to the estimated $40.64 loss due to BRD treatment (Fulton et al., 2002).

Conclusion

The administration of meloxicam to calves in this study did not improve ADG, DFI, or ADG:DFI in either XBL or STR calves. Under the conditions and limitations of this pilot study, the oral administration of meloxicam, 24 h prior to surgical castration, significantly reduced the incidence of BRD diagnosis in castrated bull calves in the 28 d post-surgical period.

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Figures and Tables

Figure 5.1. Study outline representing timeline for calves administered lactose placebo (CONT) or meloxicam (MEL; 1 mg/kg, oral) 24 h prior to either surgical castration of bulls (XBL) or sham castration of steers (STR).

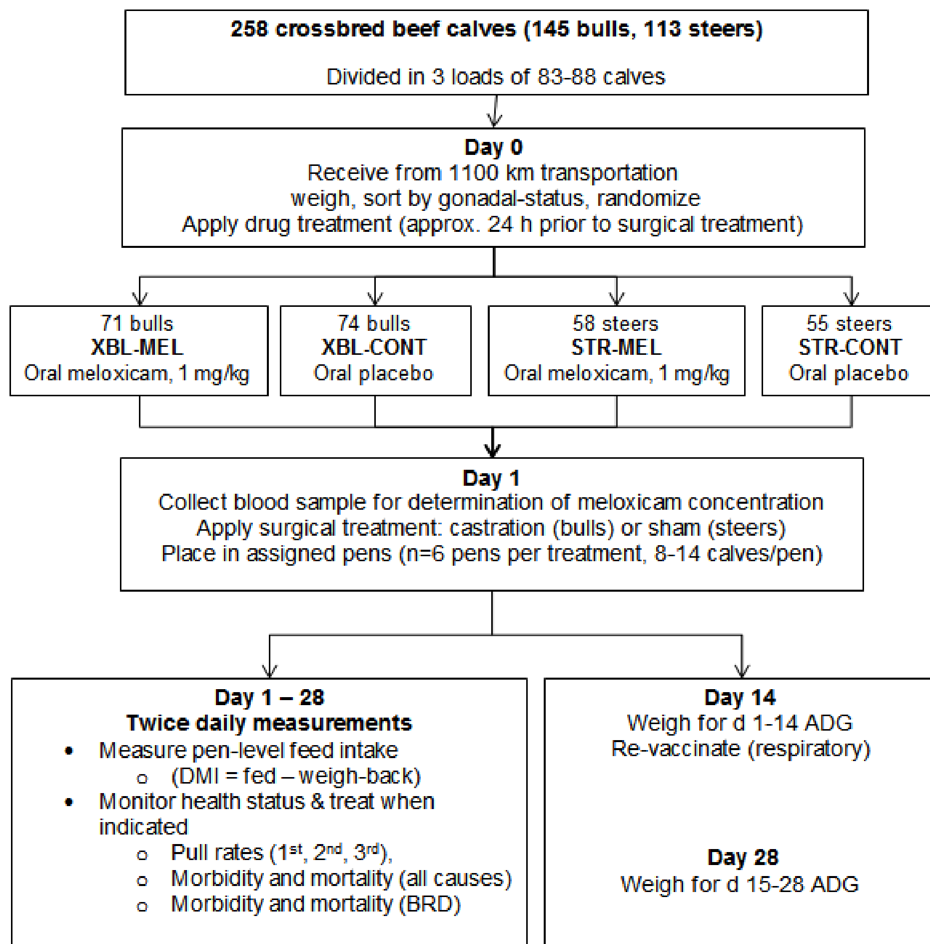

Figure 5.2. a) Dry matter intake (DMI), b) Average daily gain (ADG), and c) ADG:DMI ratio in calves administered lactose placebo (CONT) or meloxicam (MEL; 1 mg/kg, oral) 24 h prior to either surgical castration of bulls (XBL) or sham castration of steers (STR).

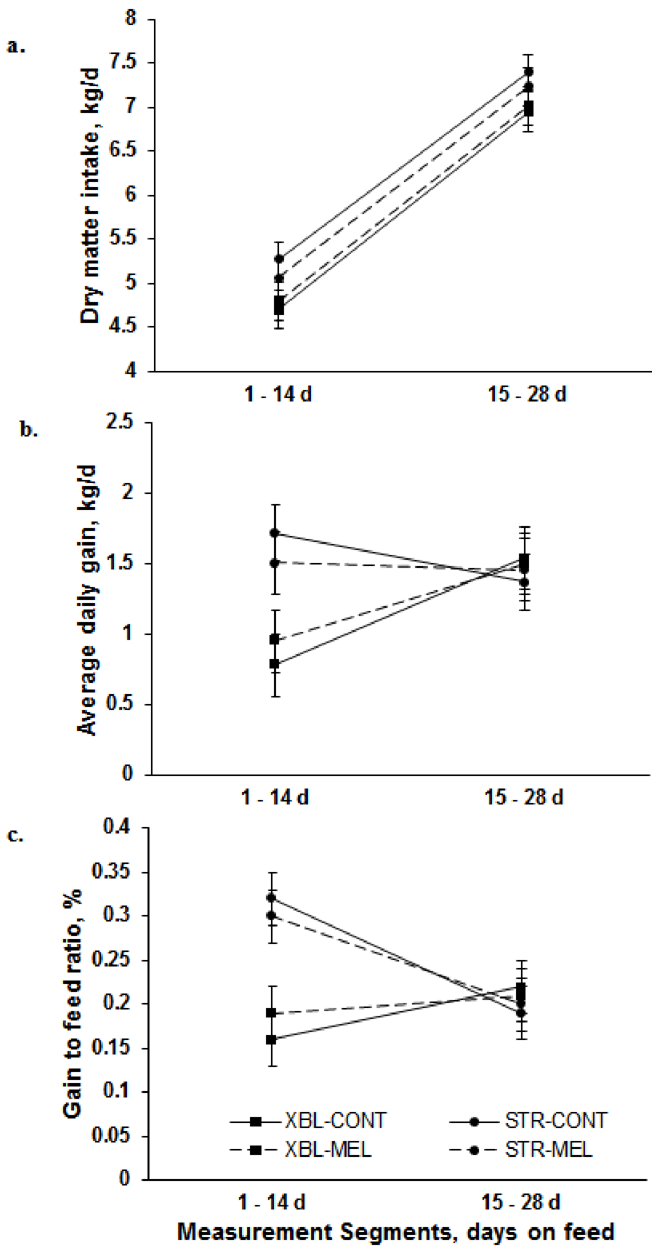

Figure 5.3. Overall rate of first pull for examination, incidence of disease for all causes, and incidence of bovine respiratory disease (BRD) in calves administered lactose placebo (CONT) or meloxicam (MEL; 1 mg/kg, oral) 24 h prior to either surgical castration of bulls (XBL) or sham castration of steers (STR).

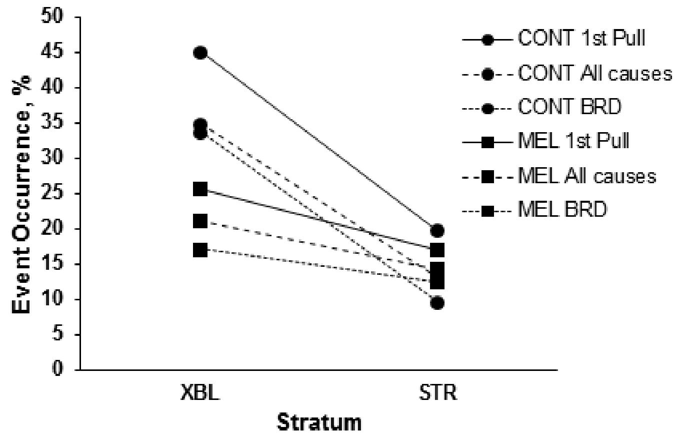

Figure 5.4. Daily incidence of bovine respiratory disease (BRD) detections in calves administered lactose placebo (CONT) or meloxicam (MEL; 1 mg/kg, oral) 24 h prior either surgical castration of bulls (XBL) or sham castration of steers (STR).

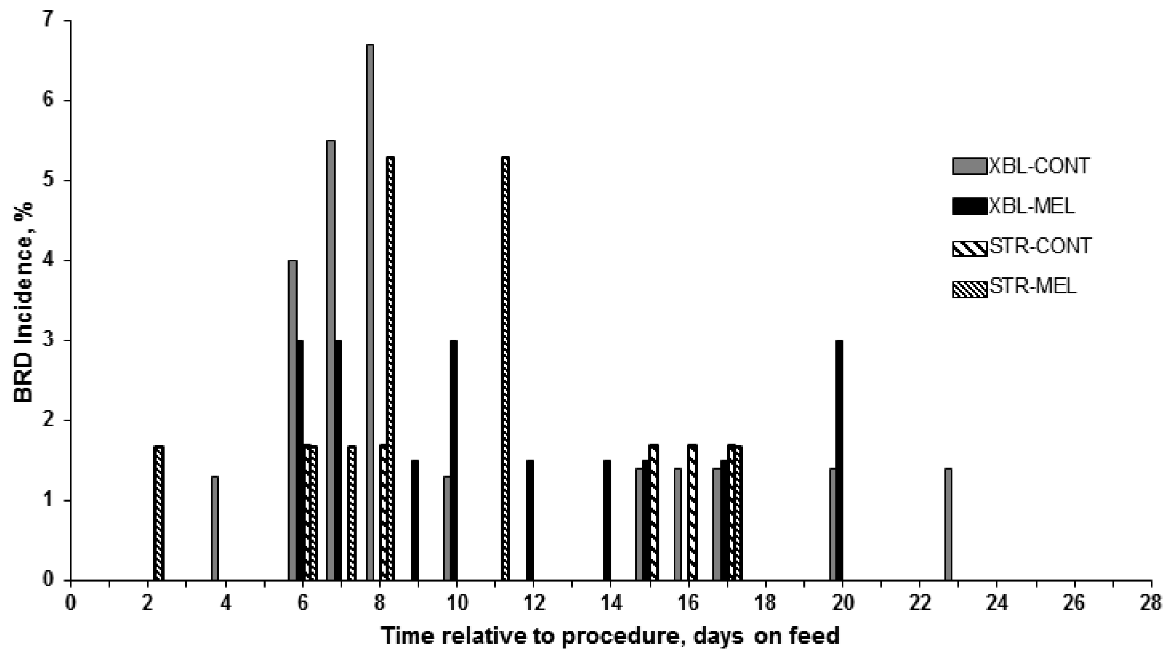

Table 5.1. Percentages of dietary components in the total mixed ration, as fed to calves.

Days on feed	1 to 7	8 to 18	19 to 28
Supplement[1], %	3	3	3
Dry-rolled corn, %	28	29	36
Wet gluten feed[2], %	30	37	37
Prairie hay, %	16	16	15
Alfalfa hay, %	23	15	9

[1]Contains 600 g/ton monensin

[2]Sweetbran, Cargill Animal Nutrition, Minneapolis, MN

Table 5.2. Depression scoring system used to determine sickness and diagnose Bovine Respiratory Disease (BRD) in calves (adapted from Perino and Apley, 1998)

Depression Score	Clinical Signs
0	Normal, no signs of depression
1	Noticeable depression without signs of weakness. Slower than pen mates but perks up when approached, raises head and actively follows observer's movements.
2	Marked depression with moderate signs of weakness, but without a significantly altered gait. Stands with head lowered, perks up when approached but soon returns to depressed stance. Moves slower than pen mates, may display signs of incoordination
3	Severe depression with signs of severe weakness such as a significantly altered gait. Stands with head lowered and raises head only when approached closely. Not easily able to stay with pen mates.
4	Moribund, unable to rise

Table 5.3. Enrollment and health statistics in long-haul transported calves, receiving either placebo (CONT) or meloxicam,1 mg/kg administered orally upon arrival (MEL). Approximately 24 h after arrival, bull calves (XBL) were surgically castrated and steers (STR) were sham-castrated.

	Treatment Group			
	Bulls (XBL)		Steers (STR)	
	XBL-CONT	XBL-MEL	STR-CONT	STR-MEL
Calves enrolled in study	74	71	55	58
Calves completing study	73	67	53	57
Removed from study	1	4	2	1
Reason for removal:				
Lameness	1	2	1	1
Chronic coccidiosis	0	1	0	0
Neurological signs	0	1	0	0
Pen Death	0	0	1	0

Table 5.4. Least squares means of average daily weight gain (ADG), daily dry matter intake (DMI) and gain-to-feed ratio (ADG:DMI) in long-haul transported calves, receiving upon arrival the treatment (Trt) of either placebo (CONT) or meloxicam, 1 mg/kg administered orally (MEL).

Approximately 24 h after arrival, calves in the bull stratum (XBL) were surgically castrated and calves in the steer stratum (STR) were sham-castrated.

Strata	XBL				STR						P-values						
Trt	Placebo		Meloxicam		Placebo		Meloxicam		SEM								
DOF[1]	1 - 14	15 – 28	1 – 14	15 – 28	1 - 14	15 – 28	1 - 14	15 – 28	1 - 14	15 – 28	Strata	Trt	DOF	Strata*Trt	Strata*DOF	Trt*DOF	Strata*Trt*DOF
ADG, kg	0.78	1.54	0.95	1.5	1.72	1.37	1.51	1.46	0.18	0.18	<0.001	0.97	0.08	0.30	0.002	0.85	0.31
DMI, kg	4.70	6.95	4.80	7.01	5.26	7.39	5.06	7.23	0.22	0.31	0.016	0.73	<0.001	0.35	0.73	0.98	0.85
ADG:DMI,%	0.16	0.22	0.19	0.21	0.32	0.19	0.30	0.20	0.03	0.03	<0.001	0.73	0.07	0.34	<0.001	0.95	0.33

[1]DOF: Days on Feed. ADG: Average Daily Gain. DMI: Daily Dry Matter Intake

Table 5.5 Least squares means of health monitoring response variables in long-haul transported calves, receiving upon arrival the treatment (Trt) of either placebo (CONT) or meloxicam, 1 mg/kg administered orally (MEL).

Approximately 24 h after arrival, calves in the bull stratum (XBL) were surgically castrated and calves in the steer stratum (STR) were sham-castrated. Contrasts between groups are not shown for the third pull (Table b.) because there were no significant differences within Strata or treatment groups.

Strata	XBL		STR		P-values			P-values of contrasts			
Treatment (Trt)	CONT	MEL	CONT	MEL							
Strata-Treatment combination	XBL-CONT	XBL-MEL	STR-CONT	STR-MEL	Strata	Trt	Strata*Trt	XBL-CONT v. STR-CONT	XBL-MEL v. STR-MEL	XBL-CONT v. XBL-MEL	STR-CONT v. STR-MEL
First Pull											
Pulls for all reasons, %	45 (11)	26 (9)	20 (6)	17 (9)	0.01	0.07	0.45	0.007	0.43	0.04	0.78
Overall morbidity, %	35 (17)	21 (12)	13 (9)	14 (11)	0.08	0.28	0.36	0.004	0.57	0.13	0.87
BRD morbidity, %	34 (15)	17 (11)	10 (6)	13 (10)	0.06	0.31	0.21	0.0006	0.68	0.027	0.67
Second Pull											
First re-pull for BRD, %	44 (14)	53 (8)	27 (16)	70 (21)	0.99	0.14	0.31				
First BRD relapse, %	33 (12)	36 (14)	33 (22)	50 (20)	0.70	0.60	0.70				

b.

	Strata		Treatment (Trt)		P-value	
	XBL	STR	CONT	MEL	Strata	Trt
Third Pull						
Second re-pull for BRD, %	30 (48)	4 (25)	22 (52)	6 (31)	0.64	0.67
Second BRD relapse, %	36 (20)	0.0001 (0.02)	0.2 (17.4)	0.04 (3.5)	0.94	0.37

Chapter 6 - Future Directions for Research

For cattle in the U.S. today to receive analgesia beyond the short duration of local anesthesia with lidocaine, veterinarians and producers must rely upon extra-label drug use (ELDU) because there is no drug approved by the FDA for that specific use in cattle. In the absence of such an approved drug, as long as the other provisions for ELDU in the Animal Medicinal Drug Use Clarification Act (AMDUCA) of 1994 are met along with published regulations in the Code of Federal Regulations (21 CFR Part 530), analgesic drugs such as meloxicam could be administered to cattle for the relief of pain.

One of the provisions in AMDUCA for ELDU is that the prescribing veterinarian must establish a substantially extended withdrawal period prior to the marketing of any portion of the animal for consumption. As discussed in Chapter 4, to make this estimation for oral meloxicam, veterinarians currently must rely upon estimates of the terminal elimination half-life from published pharmacokinetic studies in cattle.

As long as all conditions for ELDU are met, it is possible for veterinarians to administer oral meloxicam to cattle using information published to date. But for a meloxicam product, whether injectable or oral, to be approved by the FDA for marketing in the U.S., many future studies are needed. Even if a sponsor does not support and amass the data necessary to submit a New Animal Drug Application (NADA), future published research will add to the body of knowledge needed to use meloxicam safely and effectively in providing pain relief to cattle. Therefore, directions for future research should lay the groundwork for either reference by practicing veterinarians or by a drug sponsor wishing to replicate or extend the studies under the well-controlled conditions required for a NADA.

The CVM has issued a guidance document for use in designing studies to support the approval of NSAID's for use in animals (CVM 2006). Furthermore, authors from the CVM have recently published regulatory considerations intended to provide researchers and industry sponsors with the agency's current thinking on analgesic drug approval for cattle in the U.S. (Smith & Modric, 2013). These documents, within the context of federal regulations concerning NADAs (21 CFR Part 514), provide researchers with the

framework necessary to the approval of an NSAID, such as meloxicam. This chapter will discuss future research needed to fill in this framework.

To be approved by the FDA, a new animal drug must be shown to be safe and to be effective for the labeled intended use. Although for a NADA application for a specific product, there are other items to address, such as environmental impact and manufacturing considerations, because we are not looking at a specific product, this chapter will focus on studies necessary to show safety and efficacy.

Effectiveness

In the Effectiveness section of a NADA, sponsors must provide substantial evidence in the form of data from adequate and well-controlled studies which demonstrate that the drug has the intended effect when used as indicated on the product label. (21 U.S.C. § 360b(d)(1)(E)) Dose characterization is one part of this section and provides rationale for the dose, frequency, and duration of administration. The dose of oral meloxicam used in published studies in cattle thus far has been derived from the dose of the injectable product approved in other countries. Although this is a reasonable place to start, the dose must be demonstrated to be effective for the proposed indication. For the indication of providing pain relief in the form of an NSAID to calves when dehorned and/or castrated, a major difficulty is the lack of a validated model with which to measure and assess pain in that species.

As discussed in Chapter 1, current research has been aimed at evaluating various response variables which may provide a reliable and reproducible method of measuring the effectiveness of pain relief. Currently, none of these measures has been validated. Because cattle may mask pain behaviors in the presence of humans, and because scoring of those behaviors by observers is subjective and time intensive, this type of assessment is not likely to be easily validated. Objective, quantifiable assessments of behavior, such the use of accelerometers to measure postural activity are more likely to be useful than subjective counts of specific behaviors. A similar posture-assessing device which has not been reported in the literature which might be useful in measuring general attitude is one which could be applied to the ear which would monitor ear position.

Methods which "ask" the animal to show how much it hurts to put pressure on an injured area, such as pressure mats and algometers, are intriguing, however, it is important to recognize that animals may still mask the necessary behaviors such as limping on the pressure mat or moving the head away from a pressure algometer applied to a dehorned area. Furthermore, in the case of pressure algometers applied to a painful area, this measure is not totally objective because the algometer is held in the hand of the observer who determines when the animal flinches. Therefore, more research is necessary to refine the methodology by which it is measured how much pressure an animal is comfortable with. For example, the method of measuring pressure applied to the dehorned area might be improved by developing an apparatus which is attached to the head of a confined animal

Given the inherent difficulty in assessing pain behaviors in cattle, especially those that are not conditioned to the benevolent presence of humans, researchers are investigating potential biomarkers which may be more specific to pain and inflammation such as substance P.

Another avenue for research into possible biomarkers is to assess the action of meloxicam on suppressing the expression of the COX-2 enzyme following an elective surgical procedure. With this approach, the indication would be for the control of inflammation following the procedure. In a study in which an inflammatory response was induced in calves by intravenously injecting the endotoxin of Escherichia coli, LPS, a single administration of meloxicam reduced the inflammatory mediators prostaglandin E_2, prostaglandin F_{2a}, thromboxane B_2 and malonylodialdehyde compared to controls (Bednarek & Kondracki, 2006). A study measuring these inflammatory mediators at various timepoints up to 72 hours following castration or dehorning in calves treated and not treated with oral meloxicam would indicate the usefulness of these biomarkers. Such a study could also be performed as a dose titration study, with a range of doses, to determine if a lower dose is just as effective or, alternately, if a higher dose is needed to suppress the inflammatory response. Pairing a dose titration study, such as described here, with pharmacokinetic data and measurement of other pain-response variables may assist in determining the effective drug concentration. This would be helpful in determining the proper timing of drug administration.

It is also possible that meloxicam, either oral or injectable, may be developed for the indication of preventing bovine respiratory disease in the post-castration period. Although the results reported in Chapter 5 are promising, the study should be repeated in a field study with a larger number of animals under the conditions of actual use.

Safety

Because cattle are a food animal species produced for human consumption, the definition of drug safety is extended to include not only safety for the target animal (cattle), for the environment, and for the person administering the drug, but also the safety of food products for human consumption (CVM, 2006b).

Oral meloxicam is approved in the U.S. for use in humans, dogs, and cats, therefore much is already known about the safety of the drug with respect to people and select laboratory animals. This information is helpful to veterinarians in the U.S. who administer meloxicam to cattle on an extra-label basis, to governmental officials determining residue tolerance limits, and to an industry sponsor needing to amass safety data.

From public information reported in the approval documentation of the injectable meloxicam product in the European Union it can be expected that cattle administered oral meloxicam might similarly exhibit the reported occasional mild erosion of the abomasal mucosa with no major adverse reactions (EMEA, 1999). But it is possible that the oral product may not be as well-tolerated in the GI tract as the injected product. Therefore, a safety study should be conducted in which cattle are administered oral meloxicam at 0, 1, 3, and 5 times the dose established in efficacy studies. The dose should be administered at least 3 times the proposed duration. In such a study, the animals would be monitored for adverse clinical signs and, at the end of the study, animals would be sacrificed for gross and histological pathology (CVM, 2009).

Similarly, the results of residue depletion studies are reported for the injectable product, but because it is possible that orally administered meloxicam is eliminated from the various edible tissues differently, food safety studies are needed in animals of the target consumption ages, e.g. for veal and beef. In such a study, animals are dosed at the highest intended level and duration, then a portion of the animals sacrificed at intervals throughout the expected withdrawal period, and then drug concentrations are determined from edible tissue samples of muscle, fat, liver and kidney to determine elimination half-lives (CVM, 2006b). Because NSAID toxicities

may not be found during the typical Target Animal Safety study in healthy animals as described above, the CVM also recommends rigorous monitoring of field efficacy studies for adverse reactions (CVM, 2006a).

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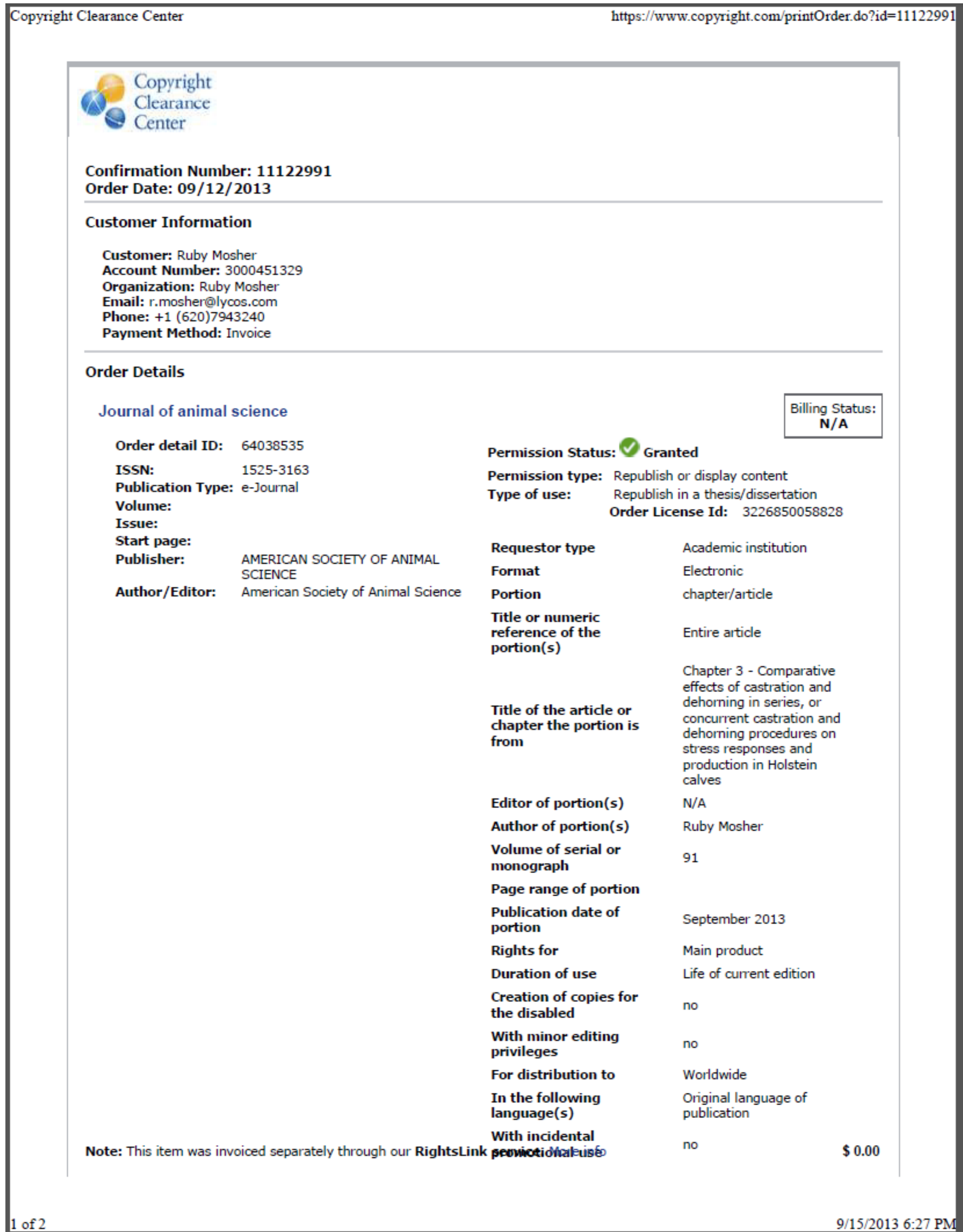

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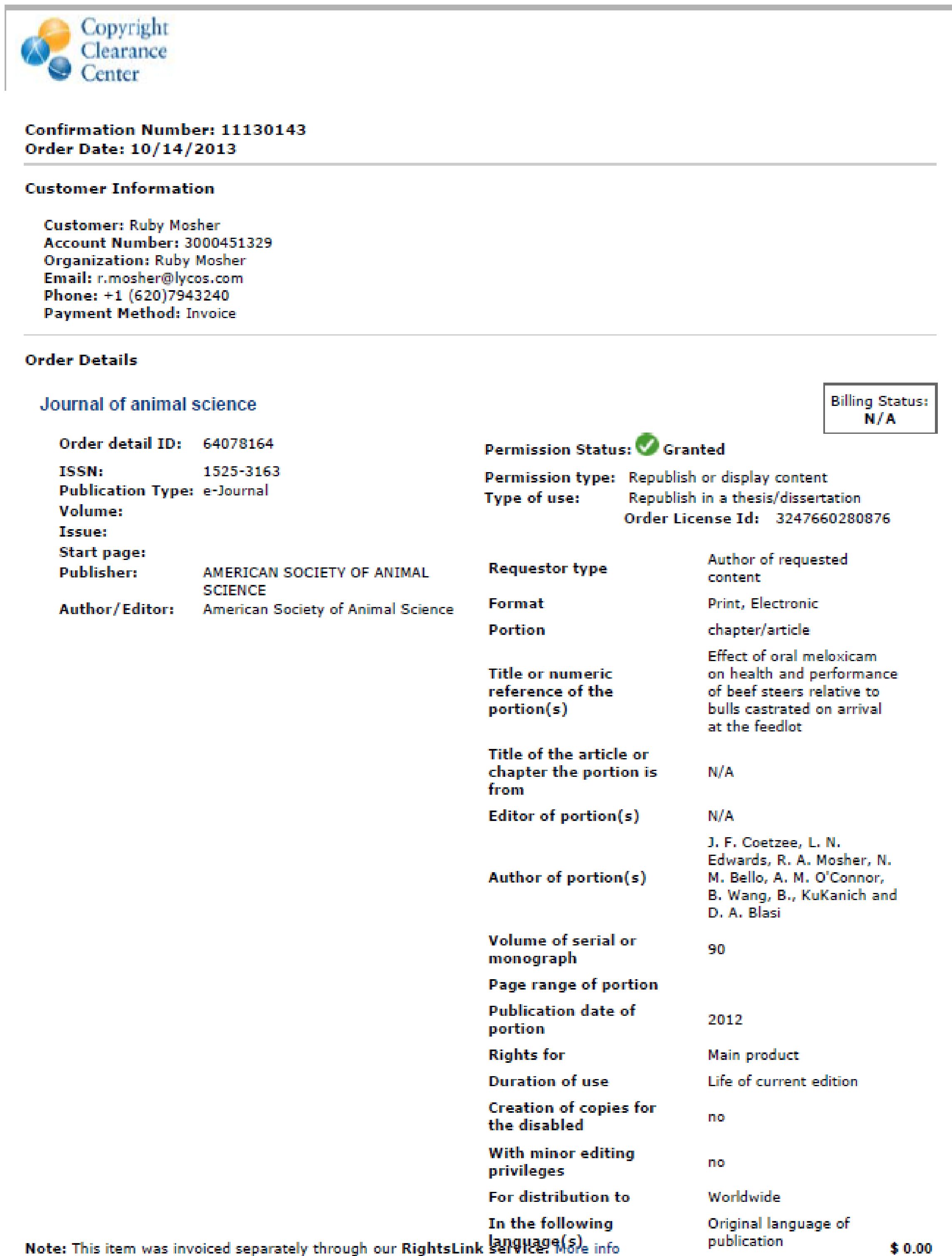
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