IDENTIFYING AND ALLEVIATING PAIN ASSOCIATED WITH ROUTINE HUSBANDRY PROCEDURES PERFORMED ON PRE AND POST WEANING DAIRY CALVES

by

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Abstract

This thesis includes two studies that assessed pain responses to husbandry procedures in order develop mitigation tools. The objective of the first trial was to identify method-related differences in behavioral pain responses in calves of two ages (6 week and 6 months) subjected to castration: surgical cut (CP; n=18), surgical cut and emasculator (CC; n=20), rubber banding (BAND; n=18), or control manipulation of the scrotum (CONT; n=20). Behavior was evaluated pre- and post-castration to record foot stamps, tail flicks, kicks, elimination, in addition to standing and lying post-castration. For 6 week calves, the probability of kicking and eliminations was greater for surgical castrates and all castrated animals, respectively. The probability of kicking was greatest for all 6 month castrates while BAND and CONT had greater probability of elimination. Both age groups increased standing post-castration. Lying decreased in 6 week calves but was unchanged for 6 month calves. Six week calves displayed more tail flicks and tended to display less foot stamps than 6 month calves. Six week calves showed a decrease in tail flicks and foot stamps in response to castration while 6 month calves showed an increase in both behaviors. CP and CC, but not BAND, resulted in less tail flicks than CONT post castration. The second trial compared the effects of preemptive analgesics administered to calves subjected to dehorning with local anesthesia. Six month Holstein steers were randomly assigned to receive one of the following treatments (n= 8/group): meloxicam (1 mg/kg PO), gabapentin (15mg/kg PO), meloxicam (1 mg/kg) and gabapentin (15 mg/kg) PO, flunixin (2.2 mg/kg IV), or a placebo. Drug, cortisol, ex-vivo prostaglandin, haptoglobin, and substance P concentrations, ocular thermography, algometry, and average daily gain were evaluated. Analgesic-treated calves had lower plasma SP concentrations and improved ADG compared with controls. Flunixin calves had reduced cortisol and ex-vivo prostaglandin concentrations for 24h compared to controls. Meloxicam treated calves showed an increase in MNT at two horn bud sites compared with the other treatments. Overall, the results provide validation of responses to noxious stimuli that can be used to develop pain alleviation for livestock.

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Chapter 1 - General Introduction

1.1 Thesis Organization

This thesis is organized with each research project as a separate chapter. Information in Chapter 1 introduces the study objectives and expected outcomes. A literature review focusing on animal pain, pain assessment, and pain mitigation is found in Chapter 2. Chapters 3 and 4 detail each research project with a specific introductory and general background information included within the research chapters. Chapter 3 focuses on the calf castration behavior research trial and is formatted according to the Journal of Dairy Science guidelines. Chapter 4 focuses on the calf dehorning research, and is formatted following the Journal of Veterinary Pharmacology and Therapeutics guidelines. Chapter 5 is a summary of the research results in addition to a general discussion and suggestions on how the results apply to the future study of animal pain and pain assessment.

1.2 Study Goals and Expected Outcomes

The first objective for this thesis was to examine subjective, objective, and threshold pain tests, including behavioral, physiological, and neuroendocrine responses to noxious stimuli as measurements of pain in cattle. The expected outcome of this thesis objective was to identify and validate pain assessment tests and biomarkers in the target species. The second objective of this thesis was to examine novel pain mitigation agents, either local anesthetics, non-steroidal anti-inflammatory drugs (NSAIDs), or gamma-aminobutyric acid (GABA) analogues for alleviating pain in cattle. The expected outcome of this objective is that the pain mitigation agents will alleviate pain as assessed by identifiable biomarkers.

Objectives of the first research trial, the castration behavior study, were to evaluate behavioral responses to different methods of castration in calves of two distinct age categories. Behavioral responses have been studied and used to quantify pain in cattle subjected to a number of procedures including castration. The expected outcome of this study was that each castration treatment would elucidate a distinct behavioral response allowing further determination of the appropriate method to employ in order to reduce excess pain. This study also compared the behavioral responses of calves from two age categories and the expected outcome was that one age category would display a markedly greater response to pain from castration.

Objectives for the calf dehorning chapter were to determine the effectiveness of flunixin, meloxicam, gabapentin, and meloxicam with gabapentin for their extended analgesia following dehorning relative to a control lidocaine cornual nerve block only. The second objective of the dehorning chapter was to evaluate biomarkers, cortisol, substance P, haptoglobin, and ex-vivo prostaglandin (PGE_2) for assessing dehorning pain in calves relative to the plasma drug concentration. In addition, non-invasive techniques including measuring neuroendocrine responses and nociception threshold tests were evaluated in relation to plasma drug concentration. The expected outcome of the first objective of this dehorning research trial was that both NSAID and GABA analogue treatments would decrease biomarker response to pain and decrease pain sensitivity compared to the control cornual nerve block of lidocaine 2% hydrochloride. The second expected outcome of this research study was that effective biomarkers and thresholds of pain would be accurately identified and assessed for dehorned calves compared to each calf's baseline concentrations and measurements.

Chapter 2 - Literature Review

2.1 Introduction

Humans have provided an important voice in determining the welfare of animals, both companion and livestock. Although many different regulations and programs have been proposed and implemented throughout the livestock industry, all have the same underlying beliefs; all animals should have: freedom from thirst and hunger, access to proper housing and health care, minimal pain and distress, and the ability to express natural behavior (Broom, 1991; Curtis, 1987; Curtis; 2007; Dawkins, 1988). In production animal agriculture, there are routine management procedures such as castration, tail docking, branding, and dehorning that cause pain thus compromising the welfare of the animals. The assessment and mitigation of animal pain is increasingly important for animal welfare research. However, because pain is an individual perception and response, it is extremely difficult to quantify (O'Callaghan et al., 2003).

The objectives of this literature review are to provide an overview of the relevant published research literature regarding pain, pain assessment, and pain mitigation, as well as to provide an in-depth background for the thesis projects evaluating the pain assessment tools for pre and post weaning dairy calf pain models, including behavioral castration research and a scoop dehorning trial.

2.2 Pain and Nociception

The International Association for the Study of Pain has defined pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (IASP, 1994). Animal pain is an aversive sensory and emotional experience representing awareness of damage or threat of damage to tissues (Fraser and Broom, 1990). In addition, pain perception changes the animal's physiology and behavior in order to reduce the likelihood of recurrence as well as to promote healing (Molony and Kent, 1997).

Pain is classified based on a number of factors including cause, location, duration, and intensity. Superficial pain results from stimulation of receptors located in the skin, while deep pain arises from underlying structures like muscles, joints, tendons, bone, and ligaments (Anil et al., 2002). Neuropathic pain results from an injury to the nervous system. Adaptive pain protects

the animal from further injury and promotes healing. Maladaptive pain is pathological pain that persists after the initiating causes have resolved (Anderson and Muir, 2005a).

Nociception is defined as the detection of noxious stimuli and the further transmission of encoded information to the brain (Kidd and Urban, 2001). As the physiologic component of pain processing and perception, nociception involves the transduction, transmission, modulation, projection, and perception of signals generated by stimulation of specialized peripheral nociceptors of the skin (Lamont, 2008; Garry et al., 2004; Anderson and Muir, 2005) as shown in Fig. 2.1. Physiologic pain initiated by noxious stimuli uses normal sensory pathways and works to guard the body from tissue damage and pathologic pain (Besson and Chaouch, 1987; Scholz and Woolf, 2002). Within the peripheral terminals of nociceptors, specialized receptors are activated which generate depolarizing currents in response to the harmful stimuli (Woolf and Salter, 2000). The signals are then transduced by cutaneous nociceptors, which then transmit the message to the first synaptic relays in the dorsal horn of the spinal cord (Kidd and Urban, 2001; Anderson and Muir, 2005). The impulses are modulated then transmitted to the afferent nerve fibers to the dorsal root ganglian of the spinal cord (Raja et al., 1988; Chen et al. 2006) stimulating the release of neurotransmitters. The neurotransmitters chemically project the nociceptive signal through the spinal tract to the brain cortex. Once in the brain, further processing of nociceptive information allows for the perception, emotional experience, memory development, and the autonomic changes associated with pain (Stasiak et al., 2003).

Pain becomes pathologic when it is associated with tissue injury and is usually the result of inflammation (Woolf and Salter, 2000; Stasiak et al., 2003). Pathologic pain typically involves peripheral and central sensitization, structural reorganization of neural elements within the central nervous system (CNS), and disinhibition (Muir and Woolf, 2001) as shown in Fig. 2.2. Pathologic pain can be categorized as nociceptive, which involves the activation of high-threshold nociceptors, or as nonnociceptive, which involves the structural reorganization and phenotypic changes of neurons (Muir and Woolf, 2001;Hunt and Mantyh, 2001; Julius and Basbaum, 2001; Woolf and Slater, 2000).

During pathologic pain, peripheral sensitization contributes to the pain and sensitivity at the site of tissue damage or injury. Tissue injury results in the release of inflammatory mediators from damaged cells including ions, bradykinin, histamine, 5-hydroxytryptamine, ATP and nitric oxide (Anderson and Muir, 2005; Kidd and Urban, 2001). Together the chemicals impact the

peripheral ends of the nociceptors, modifying the nociceptors and activating silent nociceptors resulting in a reduction in the threshold and increasing responsiveness, known as hyperalgesia (Woolf and Slater, 2000). The peripheral sensory neurons then transfer the coded information from skin, muscle, joints and the viscera through peripheral nerves to the central nervous system. In addition, the release of inflammatory cytokines stimulates the synthesis of cyclooxygenase (COX) enzymes resulting in the production of prostaglandins and other mediators (Maier et al., 1990; Anderson and Muir, 2005), which results in inflammation (Mitchell et al., 1994) likely contributing to pain perception.

Central sensitization is an increase in the excitability of neurons within the central nervous system, so that normal inputs begin to produce abnormal responses, or allodynia occurs. Furthermore, central sensitization is responsible for intense pain produced by severe damage and for the spread of pain hypersensitivity beyond an area of tissue damage. Severe tissue and nerve damage can result in chronic activation and release of action potentials, resulting in central sensitization and potentially changing the neuron (Muir and Woolf, 2001;Woolf and Slater, 2000). After a peripheral nerve injury, structural reorganization of the neuron can occur, often restoring them as low-threshold neurons, which results in subsequent pain perception of non-painful stimuli (Anderson and Muir, 2005). The extension of central sensitization from the spinal cord to the brain leads to the development of memory patterns and for quantifiable changes in behavior (Anderson and Muir, 2005). In some cases disinhibition can occur, which is caused by modulations in the dorsal horn neurons and results in a decrease of sensitivity to nociceptive input, (Anderson and Muir 2005) hypersensitivity, hyperalgesia, and allodynia (Moore et al., 2002).

Pain elicited from husbandry procedures can be divided into two categories: acute pain occurs at the time of the procedure or chronic pain, which is caused by the inflammation of the tissues that persists in the days following the procedure (Kissin, 2000). Acute pain typically has a predictable progression and duration of the healing process (Robertson, 2002; Anil et al., 2002), additionally serving as a protective function, causing the animal to attempt to withdraw from the noxious stimulus. Acute pain may also result in a reduction of activity and increasing sleep to promoting the healing process (Chapman and Gavrin, 1999). Chronic pain can result from inflammation or nerve damage and is mediated by structural, physiological and functional

changes in the central nervous system (Garry et al., 2004). Chronic pain can be described as the pain that remains once the healing time has ceased (Anil et al., 2002).

Often literature refers to the terms stress, distress, and suffering along with the discussion of pain (Anil et al., 2002). Pain is responsible for stress, which can further lead to distress and increased suffering (Clark et al., 1997a,b). Stress, in brief, can be defined as the individual being subjected to physical or mental discomfort (Muir and Birchard, 1997), which overtaxes homeostasis and reduces the animal's fitness (Broom and Johnson, 1993). Distress is defined as an aversive state in which the animal is unable to adapt to stressors and subsequent stress, thus the animal further engages in maladaptive behaviors (Committee on pain and distress in laboratory animals, 1992). Distress and suffering can be used synonymously, as they both imply the individual is exposed to persistent negative physical or mental stress (Muir and Birchard, 1997; Chapman, 1992). Physiologic stress responses may be elicited by noxious stimuli, that are predominantly emotional or physical, such as pain (Mellor and Stafford, 1999), however, stress does not always produce pain. Dividing lines between these terms is often difficult (Oldham, 1985) and they are repeatedly used synonymously in the literature when in fact they may not be exclusively indicative of pain.

Nevertheless, acute and chronic pain are capable of producing stress responses, evidenced in changes in the animals behavior, autonomic and sympathetic nervous system, as well as neuroendocrine and immunological responses (Carr and Goudes, 1999; Desborough 2000; Weissman 1999) which will be discussed further in pain assessment. Overall, pain produces alterations in the animal's homeostasis and if prolonged can result in alterations in perception, chronic stress, immune incompetence, poor quality of life, deterioration, and even death (Anderson and Muir, 2005; Chapman and Gavin, 1999).

2.3 Pain Assessment

Although there are many similarities between animals and humans regarding pain perception (Morton and Griffiths, 1985), accurate assessment of pain is more challenging in animals than in humans. Anthropomorphism, or the application of human feelings to animals or inanimate objects, is applicable in very few to zero instances when assessing pain in animals which has led to the development of more acceptable measurements such as behavioral and physiologic responses.

2.3.1 Behavioral Indicators of Pain

Animals have been proposed to respond to pain in two ways; excessive activity or lethargy (Hall et al., 1991). Another description suggests that behavioral responses to pain fall within four categories: responses that modify the animal's behavior by learning, further enabling the animal to avoid recurrence, responses that are often automatic and cause the animal to withdrawal as a form of protection, responses that minimize pain while assisting healing, and responses that are designed to elicit help or to stop another animal from inflicting additional pain (Molony and Kent, 1997; AVTRW, 1989).

Using behavior as an indicator of pain is beneficial because changes are immediate in their appearance (Mellor et al., 2000; Mellor, 1997). Many different behavioral changes have been noted and studied to provide insight into pain responses across species (Mellor et al., 1991; Molony and Kent, 1997; Wood et al., 1991; Firth and Haldane, 1999; Hay et al., 2003). Types of behavior measured and analyzed are dependent on the animal pain model and include changes in behavioral patterns, appearance, vocalization, posture, feeding, drinking, licking, shaking, restlessness, response to handling or fatigue (Anil et al., 2002; Bath, 1998; Fitzpatrick et al., 2006; Molony and Kent, 1997; Smulders et al., 2006; Stasiak et al., 2003).

Definitions and identification of specific behaviors are determined within a detailed ethogram designated as responses to pain. When establishing an ethogram for a particular procedure like castration, researchers often determine changes in the amount of normal maintenance behaviors displayed after the painful event such as standing, lying, walking, feeding, and drinking (Molony & Kent, 1997; Mitlohner et al. 2001). Specific pain-triggered behaviors can also be assessed such as excessive licking at the wound, lifting of the hind legs, foot stamps, and abnormal and excessive tail movement (Molony et al. 1995). Head rubbing or shaking and ear flicks are key behaviors associated with pain from dehorning (Faulkner and Weary, 2000). Attempts to escape from the stimulus are estimated by the amount of scrambling and collapses (Edwards et al., 2011). Even though all the behaviors constitute the ethogram for castration pain, varying responses in these behaviors have been identified in research trials. The following behaviors are those that have been used in previous research and were used in the castration research trial discussed later.

Foot Stamps

A foot stamp is classified by either a front or hind limb being lifted and forcefully placed on the ground (Schwartzkopf-Genswein et al., 1997; Mitlohner et al., 2001).

Castration has been shown to result in a decrease in the frequency of foot stamps, regardless of age, following real castration compared to manipulation of the scrotum 24 h prior (Edwards et al., 2011). When considering age at castration, a previous study on calves (6, 21, and 42d) showed the younger calves (6d) displayed significantly less foot stamping than calves in the older groups (Robertson et al, 1994).

A study by Robertson et al. (1994) found that the frequency of foot stamping was greater for banded calves than control, surgical or Burdizzo method. A previous study on 7d old calves also found the banded group displayed greater foot stamps/kicks than calves in the surgical, Burdizzo, and control groups (Molony et al. 1995). The results from another previous study by Fisher et al. (2001) found calves castrated by surgery foot stamped more than banded castrates in the hours following castration.

Tail Flicks

To be classified as a tail flick, the tail must move to the left or right of center, to the opposite side, and back to the center (Schwartzkopf-Genswein et al., 1997). Across age groups, castration resulted in a decreased frequency of tail flicks from sham manipulation to real surgical castration (Edwards et al., 2011). Calves in the same previously mentioned study by Robertson et al. (1994) found that the oldest calves (42d) displayed the greatest number of tail flicks in the 180 minutes following castration.

A greater increase in tail flicks following surgical castration (Fisher et al, 2001) was observed when compared with banding and intact yearling cattle. The frequency of tail flicks was greatest in the 1 week old banded calves in a study by Molony et al. (1995). Robertson et al., (1994) also observed that banded calves displayed higher frequencies of tail flicks compared to control, surgical, and Burdizzo.

Kicks

A kick is defined as a forceful backward or sideways extension of a hind limb (Schwartzkopf-Genswein et al., 1997; Mitlohner et al., 2001) Significant decreases of kicks in 3 and 6 month calves were observed in the observation period following surgical castration compared to the observation following manipulation of the scrotum 24h prior (Edwards et al.,

2011). However, the amount of kicking was not significantly different between observation periods for 6 week calves observed by Edwards et al. (2011).

Molony et al. (1995) found 1 week old calves castrated using rubber rings displayed a greater number of combined foot stamps/kicks than those in the control, Burdizzo, surgical and Burdizzo rubber ring combination. Conversely, Fell et al. (1986) described that 1 to 2 month old surgically castrated calves kicked during the operation but those castrated using rubber rings were quieter.

Scrambling/Collapses

Although both scrambling and collapse can be considered escape-avoidance behaviors, they each have their own definition. Scrambling is categorized as being forceful movement with 2 or more legs off the ground (Schwartzkopf-Genswein et al., 1997; Mitlohner et al., 2001). An animal falling down in the chute caused by the animal falling onto its knees or hocks is considered a collapse (Schwartzkopf-Genswein et al., 1997; Mitlohner et al., 2001).

The number of collapses in response to castration has not been heavily documented in the literature. Data by Edwards et al. (2011) has shown that 1.5M calves collapsed more than 3M and 6M calves following surgical castration. Due to the inherent nature of this behavior, research would likely benefit from including it in the pain responses ethogram.

Vocalizations

Vocalizations are considered a reliable signal of the psychological and physiological condition of the calf because the energy cost and risk of attracting predators caused by vocalization can be compensated by the high-value resources provided by the dam (Weary and Fraser, 2000;Watts et al., 2000). Vocalization (bellows or moos) in cattle and pigs are associated with pain and distress during a procedure such as castration or branding (Stafford and Mellor, 2009). Cattle that were hot iron branded displayed more vocalizations compared to controls that were restrained and not branded (Lay et al., 1992a, b; Watts and Stookey, 1999). Reported results on vocalization in response to castration in cattle are limited, however, one study found that regardless of treatment (sham manipulation or real castration), 1.5 month old calves vocalized more frequently than 3 and 6 month calves (Edwards et al., 2011).

Eliminations

Eliminations, both fecal and urine, are not often reported in livestock research as an indication of pain or stress. Gastrointestinal motility has been investigated in stress induced rats and researchers concluded that large bowel motility increases as a result of stress and subsequently increases stool output and transit speed (Mertz, 2012). Overall, the probability of elimination was low with no significant differences between ages or treatments (Edwards et al., 2011) in calves subjected to castration versus those subjected to simulated castration. Due to the limited information on this behavior, it is difficult to determine if it truly has no role as a pain indicator.

Lying and Standing

It has been established that standing and lying are among the behaviors that minimize pain and assist healing (Mellor et al., 2000). An increase in the amount of standing and lying can suggest the animal is attempting to minimize stimulation of sensitized nociceptors localized in damaged tissues (Handwerker and Reeh, 1991). An animal could lay down more versus stand or walk or may choose to stand more than walk, both which would avoid overstimulation of the scrotal area and promote healing following castration. Animals may also display abnormal standing, lying, or walking. One example is lying post-castration: normal lying occurs when the animal is in either or both an emotional or physical state of comfort. Abnormal lying, which is defined as unnatural position of the legs, may occur to reduce the area of nerves being excited and occurs when the animal is in a state of discomfort (Molony, 1995).

A previous study indicated an increase in standing behavior and a decrease in laying behavior post-castration (Edwards et al., 2011). These results are consistent with other reports of castration behavior using various methods of data capture (Ting et al., 2003; White et al., 2008; Gonzalez et al., 2010). Gonzalez et al. (2010) found that castration, banding specifically, reduced the time that 6 to 8 month old calves spent lying until 28 days post-procedure. Ting et al. (2003) monitored standing and lying postures of 11 month old calves for 6 h post-surgical castration and found higher incidence of standing postures and a lower incidence of lying postures between the control and castrated groups. Younger calves (1 week) have been shown to increase standing post-castration as compared to non-castrated control calves (Fell et al., 1986; Molony et al., 1995). Fisher et al. (2001) did not observe any significant differences in the percentage of time lying or walking between castrated and non-castrated 14 month old yearling calves on pasture.

Increases and Decreases in Behavioral Responses

A debatable topic is whether an animal will increase or decrease a maintenance behavior in immediate response to pain, but it has been suggested that either can be observed (Chapman, 1985). It has been determined that castration of calves can reduce the expression of active behaviors such as tail wagging, foot stamping, and kicking, which are often dependent on age at the time of the procedure (Edwards et al., 2011).

Individual variation within a species is another theory behind observable increases or decreases in a behavior (Robertson et al., 1994). The pain and distress ethogram developed may be associated with what environmental or physical stressors the animal has been exposed to. With further research, how an animal would respond to a painful stimulus can possibly be linked to the behavior that would be exhibited towards other stressors. For example, a highly dominant, aggressive individual may display more of a behavior, while a low aggression, submissive animal may seem to cope with a situation (Koolhaas, 2007) because that is how they would handle a social situation. Situations like these make evaluating pain difficult when using behavioral techniques alone.

In extremely painful conditions, animals may not have clinical signs of pain at least for a short period because of the secretion of naturally occurring analgesic opioid peptides that prevent overt responses, which would normally make the animal vulnerable to predation (Fraser, 1990). Some behavioral effects may be difficult to detect easily, especially when the effect of a noxious stimulus is to decrease any behavioral response (Mellor et al., 2000).

2.3.2 Biomarkers as Indicators of Pain

Biomarkers have become the focus of investigation when evaluating pain in cattle. Physiological and neuroendocrine measurements provide an additional indicator that is more objective than evaluating behavior alone. While these measurements are objective, most are components of the fight or flight responses and may be easily influenced by environment or stress (Driessen and Zarucco, 2007; Dobromylskyj et al., 2000). These physiologic factors may increase with excitement or handling and may not necessarily be indicative of pain. Recently, a few novel biomarkers are under investigation as specific responses to infection, injury, and inflammation and may be more reliable indicators of the presence of pain. Overall, all of these biomarkers can be utilized for an integrated pain assessment system (Dobromylskyj et al., 2000).

Cortisol

Acute stress results in an immediate increase in corticotrophin releasing factor that evokes the release of ACTH, and a subsequent increase in circulating corticosterone and thus cortisol is commonly used to evaluate distress (Harbuz and Lightman, 1992; Kent et al., 1993). Stressful situations increase the basal secretion of cortisol and cause episodic peaks in cortisol concentrations, making it a useful stress indicator (Minton, 1995; Grandin, 1997; Boesch et al., 2008). In addition, pain promotes activation of the hypothalamo-pituitary-adrenal axis, which is the neuroendocrine stress pathway that also results in the production of cortisol (Cardo et al., 2011).

It is important to recognize cortisol as a stress response hormone and is not necessarily always a direct biomarker of pain. Cortisol concentrations can be impacted by many procedures including handling and restraint that precede husbandry practices, thus, confounding interpretations of resulting changes in cortisol (Lester et al, 1991; Molony and Kent, 1997). Another complication is the diurnal rhythm of endogenous cortisol secretion (Hudson et al, 1975; Lefcourt et al, 1993; Thun et al., 1985). Cortisol concentrations in the relaxed animal show a circadian rhythm with a peak in the early morning followed by a decline throughout the day (Boesch et al., 2009). Without careful planning and analysis of the concentrations, the origins of amplifications in cortisol concentrations cannot accurately be determined.

Although responses vary slightly according to dehorning method, plasma cortisol concentrations increase rapidly 30 to 60 minutes after dehorning, decline slightly, plateau for 3 to 4 hours, and then return to baseline values approximately 6 to 8 hours after the procedure (McMeekan et al., 1997;McMeekan et al., 1998b; Sylvester et al., 1998a,b) as the example shown in Fig. 2.3. The initial peak in plasma cortisol concentrations is probably due to the nociceptor sensitization caused by horn amputation. The plateau and decline may represent a phase inflammation-related pain effects (McMeekan et al., 1998b).

Graf and Senn (1999) identified plasma cortisol concentration increased following cautery disbudding in non-anesthetized calves. Amputation dehorning stimulates a marked cortisol response that lasts 7–9 h. The pattern of this response is similar in calves aged 6 weeks, 3 to 4 months, and 6 months (Cooper et al., 1995; Petrie et al., 1996; McMeekan et al., 1997, 1998a,b; Sylvester et al., 1998a,b). Cauterization of the wound following scoop dehorning is a common procedure. Cauterization after scoop dehorning of 5- to 6-month-old calves marginally

reduces the plasma cortisol concentration (Sylvester et al., 1998b). Total plasma cortisol concentrations return to pre-treatment levels 9 h after dehorning and most studies show that they remain there for 24h (Sutherland et al., 2002a,b) to 36 h (Sylvester et al., 1998a), but in 4-month-old calves after castration and dehorning they were significantly elevated 48 h later (Johnston and Buckland, 1976) supporting the need to consider what happens when multiple stressors are applied to the calf.

Substance P

Substance P consists of 11 amino acids and is a prototypic neuropeptide for more than 50 neuroactive molecules (Carrasco and Van de Kar, 2003; Onuoha and Alpar, 1999). It has been documented that substance P regulates the excitability of the dorsal horn nociceptive neurons and involved in the transmission of pain, stress, and anxiety to the central nervous system (DeVane, 2001). In addition, glutamate coexists with substance P and blockers of glutamate receptors reliably reduce pain behavior and it is assumed that pain is mediated by glutamate action on dorsal horn neurons (Meller et al., 1993; Wilcox, 1991; Hunter and Singh, 1994); however the contribution of substance P is still somewhat unclear (De Biasi and Rustioni, 1988). Neurogenic inflammation, which results from peripheral release of substance P and neurokinin A (Amann et al., 1995), is almost absent in mice with alterations in the genes that encode substance P, which suggests tachykinins from nociception may be required to produce pain (Cao et al., 1998).

Furthermore, one study found that plasma SP concentrations are up to 27-fold greater in human patients with soft tissue injury than healthy controls (Onuoha and Alpar, 1999). Results from another study suggest that substance P can be useful when differentiating between the acute stress from handling and chronic stress associated with pain from husbandry procedures, such as castration (Coetzee et al., 2008) as shown in Fig. 2.4. The effect of analgesics on substance P concentrations has also been evaluated following dehorning, which is discussed later.

PGE_2

Prostanoids, like prostaglandin E2 (PGE_2), are synthetized from arachidonic acid via the COX pathway and contribute to inflammation and pain (Atsufumi, 2011). PGE_2 may have the greatest impact on processing of pain signals and is thought to result in sensitization of peripheral nociceptors coupled with enhanced pain transmission (Takada et al., 2007; KuKanich et al., 2012). A reduction in the production of prostanoids, including PGE_2 both in the spinal cord

and at the periphery, would inhibit COXs, thereby alleviating inflammatory and possibly postoperative pain (Atsufumi, 2011). PGE_2 has not been heavily studied in response to castration or dehorning, with the exception of one study by Landoni et al. (1995), which investigated the use of analgesics as pre-emptive pain mitigation, which is discussed in pain mitigation and an example shown in Fig. 2.5.

Haptoglobin

Haptoglobin is an α2-globulin recognized as an acute phase protein and is synthesized in the liver. The serum levels have been shown to increase during acute infections and inflammation as part of the acute phase reaction (Burtis & Ashwood, 1999). It is particularly valuable in those species that normally have low haptoglobin levels, such as cattle and pigs (Connor et al., 1988; Hall et al., 1992). One caveat of haptoglobin is that it could be influenced by other factors such as renal and hepatic disorders and significant changes in hemoglobin levels, so it should be evaluated with other blood indices as a measurement of pain (Nazifi et al, 2008).

Skinner et al. (1991) evaluated serum haptoglobin in inflammations and introduced haptoglobin as an inflammatory indicator in cattle. The results of one study revealed that haptoglobin was a sensitive marker in various inflammatory conditions in cattle such as digestive tract infection, abomasal displacement, acute respiratory infections and clinical mastitis (Nazifi et al, 2008). Data has shown surgical castrates produce greater plasma haptoglobin concentrations than banded animals in the days immediately following castration (Fisher et al, 2001) (Fig. 2.6). There are currently no reports regarding changes in haptoglobin concentrations following dehorning.

Thermography

Infrared thermography measures the heat emitted from a specific surface and further displays the temperature information as a pictorial representation (Dunbar et al., 2009). Furthermore, the use of infrared imaging has become a popular way to non-invasively evaluate inflammation and to an extent, pain through measurement of temperature of the associated body areas. It is proposed that epinephrine release causes changes in sympathetic tone and the adrenergic effects on cutaneous blood flow cause changes in skin temperature that can be quantified with a thermography camera. When an animal becomes stressed, the HPA axis is

activated and blood flow responses will produce changes in heat in the body (Stewart et al, 2008a,b).

Thermography evaluation of the eye is often used as a reliable indicator of changes in general body temperature (Dunbar et al., 2009). Furthermore, studies have shown that the temperature of the eye may be a good indicator of stress specifically because temperature of small areas around the posterior border of the eyelid and the caruncula lacrimalis are areas rich with capillary beds innervated by the sympathetic system (Cook et al., 2001; Cook et al., 2005; Pvalidis et al., 2002). These areas will respond to changes in blood flow, justifying the eye as a reliable indicator of heat loss and possible stress. A decrease in eye temperature suggests that sympathetic stimulation causes vasoconstriction (Stewart et al., 2008a,b). When considering an increase in eye temperature, the response may be mediated by the autonomic nervous system as suggested by a number of studies (Mellor et al., 2000; Stewart et al, 2010; Levine et al., 2001; Cook et al., 2005), which hypothesized the release of vasodilators, such as nitric oxide, in response to pain contribute to an increase in temperature.

In terms of pain and inflammation, hot iron branding (Stewart et al., 2009; Schwartzkopf and Stookey, 1997) as well as castration and disbudding (Stewart et al., 2010; Stewart et al., 2008b) have been studied to determine changes in temperature. A decrease in eye temperature observed following castration of calves has been attributed to sympathetically mediated alterations in blood flow in capillary beds (Stewart et al., 2010). One study found that eye temperature initially drops in response to a fright or an electric prod in calves (Schaefer et al, 2006). Young calves dehorned by hot-iron cauterization showed a decrease in eye temperature at 5 min post-disbudding (Fig. 2.7), and the authors suggested that the drop in temperature may be caused by sympathetic vasoconstriction and subsequent redistribution of blood flow associated with “fight or flight” (Stewart et al., 2008b).

Algometry

Pressure pain threshold is defined as the minimal amount of pressure that produces pain (Fischer, 1987; Haussler and Erb, 2006). Typically, a hand-held pressure algometer with a spring is used to gauge the pressure threshold of the area being evaluated. Presence and duration of pain are determined typically alongside the use of analgesics with pressure algometry and von Frey filaments (VF), which quantify mechanical nociceptive thresholds (MNT) as kilograms of force (kgf) and grams of force (gf) respectively (Heinrich et al., 2010). The use of pressure algometry

and VF nociceptive test measures MNTs by gradually increasing the amount of pressure until a withdrawal response is seen (Heinrich et al., 2010).

Pressure algometry has been used in cattle to assess pain associated with integument wounds (Dyer et al., 2007; Liu et al., 2009). MNT was also measured in chronically lame cattle (Ley et al., 1996) and nociceptive stimuli in sheep (Nolan et al., 1987). In a dehorning study, calves had lower MNT following dehorning compared with sham dehorning, indicating that all calves were more sensitive to the mechanical stimulus following dehorning (Heinrich et al., 2010) (Fig. 2.8).

Average Daily Gain

One of the biggest and most easily recognized economic factors that can be altered post-husbandry procedure is body weight gain. Cytokines released as a result of the inflammatory response can induce anorexia and lethargy (Johnson, 1997), negatively impacting animal health and growth. A study on yearling steers concluded that banding produced a greater suppression of growth than surgical castration as well as induced prolonged wound formation (Fisher et al., 2001). Dehorning affects weight gain negatively (Winks et al., 1977) as evidenced through a reduction in feed intake and body weight post-procedure (Faulkner and Weary, 2000; McMeekan et al., 1999). The effects have been documented during the 2–6 weeks after dehorning (Loxton et al., 1982) and up until approximately 15 weeks (Goonewardene and Hand, 1991). Behavior data can support these effects on weight gain as dehorned calves grazed and ruminated less between 24 and 48 h after dehorning than they did before or between 48 and 72h after the procedure (Stafford and Mellor, 2011). The prolonged wound healing and reduced weight gains suggest that dehorned cattle are likely to experience ongoing pain, but more long-term analgesic studies similar to Coetzee et al. (2012) which found analgesics to affect weight gain (Fig. 2.9) are required to verify this point.

2.4 Pain Mitigation

Currently, in the United States, there are no laws regulating the mitigation of pain for cattle subjected to husbandry procedures. The United Kingdom and Canada have regulations in place regarding the timing (CVMA, 1996) and treatment of husbandry procedures and also have recommendations for the use of labeled drugs for pain in livestock (DEFRA, 2003). The lack of legislation in the United States is result of the absence of reliable scientific data needed to

implement regulations of husbandry procedures. For safety reasons and due to the lack of reliable biomarkers, the United States Food and Drug Administration (FDA) has not recognized any drugs to be suitable for pain alleviation in cattle.

Although there are no strict regulations in the United States, the AVMA recommends the use of procedures and practices that reduce or eliminate pain and discomfort, including the use of approved or AMDUCA-permissible clinically effective medications such as anesthetics and analgesics (AVMA, 2011). The International Association for the study of Pain defines analgesia as the absence of pain in response to a stimulation that is predicted to be painful (IASP, 1994). Analgesia can be achieved through the use of pharmaceuticals including opioids, alpha-2 agonists, local anesthetics, and nonsteroidal anti- inflammatory drugs (NSAIDs). Studies, which will be discussed next, have indicated that the use of preoperative local anesthetics and non-steroidal anti-inflammatory agents reduce pain and distress associated with castration and dehorning.

2.4.1 Local Anesthetics

Local or regional anesthetics temporarily block the nociceptive signal generation in primary afferent terminals (Shandler, 1965). Furthermore, local anesthetics interfere with conduction of impulses by preventing generation and conduction of the nerve impulse (Riebold, 1995; Anderson and Muir, 2005). More specifically, local anesthetics block nerve fibers, B fibers or motor/touch, non-myelinated pain and temperature sensation C fibers, and A fibers or motor and proprioceptive fibers (Muir et al., 1995). Other results have suggested the G-protein-coupled receptors may be the common targets for local anesthetics as tested with lidocaine (Hollman et al., 2001).

Many sources recommend the use of local anesthetic immediately prior to dehorning which reduces avoidance behaviors such as ear flicking and head shaking in response to cauterization (Heinrich et al., 2010; AVMA, 2012b). Cauterization of the wound following the administration of local anesthesia such as lidocaine and bupivacaine were effective in reducing physiological and behavioral responses (Sylvester et al., 1998b; Sylvester et al., 2004;Sutherland et al., 2002b; Stafford and Mellor, 2005 Graf and Senn, 1999) as shown in Fig. 2.10. The suggested explanation is that the local anesthesia blocks the pain of the amputation and cautery,

and the cautery probably destroys enough nociceptors in the wound to keep the nociceptor impulse input below the pain threshold when the local anesthesia wears off.

It was observed that local anesthesia prevented an increase in plasma cortisol concentrations in 3- to 4-month-old calves undergoing dehorning only for the duration of effect of the anesthetic (McMeekan et al., 1998a). Cauterization after scoop dehorning of 5- to 6-month-old calves when combined with local anesthesia, reduced the cortisol response for 9 hours post-procedure (Sylvester et al., 1998b). This practice with a local anesthetic almost eliminated the cortisol response for 24 hours in 3- to 4-month-old calves (Sutherland et al., 2002b). Overall cortisol response was not significantly reduced, but a rise in plasma cortisol concentrations was delayed for 4 hours by administration of preoperative bupivacaine (Pulley et al., 1974). Administration of bupivacaine locally prior to scoop dehorning, followed by a second dose 4 hours later almost abolished the cortisol response for 8 hours (McMeekan et al., 1998a). Conversely, administration of lidocaine prior to electric dehorning of 7- to10- and 14- to 16-week-old calves did not significantly reduce plasma cortisol levels, suggesting that the anesthetic did not reduce stress associated with dehorning (Boandl et al., 1989).

Although local anesthesia virtually abolished behavioral indicators of pain for the duration of its action, after the anesthetic wore off calves displayed behavioral changes similar to those displayed by calves dehorned without local anesthesia and increases in cortisol concentrations return suggesting inflammatory pain for up to 24 h (Sylvester et al., 2004;Sutherland et al., 2002; Faulkner and Weary, 2000). Thus, as local anesthesia alone does not eliminate total pain following amputation dehorning, likely leaving a large amount of inflammation-related pain (McMeekan et al., 1998b). In studies using local anesthesia in combination with NSAIDS, when both lignocaine and ketoprofen are given before dehorning the cortisol response to dehorning is eliminated (McMeekan et al., 1998b), supporting that this combination alleviates acute and inflammatory, or chronic, pain. These factors support the use of a systemic analgesic along with local anesthetic.

2.4.2 Non-steroidal Anti-inflammatory Drugs (NSAIDs)

The analgesic action of nonsteroidal anti-inflammatory drugs (NSAIDs) has been explained on the basis of their inhibition of the enzymes that synthesize prostaglandins other inflammatory mediators (Mitchell et al., 1997; Curry et al., 2005). Two forms of the cyclo-

oxygenase (COX) enzyme exist in the body, COX-1 is primarily a component of normal cells and COX-2 is up regulated in inflammatory processes (Crofford, 1997). Inhibition of COX-2 activity represents the most likely mechanism of action for NSAID-mediated analgesia while some of the side effects correlated to NSAID therapies are due to the inhibition of the COX-1 enzyme isoform including gastrointestinal irritation caused by inhibiting prostaglandin synthesis in the gut (Mitchell et al., 1994). Each NSAID is dosage dependent on that target species and the objective to be attained.

Flunixin meglumine is widely used as an analgesic, anti-inflammatory, and antipyretic. The exact pharmacologic mode of action is unknown, but the analgesic action may involve blocking pain impulses by non-selectively inhibiting COX-1 and COX-2, which blocks the synthesis of prostaglandins and inhibits other local mediators of the inflammatory response. Flunixin has been previously reported to have inhibitory effects on serum thromboxane B_2 and reduction of PGE_2 concentrations in calves (Landoni et al., 1995).

Flunixin meglamine is the only NSAID approved by the FDA for use in cattle, and its approval is limited to use in treating symptoms of fever associated with mastitis and respiratory disease. Any administration of flunixin for pain relief in cattle would be extra-label use. Flunixin is approved only for intravenous (IV) delivery, which requires a skilled administrator because accidental IM injections can result in pain to the animal due to the highly irritating nature of the formulation. Additionally, the extra-label use and route of administration (IV) has caused such high numbers of violative tissue residues, that in 2007 the U.S Food and Drug Administration Center for Veterinary Medicine was prompted to issue a warning to veterinarians that flunixin should not be administered IM. In addition, the terminal plasma half-life of flunixin ranges from 3 to 8 hours, which requires administration of the drug at least once daily (Hardee et al., 1985; Odensvik and Johannsson, 1995). An NSAID with a longer half-life than flunixin may require less frequent dosing, which would be important from a production standpoint. Furthermore, an orally administered NSAID would have the advantage of avoiding injection site residues in tissues destined for human consumption. Nonetheless, flunixin dosed with caudal lidocaine has been tested and evidenced up to eight hours of visible pain relief for calves subjected to surgical castration (Fig. 2.11) (Currah et al., 2009). Another study providing preliminary results based on observed behaviors in dairy calves following dehorning showed

improved welfare outcomes for calves receiving both local anesthetic and flunixin compared to control calves (Reynolds et al., 2009).

Meloxicam has been identified as a relatively inexpensive NSAID with preferential COX-2 selectivity that has a convenient oral route of administration, a mean terminal half-life of 28 h when delivered orally, and has no identified adverse effects after a single dose of 1mg/kg per os (PO) (Coetzee et al., 2009). Meloxicam is approved for use in humans, cats, and dogs in the U.S. and the injectable form is approved for use in cattle in a number of other developed nations. A recent study found that IV meloxicam might have an effect on the behavioral, physiological, and performance responses to scoop dehorning without local anesthesia (Coetzee et al., 2012) as shown in Fig. 2.12. However, the route of administration also poses the same issue as IV flunixin, the need for skilled IV injection to avoid violative tissue residues due to IM injection. Another study investigated the use of intramuscular meloxicam to alleviate pain post-disbudding and found that it was successful in reducing physiological stress responses, including cortisol (Fig. 2.13), in 6 to 12 week old calves (Heinrich et al., 2009). In addition, meloxicam treated calves have less ear flicking than controls (Heinrich et al., 2010). On the contrary, a study on pain associated with mulesing of sheep resulted in no significant pain alleviation from subcutaneous (SC) meloxicam (Paull et al, 2008). It is possible that NSAIDs, like meloxicam, may need further pharmacokinetic investigation in order to administer a dose effective at providing analgesia for invasive husbandry procedures, such as dehorning, castration or mulesing.

2.4.3 Gamma-Aminobutyric acid Analogue

Gabapentin is a gamma-aminobutyric acid (GABA) analogue designated for treatment of neuropathic pain. The mechanism of action of gabapentin is poorly understood, however, it is thought to bind to the α2-δ subunit of voltage gated calcium channels acting pre-synaptically to decrease the release of excitatory neurotransmitters (Taylor, 2009). Neuropathic pain, such as nerve damage, is thought to be resistant to the effects of NSAIDs (Gilron et al., 2006), thus there is a need for investigation of drugs that do relieve this type of pain. Gabapentin is commonly prescribed for the treatment of chronic pain in humans, however, studies have investigated its pharmacokinetics and effects in domestic and livestock species (Vinuela-Fernándeza et al., 2007; Dirikolu et al., 2008; Kukanich and Cohen, 2009; Coetzee et al., 2010a). In addition, the

elimination half-life of gabapentin varies among species, with Coetzee et al. (2010), proposing a mean elimination half-life of 11h in cattle. Through the same investigation, it was proposed that gabapentin has the potential to mitigate chronic pain syndromes in cattle (Coetzee et al., 2010a) because plasma concentrations of greater than 2μg/mL were maintained for up to 15h (Fig. 2.14). However, investigation of gabapentin to relieve pain associated with dehorning has not been documented.

It is also possible that dehorning causes pain through both inflammatory and neuropathic types. This idea suggests investigation of the use of an NSAID dosed along with a neuropathic pain reliever like gabapentin. Experimental evidence from human studies suggests that gabapentin acts synergistically with NSAIDs, such as naproxen (Hurley et al., 2002) and diclofenac (Picazo et al., 2006), with respect to anti-nociception. An investigation in the target species, bovine, found that gabapentin powder dosed with meloxicam resulted in plasma concentrations above the minimum needed to achieve effects (Coetzee et al., 2010a).

2.4.4 Other management techniques

Despite AVMA recommendations, less than 25% of the United States veterinaries use anesthetics prior to castration and less than 20% use analgesia prior to castration (Coetzee et al., 2010b). Another recent survey found at least half of the respondents reported performing castration of beef and dairy calves < 6 months old (69.8% and 70.1% of respondents, respectively) and dehorning of beef calves < 6 months old (50.9% of respondents) without administration of analgesic drugs (Fajt et al., 2011). In addition, approximately 90% of respondents from Coetzee et al. (2010) report that they vaccinate and dehorn calves at the same time as these are castrated, suggesting additional uncontrolled pain may be experienced. Thus, there are a number of other practices and procedures that can be utilized order to reduce pain and distress from husbandry procedures.

Age

The AVMA recommends that castration should be done at the earliest age practicable. It is a common paradigm that young animals feel less pain than older animals because their pain responses are generally believed to be weaker and perhaps less obvious to an onlooker (Boesch et al., 2006). In support, a review paper found that calves castrated at 6 mo of age or older tended to have greater weight loss, decreased performance, and higher cortisol concentrations than

calves castrated at less than 6 mo old (Bretschneider, 2005). Although several studies have assessed the pain-triggered behavioral responses (i.e. foot stamps, tail flicks, kicks) to castration of calves, the research examined responses of calves in a narrow range of ages or calves well beyond puberty (Robertson et al., 1994; Molony et al., 1995; Fisher et al., 2001), thus additional examination is needed to compare a wider range of ages. Various studies have explored the impact of age on the behavioral responses to castration and found that castration in calves can reduce the expression of active behaviors such as tail wagging, foot stamping and kicking and this is often dependent upon age at the time of the procedure (Edwards et al., 2011; Robertson et al., 1994). Thus, it cannot be concluded that younger calves experience less pain because behavioral responses to acute pain differ with calf age, suggesting an age-based progression in pain responses may exist (Edwards et al. 2011) and pain responses exhibited vary according to age. Due to the lack of validated, repeatable information on identification of the ideal age at which to castrate, further investigation is warranted. Overall, the timing of castration and method employed should take into account the animal's age and weight, skill level of the technician and method used, environmental conditions, and facilities available, as well as human and animal safety (AVMA, 2012a).

As with castration, disbudding and dehorning of cattle is not currently regulated in the United States, however, in the United Kingdom, disbudding with a hot iron is preferred to dehorning and should be performed before cattle reach the 2 months of age (FAWC, 2012). The Canadian Veterinary Medical Association recommends that disbudding be performed within the first week of life (CVMA, 2012). Horns begin as buds within the skin of the poll and at approximately two months old, they attach to the periosteum of the frontal bone overlying the frontal sinus (La Fontaine, 2012). If the horns become attached to the sinus, it is likely the removal would be more invasive (La Fontaine, 2012; Ward and Rebhun, 1992) resulting in increased pain and stress when removed, thus the AVMA also recommends dehorning at a young age. Significant differences in the stress responses between ages have not been documented in the literature, however, it is important to note that the ages of calves will differ because techniques have different age windows in which they can be appropriately and effectively employed.

Method

In addition to age of the calf, the method of castration is also a consideration when attempting to minimize pain. There are a number of castration techniques utilized by the cattle industry, which include physical, chemical and hormonal. Physical castration is by far the most popular because chemical requires increased time and technical skill (Fordyce et al., 1989) while hormonal may require repeated procedures and consumer concerns exist about the method (Stafford, 2007). Physical castration can be subdivided into several methods, which include surgical (scalpel and emasculator), ischaemic methods (banding or ringing), and crushing of the spermatic cord (Burdizzo operation).

The AVMA states that elastrator rubber banding techniques have been associated with increased chronic pain and should be used under proper supervision and training (AVMA, 2008). This is in agreement with a survey of United States veterinarians which suggested that significantly more respondents believed that non-surgical methods produced more swelling, recumbency, stiffness, and anorexia than surgical methods (Coetzee et al., 2010b). Due to the associated pain, banding is discouraged except in situations where surgical castration may result in post-castration complications (AVMA, 2008). According to peer-reviewed scientific literature, the castration method that results in the least amount of pain and distress remains questionable. A study has shown that surgical techniques result in lower frequencies of abnormal behavior (Robertson et al., 1994), while another found surgical castrates to exhibit more repetitive behaviors following castration (Fisher et al., 2001). A review of the literature indicated no difference exists in stress responses between the banded and intact animals (Bretschneider, 2005). Due to conflicting results, further research is needed to develop and improve techniques that reduce pain associated with castration.

Disbudding involves destroying the horn-producing cells (corium) of the horn bud typically with a hot-iron cauterization tool, while dehorning is physical removal of the horns after they have formed from the horn bud. Physical methods of dehorning (gouge dehorning) include the use of embryotomy wire, guillotine shears, or dehorning knives, saws, spoons, cups, tubes, or rubber bands, however, the Barnes-type scoop dehorner is the most common method utilized. As discussed previously, whether disbudding or dehorning is performed is dependent upon the development of the horn. A previous study has shown that cortisol concentrations were higher in scoop-disbudded calves than in hot iron or caustic paste disbudded calves up to 6 hours post- disbudding (Stillwell et al., 2001). In addition, scoop dehorning resulted in slightly higher

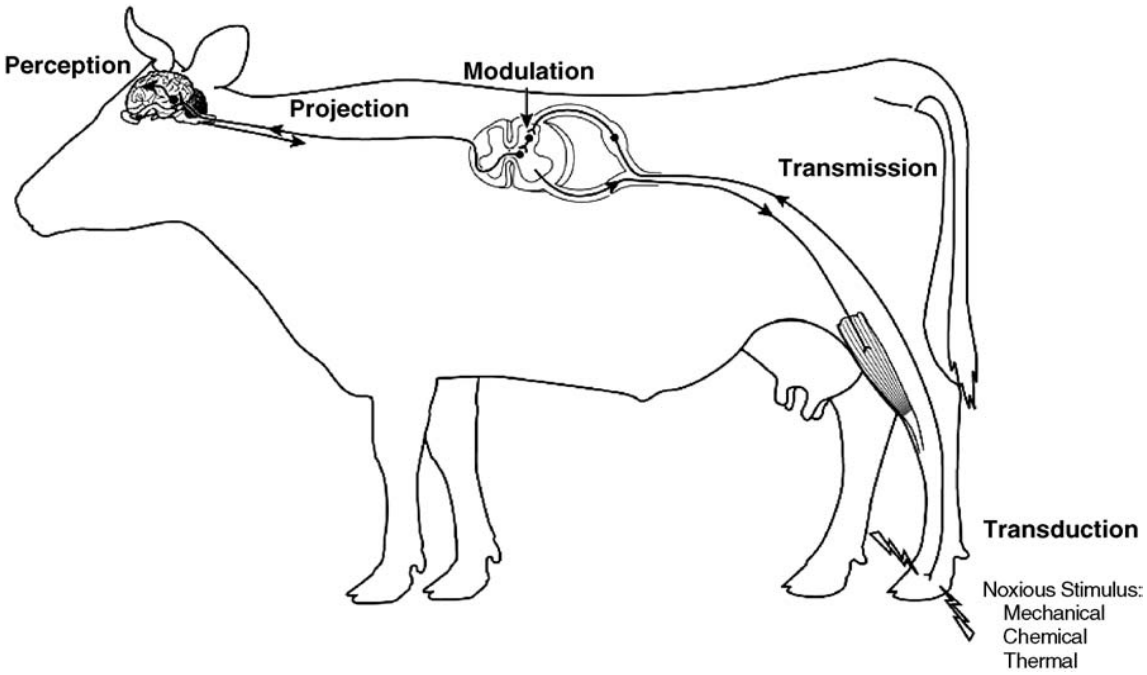
cortisol concentrations than dehorning via saw, guillotine shear, or embryotomy wire, however, there was little difference in distress displayed by 5- to 6- month-old calves in response to these methods (Stewart et al., 2008c). It is difficult to directly compare the pain responses to method because generally the method changes according to horn development (i.e. age) so more factors may contribute to the response than just the method used. Nonetheless, both disbudding and dehorning have been shown to produce pain and stress responses in calves (Stillwell et al., 2001; Vickers et al., 2005; Taschke and Folsch, 1997). As with castration, there is a need for research to identify and develop the easiest, most efficient and least painful method to dehorn cattle.

2.5 Summary

Nociception is the physiologic detection and transmission of noxious stimuli and the resulting pain associated with nociception can cause distress and impact animal welfare. In addition, poorly managed pain can result in subtle losses in productivity and profitability that can eventually mark significant economical losses. Pain can be assessed using behavioral responses, physiological measurements, nociceptive thresholds, and production measures, each of which has been studied in animal pain models. In order for anesthetics or analgesics to be approved for use in mitigating pain in cattle, pain biomarkers must be validated for use in cattle research on inherently painful procedures such as castration or dehorning. It is proposed that pain can be primarily mitigated through the use of local anesthetics, which inhibit the transmission of nociceptive signals, NSAIDs, which inhibit COX enzymes from producing inflammatory mediators, and GABA analogue, which inhibits the release of excitatory neurotransmitters. However, consistent and reliable data for the efficacy of these drugs have not been reported in order for the FDA to approve a pain mitigation drug in cattle. Furthermore, the lack of availability of approved drugs suggests other management techniques should be applied in order to reduce pain (i.e. appropriate age and least painful method), however, the data shows conflicting results as to the ideal age and method. Therefore, the goals and objectives for managing pain in cattle are vast, ranging from recognizing reliable biomarkers of pain to identifying drugs with appropriate efficacy and acceptable food safety withdrawal times. Appropriate and easily administered analgesia for husbandry procedures is required to truly manage welfare and may be of considerable economic importance in the future.

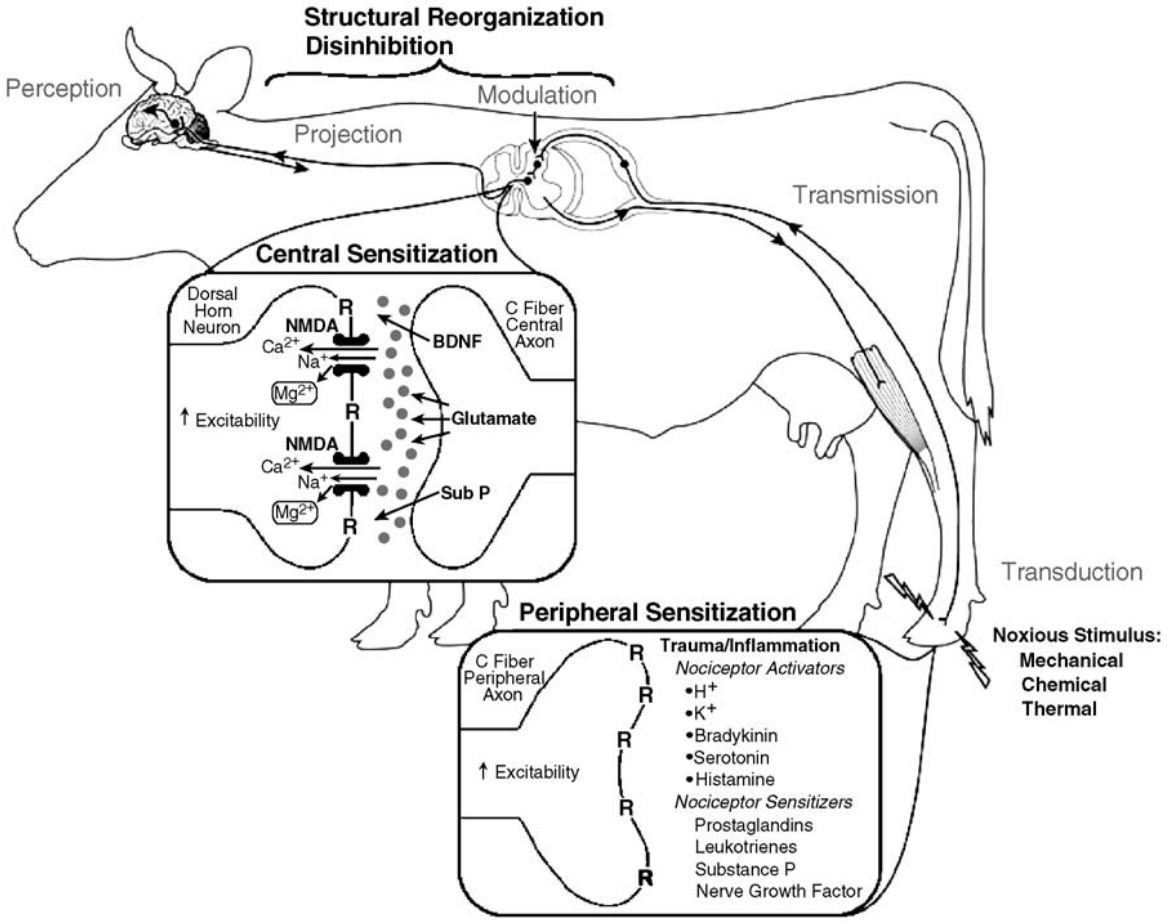
Figures and Tables

Figure 2.1. Physiologic (nociceptive) pain pathway. Noxious stimulus is transduced, transmitted, modulated, projected, and perceived. The brain also generates responses that travel via descending pathways that facilitate or inhibit (modulated) sensory input to the spinal cord



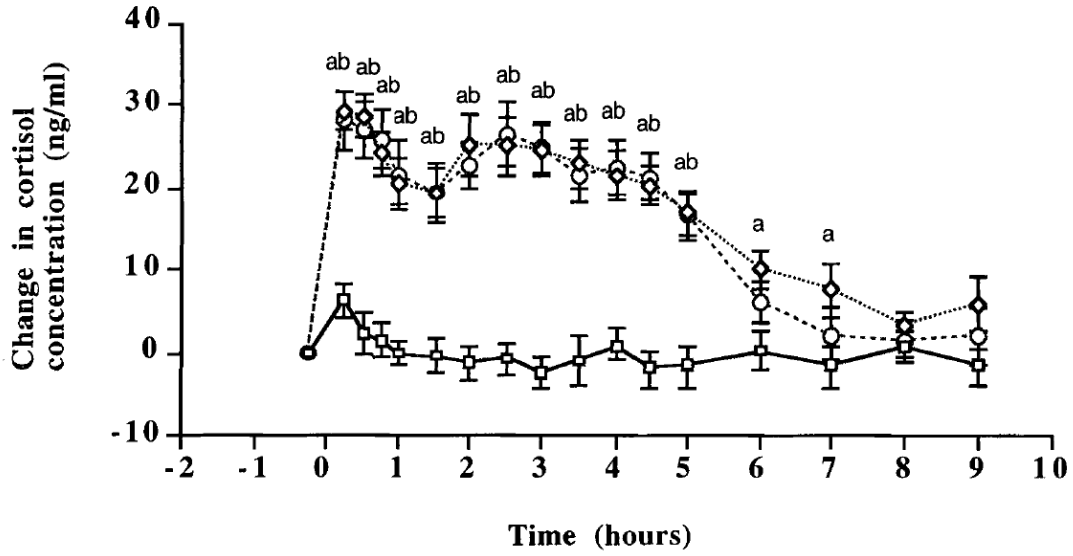
From Anderson and Muir, 2005.

Figure 2.2. Pathologic pain. Peripheral sensitization, central sensitization, structural reorganization, and disinhibition are several of the more prominent mechanisms responsible for the development of moderate, severe, and chronic pain conditions. BDNF, brain-derived neurotrophic factor; NMDA, N-methly-D-aspartate.



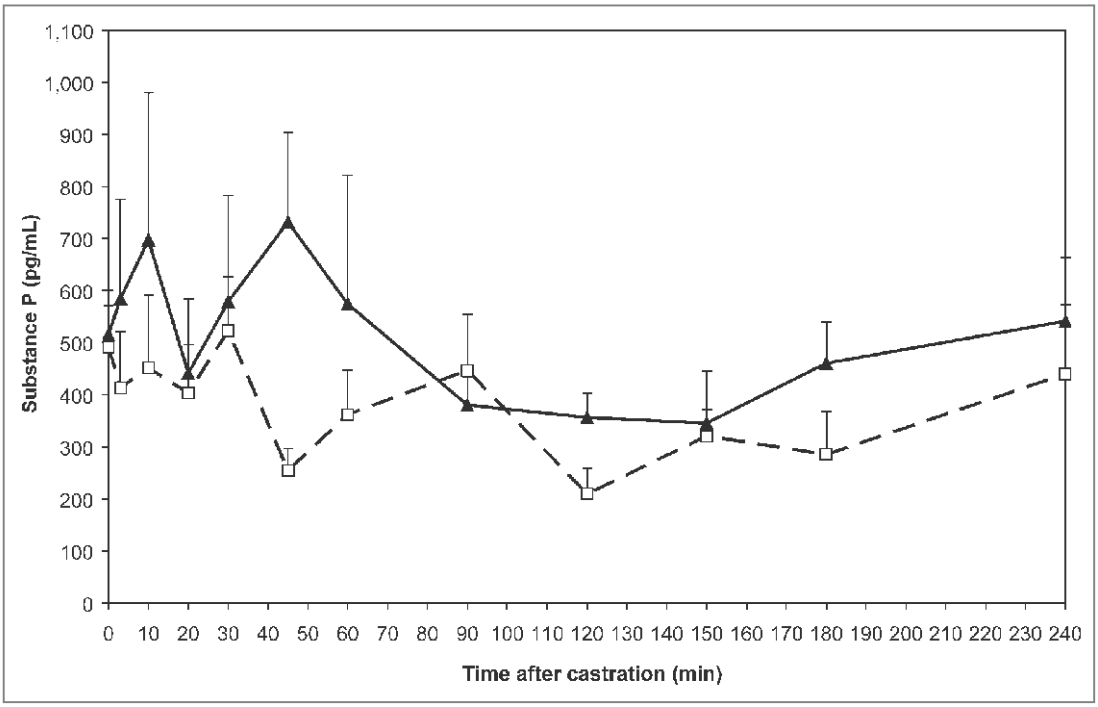
From Anderson and Muir, 2005.

Figure 2.3. Plasma cortisol concentration (nmol/L) (mean±SEM) in control (squares), shallow (diamonds), or deep (circles) scoop dehorned calves. Time 0 represents that time at dehorning. Means with superscripts are significantly different ($p<0.05$: a=shallow scoop from control; b=deep scoop from control).



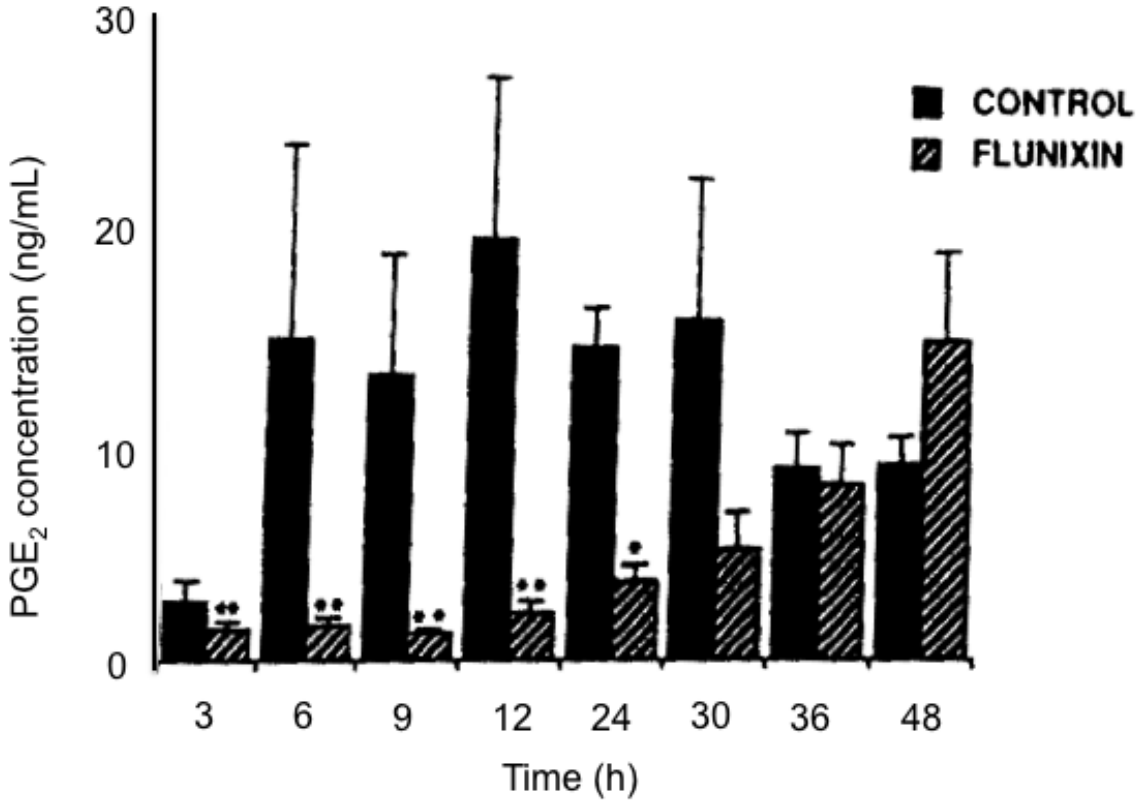
From McMeekan et al., 1997.

Figure 2.4. Mean ± SEM plasma SP concentration in beef calves (n=5/group) after surgical castration (black triangles) or simulated castration (white squares). Time of castration or simulated castration was designated as time 0. Concentrations different significantly (P=0.042; repeated-measures ANOVA) between groups.



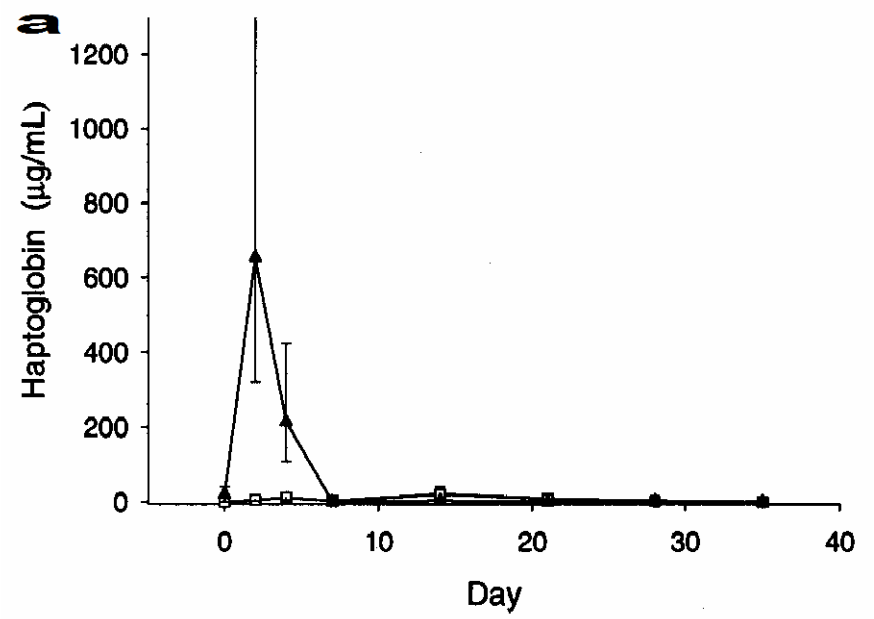
From Coetzee et al., 2008

Figure 2.5. Time course of exudate prostaglandin E_2 in saline and flunixin treated calves. Values are mean ± SEM (n=8).



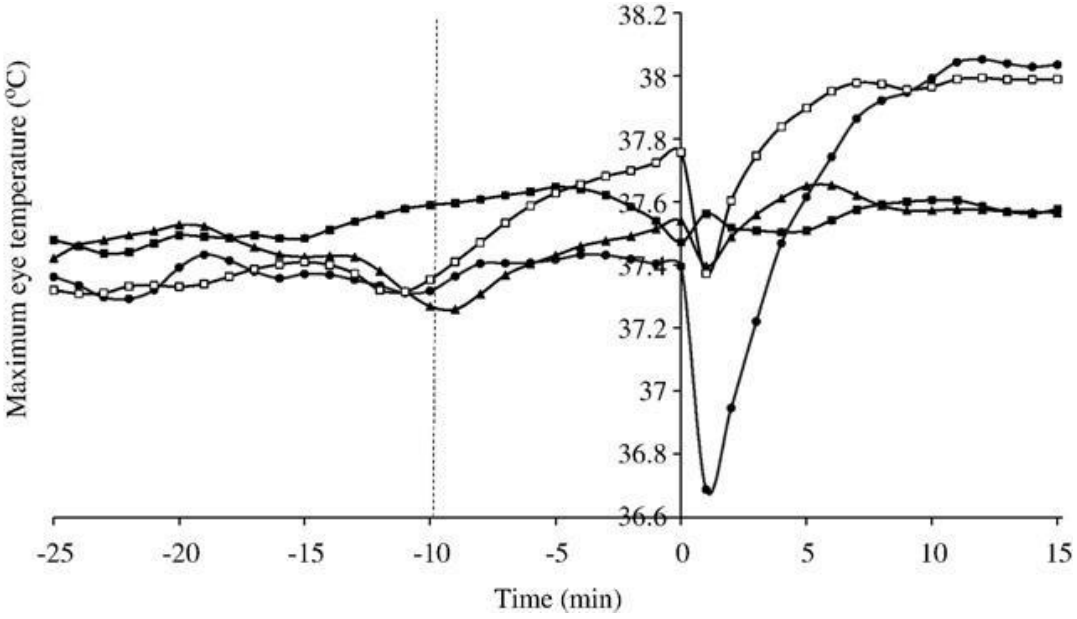
From Landoni et al., 1995.

Figure 2.6. Plasma haptoglobin concentrations following surgical (triangle) or banding (square) castration of 9-month old cattle. Time 0 represents time of castration.



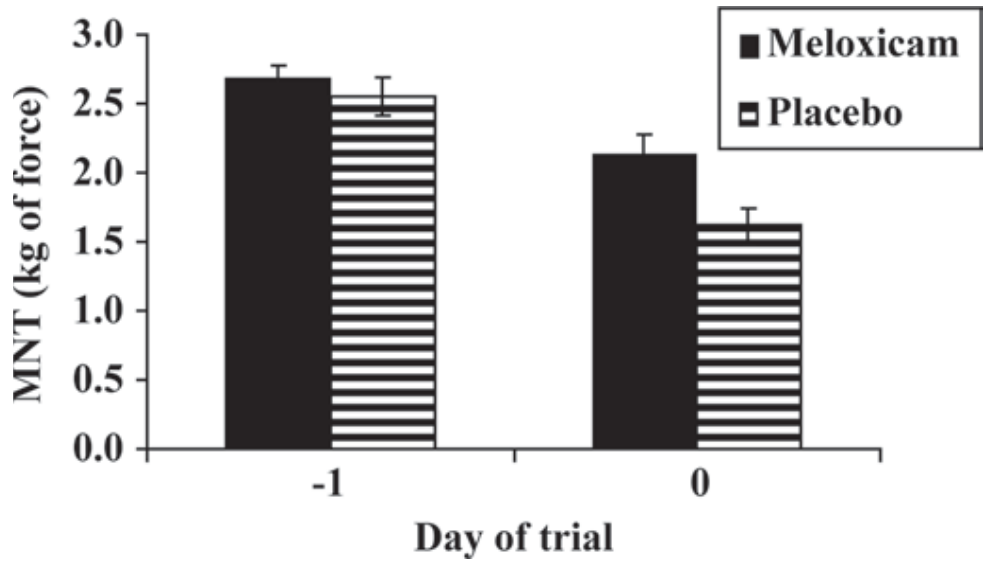
From Fisher et al., 2001.

Figure 2.7. Maximum eye temperature (°C) during the 40 min sampling period for control (closed square), local anesthetic control (closed triangle), disbudding with local anesthetic (open square), and disbudding without local anesthetic (closed circle). Lines were smoothest using a loess smoother separately for each animal pre and post disbudding. The dashed vertical line indicates the time that local anesthetic or the sham procedure was administered and 0 min indicates the time of treatment.



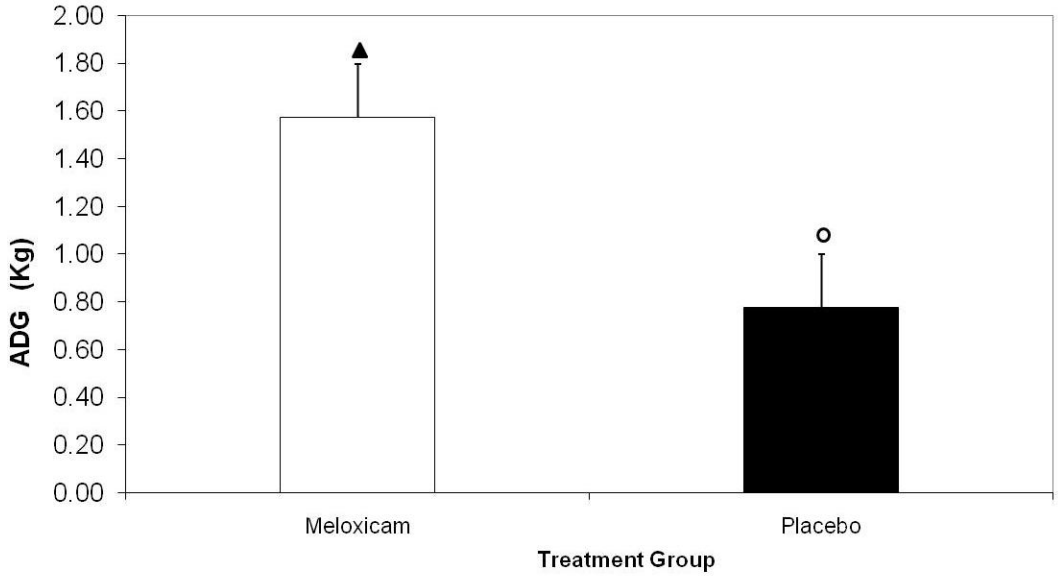
From Stewart et al., 2008b

Figure 2.8. Mean (±SE) mechanical nociceptive threshold (MNT, kg of force), as measured by pressure algometry following sham (d -1) and actual dehorning (d 0). There was no difference in mean MNT between treatments on d -1 (P>0.05). Change in MNT between d -1 and d 0 was greater in control calves (P=0.0004).



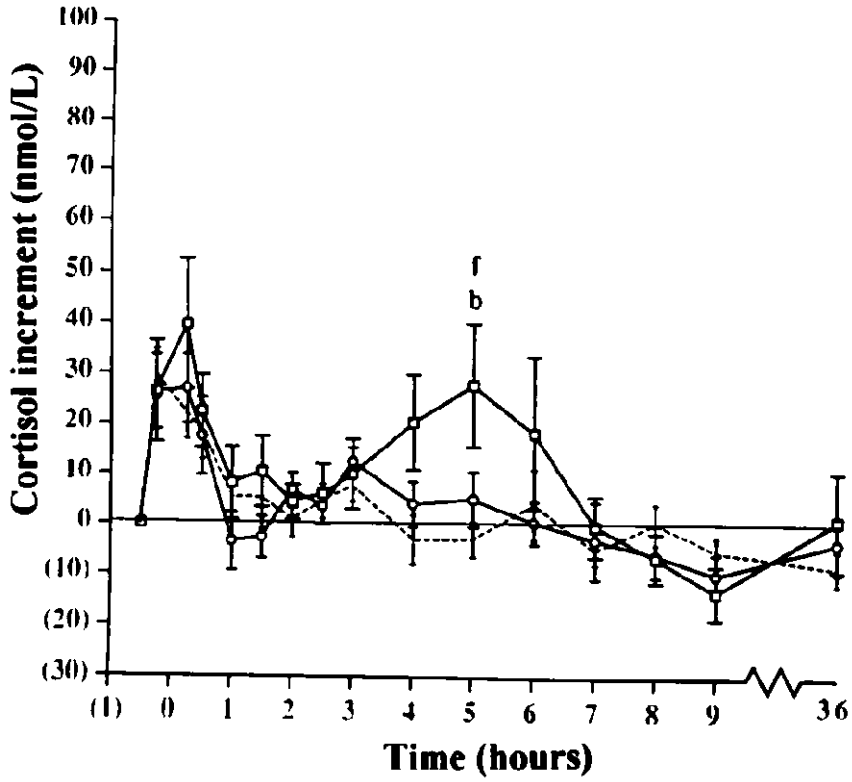
From Heinrich et al., 2010

Figure 2.9. Mean Average Daily Gain (ADG) (Kg) (+/- SEM) 10 days post-dehorning after placebo or meloxicam administration at 0.5 mg/kg IV prior to dehorning. Columns not connected by a symbol of the same shape and color are significantly different (P<0.05).



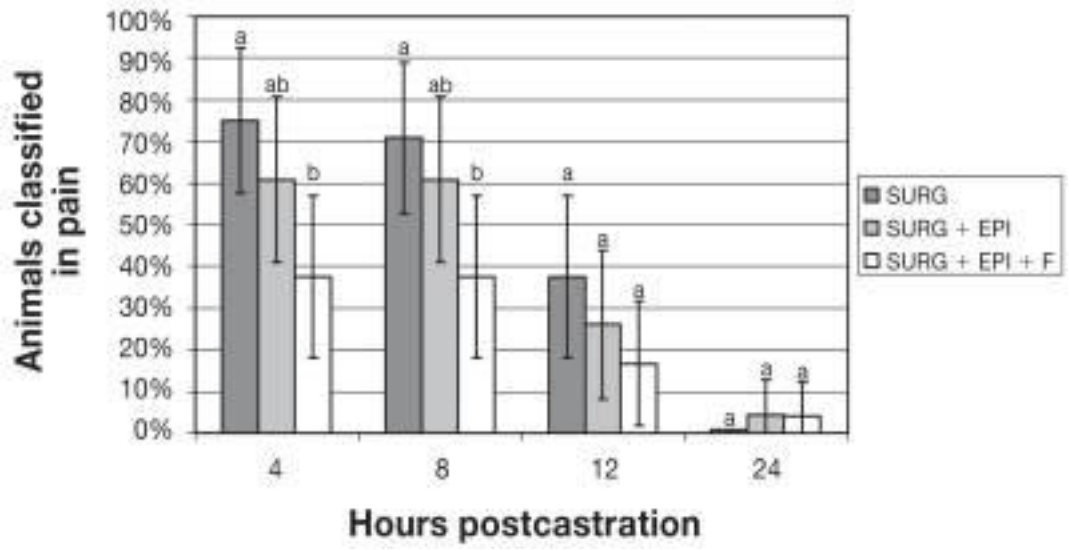
From Coetzee et al., 2012.

Figure 2.10. Changes in the mean plasma cortisol concentrations. Control handling (broken line), local anaesthetic control (diamond), scoop (squares), and scoop and cautery (circles) calves. e= significantly different (P<0.05) from scoop and cautery; b= significantly different (P<0.05) from local anaesthetic control; f= significantly different (P<0.05) from local anaesthetic scoop and cautery. Values are derived from actual plasma cortisol concentrations minus the pretreatment concentration.



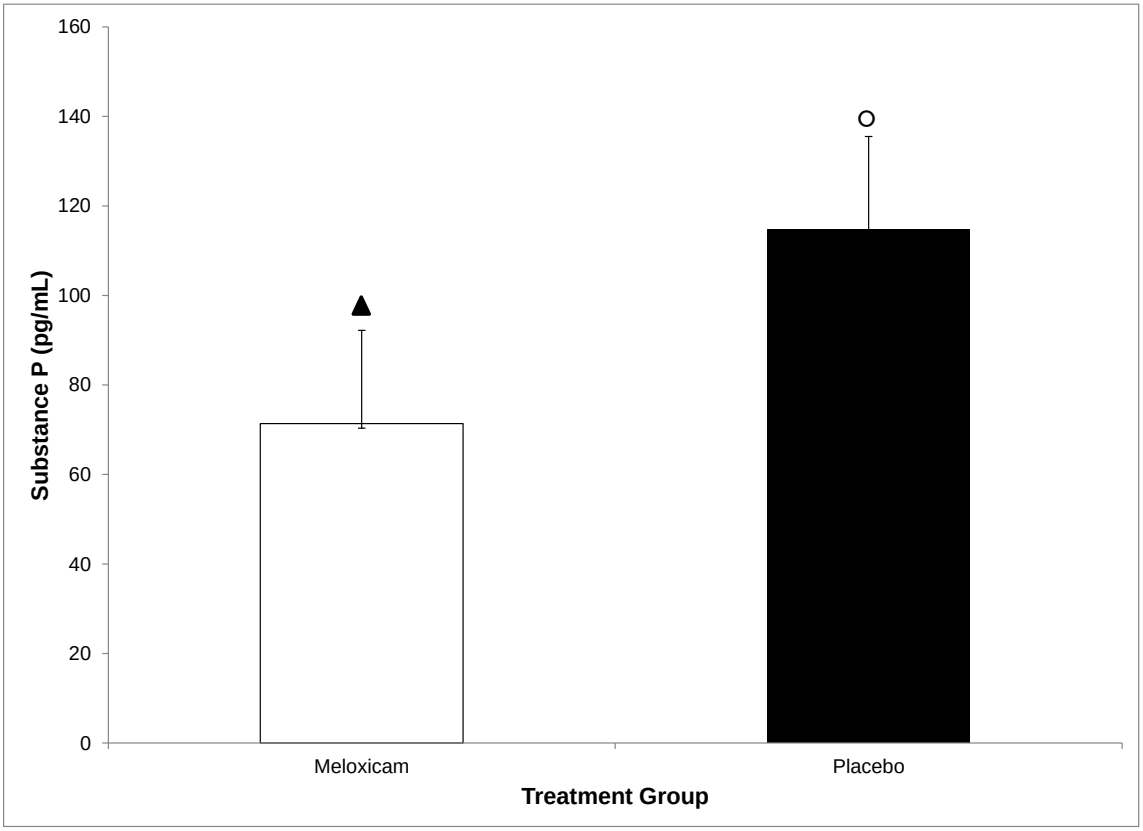
From Sylvester et al., 1998b.

Figure 2.11. Comparison between treatment subgroups of the proportion of animals (95% CI) classified in pain at 4, 8, 12, and 24 h postcastration (SURG — surgical castration; SURG + EPI — surgical castration following lidocaine with epinephrine caudal epidural anesthesia; SURG + EPI + F — surgical castration following flunixin meglumine and lidocaine with epinephrine caudal epidural anesthesia). Bars within observation times with different letters are statistically different from one another ($P < 0.05$).



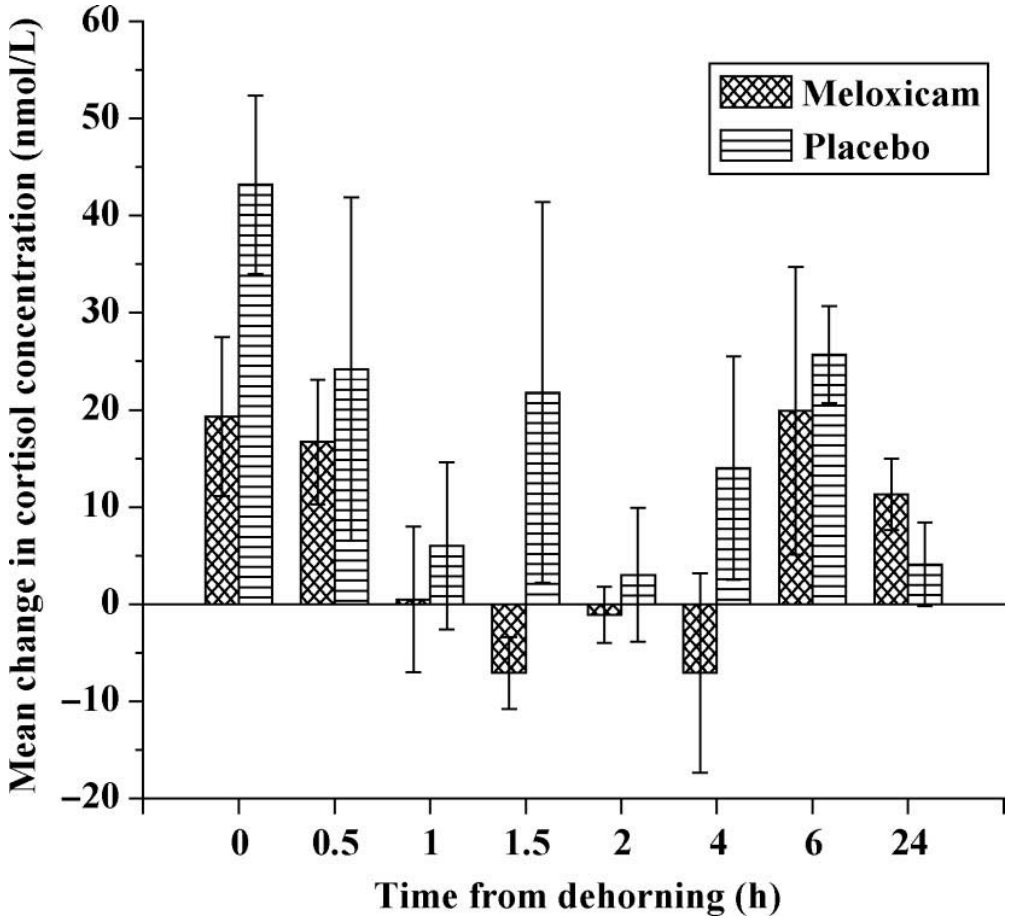
From Currah et al., 2009

Figure 2.12. Mean (± SEM) plasma Substance P concentrations (nmol/L) in calves receiving 0.5 mg/kg meloxicam or placebo IV immediately (< 30 s) prior to dehorning. Columns not connected by a symbol of the same shape and color are significantly different (P<0.05).



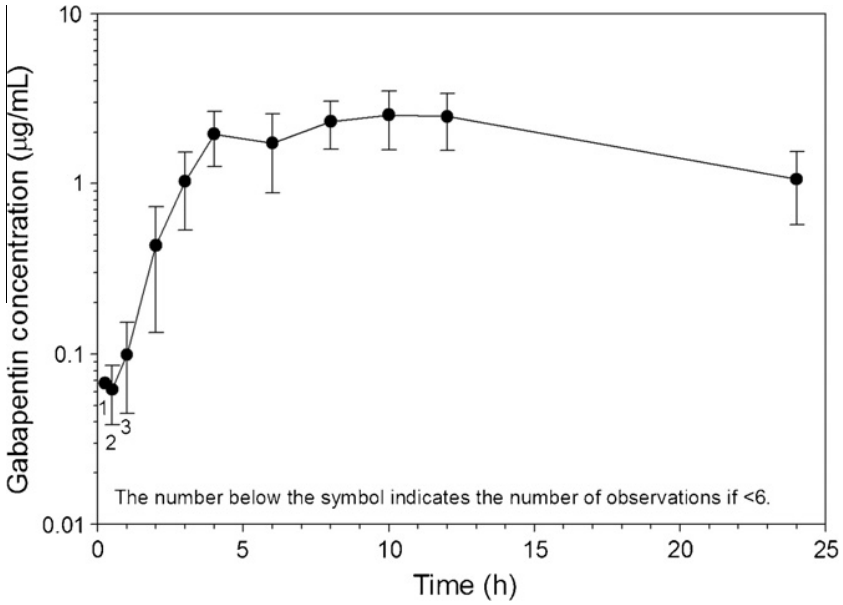
From Coetzee et al., 2012

Figure 2.13. Mean (±SE) change in serum cortisol concentration between sham and actual dehorning (treatment x time interaction, P=0.006). Increase in cortisol from d -1 to 0 was less in meloxicam-treated calves from 0 to 6h. There was no treatment difference at 24h.



From Heinrich et al., 2009.

Figure 2.14. Mean plasma concentrations of gabapentin (±standard deviation) following single 10 mg/kg PO administration in calves. The numbers in parenthesis are the number of observations above the limit of quantitation (LOQ) of the assay if the number of observations was less than 6.



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Chapter 3 - A comparison of the effect of surgical and non-surgical castration on the behavior of 6 week and 6 month old Holstein calves

Paper in preparation for submission to the *Journal of Dairy Science*

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Abstract

The objective of this study was to identify method-related differences in behavioral pain responses in pre and post weaning calves. Calves in two age categories (6 week and 6 mo) were subjected to one of four castration methods: surgical cut (CP; n=18), surgical cut and emasculator (CC; n=20), rubber banding (BAND; n=18), or control manipulation of the scrotum (CONT; n=20). The behavior of all calves was assessed pre- and post-castration for approximately 30 min total to record foot stamps, tail flicks, kicks, collapses, vocalizations, scrambling and elimination. Behavior was also monitored by video camera for 12 h on d 1,2, and 3 post-castration and reviewed for the percentage of time spent standing and lying. There were no significant castration method related effects on the frequency of tail flicks (p=0.96) or foot stamps (p=0.33) nor for the percentage of time spent standing (p=0.72) or lying (p=0.64) for the 6 week old calves. The probability of kicking was greater for 6 week CP (0.10) and CC (0.27) than the other groups proposing greater acute pain is produced from surgical castration. The probability of elimination was greater for 6 week castrated calves than the control animals (BAND, 0.20; CC, 0.07; CP, 0.19). The percentage of time 6 week calves spent standing significantly increased, while the percentage of time lying significantly decreased from d 1 to 2, both suggesting the calves' attempt to avoid over stimulating the scrotal area. The 6 week calves stood more in the middle hours of the day and lie down more in the early and late hours of the day which can be attributed to the natural diurnal pattern and feeding schedule for the animals. Tail flicks (p=0.96) and foot stamp (p=0.33) frequencies of calves in the 6 mo age group were not influenced by castration method nor were the percentage of time spent standing (p=0.50) and

lying (p=0.51). The 6 mo BAND (0.27), CC (0.19), and CP (0.31) had a greater probability of kicking than the control while only BAND (0.50) and CONT (0.07) had a greater probability of eliminating than the surgical castrated groups, likely due to individual variability. A significant increase in the percentage of time spent standing was observed in 6 mo old calves signifying the acknowledgement of damaged tissue and attempts to avoid overstimulation, however, lying was not significantly different between any of the days. The standing and lying patterns of 6 mo calves were influenced by the feeding schedule as they spent less time standing in the morning hours than in the afternoon. When comparing age, 6 week calves displayed greater tail flicks per minute than did 6 mo (p=0.003) and tended to display less foot stamps (p=0.07). In addition, the 6 week calves displayed significantly less tail flicks (p=0.03) and foot stamps (p=0.01) in response to castration while the 6 mo calves showed an increase in tail flicks and foot stamps in response to castration, indicating specific behavioral measurements are dependent upon age. Regardless of age, CP (p=0.007) and CC (p=0.02), but not BAND (p=0.30) calves had fewer tail flicks than control in the post-castration period, signifying surgical castration produces behavioral responses to acute pain and stress. The results add to the body of work to validate behavioral responses to noxious stimuli that can be used for the future development of a cost-effective analgesia to mitigate pain in livestock.
(Key words: calf, castration, behavior, pain)

Introduction

There are approximately 8 million bull calves castrated annually in the United States (Coetzee et al., 2010) in order to reduce unwanted pregnancies, decrease aggressive behavior, and modify carcass quality (Tarrant, 1981; AVMA, 2011; Stafford and Mellor, 2005; Seideman et al., 1982). Castration is not currently regulated in the United States, however, the American Veterinary Medical Association (AVMA) recognizes the importance of castration for animal and human safety, acknowledges the pain and stress associated with the procedure (Stafford and Mellor, 2005), and recommends the use of procedures that reduce or eliminate pain and suffering.

The AVMA recommends that calves should be castrated at the youngest age practical. A paradigm exists that young animals feel less pain than older animals because their pain responses are generally believed to be weaker and perhaps less obvious to an onlooker (Boesch et al.,

2006). Despite AVMA recommendations to castrate at a young age, castration of bulls past weaning age is often observed in attempts to maximize effects of endogenous androgen hormones on growth and performance as evidenced in a survey of cattle veterinarians conducted by Coetzee et al., (2010).In the aforementioned survey, 95% of the respondents indicated that they castrate light weight calves (90 - 270 kg) and approximately 89% of castrate heavy weight calves (>270 kg). The same survey indicated that approximately 90% of respondents castrate some perinatal calves (calves weighing less than 90 kg). However, the respondents indicated that the actual number of calves castrated was relatively small with 21% indicating that they only castrate between 1 and 24 perinatal beef calves per year and 29% indicating that they do not castrate any perinatal dairy calves.

The application of anesthetics or analgesics is a second AVMA recommendation, however, less than 20% of United States veterinarians use analgesia prior to castration (Coetzee et al., 2010). In addition, United States members of the American Association of Bovine Practitioners reported they do not provide analgesic drugs to approximately 70% of calves castrated at less than 6 months of age (Fajt et al., 2011). The reasoning behind the lack of application of anesthetics and analgesics is likely multi-faceted, however a main contributor is the absence of Food and Drug Administration (FDA) approved, cost-effective analgesics for use in cattle. For analgesic compounds to be approved for use in the United States, researchers need to identify and validate methods of pain assessment. This, in combination with the growing desire to minimize animal suffering, has promoted the investigation of various behavioral and physiological measurements as potential biomarkers of pain to ultimately develop effective pain management techniques to be used during routine management procedures.

Because castration at a young age is not widely acknowledged and analgesics are not extensively utilized, employing the least painful method of castration becomes increasingly important. The three types of castration methods used on cattle in the United States include surgical (scalpel and emasculator), ischaemic methods (banding or ringing), and crushing of the spermatic cord (Burdizzo operation). A survey of United States veterinarians conducted by Coetzee et al (2010) identified 57% of respondents most commonly utilize surgical castration with a scalpel followed by testicular removal by twisting (calves <90 kg) or an emasculator (calves >90 kg). Comparison between non-surgical and surgical castration methods in perinatal, light weight and heavy weight calves suggested that significantly more respondents believed that

non-surgical methods produced more swelling, recumbency, stiffness, anorexia, and wound infection than surgical methods (Coetzee et al., 2010). According to peer-reviewed scientific literature, the castration method that results in the least amount of pain and distress is questionable (Molony et al., 1995; Robertson et al., 1994; Fisher et al., 2001; Bretschneider, 2005), thus further research of behavioral and physiological responses is required.

Previous researchers have developed an ethogram to measure specific pain-triggered behaviors (Molony et al. 1995; Mellor et al., 1991; Molony et al., 1993; Kent et al., 1998; Thornton, 1999; Edwards et al., 2011). As a result, the castration ethogram typically contains the following behavioral measurements: foot stamps, abnormal and excessive tail movement, kicks, and collapses or scrambling. Changes in the amount of normal maintenance behaviors displayed after the painful event, such as standing and lying (Molony & Kent, 1997; Mitlohner et al. 2001), were also measured.

Due to the lack of validated, repeatable information on identification of pain responses, which is needed for the development pain mitigation techniques, further investigation is warranted. Thus, the objective of this study was to evaluate the effects of age and method of castration on behavioral responses of bull calves. Calves in two age categories (1.5 and 6 months of age) were subjected to one of four castration methods: surgical cut, surgical cut and emasculator, elastic banding, or control (manipulation of the scrotum). Behavior pre- and post-castration was evaluated to determine if a noxious stimulus, i.e. castration, alters an animal's behavioral responses, namely chute behavior responses (e.g. foot stamps, abnormal and excessive tail movement, kicks, and collapses or scrambling) and pen behavior response (e.g. standing and lying).

Materials and Methods

Animals, Housing, and Husbandry

Prior to the initiation of this experiment, all animal use, handling, and sampling techniques described herein were approved by the Kansas State University Animal Care and Use Committee (Protocol #2831). Following industry standard, analgesia was not provided to calves during the castration procedure and rescue analgesia was not deemed necessary for any calves following the procedure.

Forty Holstein bull calves aged 6 weeks (28-55 kg BW) and 40 Holstein bull calves aged 6 mo (107-197 kg BW) were enrolled in the study and housed at the Kansas State University Beef Cattle Research Center. Upon arrival, all calves received a single subcutaneous dose of oxytetracycline 300 mg/mL (NoromycinTM 300 LA, Norbrook Inc., Lenexa, KS), a single subcutaneous dose of an eight-way clostridial vaccine (Covexin® 8, Intervet/Schering-Plough Animal Health, Summit, NJ), and a single subcutaneous dose of a bovine rhinopneumonitis vaccine (Bovi-Shield GOLD® 5, Pfizer Animal Health, New York, NY) to prevent common bovine disease. A topical pour-on comprised of 5% permethrin and 5% piperonyl butoxide (Ultra Boss® Pour-On Insecticide, Intervet/Schering-Plough Animal Health, Summit, NJ) was applied to all calves upon arrival and repeated as needed for fly control. The 6 week calves were given 14 d for acclimation and 6 mo calves were given 6 d for acclimation. Calves were weighed on arrival, at treatment, and 4 d post-procedure on an in-chute scale (Smartscale 500 USA, Gallagher Group Ltd., Hamilton, New Zealand).

The 6 week calves were individually housed in 1.5 by 6 m stalls with concrete floors with half of the stall shaded by a tin roof. The 6 mo calves were group housed (4/pen) in 4.3 by 8.5 m pens with concrete floors. A mesh cover shaded half of each pen. The 6 week calves received 340 g of milk replacer powder mixed with 2.18 L of water in calf bottles (Land O'Lakes Instant Amplifier Max, Land O'Lakes Inc., Shoreview, MN) at 5:00 h and 17:00 h each day. In addition, all calves were fed 0.68 kg of a calf starter diet per day composed of rolled corn, soybean hulls, dry distiller's grains, and soybean meal. Six-month-old calves were fed 10.10 kg of a growth diet at approximately 13:00 each day composed of brome hay, dried distillers grains with solids, steep-monocalcium phosphate, and soybean hulls. Water was offered ad libitum via an automatic waterer for 6 mo calves and secured water buckets for 6 week calves.

Treatment Groups and Experimental Procedures

The information presented in this study represents a portion of a larger study that included the measurement of additional stress indicators (Dockweiler et al., submitted). Some of these measures will be described in brief in the following sections as some of them impacted experimental procedures and behavioral data collection.

Calves were blocked by age and arrival weight and each was randomly submitted to one of four castration treatments: control (CONT; n=20), banding (BAND; n=18), cut and clamp

(CC; n=18), and cut and pull (CP; n=20). The CONT treatment consisted of scrotal manipulation only. The BAND treatment involved scrotal manipulation followed by the application of a latex band around the scrotum using an elastrator tool. The CC treatment consisted of scrotal manipulation followed by a scrotal incision with a scalpel and clamping of the spermatic cords with an emasculator. CP treatment consisted of scrotal manipulation followed by a scrotal incision with a scalpel and pulling of the testicles until rupture of the spermatic cords. Prior to all treatments, the scrotum was washed with an iodine solution (TriadineTM Povidone-Iodine USP Prep Solution, Triad Disposables Inc., Brookfield, WI) and tools were thoroughly sterilized with 70% isopropyl alcohol (Isopropyl Rubbing Alcohol 70%, Vi-Jon Inc., St. Louis, MO). All treatments were performed by the same experienced operator to minimize inter-individual variation.

Data collection took place over a two-week period and calves were randomly assigned (Microsoft Excel 2010, Microsoft Corporation, Redmond, WA) to a study day with one treatment per age group represented each day (eight calves per day, four 6 week and four 6 mo). Calves belonging to each castration day were housed together, either in the same (6 mo) or adjacent (6 week) pens. The treatment sessions began at 6:00 h and ended at 12:00 h and each calf remained in the chute for approximately 30 min. All treatments took place in a hydraulic, double-alley squeeze chute with a belly bar (Daniels Manufacturing Co., Ainsworth, NE). Upon entrance into the chute, each calf's head was restrained with a halter to facilitate electroencephalogram (EEG) measurement. Briefly, calves were equipped with 12 EEG electrodes placed transcutaneously on the scalp. During EEG set-up, heart rate monitors and accelerometers were applied to each calf. Blood sampling was also performed during this study to measure plasma cortisol and substance P. One day before castration, each calf was fitted for a jugular catheter on the left side of the neck. Catheters were secured to each calf's neck with suture and adhesive. Blood was sampled from each calf at the following time points: baseline, 5, 10, 20, 30, 40, 50, 60, 120, 480 min and 3 d post-castration. The results from these data collections are reported in another manuscript (Dockweiler et al., submitted).

Behavioral Data Collection

Assessment of Behavior in the chute

The behavior of all calves was assessed during the restraint period (approximately 30 min) by one researcher. The observer was positioned at the rear of the chute in view of the rear end of the calf. Foot stamps, vocalizations, scrambling, collapses, kicks, and eliminations were continuously recorded during the procedures and their characterization is noted in Table 3.1. Additionally a video camera (Sanyo Xacti, Panasonic, San Diego, CA) was used to record all the experimental sessions. Video footage was reviewed after the study completion by one observer to count tail flicks. The behavior was recorded during pre-castration (approximately 5-10 min) and post-castration (20 min) periods. To accurately compare the castration periods, all original observations tallied were divided by the number of minutes in each castration period in order to calculate the frequency of each behavior per minute.

Assessment of behavior in the home pen

Behavior of all 6 week calves was monitored in their home pens using stationary cameras and an 8-channel digital video recorder (Swann 4CH Digital Video Recording System, Swann Communications USA, Inc., Santa Fe Springs, CA). Cameras were mounted approximately 2.5 m above ground level, angled to encompass the majority of each pen. Due to the limited nature of the camera set-up, only 32 of the 6 week calves were recorded in the home pens. One camera and one recorder (First Alert Wireless Digital System, Lehigh Consumer Products, LLC, Macungie, PA) were used to monitor each pen of 6 mo calves. The camera was mounted approximately 2.5m above ground level and recorded all the calves in the individual pen.

Upon completion of the study, one researcher reviewed the video footage for each of the age groups. Behavior was monitored from 6:00 h to 19:00 h. Standing and lying behaviors were recorded and each category is described in brief in Table 3.1. Instantaneous sampling with a 5 min interval length was used to quantify the percentage of time spent standing and lying for each hour (Mitlohner et al., 2001). Each behavior was tallied in each hour to obtain a total count that was then divided by the total number of observations recorded per hour (12). The value was then multiplied by one hundred to obtain a percentage of the behavior displayed per hour.

Statistical Analysis for Chute Behavior

Testing normality of residuals for the chute behavior response variables (tail flicks, foot stamps, vocalization, collapses, scrambling/escape attempts, kicks, and eliminations) indicated

that all response variables were not normally distributed. Instead of transforming data to try to achieve normality, generalized linear mixed models (GLMM) were used (Littell, 2006).

The specific fitted GLMMs used were: 1) a gamma distribution with log link function for tail flicks and foot stamps; 2) a generalized Poisson distribution with log link function for counts of vocalizations, collapses, and scrambling/escape attempts; and 3) a binary distribution with logit link function for occurrence (yes/no) of kicks and eliminations. Due to the limited occurrence of vocalizations, collapses, and scrambling/escape attempts, neither the generalized Poisson nor binary distribution could appropriately fit the data, thus they were excluded from the results and discussion.

The overall experiment was a randomized complete block design with a split-plot in time (castration period). Date was the blocking factor and treated as random. The whole plot treatment structure is a 2(Age)*4(treatment) factorial and the split-plot factor was castration period. Therefore, all statistical models used for analysis included the fixed effects of treatment (CONT, BAND, CC, and CP), age (6 mo and 6 week), and castration period (Pre and Post), as well as all 2- and 3- way interactions, and the random effects of date (10 study days), date*(age + treatment), and residual.

All statistical models were fitted using the GLIMMIX procedure of SAS (Version 9.2, SAS Institute, Cary, NC). When analyzing tail flicks and foot stamps F-tests (using a significance level of 0.05), means and confidence intervals were calculated for the age, treatment, castration period main effects and the treatment by castration period interaction. If the treatment by castration period interaction was significant, pairwise comparisons were performed to compare different castration methods within each castration period. If the Treatment main effect was significant, pairwise comparisons were performed to compare different castration methods. When analyzing kicks and eliminations, data were sparse and was fit using a binary distribution. All pairwise comparisons were conducted using the Bonferroni adjustment to avoid inflation of Type I error rate due to multiple comparisons.

Statistical Analysis of Pen Behavior

Percentages of standing and lying in the home pens were analyzed separately by age due to differences in sample size, feeding times, housing arrangement, and pen cleaning. The visual inspection of residuals (normal probability plot) implied that the normal assumption was

appropriate for the standing percentages and lying percentages for 6 mo and 6 week calves. In addition, the central limit theorem supported that the F-tests on means and confidence intervals were valid for the large sample size. Thus, a general linear mixed model was fitted to the response "percentage" for each age level.

For both 6 mo and 6 week calves, the overall experiment was a completely randomized design with a split-plot in time (Day and Hour). The whole plot treatment structure is one-way with the factor Treatment, and the split-plot treatment structure is 3(Day)*13(Hour) factorial. The statistical model used for analysis included the fixed effects of treatment (CONT, BAND, CC, and CP), day (1, 2, and 3), and hour (6:00 to 17:00), as well as all 2- and 3- way interactions, and the random effects of Calf ID nested within Treatment and residuals. Several different variance-covariance structures of the data were specified, but would not converge, so data were assumed independent.

The statistical models were fitted using the GLIMMIX procedure of SAS (Version 9.2, SAS Institute, Cary, NC) under a Gaussian distribution with identity link function. For both 6 mo and 6 week calves, F-tests (using a significance level of 0.05), means and confidence intervals were calculated for the treatment, day, hour main effects and the day by hour interaction. If the day by hour interaction was significant, pairwise comparisons were performed to compare different hours within each day. If day or hour main effect was significant, pairwise comparisons were performed to compare different days or different hours. All pairwise comparisons were conducted using the Bonferroni adjustment to avoid inflation of Type I error rate due to multiple comparisons.

Results and Discussion

Behavior has been extensively studied to quantify an animal's response to pain and distress (Mellor et al., 1991; Molony and Kent, 1997; Wood et al., 1991; Firth and Haldane, 1999; Hay et al., 2003). The objective of the present study was to identify and compare behavioral responses before and following castration. To our knowledge, the present research is the first study to directly compared method, age and before and after castration under the same experimental conditions. Although several studies have assessed the pain-triggered behavioral responses (i.e. foot stamps, tail flicks, kicks) to castration of calves, the previous researchers determined responses of calves in a narrow range of ages or calves well beyond puberty (Molony

et al., 1995; Fisher et al., 2001) and only monitored responses after castration with no restraint. Thus, the present study incorporated a range of ages in order to capture any true differences in responses due to age and compared the calf's baseline response (response to being handled and restrained) to the post noxious stimulus response.

Chute Behavior

The chute behavior was recorded prior to and immediately following castration on d 0. Prior to data collection, four 6 week (2 CP and 2 BAND) animals were excluded from the study due to clinical issues.

Foot Stamps

A tendency was evidenced for the frequency of foot stamps to be different between treatments from pre to post castration ($p=0.07$). No treatment ($p=0.92$), age and treatment interaction ($p=0.33$), or castration period ($p=0.08$) effects were observed (Table 3.2). There was a slight tendency for 6 week to display less foot stamps per minute than 6 mo calves ($p=0.07$) and the frequency of foot stamps was significantly different between ages from pre- to post-castration ($p=0.01$) (Fig.3.1) suggesting that younger calves displayed fewer foot stamps following castration while older calves displayed more foot stamps compared to the pre-castration period.

Robertson et al. (1994) compared the behavior of calves in different age groups following surgical, rubber ring, or Burdizzo castration and found the youngest group of calves (6 d) showed significantly less foot stamping than calves in the older groups (21 and 42 d) post castration. The results from the present study and those from Robertson et al. study (1994) indicate that foot stamps change in response to castration. There is decreased foot stamping in younger animals and a possible increase in older animals. Behavioral expression of the foot stamp may change with age.

Tail Flicks

Overall, there was no effect of treatment on the frequency of tail flicks per minute ($p=0.38$). In addition, no effect of age*treatment ($p=0.96$) or castration period ($p=0.93$) was found (Table 3.2).

An effect of age was found for frequency of tail flicks (p=0.0030) with 6 week calves displaying a greater number of tail flicks than 6 mo. A previous study conducted by Robertson et al. (1994) found that younger calves (6 d) displayed fewer tail flicks than the older age groups. The youngest age in the present study corresponds closely with the oldest age (42 d) in Robertson et al. (1994), suggesting that both studies observed 6 week old calves to display a greater number of tail flicks. An interaction of age and castration period was evidenced (p=0.03) with the 6 week calves displaying a decrease in tail flicks while the 6 mo calves displayed an increase in tail flicks from pre to post castration (Fig.3.2). Due to the varying responses, it is probable that this particular behavioral response is dependent upon the age at castration.

In addition, a treatment and castration period interaction (p=0.0008) (Table 3.2.) evidenced that calves in the CC (p=0.02) and CP (p=0.007) had significantly less tail flicks post castration than during pre castration as compared to calves in the control group. Banded calves were not different from control (p=0.29). The findings suggest that surgical castration decreases the likelihood of an active behavioral response (i.e. a tail flick), while banding has an effect similar to manipulation of the scrotum. This is similar to results obtained by Fisher et al. (2001), who found minimal difference between behavioral responses of banded and control yearling calves in a 1 h and 15 min observational period. Contrary to the present results, previous studies have found the frequency of tail flicks was greatest in the banded calves (Molony et al., 1995; Robertson et al., 1994). The behavioral monitoring period was longer than the current trial as Robertson et al. (1994) monitored for 180 min post-castration and Molony et al. (1995) monitored for 3 h immediately post-castration, 3 h for 4 days and again on every third day for 48 d following castration. It is suspected that banding does not produce substantial acute pain in response to castration, but rather arises as chronic pain resulting from inflammation and occasionally sepsis in the days following castration (Molony et al., 1995). This is a likely explanation for the acute responses to surgical castration, lack of acute responses to banding, and an observational period too short to capture the chronic responses of animals that were banded in both the present study and Fisher et al. (2001).

According to our results, surgical castration appears to result in a decrease of tail flicks, while a greater increase in tail flicks following surgical castration was observed in Fisher et al. (2001) when compared with banding and intact yearling cattle. It has been proposed that behaviors may increase or decrease in response to painful or stressful stimuli (Chapman, 1985),

therefore the literature that exists on immediate behavioral responses to castration does not completely identify a behavioral ethogram that will be consistently observed in all calves, all ages, and all situations. The differences in the two studies are likely multifaceted; however, it could be due to a natural physiologic occurrence involving analgesic opioid peptide release which result in the animal's inhibition of behavioral responses that may cause it to be viewed as an injured, weak and easy target for predators (Fraser and Broom, 1990). Why the animals in Fisher et al. (2001) did not experience a similar response is unclear, but could be due to age (6 week and 6 mo vs. 14 mo) or environment (retained in the chute/human presence vs. open pasture). Calves may hide their pain in the presence of a predator in an effort to mask their vulnerability and because humans may be perceived as predators, an animal may not manifest its pain in front of them (Federation of Animal Science Societies, 1999). This difficulty has led to the development of alternative monitoring techniques including, video (Overton et al., 2002; Edwards et al., 2011), accelerometers (Muller and Schrader, 2003; Robert et al., 2009), and pedometers (O'Callaghan et al., 2003; Haley et al., 2005), which can monitor activity constantly and are small, noninvasive devices that should not influence natural behavior patterns (White et al., 2008).

Kicks

When analyzing kicks, data were sparse and the binary distribution fit was not ideal, with relatively low probability of kicking, so caution is needed when interpreting statistical results. The probability of a calf kicking, pre or post castration is represented for 6 week and 6 mo calves in each treatment, which can be found in Table 3.3.

The probability of 6 mo calves kicking was higher in all castrated groups compared to CONT animals following castration, suggesting that kicking may be a reliable response to a noxious stimulus in older calves. For 6 week old calves, CC and CP had high probability of kicking compared to BAND and CONT, suggesting that surgical castration has a higher probability of producing behavioral responses (i.e. kicking) that are indicative of acute pain or distress than does application of an elastic ring or scrotal manipulation. The results of the present study contrast those in Molony et al. (1995), which found 1 week old calves castrated using rubber rings displayed a greater number of combined foot stamps/kicks. Molony et al. (1995) utilized a longer observational period (3h immediately post-castration, 3h for 4days and again on every third day for 48d following castration) to calculate the frequency of kicks, which allowed

for the capture of the chronic pain responses to banding and contributes to the differing results. The current study also contrasts with another previous study, which found significant decreases of kicks in 3mo and 6 mo calves during the observation period following surgical castration (Edwards et al., 2011). However, the previous study did not capture responses to castration immediately before and after but rather compared responses to an observation period following manipulation of the scrotum 24h prior to castration (Edwards et al., 2011).

Eliminations

When analyzing eliminations data were sparse and the binary distribution fit was not ideal, so caution is needed in interpreting statistical results. The probability of an elimination occurring pre or post castration is represented as a proportion for 6 week and 6 mo calves in each treatment, which can be found in Table 3.3.

The probability of elimination was greater for 6 week BAND, CP, and CC calves post-castration compared to control, suggesting elimination to be a response of pain and distress following castration for this age group. The probability of eliminations occurring was greatest for 6 mo BAND calves post-castration compared to the other three treatments. In addition, CONT calves also had a probability of eliminating post castration higher than the surgical castration methods. The fact that CONT and BAND but no surgical castration method had a high probability of elimination could be due to individual variability.

A previous study found no differences in the number of eliminations between age groups 1.5, 3, and 6 months surgically castrated (Edwards et al., 2011). Furthermore, the variability of the elimination response is likely to due individual variability and different diets (milk replacer vs. mixed ration), which questions the use of elimination as a valid behavioral measurement of pain and distress when comparing two distinct age categories.

Pen Behavior

The pen behavior results will be discussed in terms of d1 being one day post-castration. Day 1 will not be the same calendar day for each calf (e.g. d1 for calf #11 was June 8 and d1 for calf #86 was June 18). There were problems with residual normality testing; since the sample size was so large (1560 observations for standing percentages and lying percentages at each level of age) the power of the tests was high, and thus the test results need to be interpreted with caution.

Standing and Lying

Evidence of significant effects of day ($p<0.0001$), hour ($p<0.0001$), and day*hour interaction ($p=0.02$) were identified in the percentage of time 6 week calves spent standing. No differences were apparent between treatment groups in the percentage of time spent standing ($p=0.72$). The percentages of time 6 week calves spent standing on d 1, 2, and 3 post-castration are reported in Table 3.4. Day by hour interactions are displayed in Table 3.5. Significant effects of day ($p<0.0001$), hour ($p<0.0001$), and day*hour interaction ($p=0.0002$) were identified in the percentage of time 6 mo calves spent standing. No differences were apparent between treatment groups ($p=0.50$). The percentages of time 6 mo calves spent standing on d 1, 2, and 3 post-castration are reported in Table 3.4, while day by hour interactions are reported in Table 3.5.

It has been established that standing is a behavior that minimizes pain and assists healing (Mellor et al., 2000). A voluntary change in posture, such as increased standing after castration, may be the exhibited in order to avoid or reduce stimulation of hyperalgesic tissues (Molony and Kent, 1997; Handwerker and Reeh, 1991). This model was evidenced in the present study as there was a significant increase in standing in both age groups from day 1 to day 3 signifying the animal's attempts to avoid sensitizing the area. In addition, our results are in agreement with a previous study, which found no differences in standing between handled, Burdizzo, surgical, and rubber ring castrated calves (Molony et al., 1995) in the 48 d following the procedure.

For the percentage of time 6 week calves spent lying, there were significant effects of day ($p<0.0001$), hour ($p<0.0001$), and day*hour ($p=0.0031$) but no treatment effect ($p=0.64$). The percentages of time 6 week calves spent lying on d 1, 2, and 3 post-castration are reported in Table 3.4. The percentage of time 6 week calves spent lying down decreased when comparing d 1 to d 2 and 3. On d3, the calves increased in the percentage of time spent lying down compared to day 2, but the percentage was still significantly less than on d3. Day by hour interactions for the percentage of time 6 week calves spent lying per hour are reported in Table 3.5. The percentages of time 6 mo calves spent lying on d 1, 2, and 3 post-castration were not significant and are reported in Table 3.4. Although there was no treatment effect ($p=0.51$), hour ($p<0.0001$) and day*hour ($p=0.0004$) had significant effects on the percentage of time 6 mo calves spent lying. Day by hour interactions are shown in Table 3.5.

Lying is also considered to be a behavior that assists healing (Mellor et al., 2000). An animal could lay down more versus stand or walk in order to avoid further damaging tissue and

further promote the healing process. Overall, the lack of treatment effect is in agreement with a previous study which found no difference in the percentage of time spent lying post surgical or banding castration (Fisher et al., 2001). A lack of differences between castrated animals and control is puzzling, however, it has been documented that control calves whose behavior was monitored using accelerometers for approximately 2 d, stood numerically more after castration (handling and scrotal manipulation) than before castration (White et al., 2008). The study also found that there were no significant differences between surgical castrates and control animals (White et al., 2008), suggesting that in the days immediately following castration, handling and scrotal manipulation have similar behavioral effects as true castration.

The data on the percentage of time standing and lying for the 6 week calves changed throughout the three-day recording. On d 1 and 2, the data suggests the calves stood more in the middle hours of the day and lay down more in the early and late hours of the day. However, on d 3 the 6 week calves appeared to spend an even percentage of time standing (an average of 35.5± 2.37%) and an even percentage of time lying (an average of 56.7± 4.28%) throughout the day with an increase in the last hour. With exception of d 3, the present study captured calves displaying a diurnal pattern of standing and lying (which coincides with feeding patterns), which has been previously documented in cattle (Kilgour et al., 1975; Kondo et al., 1984; Overton et al., 2002; Cook et al., 2005).

In the present study, 6 mo calves spent less time standing in the morning hours than in the afternoon on all three recording days. The observed increase in standing in the afternoon hours likely coincides with the feeding schedule, which was approximately around 1:00pm each day. It is likely that the once-a-day feeding schedule and the limited 12 hours of behavioral data contributed to the absence of a normal diurnal pattern of standing and lying in the 6 mo calves.

The results for percentage or time spent standing and lying are consistent with other reports of post-castration behavior using various methods of data capture, such as pedometers, accelerometers, and automatic telemetry (Ting et al., 2003; White et al., 2008; Gonzalez et al., 2010). Although studying castration via the banding method only, Gonzalez et al. (2010) found that castration reduced the time that calves (6 to 8 months old) spent lying until d 28 post-procedure. Edwards et al. (2011) evaluated castration of 1.5, 3, and 6 mo calves and also found calves spent more time standing than lying following surgical castration. Ting et al. (2003) monitored standing and lying postures of 11 month old calves for 6 h post-surgical castration and

observed a higher incidence of standing postures and a lower incidence of lying postures between the control and castrated groups. Younger calves have been shown to increase standing post-castration as compared to non-castrated control calves (Fell et al., 1986; Molony et al., 1995).

Although the present study was unable to detect differences among castration treatments in the days following castration, the percentage of time spent standing and lying is still of important consideration when evaluating the welfare of the animals. It has been proposed that cattle spend, on average, approximately half of a 24h day lying down (Johnstone-Wallace and Kennedy, 1944; Arnold, 1984). Researchers have found that cattle deprived of the opportunity to lie down have greater acute increase in cortisol concentrations, reduced responses to ACTH challenges, and reduced concentrations of circulating growth hormone compared with free-lying conspecifics (Munksgaar and Lovendahl, 1993; Fisher et al., 2002). The research on time spent lying is done mostly on dairy cows, where cow comfort is crucial and pertains more to chronic distress. Nevertheless, if calves experience increased amounts of standing, significant distress can arise with potential for the development of further issues. Furthermore, according to the results obtained here, researchers would benefit from additional examination of behavioral responses to castration (specifically standing and lying) to evaluate the therapeutic abilities of analgesics and anesthetics that would allow calves to avoid abnormal increases and decreases in behavioral responses.

Conclusion

Despite the complex factors influencing behavior including environment, temperament, and previous experience, assessment of behavior may give a good indication of an animal's reaction to a painful stimulus (Morton and Griffiths, 1985). The behaviors measured in the present study are among those that a calf would display under normal conditions (i.e. standing, lying, and eliminations) in addition to those behaviors that may not occur frequently (i.e. kicking, excessive tail movement) (Schwartzkopf-Genswein et al. 1997; Mitlohner et al. 2001; Robertson et al., 1994; Molony et al., 1995). The study design allowed for detection of pain response by making a comparison of the normal occurrence of these behaviors (pre-castration) to the frequency of the behavior following a noxious stimulus (post-castration), which has not been previously reported. Although an exact quantification of intensity of a noxious stimulus cannot

be found from every event and animal (Lester et al., 1996; Stafford and Mellor, 1993), the present study also allowed for comparison of a wider range of ages than previously reported and assessment of common castration methods.

The age at which calves experience the least behavioral responses to pain cannot be identified by these results, but responses according to age were identified. It appears that 6 week old calves exhibit certain behaviors more than older calves (i.e. tail flicks) but may display less of another behavior than 6 mo calves (i.e. foot stamps). As suggested by Chapman et al. (1985) behavioral responses can increase or decrease which was identified in the 6 week calves decrease and 6 mo calves increase in frequency of foot stamps and tail flicks. Overall, the results indicate the frequencies of specific behavioral pain responses are dependent upon age at the time of the procedure. Although surgical castration manifests a number of painful responses, an overall least painful treatment cannot be established from the present study because all methods of castration produced one or more behavioral responses indicative of pain. In addition, future research would benefit from monitoring the animals for an extended period of time in order to capture all responses that may arise with the chronic pain of banding.

While the intensity of pain from castration is uncertain, pain and distress do result from castration and further reduce the welfare of calves. The pain and distress can be estimated through behavioral research, as performed in this study, and further incorporated into pain models. The results obtained here add to the body of work to validate behavioral responses to the noxious stimuli in hopes to find the least painful method to utilize and to develop a cost-effective analgesia to mitigate the pain.

Figures and Tables

Figure 3.1. Number of foot stamps per minute in 6 week and 6 month calves subjected to different methods of castration. There was a tendency for 6 week calves to display less foot stamps than 6 mo (p=0.07). An age by castration period interaction was observed (p=0.014).

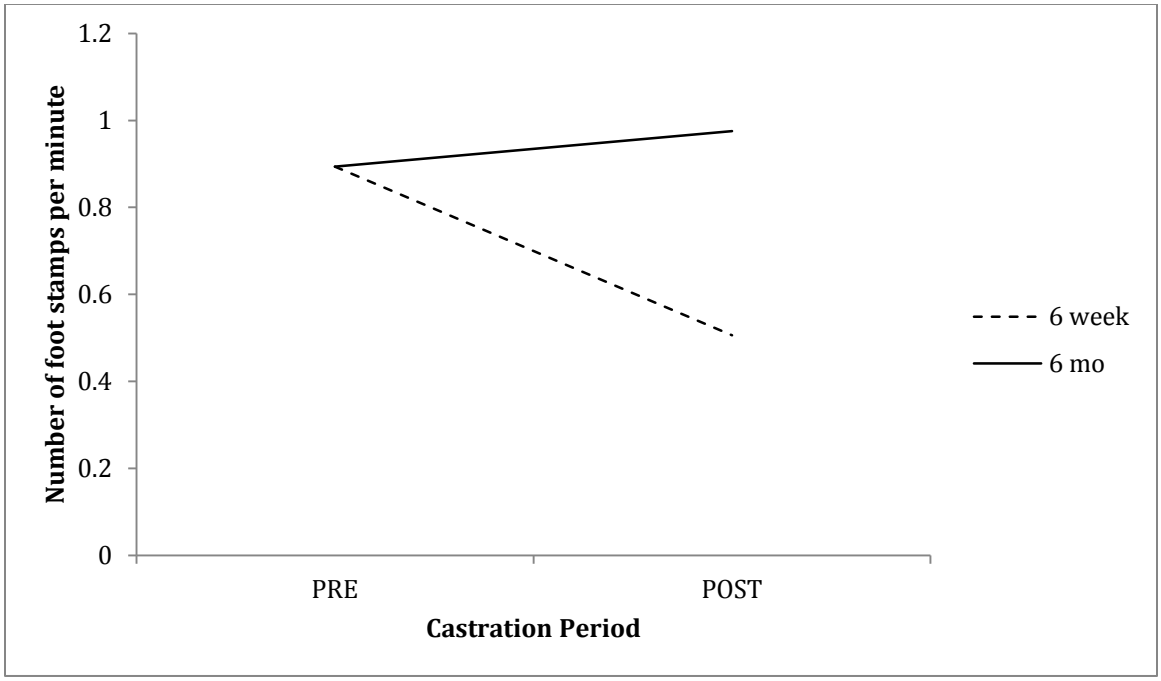

Figure 3.2. Number of tail flicks per minute in 1.5 and 6M calves subjected to different methods of castration. Six week calves displayed a greater amount of tail flicks than did 6 mo (p=0.003). An age by castration period interaction was observed (p=0.03).

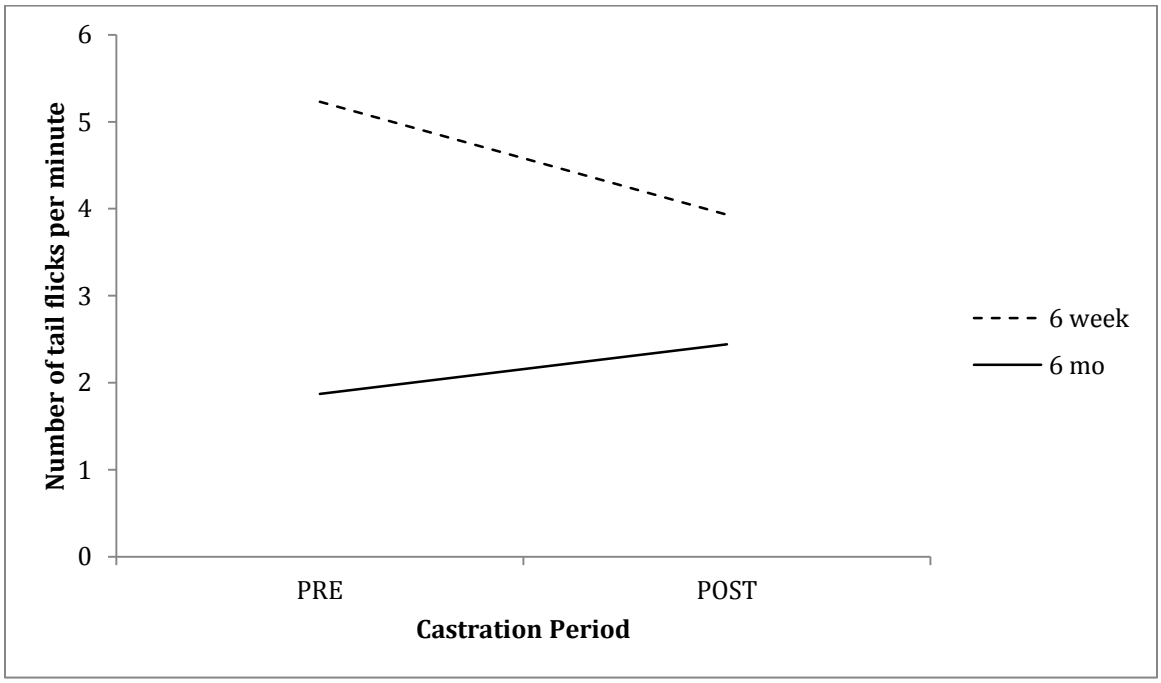

Table 3-1. Definitions of behavioral measurements. These definitions were adapted from Schwartzkopf-Genswein et al. (1997) and Mitlohner et al. (2001).

Behavioral measurement	Definition
Chute behavior	
1. Foot stamp	1. Either a front or hind limb is lifted and forcefully placed on the ground.
2. Tail flick	2. The tail moves to the left or right of center, to the opposite side, and back to the center.
3. Vocalization	3. All vocal sounds. Duration was not measured.
4. Scrambling	4. Forceful movement of the body/attempt to escape; 2 or more legs off the ground and quickly placed back on the ground (running in place).
5. Collapse	5. An animal falling down in the chute caused by the animal falling onto its knees or hocks. The duration of the collapse was not recorded.
6. Kick	6. A forceful backward or sideways extension of a hind limb.
7. Elimination	7. Urination or defecation.
Home-pen behavior	
1. Standing	1. An animal is inactive and in an upright position. No locomotion.
2. Lying	2. An animal has body contact with the ground.

Table 3-2. Mean (SEM) frequencies per minute of observed foot stamps and tail flicks recorded while 6 week and 6 mo calves were restrained for approximately 30 min total in the chute prior to (Pre) and following (Post) one of four castration methods: manipulation of the scrotum (CONT), banding (BAND), surgical cut and clamp with an emasculator (CC), and surgical cut and pull (CP). P-values for age, treatment, castration period (period), age x treatment (A*T), age x castration period (A*P), treatment x castration period (T*P), and age x treatment x castration period (A*T*P) were considered significant at ($P<0.05$).

	Age				**Methods**								**P-value**						
	6 week		**6 mo**		**CONT**		**BAND**		**CC**		**CP**		**Age**	**Treatment**	**Period**	**A*T**	**A*P**	**T*P**	**A*T*P**
	Pre	**Post**	**Pre**	**Post**	**Pre**	**Post**	**Pre**	**Post**	**Pre**	**Post**	**Pre**	**Post**							
Foot Stamp	0.9 (0.2)	0.5 (0.1)	0.9 (0.1)	1.0 (0.1)	0.7 (0.2)	0.7 (0.1)	0.7 (0.2)	0.9 (0.7)	1.1 (0.3)	0.6 (0.1)	1.0 (0.3)	0.7 (0.2)	0.07	0.91	0.09	0.33	0.014	0.07	0.67
Tail Flick	5.2 (1.0)	3.9 (0.8)	1.9 (0.4)	2.4 (0.5)	2.1 (0.5)	2.9 (0.8)	2.3 (0.6)	4.7 (1.3)	3.3 (0.9)	2.1 (0.5)	5.9 (1.8)	3.1 (0.9)	0.003	0.38	0.93	0.95	0.03	0.0008	0.12

Table 3-3. The 95% lower mean confidence limit (LCL), mean (M), and 95% upper mean (UCL) confidence limit proportion of kicking in 6 week and 6 mo calves prior to (Pre) and following (Post) control (CONT), banding (BAND), cut and clamp (CC), or cut and pull (CP) castration.

	6 week							**6 mo**					
	Pre			**Post**				**Pre**			**Post**		
KICKING	**LCL**	**M**	**UCL**	**LCL**	**M**	**UCL**		**LCL**	**M**	**UCL**	**LCL**	**M**	**UCL**
CONT	0	0	0	0	0	0		0	0	0	0	0	0
BAND	0.02	0.10	0.53	0	0	0		0	0	0	0.07	0.27	0.66
CC	0.01	0.08	0.47	0.08	0.27	0.65		0.01	0.09	0.52	0.04	0.19	0.62
CP	0.02	0.10	0.55	0.02	0.10	0.55		0	0	0	0.08	0.31	0.71
ELIMINATIONS													
CONT	0	0	0	0	0	0		0.01	0.07	0.51	0.01	0.07	0.51
BAND	0.03	0.20	0.67	0.03	0.20	0.67		0.01	0.07	0.51	0.17	0.50	0.83
CC	0	0	0	0.01	0.07	0.49		0	0	0	0	0	0
CP	0.01	0.08	0.60	0.03	0.20	0.68		0	0	0	0	0	0

Table 3-4. Mean (±SE) percentage of time spent standing or lying per day in either 6 week or 6 mo old calves subjected to different methods of castration. Rows not connected by the same letter are significantly different (P<0.05).

	Day 1	**Day 2**	**Day 3**
Standing			
6 week	25.9± 2.37[a]	39.7± 2.37[b]	35.5± 2.37[c]
6 mo	50.7± 1.88[a]	49.5± 1.88[a]	59.2± 1.88[b]
Lying			
6 week	64.5± 4.28[a]	51.7± 4.28[b]	56.7± 4.28[c]
6 mo	32.3± 2.49	31.5± 2.49	30.2± 2.49

Table 3-5. Mean percentages of time spent standing and lying in 1.5 and 6 mo calves subjected to castration. Comparison of hours within an observation day (1, 2, or 3 post-castration). Rows not connected by the same letter are significantly different (P<0.05).

6 week	**Hour**													
Standing	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**SEM**
Day 1	2.9^{d}	28.0^{ac}	16.4^{ad}	18.1^{ad}	24.5^{a}	26.5^{a}	30.9^{ac}	19.1^{a}	23.5^{a}	25.9^{c}	42.1^{a}	59.0^{b}	18.8^{a}	5.8
Day 2	20.2^{f}	39.4^{abde}	41.1^{abde}	27.4^{aef}	46.8^{bc}	28.8^{adef}	41.2^{ab}	56.7^{c}	38.7^{abe}	53.5^{bc}	43.2^{bcd}	52.2^{bc}	27.3^{af}	5.8
Day 3	33.4^{c}	40.8^{bc}	30.1^{c}	31.1^{ac}	36.9^{c}	38.5^{c}	31.5^{ac}	32.0^{ac}	34.8^{c}	39.4^{bc}	40.5^{bc}	53.9^{b}	17.8^{a}	5.8
Lying														
Day 1	82.2^{g}	55.2^{bd}	68.2^{acdefg}	63.8^{acdef}	59.1^{def}	56.4^{df}	59.5^{cdf}	77.1^{ag}	74.4^{aceg}	53.5^{bd}	69.9^{afg}	40.5^{b}	78.8^{ag}	6.8
Day 2	74.3^{e}	51.38^{acd}	47.7^{bdf}	63.6^{ace}	50.6^{acd}	59.5^{acde}	53.8^{cdf}	34.2^{b}	56.7^{af}	39.2^{bd}	41.3^{bd}	38.5^{bd}	61.8^{ae}	6.8
Day 3	55.9^{c}	53.2^{c}	56.5^{c}	59.5^{c}	55.6^{c}	54.6^{c}	58.3^{c}	61.2^{c}	60.9^{c}	54.4^{c}	56.6^{c}	33.1^{b}	77.3^{a}	6.8
6 mo	**Hour**													
Standing	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**SEM**
Day 1	32.9^{c}	34.7^{c}	37.0^{c}	30.1^{c}	40.2^{c}	38.1^{c}	55.4^{a}	61.7^{a}	76.1^{b}	68.9^{ab}	63.9^{ab}	60.5^{a}	59.7^{a}	5.3
Day 2	21.8^{de}	31.9^{def}	63.2^{b}	49.4^{ab}	34.5^{e}	51.8^{ab}	59.7^{ab}	54.7^{ab}	61.3^{bc}	59.4^{ab}	45.8^{af}	54.8^{ab}	54.3^{ab}	5.3
Day 3	34.1^{a}	42.4^{ac}	55.7^{bc}	61.9^{bdf}	46.0^{ac}	54.3^{bc}	53.2^{cf}	56.1^{bc}	87.2^{e}	71.1^{d}	65.3^{bdf}	68.0^{bd}	73.9^{ef}	5.3
Lying														
Day 1	45.4^{be}	49.4^{b}	36.7^{abd}	41.8^{bde}	30.6^{adf}	31.8^{acdef}	24.4^{af}	24.1^{af}	18.5^{f}	24.4^{acf}	31.4^{acdef}	31.3^{acdef}	29.9^{acdf}	5.5
Day 2	66.3^{f}	48.5^{g}	25.8^{ace}	30.1^{ade}	42.6^{dg}	21.7^{ce}	21.1^{ce}	25.5^{ace}	14.1^{bc}	14.9^{bc}	39.1^{ad}	29.1^{ade}	30.6^{ade}	5.5
Day 3	38.8^{de}	36.5^{de}	29.5^{acde}	29.7^{acde}	39.3^{de}	37.9^{de}	42.5^{df}	34.2^{cef}	7.1^{b}	20.1^{ab}	28.7^{acef}	27.7^{ace}	20.3^{abc}	5.5

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Chapter 4 - The pharmacokinetics and impact of meloxicam, gabapentin, and flunixin in post-weaning dairy calves following dehorning with local anesthesia

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Abstract

Approved analgesic compounds in cattle are not currently available in the United States due to the lack of validated pain assessment methods and marker residue depletion studies.. In this study, we compare the pharmacokinetic parameters and effect of preemptive analgesics administered to calves subjected to dehorning with local anesthesia. Holstein steers were randomly assigned to receive one of the following treatments *per os* (PO) or intravenously (IV) (n= 8/group): meloxicam (1 mg/kg PO), gabapentin (15mg/kg PO), meloxicam (1 mg/kg) and gabapentin (15 mg/kg) PO, flunixin (2.2 mg/kg IV), or a placebo. Plasma drug, , haptoglobin and substance P (SP) concentrations, serum cortisol concentrations, ocular thermography, mechanical nociceptive threshold (MNT), average daily gain (ADG) and ex-vivo prostaglandin (PGE_2) synthesis was evaluated. Data were analyzed using mixed effects models and non-compartmental pharmacokinetic analysis. Meloxicam, gabapentin, and meloxicam with gabapentin at the present doses did not reduce cortisol or PGE_2 concentrations. Analgesic-treated calves had significantly lower plasma SP concentrations and improved ADG compared with controls. Flunixin calves had reduced circulating cortisol and PGE_2 concentrations for 24h compared to controls. Meloxicam treated calves showed an increase in MNT at two horn bud sites compared with the other treatments. Analgesics improved ADG and reduced biomarkers of pain but effects differed by compound and route of administration.

Keywords: calves, dehorning, meloxicam, gabapentin, flunixin

Introduction

Dehorning is a routine management practice performed on dairy cattle operations worldwide (Stafford and Mellor, 2011). Dehorning is not currently regulated in the United States, however, it is recommended by the American Veterinary Medical Association (AVMA) that calves should be dehorned at the youngest age practical (AVMA, 2012). The AVMA also recommends the use of practices which reduce pain associated with dehorning (AVMA, 2012), however, a survey of North-Central and North-Eastern United States dairy producers found that only 12.4% of dairy owners use local anesthetic nerve blocks and only 1.8% provide systemic analgesia at the time of dehorning (Fulwider *et al.*, 2008). The reasoning behind the lack of application of anesthetics and analgesics is likely multi-faceted, however a main contributor is the absence of Food and Drug Administration (FDA) approved analgesics for use in cattle.

Despite the benefits of dehorning cattle, the tissue damage from husbandry procedures has been shown to cause acute pain and distress, which is indicated by changes in plasma cortisol (Petrie *et al*., 1996; Sutherland *et al*., 2002), changes in behavior (Sylvester *et al*., 2004), fluctuations in eye temperature (Stewart *et al*., 2008), electroencephalography (EEG) (Bergamasco *et al*., 2011; Gibson *et al.*, 2007), heart rate (Von Borell *et al*., 2007), weight gain (Laden *et al*., 1985), substance P (Coetzee *et al*., 2008), and haptoglobin (Fisher *et al*., 2001). Many sources recommend the use of local anesthetic prior to dehorning which reduces behavioral and physiological pain responses (AVMA, 2012; Sutherland *et al*., 2002; Graff and Senn, 1999; Faulkner and Weary, 2000). However, upon the elimination of the anesthetic dose, pain responses return (Sutherland *et al*., 2002; Graf and Senn, 1999) which necessitates the investigation of extended pain alleviators.

The administration of non-steroidal anti-inflammatory drugs (NSAIDs) has been shown to provide extended postoperative analgesia for calves subjected to castration or hot-iron dehorning (Andersen and Muir, 2005; Duffield *et al*., 2010). Flunixin meglumine is the only NSAID approved by the United States FDA for IV delivery in cattle and is limited by label to treating signs of fever and endotoxemia associated with mastitis and respiratory disease. Furthermore, the terminal plasma half-life of flunixin ranges from 3 to 8 hrs (Hardee *et al*., 1985; Odensvik and Johannsson, 1995), which requires administration of the drug at least once daily. An orally administered NSAID, like meloxicam, with a longer half-life (28 h) may require less frequent dosing (Coetzee *et al*., 2009), which would be important from a production standpoint.

Previous literature has investigated the use of IM meloxicam to alleviate pain post-disbudding or hot iron cauterization only, and found that it was successful in reducing physiological and behavioral stress responses (Heinrich *et al*., 2009; Heinrich *et al*., 2010). On the contrary, a study of reducing the pain associated with mulesing (removal of wool bearing skin around the buttocks) of sheep resulted in no significant pain alleviation from SC (0.05mg/kg) meloxicam (Paull *et al*., 2008). It is likely that the efficacy of the drug is dependent upon the route of administration, promoting the development of an effective and easily administered dose. Neuropathic pain is thought to be resistant to the effects of NSAIDs, thus there is a need for investigation of drugs, such as gabapentin, that are designated for the treatment of nerve damage. Gabapentin is commonly prescribed for the treatment of chronic pain in humans, moreover, it has been previously documented that gabapentin has an elimination half-life of 11h in cattle with the potential to mitigate chronic pain syndromes, such as lameness, in cattle (Coetzee *et al*., 2011). It is also possible that dehorning causes both inflammatory and neuropathic pain , suggesting that concurrent dosing of an NSAID and a neuropathic pain reliever may provide superior analgesia. An investigation in the target species, bovine, found that gabapentin powder dosed with meloxicam PO resulted in plasma concentrations above the minimum concentration believed to provide analgesic effects (Coetzee *et al*., 2011).

The objective of this study was to determine the pharmacokinetic parameters of meloxicam, gabapentin, meloxicam with gabapentin administered PO, and flunixin administered IV to calves in order to compare drug effects on biomarkers of pain and distress following scoop dehorning. The variables investigated included plasma drug concentrations, serum cortisol concentrations, ex-vivo PGE_2 synthesis, plasma haptoglobin concentrations, plasma substance P concentrations, ocular thermography, MNT as measured through pressure algometry, and ADG. A primary goal of the study was to develop a cost effective analgesic regimen for use in cattle that will have a short meat withdrawal time and is easily administered by lay persons.

Materials and Methods

Prior to the initiation of this experiment, the Kansas State University Animal Care and Use Committee approved all animal use, handling, and sampling techniques described.

Animals, Housing, and Feeding

Forty-five, six month old, Holstein steers ranging in weight from 111 to 197 kg were obtained from Northeast Kansas in June 2011 and shipped to the Kansas State University Beef Cattle Research Center where they were housed for the duration of the study. All calves received physical examinations and were given a commercial (eight-way clostridial vaccine Covexin 8, Intervet/Schering-Plough Animal Health, Summit, NJ), a bovine rhinopneumonitis preventative (Bovi-Shield GOLD 5, Pfizer Animal Health, New York, NY), oxytetracycline (Noromycin 300 LA, Norbrook Inc., Lenexa, KS), and a topical pour-on comprised of 5% permethrin and 5% piperonyl butoxide (Ultra Boss, Inetervet/Schering-Plough Animal Health, Summit, NJ), all administered per label instructions for dosage and route of administration.

All calves were maintained in open concrete pens with half of each pen shaded by a mesh cover. Each pen was 4.3 by 8.5 m and held 5 animals. One animal per treatment was assigned to the pen they would be housed in at random, using Excel random number generator (Excel, Microsoft Works 2010, Microsoft, Redmond, WA). Water was offered *ad libitum* via automatic waterers. Calves were fed 10.10kg of a growth diet at 13:00 h each day.

Treatment Allocation and Drug Administration

Forty steers were randomly assigned to one of five treatment groups as described in Table 4.1. Calves were ranked in ascending order of bodyweight, blocked into cohorts of 5 calves, and within each cohort, calves were assigned a random number (Excel, Microsoft Works 2010, Microsoft, Redmond, WA). Random numbers were then assigned (Excel, Microsoft Works 2010, Microsoft, Redmond, WA) to treatment groups to ensure that bodyweight was equally distributed between treatment groups. All steers were then given a random number in order to determine the order in which they would be dehorned. This study was conducted on two consecutive days in June 2011, with 20 steers dehorned per day.

Jugular Catheterization

One day before dehorning (d-1), 14G x 140mm jugular catheters (Abbocath-T, Abbott Ireland, Sligo, Republic of Ireland) were placed in the shaved left side of the neck of the calves. Catheter patency was maintained with approximately 3mL heparin saline flush (4U/mL).. All catheters were removed following the last blood sample collection on d 0 to prevent infection from occurring.

Dehorning Procedure

All animals were dehorned in a hydraulic, squeeze chute with a belly bar to prevent recumbency (Daniels Manufacturing Co., Ainsworth, NE). All animals to be dehorned were brought from home pens to the crowding pen and placed in the previously established procedure order. It is documented that performing dehorning with a local anesthetic reduces acute stress responses, thus all calves were given the local anesthetic lidocaine as a cornual nerve block (Stafford and Mellor, 2011; Faulkner and Weary, 2000). Upon entrance from the crowding pen to the alleyway preceding the chute, one veterinarian restrained and administered each calf a 2% lidocaine hydrochloride cornual nerve block (3 mL per horn) ten min before dehorning. All oral and IV drug dosing was given at one min prior to dehorning by another veterinarian blinded to the treatments.

Horns were removed with a Barnes-type scoop dehorner followed by the cautery of the blood vessels via a hot iron according to industry standards. Following cauterization, blood stop powder (Vedco, Inc., St. Joseph, MO) was applied. After processing, calves were haltered and tied in sorting pens just beyond the chute for approximately 2 h and then moved to shaded study pens for an additional 8 h. Following 12 h, all steers were moved back to their home pens. All pens provided free access to water for the duration of data collection and the experimental design was controlled as much as possible for stress of handling.

Blood sample collection

Time points for blood collected were baseline -10 min (TB; true baseline with no drug in system and prior to dehorning), in the chute (TC; after lidocaine had been administered but before dehorning),at 5 min and at 0.5, 1, 2, 4, 6, 8, and 12 h post dehorning. The 5 min blood sample was collected, then the calf was released from the chute and taken to the sorting pens where the 0.5, 1, 2 h samples were obtained. The remaining blood samples were collected after the calves had been moved to the study pens. Calves were processed through the chute to obtain samples from 24 through 168 h. Blood samples for cortisol, drug concentration, and substance P analysis were obtained at all time points. Samples for haptoglobin and ex-vivo prostaglandin E2 were obtained at TB, 5 min and 6, 12, 24, 48, 72, 96, 120, 144, and 168 h..

Blood samples taken up to 12 h post-procedure were collected from all treatment groups using the catheters. Blood samples collected at 24 through 168 h were obtained via jugular

venipuncture. Catheter patency was maintained using heparin saline flush (4U/mL), thus an initial 5 mL of blood was withdrawn from the catheter and discarded, followed by collection of sample blood and transfer to evacuated tubes. For cortisol and haptoglobin analysis, blood was placed into a serum clot activator tube (Vacuette 6mL Z Tubes, Greiner Bio-One, Kremsmünster, Austria). Blood for drug concentration and ex-vivo prostaglandin determination was placed in a lithium heparin tube (Vacuette 6mL LH Tubes, Greiner Bio-One, Kremsmünster, Austria). The substance P analysis required each tube to contain 300µL benzamidine to act as a protease inhibitor prior to blood collection in the ethylenediaminetetraacetic acid (EDTA) K3 tube (Vacuette 6mL K3E Tubes, Greiner Bio-One, Kremsmünster, Austria). A 20mM solution of benzamidine was prepared in water and 300µL was added to each tube for a final concentration of 1mM benzamidine in whole blood to act as a protease inhibitor. The EDTA K3 tubes and lithium heparin tubes were stored on ice and the serum clot activator tubes were stored at room temperature prior to centrifugation. Blood samples were centrifuged for ten min at 1500g within thirty min of collection. Plasma and serum was then harvested and frozen at - 70° C until analysis.

Cortisol Analysis

Serum cortisol concentrations were determined as previously described in cattle (Coetzee *et al*., 2007) using a solid-phase competitive chemiluminescent enzyme immunoassay and an automated analyzer system (Immulite 1000 Cortisol, Siemens Medical Solutions Diagnostics, Los Angeles, CA). A sample volume of 100 µL was used in each assay well. The reported calibration range for the assay is 28 to 1,380 nmol/L with an analytical sensitivity of 5.5 nmol/L.

Haptoglobin Analysis

Haptoglobin analysis depends on the peroxidatic activity of hemoglobin. By adding hemoglobin in excess to serum samples, it binds to haptoglobin and becomes resistant to acid inactivation. In contrast, the peroxidatic activity of free hemoglobin is lost. The plasma haptoglobin concentration is calculated using a standard curve prepared by incubating known amounts of hemoglobin with a serum sample containing a concentration of haptoglobin greater than 150mg/dL (Smith *et al*., 1998).

Substance P Analysis

Blood for substance P analysis was collected into EDTA K3 tubes containing benzamidine. Plasma substance P concentrations were determined in duplicate using a validated method as previously described (Coetzee et al., 2008). Briefly, Substance P was 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 according to the manufacturer's instructions in the Substance P enzyme linked immunosorbent assay (ELISA) kit (Substance P ELISA Kit, Assay designs, Ann Arbor, MI). The coefficient of variation between triplicate bovine samples at each fortified SP concentration was < 30%. The linear regression line fit between the three points at each of three control concentrations had a correlation coefficient of 0.99.

Ex-vivo Prostaglandin Analysis

Analysis of ex-vivo prostaglandin synthesis at each time point, 3 mL of whole blood were incubated in glass tubes for 24 h at 37 °C with LPS (diluted in PBS) used at 10 μg/ml to stimulate PgE2 production operated by monocytes as described by Brideau *et al*. (2001) for an ex-vivo analysis of PGE_2. At the end of incubation, all samples were centrifuged at 400*g* for 10 min at room temperature. The resulting supernatant was stored at -80 °C until the determination of *ex-vivo* plasma PGE_2 levels using a commercially available standard ELISA kit (Prostaglandin E_2 Kit, Cayman Chemical Company, Ann Arbor, MI) (Fuchs *et al*., 2002). This assay is based on the competition between PGE_2 and a PGE_2-acetylcholinesterase (AChE) conjugate (PGE Tracer) for a limited amount of PGE Monoclonal Antibody (Prostaglandin E_2 EIA Kit – Monoclonal. Cayman Chemical Item No. 514010. Kit booklet).

Pressure Algometry

Pain sensitivity was measured before and after the dehorning procedure using a pressure algometer (Wagner Force One FDIX, Wagner Instruments, Greenwich, CT) at time points TB, 1, 2, 4, 6, 8, and 12 h. The device was equipped with a round rubber tip measuring 1 cm in diameter. The amount of pressure a calf tolerated was measure in kilograms of force (Kgf) over the area of the rubber tip. The calves were restrained with a halter in the study pens for time points 1, 2, 4, 6, 8, and 12 h post-dehorning. All algometry measurements were taken as

described by Heinrich (2010) by the same researcher. The researcher placed a hand lightly on the calf's right poll until the animal habituated to being touched and stood still in a relaxed posture. The hand was slowly removed and replaced with the algometer rubber tip placed directly beside the horn bud, such that rubber tip covered the cautery wound and the edge of the normal tissue. Pressure was applied perpendicular to the poll at a rate of approximately 1Kgf/s until the calf withdrew its head. The same procedure was followed for the right horn (RH) and left horn (LH). Three sites around each horn bud were measured, in alphabetical order, as shown in Fig. 4.1.

Ocular Thermography

Changes in corneal temperature were measured using an infrared system (ThermaCAM P65HS, FLIR Systems, Wilsonville, OR). An image was taken of the eye at baseline, immediately after dehorning, and at 720 min post-dehorning by one researcher blinded to the treatments. Images were analyzed for changes in temperature using research grade software (Thermacam Researcher Pro 2.8 SR-1, FLIR Systems, Wilsonville, OR). Variables included maximum temperature, minimum temperature, and average temperature, and the difference between the maximum and minimum temperature, analyzed from a circular designated area of the eye.

Plasma drug analysis

Plasma concentrations of meloxicam (mass-to-charge ratio *(m/z)* 352.09→114.90) and gabapentin (*m/z* 172.1→154.1) 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 (50 µL) were added to 200 µL of internal standard (piroxicam 0.5 µg/mL in methanol, *m/z* 332.12→95.10 and pregabalin 5 µg/mL, *m/z* 160.00→142.00) in methanol with 0.1% formic acid to precipitate the proteins. The samples were vortexed for 5 s and centrifuged for 10 min 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 100% B from 0 – 0.5 min with a linear gradient to 50% B at 2.5 min which was maintained until 5 min, followed by a linear gradient to 100% B at 5.5 min with a total run time of 8 min. Separation was achieved with a C18 column (ACE C18AR, 150 mm x 2.1 mm x 5 µm, MAC-MOD Analytical, Chadd's Ford, PA, USA)

maintained at 40 C. The standard curve was linear from 0.01 to 5 μg/mL for meloxicam and from 0.25 to 10 μg/mL for gabapentin. The standard curves were accepted if the correlation coefficient exceeded 0.99 and predicted values were within 15% of the actual values. The accuracy of the assay was 98 ± 10% of the actual value and the coefficient of variation was 6% determined on replicates of 5 each at 0.01, 1, and 5 μg/mL for meloxicam. The accuracy of the assay was 98 ± 5% of the actual value and the coefficient of variation was 5% determined on replicates of 5 each at 0.25, 2.5, and 10 μg/mL for gabapentin.

Plasma concentrations of flunixin (*m/z* 297.14→264.00) were also determined with high-pressure liquid chromatography and mass spectrometry. Plasma samples or standards (50 μL) were added to 200 μL of internal standard (piroxicam 0.5 μg/mL in methanol, *m/z* 332.12→95.10) in methanol with 0.1% formic acid to precipitate the proteins. The samples were vortexed for 5 s and centrifuged for 10 min 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 min with a linear gradient to 50% B at 2.5 min which was maintained until 5 min, followed by a linear gradient to 85% B at 5.5 min with a total run time of 8 min. Separation was achieved with a C18 column (Supelco Discovery 50 mm x 4.6 mm x 5 uM, St. Louis, MO, USA) maintained at 40 C. The standard curve was linear from 0.01 to 55 μg/mL. The standard curves were accepted if the correlation coefficient exceeded 0.99 and predicted values were within 15% of the actual values. The accuracy of the assay was 100 ± 10% of the actual value and the coefficient of variation was 6% determined on replicates of 5 each at 0.01, 1, and 25 μg/mL.

Pharmacokinetic Analysis

Pharmacokinetic analyses were performed with computer software (WinNonlin 5.2, Pharsight Corporation, Mountain View, CA, USA). The variables calculated included area under the curve from time 0 to infinity (AUC 0-INF) using the linear trapezoidal rule, area under the first moment curve from time 0 to infinity (AUMC 0-INF), plasma clearance per fraction of the dose absorbed (Cl/F), apparent volume of distribution (area method) per fraction of the dose absorbed (Vz/F), first-order rate constant (Lz), terminal half-life ($T_{½}$), and mean residence time extrapolated to infinity (MRT 0-INF). The percent of the area under the curve (AUC)

extrapolated to infinity (AUC extrapolated) was determined. The maximum serum concentration (CMAX) and time to maximum serum concentration (TMAX) were determined directly from the data.

Data Analysis and Statistics

Hypothesis tests were conducted using JMP 9.0.0 analytical software (SAS Institute, INC, Cary, NC, USA). The mean ± standard errors of the means (SEM) were calculated for each outcome variable at each time point. Data that demonstrated a departure from normality (cortisol, haptoglobin, PGE_2 and substance P) were log transformed prior to statistical analysis. Repeated measures data were analyzed using a univariate split-plot approach. In order to compare the effect of different treatments on the outcome variables, a random effects-mixed model was constructed with treatment, time, time*treatment interaction designated as fixed effects. In this model, animal nested in treatment was designated as a random effect to account for the between subject effects. Horn length and horn diameter were included as a covariate in the model, where applicable. Statistical significance was designated a priori as $p<0.05$. In order to compare the effect of analgesic drug administration on the outcome variables, a random effects-mixed model was constructed with analgesia (yes/no), time, time*analgesia interaction designated as fixed effects. In this model, animal nested in treatment was designated as a random effect to account for the between subject effects. In the case of significant differences among all groups a Wilcoxon rank sum test was used for post-hoc comparisons between each treatment group and the controls, at each sampling time. Statistical significance was again designated a priori as $p<0.05$. Areas under the time-effect curves (AUEC) were determined as previously described (Leon-Reyes *et al*., 2008) with the commercially available software program GraphPad Prism Version 5.04 (GraphPad Software, La Jolla, CA). using the linear trapezoidal rule. Analysis of variance (ANOVA), using Tukey's test, was employed to evaluate differences, with statistical significance considered at $p<0.1$.

Results

Pharmacokinetic parameters

No adverse effects were noted after PO administration of meloxicam or gabapentin alone or in combination. In addition, no adverse effects were apparent after IV administration of

flunixin. Pharmacokinetic parameter estimates are summarized in Table 4.2 and include the median and range. Plasma concentrations of each treatment are displayed in Figure 4.2a-e. The median dose of meloxicam, gabapentin, meloxicam in combination, and gabapentin in combination was 1.00 mg/kg (0.98-1.05 mg/kg), 14.9 mg/kg (14.7-15.1 mg/kg), 0.99 mg/kg (0.96-1.03 mg/kg), and 15.1 mg/kg (14.8-15.2 mg/kg), respectively. The area under the plasma-concentration time curve (AUC) was 7.9 hr*µg/mL (6.5-9.0 hr*µg/mL), 78 hr*µg/mL (41.3-93.4 hr*µg/mL) and 87.2 hr*µg/mL (59.2-134.9 hr*µg/mL) for flunixin, meloxicam and gabapentin, respectively. The terminal half-lives of flunixin, meloxicam and gabapentin were 6.0 h (3.4-11.0 h), 16.7 h (13.7- 21.3 h) and 15.3 h (11-32.9 h), respectively. The median and range volume of distribution (Vz) for flunixin was 2.28 L/Kg (1.27-4.46 L/Kg). The Vz/F for meloxicam and gabapentin were 0.338 (0.285-0.479) L/kg and 4.45 (2.94-6.79 L/Kg, respectively. When co-administered with meloxicam, gabapentin showed higher CMAX values (4.1 µg/mL; 2.3-6.5 µg/mL) than when the drug was given alone (2.7 µg/mL; 2.2-4.0 µg/mL) but this difference was not statistically significant.

Cortisol Concentration

There was time effect on serum cortisol concentrations ($p<0.0001$) (Fig. 4.3). An expected significant increase at 5 min post-dehorning was observed ($p<0.0001$). The increase was followed by a subsequent significant decrease at 0.5 h ($p=0.0069$). Serum cortisol continued to decrease at 1 h ($p<0.0001$) and 2 h ($p<0.0001$), however, a steady increase from was observed from 2 to 4 h ($p=0.0485$) and 6 h ($p<0.001$). There was also a significant increase in cortisol when comparing 12 to 24 h ($p<0.0001$). Serum cortisol concentrations peaked for all treatment groups at 0. 083 h (Fig. 4.3). A treatment effect was found, with the flunixin treatment group exhibiting a significantly lower mean serum cortisol concentration of 29.89±8.76 μg/mL compared to the control concentration of 60.79± 9.39 μg/mL ($p=0.045$). There was no effect of any treatment on cortisol CMAX ($p=0.62$) or TMAX ($p=0.58$). In addition, when investigated as analgesic versus no analgesic, there was no significance.

Haptoglobin Concentration

There was a time effect on the haptoglobin concentration ($p < 0.0001$), which displayed an increase in plasma concentration over time and remained elevated above the baseline for 168 h post-dehorning (Fig. 4.4). A significant increase in plasma haptoglobin concentrations was

observed when comparing 24 to 48 h (p=0.014). No difference was found between treatment and no significant treatment*time effect. No significant effect was found when considering overall analgesic versus no analgesic.

Ex-vivo Prostaglandin Synthesis

Ex-vivo plasma PGE_2 concentrations measured at each time point in the different treatment groups are shown in Figure 4.5. A significant decrease in *ex-vivo* plasma PGE_2 concentrations was observed at 5 min (p=0.0003), 6 (p=0.0003) and 12 h (p=0.008) after treatment in the flunixin group compared with controls. Even though a slight decrease in *ex-vivo* PGE_2 concentration was found also in the meloxicam treated group at 6 h, no significant differences were recorded by comparing each time point with control group values (Wilcoxon rank sum test p>0.05 in all cases). In addition, over all there was no significant effect when considering analgesic versus no analgesic.

Substance P

Due to issues in retrieving adequate blood for evacuated tubes, sampling was discontinued for a number of 8 (16 samples) and 12 (20 samples) h time points. Samples were analyzed for plasma substance P and no evidence was found for differences between treatment groups, time, or treatment*time interaction. However, when analyzed as analgesic versus no analgesic, a significant effect was found (p=0.02). The mean plasma (±SD) concentrations of substance P were significantly lower in calves given analgesic (63.35±21.25pg/mL) than those who did not receive analgesia (137.29±42.97pg/mL).

Thermography

No evidence was found for differences in minimum, maximum, or average corneal temperature between treatment groups, thus each of the outcomes were insensitive to drug effects. There was a time effect (p=0.0019) and a covariate horn length effect (p=0.0385) for the minimum temperature. A significant decrease was observed in the minimum eye temperature from 5 to 720 min post dehorning (p=0.0006) (Fig. 4.6).

Maximum temperature demonstrated a time effect (p=0.011) and a covariate horn length effect (p=0.0397). Maximum eye temperature was significantly increased from the -10 min recording compared to 5 min post procedure (p=0.01). When comparing 5 min post-dehorning,

the 720 min maximum eye temperature was significantly decreased (p=0.012) (Fig. 4.6). No effect of analgesia versus no analgesia was shown.

The difference between the maximum and minimum temperature of the eye showed a time effect (p<0.0001). The difference between maximum and minimum temperature at 5 min was greater than the difference at -10 min (p=0.0108). At 720 min post dehorning the difference between the maximum and minimum temperature was less than that displayed at 5 min (p<0.0001) (Fig. 4.6). No difference was detected between analgesia versus no analgesia.

Upon evaluating average eye temperature, evidence of a time effect (p=0.017), covariate effects of horn diameter (p=0.036) and length (p=0.029) for average corneal temperature were observed. There was a significant increase in eye temperature when comparing -10 to 720 min post castration. No effect was found when analyzed as analgesia versus no analgesia.

Algometry

The variables were analyzed for MNT Kgf for each horn through pressure algometry. A decrease in the amount of force tolerated with increased time post-dehorning was observed for both the RH (p<0.0001) LH (p<0.0001) (Fig. 4.7). A significant decrease in the amount of force tolerated to the horn area was observed at 1 h (RH: p<0.0001 LH: p=0.0087), 2 h (RH: p=0.017; LH: p=0.0001), 4 h (RH: p=0.0046; LH: p=0.01) and 8 h (RH: p=0.035; LH: p=0.002). No effect of treatment or treatment*time was found for either horn.

There was a significant effect of the site of measurement (A – F) on the left and right horn (Fig. 1) on the mean MNT determined after dehorning with or without treatment with flunixin, meloxicam and gabapentin (p<0.0001). Therefore, the data collected at each site was analyzed separately. There was an effect of time on all 6 sites with MNT decreasing significantly after dehorning (p<0.0001). There was no evidence of a treatment effect or a time by treatment interaction at site A, B, C and D. However, the MNT at site E was significantly greater in the meloxicam-treated calves compared with the control calves (P=0.0128). Furthermore, the meloxicam treated calves tended to have a higher MNT compared with the control calves at Site F (P=0.058) (Table 4.3).

Similarly there was a significant effect of the site of measurement (A – F) on the left and right horn on the mean MNT determined after dehorning with or without analgesia (P<0.0001). Therefore, the data collected at each site was analyzed separately. As expected, there was an

effect of time on all 6 sited with MNT decreasing significantly after dehorning (P<0.0001). There was no evidence of a treatment effect or a time by treatment interaction at site A, B, C and D. However, the MNT at site E was significantly greater in the calves that received analgesia compared with the control calves (P=0.046). Furthermore, the analgesic- treated calves tended to have a higher MNT compared with the control calves at Site F (P=0.074). (Fig. 4.8)

Average Daily Gain

All steers were weighed on d0 and again on d7. Evidences of a treatment effect were observed (p<0.0001) which are noted in Table 4.4. MEL+GBP treated calves gain was significantly greater than when the drugs were given alone (versus MEL p=0.0239; versus GBP p=0.006). Flunixin (p<0.0001) and MEL+GBP (p=0.002) calves gain was greater than that of control calves. Flunixin also was significantly greater than GBP (p=0.0132). In addition, a significant effect was found between analgesic treatment versus no analgesia (p<0.0001) indicating that calves receiving analgesia gained more (1.2 kg) than calves who received the placebo (0.32 kg).

Discussion

A growing concern for animal welfare has led to an increased need for knowledge on which research topics to emphasize and how to better address consumer demands and still accommodate producer capabilities. Consumer concern primarily drives this type of research as well as, to a lesser extent, the threat of economic loss (Bicalho *et al*., 2008; Thrift *et al*., 1974; Pinchak *et al*., 2004). The FDA Center for Veterinary Medicine guidance for the development of effectiveness data for analgesics recommends that validated methods of pain assessment must be used in order for a drug to be labeled as an analgesic in the proposed species. The findings in this study add to the work done using meloxicam alone and to compare the use of meloxicam alone or in combination with gabapentin or placebo against a non-selective COX inhibitor, flunixin.

When considering the use of NSAIDs for pain relief, the pharmacokinetic properties of IV flunixin in this study are in agreement with those previously reported in calves (Landoni *et al*., 1995). The elimination half-life of flunixin in calves is longer than that reported for other domestic species (Lees *et al*., 1991; Lees *et al*., 1987; Hardie *et al*., 1985). Evaluation of meloxicam, revealed that a mean CMAX of 1.9 µg/mL (range 1.3 to 2.1 ug/mL) occurred

approximately 24 h (range 12 to 24 h) following oral administration. The mean TMAX in the present study is longer than those previously reported and lower mean AUC values were observed (Coetzee *et al*., 2009; Coetzee *et al*., 2011; Mosher *et al*., 2011). In the previously mentioned studies, the method of oral administration was a suspension of crushed tablets in water, whereas in our study meloxicam tablets were administered whole and placed in a bolus filled with whey powder. The dosing method could explain the differences in pharmacokinetic measurements. Inter-study random variability could also contribute to any perceived difference.

The pharmacokinetic estimates for gabapentin are similar to those previously reported (Coetzee *et al*., 2011). The plasma terminal half-life of gabapentin in calves was longer than previously reported in horses (Dirikolu *et al*., 2008) and dogs (KuKanich and Cohen, 2009). It was observed that the terminal half-life of gabapentin was numerically longer than the 8.12 h reported previously (Coetzee *et al*., 2011) when co-administering gabapentin with meloxicam. This may be due to individual variability or differences in formulation, however, further investigation is needed.

Cortisol is a common corticosteroid hormone used as an indicator of stress responses, and to some extent, pain, since pain by definition is associated with a stress response. In the present study, the response to dehorning was marked by a rapid rise in cortisol concentrations that peaked within several minutes followed by a rapid decline to a plateau. Following the decline, an increase in cortisol was shown, agreeing with previous reports that local anesthetic merely delays the onset of increased cortisol concentrations (Sutherland *et al*., 2002; McMeekan *et al*., 1998). It appeared that a significant increase in cortisol concentrations occurred at 24 h, suggesting there was substantial stress occurring at this time point. However, the last sampling point of the day occurred at 12h and calves were returned to the home pens, then brought back up to the chute area for the 24 h blood sample, thus the stress of handling could attribute to this increase which is not necessarily evidence of pain. A marked difference in the serum cortisol concentration between the flunixin and control groups, but no effect of orally dosed drugs, is likely due to IV administration. IV administration allows for quicker achievement of therapeutic plasma concentrations of the drug versus PO administration of meloxicam and gabapentin, which requires a longer period to establish analgesic activity.

Haptoglobin, an acute phase protein, which is normally found at low concentrations in cattle and synthesis increases during an acute infection or inflammation, which has been

documented in response to weaning, transportation, and castration (Connor *et al*., 1988; Lomborg *et al*., 2008; Fisher *et al*., 2001). Although our results show no reduction of haptoglobin levels from the use of pain alleviators, the use of haptoglobin in pain and stress research is supported, as it appears levels were elevated for at least 7 d, indicating persistent inflammation present well beyond dehorning. Although there is no previously reported literature on haptoglobin responses to dehorning, other literature on treating metritis in cows and alleviating pain of mulesing in sheep suggests there is no effect of meloxicam or flunixin on haptoglobin responses (Drillich *et al*., 2007; Paull *et al*., 2008). Results from previous studies on cattle subjected to castration showed reduced haptoglobin concentrations when provided carprofen or ketoprofen, respectively (Ting *et al*., 2003; Pang *et al*., 2006). The cause of the varying results are unknown, however, a difference in the tissues implicated in the procedure , husbandry procedure, individual variability, and pharmacokinetic variability are likely explanations for these equivocal result.

Suppression of PGE2 concentrations synthesized ex vivo is thought to reflect drug effects at the site of inflammation following surgery (Brideau *et al.,* 2001; Donalisio *et al.,* 2009; Giorgi *et al*., 2010). Therefore, this aspect of the trial likely simulates the effect of the drug at a local level. Our data indicate that flunixin administered IV suppresses the synthesis of PGE_2 *ex-vivo* at 4 minutes and 6, and 12 h post-treatment which is in agreement with previously report which showed reduced eicosanoid PGE_2 concentrations in calves (Landoni *et al*., 1995). In the present study, flunixin inhibited *ex-vivo* blood PGE_2 levels up to 12 h which is longer than the period of visible analgesia observed previously in castrated beef calves (Currah *et al*., 2009). Further research is needed to more clearly define the relationship between inhibition of PGE2 production ex-vivo and outward signs of analgesia. Although flunixin appeared to reduce stress and inhibit *ex-vivo* PGE_2 synthesis over the first 24h , IV administration still poses the risk of accidental perivascular injection if administered by an experienced operator, This may resultin injection site residues in tissues destined for human consumption (Smith, 2008).

Substance P is released by sensory nerves and nonneural sources and has an important function in the transmission of painful stimuli (Rameshwar *et al*., 1993). A recent dehorning study found that calves administered meloxicam IV had a lower mean plasma substance P concentration compared to the control calves (Coetzee *et al*., 2012). Although no individual drug effects of substance P were observed in the present study, our results support previous trials

demonstrating that calves receiving an analgesic had lower substance P concentration that the calves given a placebo.

The use of thermography to evaluate a painful stimulus has been evaluated in a number of studies (Schwartzkopf and Stookey, 1997, Stewart *et al*., 2008; Stewart *et al*., 2010;). It has been proposed that the maximum eye temperature signifies a true temperature reading that is not as easily impacted (as other measurements) by hair, dirt, and other surroundings that may be unintentionally detected in the image (Stewart et al., 2005). An increase in maximum eye temperature from baseline to 5min post-dehorning indicates that a stress response is occurring and the increase is likely mediated by the autonomic nervous system as suggested by a number of studies (Stewart *et al*., 2010; Mellor *et al*., 2000). The lack of analgesic effect suggests that the temperature change may be largely indicative of stress and cannot be used accurately and solely as a pain indicator.

A pressure algometer can determine the minimal amount of pressure that produces a pain response and has been effectively utilized to evaluate the therapeutic effects of analgesics on disbudding pain (Heinrich *et al*., 2009; Heinrich *et al*., 2010). Although no specific treatments were able to reduce the sensitivity of the entire horn area, a decrease in the MNT (KgF) was observed at 1 h indicating a reduction in the desensitizing effects of the local anesthetic (Sutherland *et al*., 2002; McMeekan *et al*., 1998; Sylvester *et al*., 1998) followed by subsequent increases in horn sensitivity for at least 12 h.

Differences in specific treatment detected at individual horn sites measured are likely due to the order of measurements or differences in site sensitivity. Site A was always measured first, and because the animal was on a halter, it may have been fractious to the presence of the person with the instrument. As more measurements were taken, the animals were likely less fractious, thus responding less to the person and more to the pressure. Although a previous study had suggested to avoid this issue by having the researcher place a hand lightly on the poll until the calf habituates to being touched and stands still in a relaxed posture (Heinrich *et al*., 2010), our results have shown that a different design may be required for future studies to account for behavioral responses in older animals in order to distinguish aversive behavior due to the algometer.

A previous study found that meloxicam treated calves displayed less sensitivity to pressure algometry 4 h post-procedure (Heinrich *et al*., 2010). The differences found between the

previous and present study could be due to the route of administration (IM versus PO) or due to the procedure performed (Cauterization only versus Scoop dehorning in older calves). Scoop dehorning is likely more invasive, stressful, and produces greater pain than cauterization due to the development of the horn from the bud and attachment to the frontal sinus. Due to the lack of definitive drug effects on scoop dehorning in the present study, it could be recommended to cauterize the horn before growth and attachment occur to avoid the excess pain of dehorning. This concept warrants further investigation as to the effects of two different procedures and the use of pain alleviation strategies.

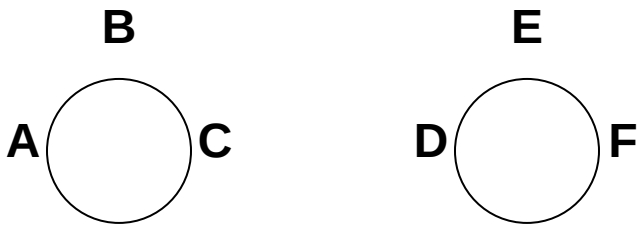
Reduced weight gain has been observed during the first 6 weeks after physical dehorning (Loxton *et al*., 1982), which suggests decreased animal welfare and subtle economic losses. In the present study, it appears that the combination treatment animals (MEL+GBP) gained more than those receiving individual drugs and CONT, suggesting both neuropathic and inflammatory pain may exist and the drugs react synergistically as previously suggested in humans (Hurley et al., 2002; Picazo et al., 2006). In addition, FLU gained more than CONT and GBP alone, suggesting that a NSAID has a greater influence on gain than gabapentin and treatment of neuropathic pain alone is not as effective. Analgesic treated calves gained around 1kg more than calves given a placebo, which supports the results of a previous study which found meloxicam treated calves gained more than placebo-treated calves (Coetzee *et al*., 2012). Although the gain observed in this study was likely of short duration, it is of interest for future research to determine if any differences exist over a longer period of time and if there are any differences in feed consumption.

In conclusion, oral meloxicam or gabapentin did not specifically reduce plasma physiological indicators of pain association with scoop dehorning. Although IV flunixin was effective in reducing cortisol-marked stress and *ex-vivo* PGE_2 inflammation for 24h, the pain alleviating effects of flunixin were not significantly greater than those exhibited by the other drugs. Based on previous literature, it is likely that our chosen dosage and route of administration was not adequate to reduce pain from scoop dehorning, thus alternatives should be investigated. I Calves subjected to dehorning would benefit from an extended analgesic as it was observed that calves given analgesia had a lower substance P concentration, a higher pain threshold at specific horn sites, and maintained a higher growth rate than calves given a placebo. Although analgesic effect on haptoglobin and thermography was absent from the results, the data of the present study

provide a better understanding of the level and duration of the stress and pain that is produced by scoop dehorning. Future research would benefit from incorporating these specific measurement tools into pain models to help determine the efficacy and therapeutic levels required for a drug to be classified as a pain reliever from scoop dehorning.

Figures and Tables

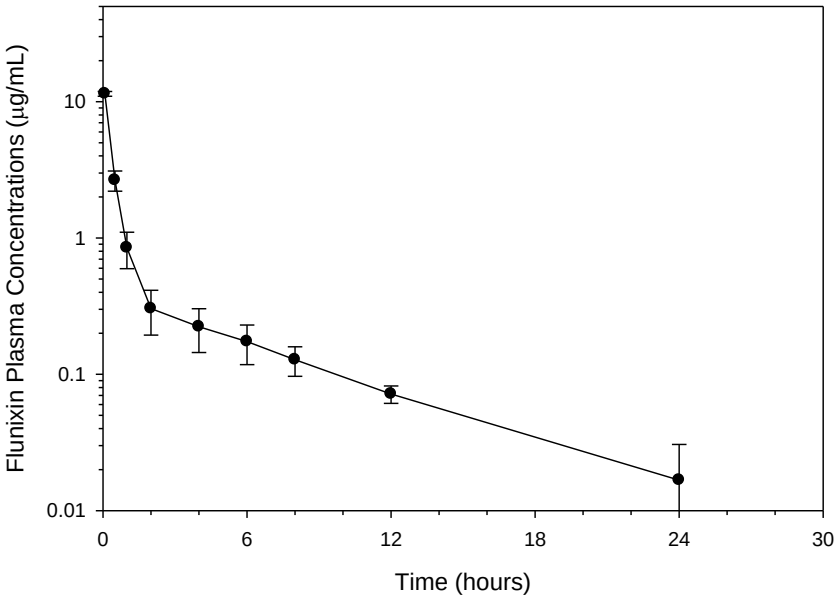
Figure 4.1. Locations around the horn bud measured by pressure algometry. Sites were labeled alphabetically to indicate the order in which they were measured.



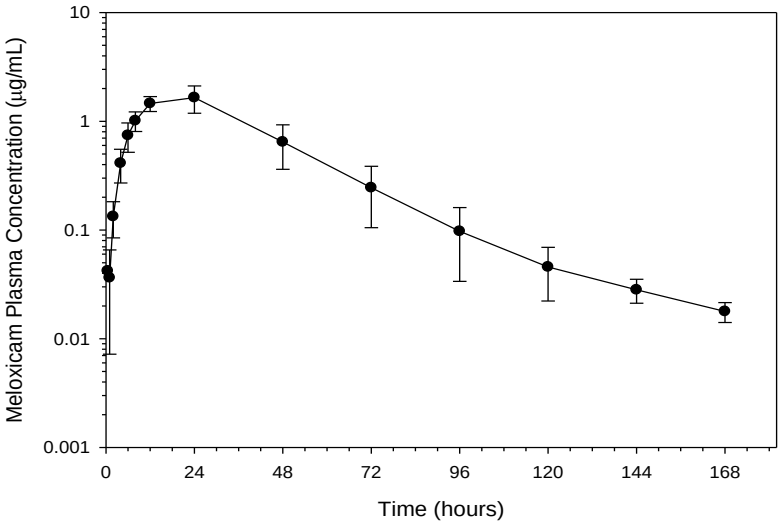
Right Horn Left Horn

Figure 4.2a-e.

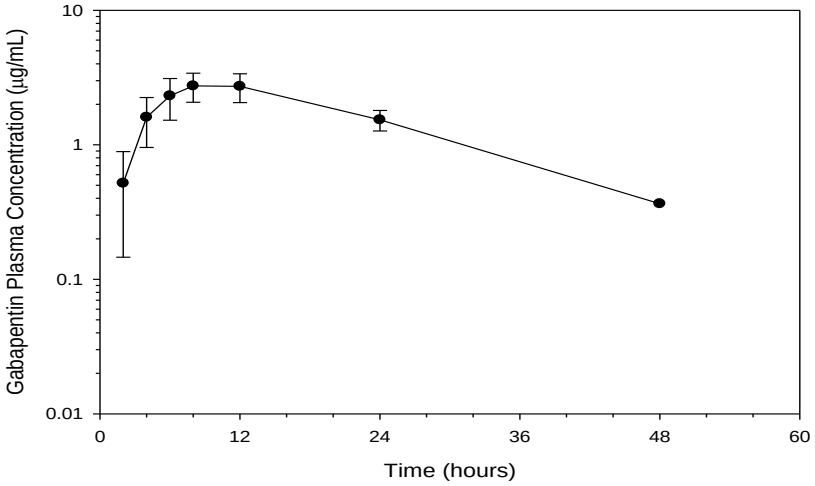
a.) Plasma flunixin concentrations (mean ± SEM) after IV administration of the drug in calves at a dose rate of 2.2 mg/kg BW (n=5, excluding animal 62 in which the samples were mis-labeled).



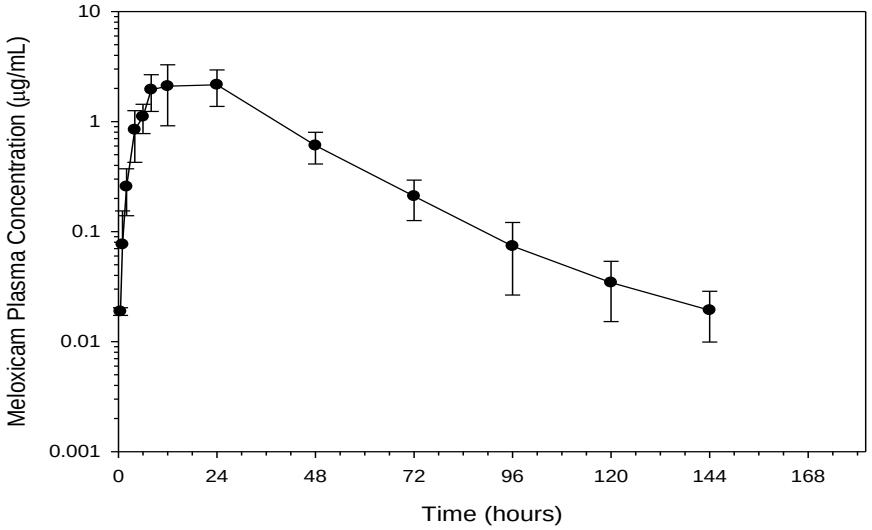
b.) Plasma meloxicam concentration (mean ± SEM) following single oral dose of 1 mg/kg BW to calves.



c.) Mean values ± SEM of the plasma gabapentin concentrations after oral administration of the drug at 15 mg/kg BW in calves.



d.) Plasma meloxicam concentration (mean ± SEM) following a single oral administration of 1 mg/kg BW meloxicam with 15 mg/Kg BW dose of gabapentin in calves.



e.) Plasma gabapentin concentration (mean ± SEM) following the oral administration of 15 mg/kg BW gabapentin in calves with 1 mg/Kg BW dose of meloxicam in calves.

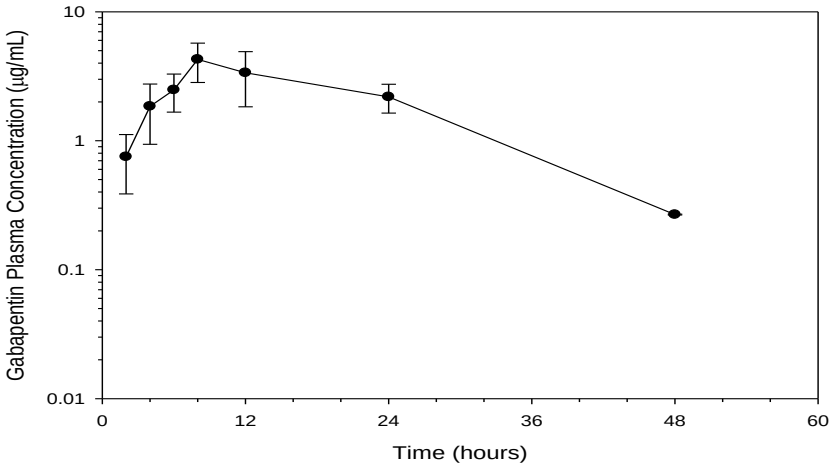


Figure 4.3. Effect of placebo (CONT), flunixin (FLU), gabapentin (GBP), meloxicam (MEL), and meloxicam with gabapentin (MEL+GBP) treatments on serum cortisol values of dehorned calves. Overall, the FLU treatment group had lower plasma cortisol concentration than CONT (p=0.045). * Indicates a significant increase in plasma cortisol concentrations, while † indicates a significant decrease from the previous time point (p≤0.05).

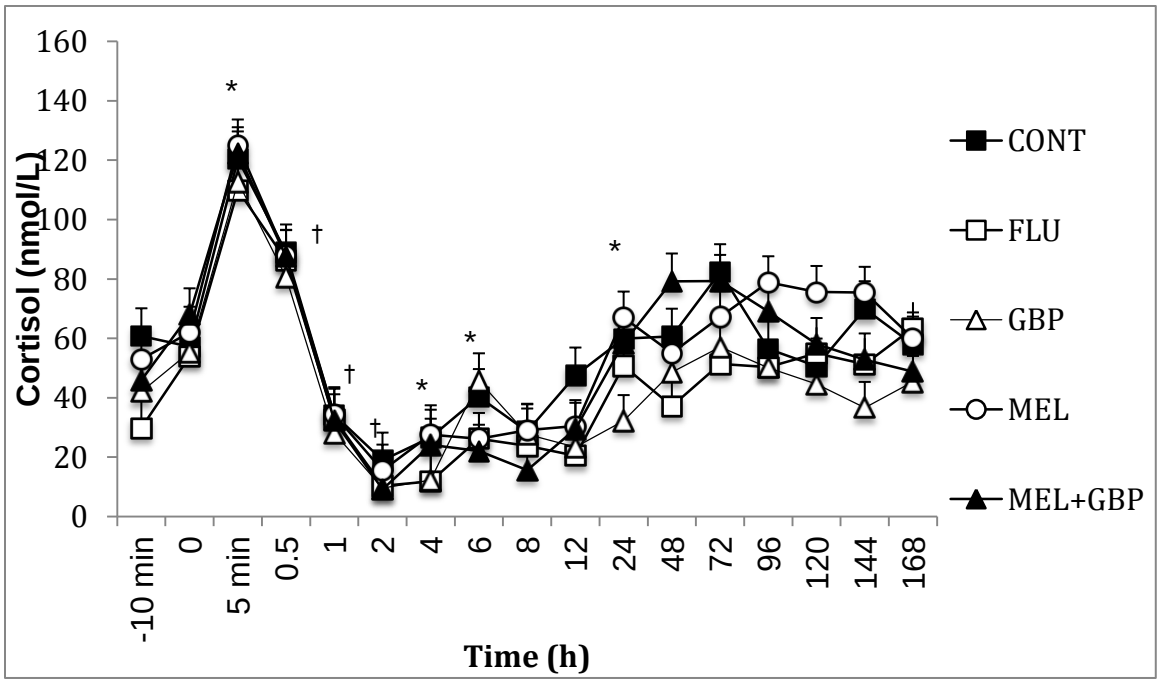


Figure 4.4. Effect of placebo (CONT), flunixin (FLU), gabapentin (GBP), meloxicam (MEL), and meloxicam with gabapentin (MEL+GBP) treatments on plasma haptoglobin values of dehorned calves. Following dehorning, plasma haptoglobin increased over time and remained elevated above baseline for 7d. No treatment significantly suppressed plasma haptoglobin concentrations. * Indicates a significant increase in plasma haptoglobin between 24 and 48 h (p=0.0137).

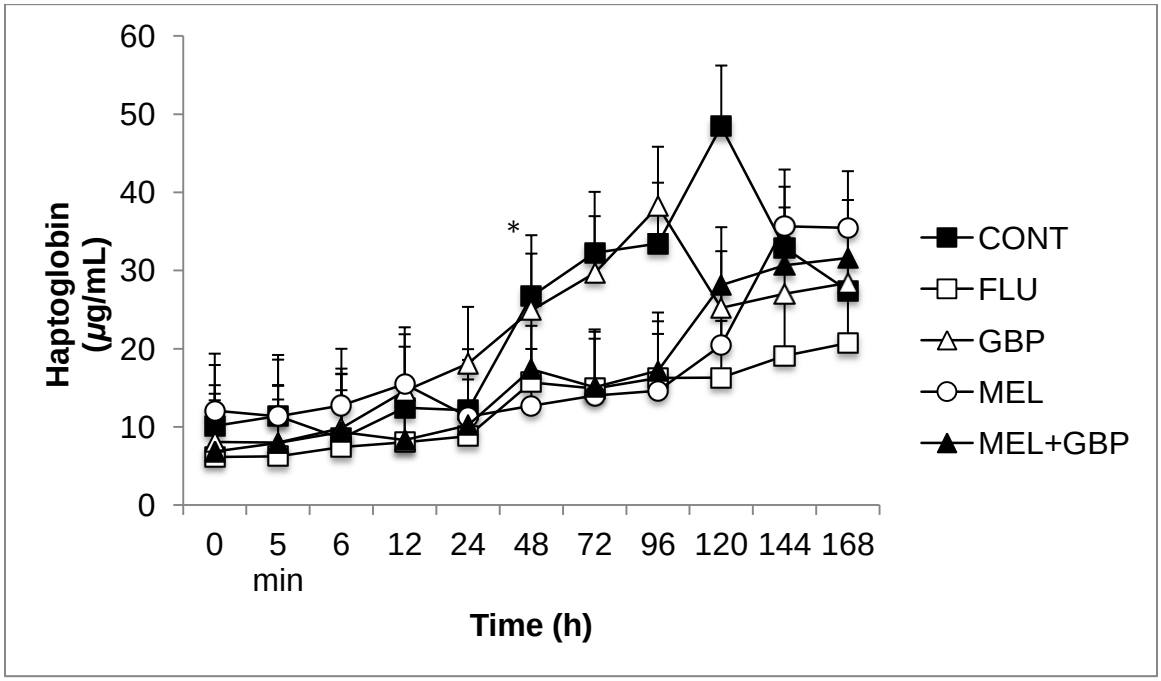


Figure 4.5. Effect of placebo (CONT), flunixin (FLU), gabapentin (GBP), meloxicam (MEL), and meloxicam with gabapentin (MEL+GBP) treatments on ex-vivo prostaglandin (PGE^2) synthesis of dehorned calves.

* Indicates $p<0.01$ in control vs. treated calves.

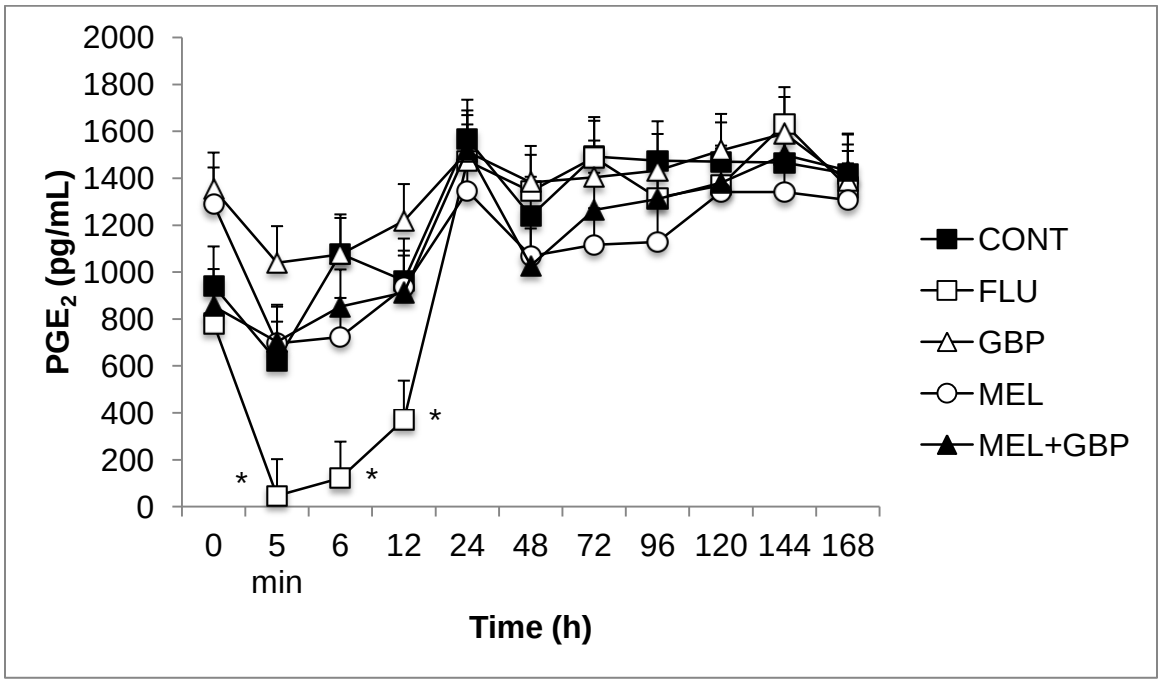


Figure 4.6. Change in eye temperature (°C) ± SEM over time including minimum (MIN), maximum (MAX), average (AVG), and the difference between maximum and minimum temperature (DIFF). Significant differences ($p \leq 0.05$) between time points are distinguished by different letters (a, b, and c).

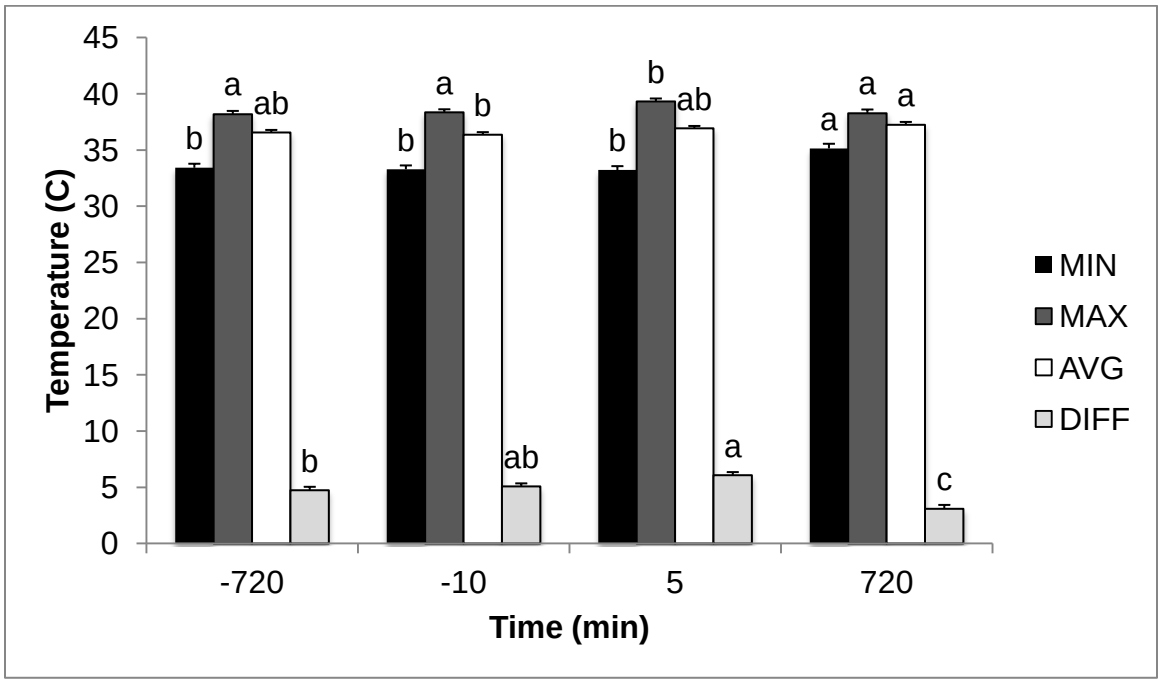


Figure 4.7. Mean (±SE) mechanical nociceptive threshold (MNT, kg of force), as measured by pressure algometry pre-dehorning (-10 min), after lidocaine administration (0), and following dehorning (1-12 h) for both the right (RH) and left (LH) horns.

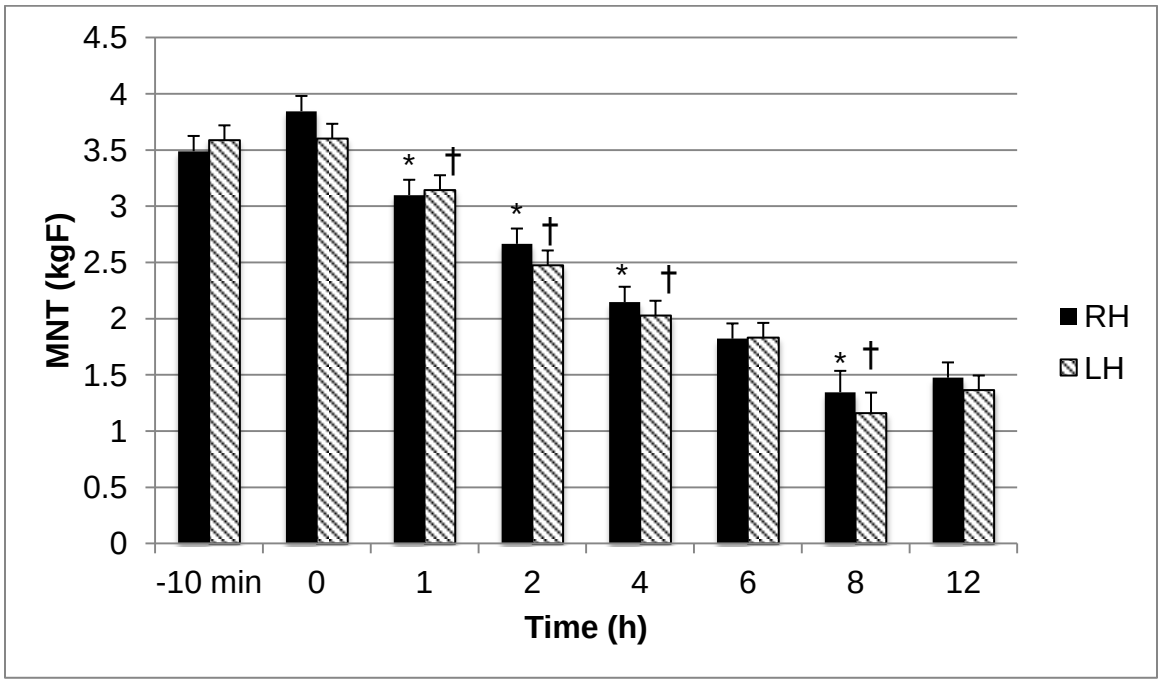
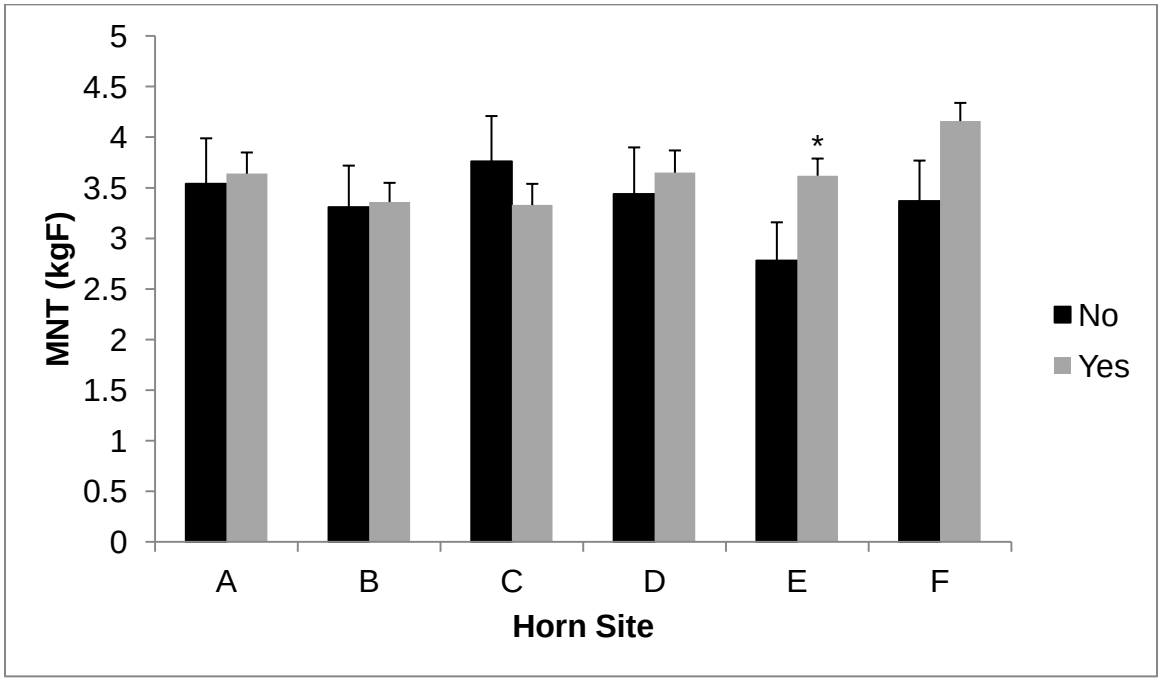


Figure 4.8. Effect of analgesia (YES) or no analgesic (NO) treatment on mean mechanical nociceptive threshold (MNT) at specific sites designated in Fig.4.1 following scoop dehorning in calves. The MNT at site E was significantly greater in the analgesia-treated calves compared with the placebo treated calves. Furthermore, the analgesia treated calves tended to have a higher MNT compared with the control calves at Site F. * Indicate differences between treatment groups ($p<0.05$).



Treatment	Agent Administered PO	Dose	Agent Administered IV	Dose
CONT	Whey Bolus Spray Dried Pasteurized Whey, Kraft, Hartford, CA Porcine Hard Gelatin Capsules, Torpac, Inc., Fairfield, NJ	-	Saline	2.2 mg/kg
MEL	Meloxicam tablets USP 15 mg [NDC 29300-125-01] Lot # GMMH10078 Unichem Pharmaceuticals USA Inc. Rochelle Park, NJ	1 mg/kg	Saline	2.2 mg/kg
GBP	Gabapentin capsules 100 mg, Amneal Pharmaceuticals, Lot #HA39811 Hauppauge, NY 300 mg, Greenstone Brand, Lot #V110200 600mg, Greenstone Brand, Lot #V110370 Peapack, NJ	15 mg/kg	Saline	2.2 mg/kg
MEL+GBP	Meloxicam tablets Gabapentin capsules	1 mg/kg 15 mg/kg	Saline	2.2 mg/kg
FLU	Whey Bolus	-	Flunixin meglumine Banamine – Flunixin Meglumine Injectable Solution Schering-Plough Animal Health Summit, NJ	2.2 mg/kg

Table 4-1. Experimental treatments. Weights were obtained one day (d -1) prior to dehorning in order to calculate individual dosages. Meloxicam tablets and gabapentin capsules were given in a bolus filled with whey to mask the treatment. Each steer was subjected to the same handling procedures including an intravenous injection into the right jugular vein and oral dosage of the bolus using a balling gun.

Parameter	MEL	GBP	MEL/GBP	GBP/MEL	FLU
Dose (mg/kg)	1.00 (0.98-1.05)	14.9 (14.7-15.1)	0.99 (0.96-1.03)	15.1 (14.8-15.2)	-
AUC (hr*ug/mL)	78 (41.3-93.4)	87.2 (59.2-134.9)	91.4 (48.1-146.2)	122.8 (61.9-142.3)	7.9 (6.5-9.0)
Cl	-	-	-	-	4.7 (4.1-5.7)
Cl/F (mL/min/kg)	0.218 (0.177- 0.403)	2.87 (1.82-4.24)	0.18 (0.11-0.33)	2.06 (1.73 – 4.06)	-
C0	-	-	-	-	15.3 (14.5-16.2)
Cmax (ug/mL)	1.9 (13-2.1)	2.7 (2.2-4)	2.3 (1.4-4.6)	4.1 (2.3-6.5)	-
T ½ (hr)	16.7 (13.7- 21.3)	15.3 (11-32.9)	14.6 (11.9-24.5)	13.2 (10.4-23.3)	6.0(3.4-11)
MRT (hr)	34.2 (26.2-42.3)	26.6 (19.8-51.2)	29.4 (23.9-33.4)	23.7 (18.3-37.1)	3.2 (2.0-5.9)
Tmax (hr)	24 (12-24)	8 (6-12)	18 (8-24)	8 (8-12)	-
Vz (L/kg)	-	-	-	-	2.28 (1.27-4.46)
Vz/F (L/kg)	0.338 (0.285-0.479)	4.45(2.94-6.79)	0.25 (0.13-0.40)	3.4(1.6-3.7)	-

Table 4-2. Pharmacokinetic parameters of meloxicam (MEL) (1mg/kg), gabapentin (GBP) (15 mg/kg), meloxicam in combination (MEL/GBP) (1mg/kg), and gabapentin in combination (GBP/MEL) (15mg/kg) following oral administration in calves. In addition, pharmacokinetic parameters of flunixin (FLU) (2.2 mg/kg) are shown following IV administration in calves. Vz/F = Volume of distribution per fraction of dose absorbed. MRT = mean residence time extrapolated to infinity.

Site	Mean ±SEM MNT (kg)					P-values		
	CONT	FLU	MEL	GBP	MEL+ GBP	TRT	Time	Time*TRT
A	3.53±0.45	3.45±0.42	3.47±0.42	3.26±0.42	4.37±0.42	0.37	<0.01	0.93
B	3.30±0.41	3.37±0.38	2.7±0.38	3.5±0.39	3.88±0.38	0.29	<0.01	0.53
C	3.76±0.45	2.86±0.42	3.44±0.42	3.49±0.43	3.53±0.42	0.65	<0.01	0.58
D	3.43±0.47	3.40±0.44	3.82±0.44	3.69±0.44	3.70±0.44	0.95	<0.01	0.38
E	2.78±0.38[a]	3.03±0.36	4.33±0.36[b]	3.93±0.36	3.21±0.36	0.01	<0.01	0.14
F	3.37±0.40	3.63±0.38	3.90±0.38	4.27±0.38	4.85±0.38	0.06	<0.01	0.44

Table 4-3. Effect of placebo (CONT), flunixin (FLU), gabapentin (GBP), meloxicam (MEL), and meloxicam with gabapentin (MEL+GBP) treatments on mean mechanical nociceptive threshold (MNT) at specific sites designated in Fig.4.1 following scoop dehorning in calves. The MNT at site E was significantly greater in the meloxicam-treated calves compared with the control calves. Furthermore, the meloxicam treated calves tended to have a higher MNT compared with the control calves at Site F. [a, b] Indicate differences between treatment groups ($P<0.05$).

Average Daily Gain (kg)				
Treatment	ADG (kg)	Std. Error (kg)	Comparison	
			Treatments	P-value
CONT	0.2653074	0.21193004	MEL+GBP vs CONT	<0.0001
FLU	1.5178582	0.19824238	FLU vs CONT	0.0002
GBP	0.6250007	0.19824238	MEL+GBP vs GBP	0.0006
MEL	0.9107147	0.19824237	FLU vs GBP	0.0132
MEL+GBP	1.7500009	0.19824241	MEL+GBP vs MEL	0.0239

Table 4-4. Effect of placebo (CONT), flunixin (FLU), gabapentin (GBP), meloxicam (MEL), and meloxicam with gabapentin (MEL+GBP) treatments on gain (kg) in dehorned calves. Calves co-administered (MEL+GBP) achieved significantly greater gain than the control group and when administered the drugs alone. Flunixin treated calves gained more than the control animals.

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Chapter 5 - General Summary and Conclusions

The first objective for this thesis was to examine subjective, objective, and threshold pain tests, including behavioral, physiological, and neuroendocrine responses to noxious stimuli as measurements of pain in cattle. The second objective of this thesis was to examine novel pain mitigation agents, either local anesthetics, non-steroidal anti-inflammatory drugs (NSAIDs), or gamma-aminobutyric acid (GABA) analogues for alleviating pain in cattle. The final chapter of this thesis serves to review the results of the entire project as well as to outline the limitations of the present work that future research can potentially modify when designing additional experiments.

The third chapter of the thesis was an assessment of behavioral responses of dairy calves to different methods of castration, including banding, surgical cut and pull, and surgical cut followed by the use of an emasculator. In addition, the behavioral responses of calves in two distinct age categories, 6 week months and 6 months, were evaluated.

When comparing age, 6 week calves displayed greater tail flicks per minute and tended to display less foot stamps than did 6 mo. In addition, the 6 week calves displayed significantly less tail flicks and foot stamps in response to castration while the 6 month calves showed an increase in tail flicks and foot stamps in response to castration, indicating specific behavioral measurements are dependent upon age. Future research would benefit from including ages in-between the two studied here to confirm the hypothesis. The surgical cut and emasculator method and the surgical cut and pull method resulted in significantly less tail flicks when comparing pre- to post-castration than control manipulation of the scrotum, however, banding was not significantly different than the control group, proposing surgical castration produces greater acute behavioral responses. It cannot be concluded that banding does not produce painful responses because it has been suggested to produce greater chronic effects than acute (Molony et al., 1995). Future research would benefit from including a longer behavioral observation period to capture any chronic pain responses associated with the banding method.

It appears that kicking is a possible indicator of pain and stress in 6 month calves, with the probability of kicking found to be higher in all castrated groups compared to the control animals. The 6 week surgical castrates had high probability of kicking compared to banding and control animals, again proposing that surgical castration has a higher probability of producing

behavioral responses. The data did not allow for calculation of the frequency of kicking and eliminations, thus probability was calculated. Careful interpretation of the results for kicking and elimination is required as the probabilities were low. Future research would benefit from incorporating a larger sample size or increased observation time to obtain an accurate assessment of these two behaviors.

The present research was unable to analyze scrambling, collapses, or vocalizations due to the limited frequency of those behaviors. Scrambling and collapses are typically considered to be escape-avoidance behaviors, and are usually recorded as a subjective score, however, the accuracy or consistency between and within observers may be questionable (Stookey et al., 1994). Future research would benefit from quantifying the force and duration of the escape-avoidance response, which would eliminate the subjectivity, provide adequate data to analyze, and allow for effective cross-study comparisons to be made. In addition, frequency of vocalizations may be influenced by the individual perceptions of the situation and being singled out from the herd (Watts and Stookey, 2000), thus making it difficult to evaluate vocalization in response to pain. Future research would benefit from recording the duration of the vocalization or timing in comparison to the procedure, but should keep in mind vocalization may not be solely indicative of pain.

A significant increase in the percentage of time spent standing was observed for both age groups from day 1 to day 3 signifying the animal's attempts to avoid sensitizing the injured area. Lying was not significantly different for 6 month old calves but the percentage of time spent lying in 6 week calves was significantly decreased from day 1 to day 2 post castration with a slight increase on d3, suggesting response to pain varies according to age. It would be useful for the animals to be fed and housed in a similar manner in order for ages to be directly compared in future trials. There were no significant treatment effects for standing or lying in either age group. Future research may benefit from selecting an alternative recording method, such as accelerometers and pedometers, which may be more sensitive and able to detect changes between treatments.

Behavioral changes can be detected immediately serving as an additional indicator of pain and stress that supports physiological changes. The appropriate age and method to utilize could not be determined from the research trial, however, the results add to the body of work to

validate behavioral responses to noxious stimuli that can be used for the future research and development of a cost-effective analgesia to mitigate pain in livestock.

The fourth chapter of this thesis evaluated the effectiveness of flunixin (FLU), meloxicam (MEL), gabapentin (GABA), and meloxicam with gabapentin (MEL+GABA) for their extended analgesia following dehorning relative to a control lidocaine cornual nerve block only. This study was also conducted to evaluate biomarkers, cortisol, substance P, haptoglobin, and ex-vivo prostaglandin (PGE2) for assessing dehorning pain in calves relative to the plasma drug concentration. In addition, non-invasive techniques including measuring neuroendocrine responses and nociception threshold tests were evaluated in relation to plasma drug concentration.

Although some NSAIDs have been previously described as adequate pain alleviators in response to dehorning and disbudding, MEL at the current dosage and per os (PO) route of administration does not appear to specifically reduce the particular physiological responses examined in this study. Previous studies have used IM (Heinrich et al., 2009, 2010) or IV (Coetzee et al., 2012) administration of MEL for painful husbandry procedures, thus it appears there is limited effect when administering MEL PO in cattle. It is advantageous for future research to examine another route of administration in order to determine the pain mitigation effects of NSAIDs and other analgesics.

MEL was able to influence MNT at specific sites around the horn bud, however, this result is likely due to the order in which the measurements were taken. It is likely that the animal had been initially fractious to the researcher performing the measurements, resulting in decreased MNT in the first few sites and habituation in the later measurements. Although previous studies have suggested that young animals can be acclimated to the procedure before beginning, it should be the strategy of future research to investigate a different method of familiarization to the procedure for older calves due to differences in behavioral responses.

FLU was able to reduce cortisol-marked distress and inhibit PGE2 synthesis for 24h. However, cortisol is not a true indicator of pain, thus FLU may not be reducing pain from the dehorning procedure, rather just the distress of handling. A reduction in PGE2 synthesis demonstrates the non-selective COX characteristics of FLU. COX-2 selective NSAIDs, like MEL are less effective at inhibiting ex-vivo prostaglandin synthesis. It would be beneficial for

future studies to examine other biomarkers outside of COX inhibition in order to encompass the total pharmacokinetics (PK) and pharmacodynamics (PD) of NSAIDS in response to dehorning.

Including a non-selective COX inhibitor, like flunixin IV, allowed for comparison of its effects to a delayed and extended therapeutic drug, like MEL or GABA. Although FLU achieves earlier therapeutic concentrations and immediate pain relief, the short duration of the therapeutic effect makes it a less desirable treatment, as multiple doses would be required to fully alleviate pain from dehorning.

This study was able to demonstrate the potential reliability of substance P as an indicator of pain. Although, no specific drugs were able to reduce substance P concentrations, calves treated with an analgesic had lower concentrations, suggesting analgesics have the ability to alleviate pain from dehorning and recommending substance P to as a biomarker. Substance P appears to be an important biomarker, which can be incorporated into future pain research and validation of drugs for pain alleviation in livestock.

Reduced weight gain has been observed following dehorning, which can become economically important in older calves. It was observed that calves given an analgesic gained significantly more than calves that received a placebo. The present study was limited to evaluation of gain over a 7d period, thus it would be beneficial for future studies to evaluate gain over a longer period of time as well as feed intake and efficiency.

No differences between treatments were detected as tested in haptoglobin concentrations or ocular thermography, suggesting that the methods were not sensitive or robust enough in this particular experiment. It is possible that only this particular study was unable to detect changes and further research should not disregard these previously validated measurements (Fisher et al., 2001;Stewart et al., 2008).

In conclusion, regardless of age or treatment, castration and dehorning cause stress and pain that can impact the welfare of the calf and if not adequately managed and mitigated can cause subtle economic losses. Research leading to new or improved techniques that can reduce or eliminate pain and distress, identification of valid biomarkers and cost-effective analgesics and anesthetics is key to the well being and success for both the animal and the producer.

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