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**The Use of Thermal Nociceptive Threshold Testing
to Assess the Effect of Analgesic Drugs on the Pain
Response of Dairy Cattle**

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Abstract

Painful procedures are routinely performed on cattle and the use of analgesia can abate this pain. Thermal nociceptive threshold (TNT) testing is used to assess pain sensitivity and the effect that painful conditions and analgesia have on this. However, little work has used TNT testing in cattle for these purposes. This research was carried out to determine if TNT testing could be used to assess the effects of analgesic drugs in both pain-free cattle and those that had undergone liver biopsy.

A carbon dioxide laser was used as the noxious thermal stimulus. In the first experiment, the effects of an alpha2-adrenoreceptor agonist (medetomidine) and a non-steroidal anti-inflammatory drug (ketoprofen) were compared with the effect of saline on TNTs of pain-free cattle. TNTs were measured 20 minutes before treatments were administered, then again at 20, 40 and 60 minutes after treatment. Medetomidine significantly increased the cows' TNT at 60 minutes post-treatment. This increased TNT may be due to the central analgesic properties of the drug. Ketoprofen had no effect on TNTs.

In the second experiment, TNTs were measured to determine if different analgesic protocols moderated central sensitisation that may have occurred after liver biopsy. Behavioural observations were also used to assess pain in the post-biopsy period. Cows were assigned into one of four groups: control (local anaesthetic (LA) + sham-biopsy); LA + biopsy; LA + ketoprofen + biopsy; LA + meloxicam + biopsy. TNTs were measured 1 day before liver biopsy was performed, and once daily on the 3 days post-biopsy. Behavioural observations were made in the 4 hours after biopsy and on the 3 days post-biopsy. TNTs of biopsied cows did not differ from sham-biopsy cows. This may be because liver biopsy did not induce central sensitisation, or because the TNT method used did not reflect localised hyperalgesia. Behaviour also did not differ between treatment groups. These findings suggest that liver biopsy as it was performed here does not induce significant pain in cattle.

It is concluded that TNT testing may be useful to investigate the effects of some analgesics on the acute pain response of pain-free cattle, but it has not been useful in demonstrating central sensitisation after liver biopsy. Further development and refinement of the methodology is required in order for this technique to be of future use for similar research in cattle.

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Abbreviations

AP	Action potential
CO ₂	Carbon dioxide
COX	Cyclooxygenase
DH	Dorsal horn
DHN	Dorsal horn neurons
DoA	Duration of action
IM	Intramuscular; intramuscularly
IP	Intraperitoneal; intraperitoneally
IT	Intrathecal; intrathecally
IV	Intravenous; intravenously
Kg	Kilogram
LA	Local anaesthetic
MNT	Mechanical nociceptive threshold
NSAID	Non-steroidal anti-inflammatory drug
NT	Nociceptive threshold
NTT	Nociceptive threshold test/testing
PAF	Primary afferent fibre
PG	Prostaglandin
PK	Pharmacokinetics
PO	Per os (oral administration)
SIH	Stress-induced hypoalgesia
SRT	Spinoreticular tract
STT	Spinothalamic tract
T _{max}	Time to maximum plasma concentration (of drugs)
TNT	Thermal nociceptive threshold

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1 Introduction

Pain is a noxious experience, both physically and emotionally. Few people doubt that pain impacts negatively on a person's well-being, whether of short or long duration. It is now recognised that other mammalian species experience pain in a similar way to humans, and we infer from this that their welfare state is also negatively impacted by its occurrence. Increased understanding and awareness of pain in these animals means that most people now agree we are obliged to alleviate pain in these animals when it occurs. Most developed nations now have recommendations or legislation for minimising animal pain and its associated distress.

In order to alleviate pain, we must first be able to recognise and categorise it. This presents a significant challenge when dealing with species that cannot verbally communicate their experience to us. Pain assessment in farm animals has primarily focused on techniques that evaluate physiological responses (such as changes in hormone levels) or behavioural alteration. These types of responses indicate that pain in farm animals occurs after many routine husbandry procedures.

Another method of pain assessment is thermal nociceptive threshold (TNT) testing, which is one type of nociceptive threshold testing (NTT). These techniques evaluate what is colloquially termed "pain sensitivity": how animals or humans respond to an acutely painful stimulus before and after different types of experimental interventions. Such methods have been widely used in human medical research on both human and animal (particularly rodent) subjects. While there has been some application of TNT testing to pain assessment in veterinary species, many potential uses have not yet been investigated. As this tool is a relatively unobtrusive way of assessing pain in farm animals it seems pertinent to explore which aspects of pain physiology and pharmacology it might be best suited to.

The overall objective of this research was to evaluate if TNT testing was useful for assessing the effect of several analgesic drugs on different types of pain in cattle. TNT testing has been mostly used in this species to determine nociceptive thresholds (often called "pain thresholds") of healthy cows and calves or to identify the occurrence of stress-induced changes in pain sensitivity in cows. Only very few studies have examined the effect of analgesia on TNTs of pain-free cows, or the effect of illness or injury on the TNTs of cows. No studies have used TNT testing in cattle to assess how analgesics might influence alterations in pain sensitivity after a painful procedure. Thus, it was undertaken here to further examine how analgesia might affect

TNTs of healthy cattle without pre-existing pain, and TNTs of cattle that had undergone a painful procedure.

This thesis commences with a literature review. This provides an overview of the physiology of pain, and then considers the work that has occurred to date on the assessment and alleviation of pain in cattle. Chapter 2 investigates if the acute pain response of healthy cows with no pre-existing pain or inflammation was altered by the central analgesic properties of 2 different classes of drugs. To assess this, medetomidine and ketoprofen were given and repeated measures of TNTs were made in the hour after administration. Chapter 3 examines whether TNTs of cows that underwent a painful procedure were altered due to central sensitisation, and whether different analgesic protocols influenced any such alteration. Liver biopsy was performed in cattle that received one of three analgesic protocols, and their TNTs and behaviour were compared to sham-biopsied animals. The thesis concludes with a general discussion of the relevant findings of this research. Important results and limitations are identified, and potential areas of future work are indicated.



Grazing Cows, Spring Afternoon

2 Literature Review

2.1 Introduction

During the last four decades, much has been learned about animal pain. Modern opinion is that non-human mammals feel pain. Much of the knowledge of human pain management has come from studies of other species, and the use of these for such research is an acknowledgement of the similarities between humans and other mammals (Johnson, 2008). Comparative anatomy and physiology indicates that the nervous pathways, receptor systems and neurotransmitters required for the sensory component of pain exist in both man and other mammals (Livingston, 2010). As awareness about the existence of animal pain increases, so too does the concern about the impact that pain may have on the welfare of animals. A growing movement toward improved welfare for farm animal species has seen the issue of pain and its alleviation in these animals come under increasing scrutiny.

Cattle may experience pain due to a number of causes. Lameness and mastitis are the two most significant conditions in dairy cattle that are painful (Stafford, 2012). Routine husbandry procedures such as castration, dehorning and branding elicit physiological and behavioural changes that indicate these are painful (Lay et al., 1992a; Stafford & Mellor, 2005a, 2005b).

From an ethical perspective, the pain and suffering experienced by animals under our care should be minimised. This is one aim of the veterinary profession (Anonymous, 2011), but applies equally to farmers and all those involved in the animal production system (e.g. transporters, processors). Many countries have codes of animal welfare that detail expected minimum standards of care (Anonymous, 2010) and recommendations on the use of analgesia/anaesthesia when performing painful procedures such as castration or dehorning (Anonymous, 2005). In addition, legislation in most developed nations sets down the legal requirements and obligations of those caring for animals (Anonymous, 1999).

From an economic perspective there are also good reasons to minimise pain and associated suffering in farm animals. Firstly, pain can significantly decrease productivity in cattle. For example, pain caused by lameness will decrease milk yield (Pavlenko et al., 2011). Therefore farmers should treat such conditions promptly to gain maximal production. Secondly, public perceptions of animal welfare can influence consumer behaviour. Public concern for improved animal welfare is increasing throughout the world, and comes primarily from urbanised

populations (Caporale et al., 2005). As a consequence, consumer demand for products derived from systems with positively perceived welfare status may continue to increase, as these people make purchasing decisions based on their attitudes to welfare (Toma et al., 2011).

Globalisation means that producers wanting to export cannot afford to ignore consumer concerns in an importing country (Thiermann & Babcock, 2005). International market access may therefore be affected by the importing country's perception of animal welfare in the management systems of a producing country.

Pain alleviation in farm animal species has been hindered in part by a lack of efficacious analgesics. Until the development of the non-steroidal anti-inflammatory drugs (NSAIDs) in the 1980s, veterinarians had very few analgesic agents available for use in ruminants (Stafford, 2012). The consequence of this may have been that pain in their patients was ignored or underestimated because of their limited ability to manage it (Stafford & Mellor, 2007). In addition, the belief of some that these animals do not sense pain as humans do, coupled with our poor ability to recognise pain in stoic prey species led to a reluctance to treat painful situations (Livingston, 2010). As a result, provision of analgesia during most routine husbandry and surgical procedures of cattle has been non-existent or inadequate. Although the use of analgesia in cattle is now increasing, the rates of use are still low for many painful procedures (Fajt et al., 2011; Hewson et al., 2007), especially when compared to use in companion animal species (Williams et al., 2005).

As noted above, perhaps one of the biggest barriers to widespread use of analgesia in cattle is our inability to assess the pain experienced by them. The subjective experience of pain can only be inferred by our observation and interpretation of measurable responses. Valid and reliable methods of pain assessment are required if we are to progress our understanding of pain in animals (Livingston, 2010). Many authors are in agreement that the development of species-specific assessment techniques is the first step in formulating appropriate strategies for pain alleviation in farm animals (Anil et al., 2005; Stafford & Mellor, 2007; Weary et al., 2006). Such developments would allow for improved pain alleviation in animals as we are better able to determine the significance of a presumed painful experience, or the efficacy of any pain management strategy. The aims of this literature review are:

1. To briefly describe the physiology and pathophysiology of pain.
2. To outline the methods used for the assessment of pain in cattle.
3. To examine the evidence for acute pain and its alleviation in cattle.

2.2 Pain

Pain is defined by the International Association for the Study of Pain (IASP) as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage” (Merskey et al., 1979). The ability to perceive pain is a feature of highly evolved animals, such as mammals, and an important component of homeostasis. The function of pain is to warn of occurring or impending tissue damage, thus eliciting immediate withdrawal or escape behaviours, whilst the experience of pain provides a learning opportunity so that future behaviour is moderated to avoid similar pain-causing circumstances (Mellor et al., 2000).

Understanding pain in another human largely hinges on the subject’s ability to communicate their experience; an issue partly resolved when language is used. If we consider pain in other species (or non-verbal humans), the absence of common language makes the above definition of pain less useful. There has since been some effort to give a definition to pain as it might apply to other animal species (Anderson & Muir, 2005b; Zimmerman, 1986), with the following interpretation proposed by Molony and Kent (1997) being widely accepted: “animal pain is an aversive sensory and emotional experience representing an awareness by the animal of damage or threat to the integrity of its tissues. It changes the animal’s physiology and behaviour to reduce or avoid the damage, to reduce the likelihood of recurrence and to promote recovery”.

2.2.1 Neurophysiology of Pain

Within both definitions of pain given above there are two key concepts: nociception, the objective sensory aspect, and feeling pain, the subjective emotional aspect. A description of these two aspects under normal physiological conditions is given below.

2.2.1.1 Nociception

The detection of noxious and innocuous stimuli occurs at the peripheral level via free nerve endings of myelinated A-fibres and unmyelinated C-fibres. These fibres may respond specifically to mechanical, thermal or chemical stimuli, or may be polymodal. The A-fibres are further classified as A δ - or A β -fibres. Under normal physiological conditions, all 3 fibre types transmit information about non-noxious stimuli, but only C-fibres and A δ -fibres signal noxious input (Millan, 1999). A comparison of their properties is presented in Table 2.1

Activation of the nociceptors results in the generation of action potentials (APs) which are conducted along these primary afferent fibres (PAFs) towards the pain processing centres in the brain. These afferent signals are processed and/or modulated at various sites between the peripheral receptor and the brain.

The first stage of sensory processing of incoming noxious signals occurs in the dorsal horn (DH) of the spinal cord, where “gating” of the signals modulates transmission from the PAFs to central systems (Melzack & Wall, 1965). This refers to the balance of activity between the small nociceptive PAFs and the large somatosensory nerves: when activity in the large fibres is greater, the “gate” is “closed” and painful signals are prevented from passing beyond the spinal cord. When nociceptive input is greater, the “gate” is “open” and pain signals are transmitted to the brain. It is also likely that a second level of gating exists at the afferent input to ascending dorsal columns (Livingston & Chambers, 2000), via which the noxious signals are carried up to the higher processing centres of the brain, where the feeling of pain is transduced.

Table 2.1: Properties of C- & A-fibres (derived from Millan, 1999 and Raja et al., 1999)

C-fibres	A δ -fibres		A β -fibres
<ul style="list-style-type: none"> - Unmyelinated - 0.4-1.2 μm, thin - Slow conduction velocity - Secondary "dull", aching pain - Difficult to localise pain due to lack of direct connection with cerebral cortex - Synapse in laminae I, II & V of dorsal horn 	<ul style="list-style-type: none"> - Myelinated - 2-6 μm, medium thickness - Intermediate conduction velocity - First acute "sharp" pain - Accurate location of pain due to connections with cerebral cortex allows reflex withdrawal - Synapse in laminae I & II of dorsal horn 		<ul style="list-style-type: none"> - Myelinated - >10 μm, thick - Fast conduction velocity - Synapse in laminae III-VI of dorsal horn
<ul style="list-style-type: none"> - Polymodal - High activation threshold - Engaged by low noxious skin heating rates - "Silent" nociceptors: chemosensitive receptors, unresponsive to acute noxious stimuli; activated by chemical mediators during injury/inflammation; respond to thermal and mechanical stimuli once activated 	Type I	Type II	<ul style="list-style-type: none"> - Low threshold mechanoreceptors - Only mediate innocuous "touch" sensations in normal states
	<ul style="list-style-type: none"> - Polymodal - High-threshold mechanoreceptors - High response threshold to short or low intensity heat stimuli - BUT may be sensitised by repeated thermal stimuli 	<ul style="list-style-type: none"> - Mostly insensitive to mechanical stimuli - Lower threshold to noxious heat than Type I 	

2.2.1.2 Pain Perception

The ascending nerve fibres are organised into a number of pathways which carry the noxious signals forward to the brain, the main ones being the spinothalamic tract (STT) and spinoreticular tract (SRT) (Livingston & Chambers, 2000). These then synapse in the brain at various levels, the most important of which is the thalamus. (Millan, 1999). Signals are then referred to the cerebral cortex where they are interpreted as “pain”. This results in a signal transmission back to the spinal cord via descending control systems. Some of the ascending A δ -fibres terminate directly in the reticular areas of the brain stem, which control the levels of arousal and brain activity (Hall, 2011c). This system can elicit immediate activity in the cerebral cortex and is an important link between pain impulses and physiological stress responses involving both the sympathoadrenal system (SAS) and the hypothalamic-pituitary-adrenocortical (HPA) axis (Hall, 2011a, 2011b).

The prefrontal cortex is key to the emotional component of pain (Millan, 1999). Thus, because most non-human species have a relatively small prefrontal cortex, the suggestion has been made that pain in these species is perceived purely as a stimulus and has no emotional aspect (Bermond, 2001). This assertion assumes that the capacity for pain perception is determined by size of the prefrontal cortex alone, but it is possible that other areas have an analogous role in non-human species (Flecknell, 2000). Indeed, despite gross anatomical differences, it appears that mammalian and avian brains share similar circuitry required for a high level of conscious awareness (Butler & Cotterill, 2006), a property which is thought to indicate that these animals are capable of experiencing pain (Bateson, 1991)

2.2.1.3 Descending Modulation of Pain

The descending control systems are responsible for regulating the on-going input from the nociceptive afferent fibres. Both inhibition and facilitation of pain signals occur via these pathways. The sites of origin in the brain and the neurotransmitters used may be common to both activities (Millan, 2002). These pathways enter the spinal cord via the dorsolateral and ventrolateral funiculi and can modulate nociception by interacting with PAF terminals, projection neurons, intrinsic excitatory interneurons and terminals of other descending pathways (Millan, 2002).

One example of such pain modulation is the stress-induced hypoalgesia seen in cattle in response to social stressors (Herskin et al., 2007; Herskin et al., 2004). This occurs due to descending inhibition, which results in a reduced perception of pain (Livingston & Chambers,

2000). Functionally, this change is thought to allow unimpeded or enhanced performance during times of stress, danger or tissue damage (Millan, 2002). A more detailed consideration of this area of pain physiology is beyond the scope of this review, and the reader is referred to the comprehensive review of Millan (2002) for further information.

2.2.2 Pathophysiological Pain States

The previous section outlined the transmission and perception of noxious signals under normal physiological conditions, where pain functions as a warning signal to prevent tissue damage. However, when events such as injury or inflammation occur, enhanced sensitivity to pain may ensue. These altered pain states are a consequence of modified pain signals. Modification can occur at the peripheral level of the nociceptor, resulting in peripheral sensitisation, and the central sites of the dorsal horn (DH) and brain, leading to central sensitisation. A description of the development of peripheral and central sensitisation is given, followed by how these altered pain states manifest in the form of hyperalgesia.

2.2.2.1 Peripheral Sensitisation

Tissue damage (e.g. from injury or surgical procedures) results in the release of inflammatory mediators from the damaged cells, inflammatory cells and the PAF. These mediators are vast in number and include prostaglandins, cytokines, histamine, cyclooxygenase and growth factors (Millan, 1999). Some of these directly activate the nociceptors, while others sensitise the nerve terminals (Dolan & Nolan, 2008). The consequence is sensitisation of the PAF, where stimuli that were previously of insufficient intensity can cause activation of the nociceptor.

The inflammatory mediators can also activate the “silent” C-fibres. Thus, in addition to the amplified response of normally active PAFs, the afferent input to the DH is further enhanced by the contribution of these newly recruited units (Millan, 1999). The combination of the sensitised PAFs and the activated “silent” fibres leads to an altered pain response in the animal, whereby it becomes more sensitive to stimuli of less intensity because of the nociceptors’ lowered activation thresholds, increased spontaneous activity, and increased responses to suprathreshold stimuli (Raja et al., 1999).

2.2.2.2 Central Sensitisation

Central sensitisation arises from sustained activity of the peripheral nociceptors, either due to tissue injury or inflammation, which leads to increased responsiveness of the dorsal horn neurons (DHNs). It has a major role in the development of acute post-operative pain (Dolan &

Nolan, 2008), and can have a protective function by helping minimise the exposure of damaged tissue to further injury while healing occurs (Doubell et al., 1999) Central sensitisation occurs in two forms (Woolf & Salter, 2006):

Homosynaptic Central Sensitisation (“Wind-up”)

In this instance, only activated synapses show a change in activity. During repeated stimulation an increase in APs generated from the DHNs exists over the time-course of the stimulus and terminates when the stimulus ends. The net result is increased pain with each repetition of the noxious stimulus, even though the stimulus intensity is unchanged.

Heterosynaptic Central Sensitisation

This includes homosynaptic sensitisation as well as additional synapses not originally activated by the noxious stimulus. After induction, normally sub-threshold inputs activate the DHNs due to an increased synaptic efficacy. It can occur within seconds of an appropriate conditioning stimulus, and outlasts the nociceptor barrage.

Central sensitisation occurs due the release of several CNS neurotransmitters. Intracellular signalling cascades which alter neuronal plasticity are then activated (Vinueza-Fernandez et al., 2007). The manifestations of central sensitisation are three-fold: a reduction in the activation threshold, an increased responsiveness of DH neurons, and an expansion of receptive fields of the nociceptors (Doubell et al., 1999).

2.2.2.3 Hyperalgesia – The Effect of Sensitisation

Hyperalgesia is an increased magnitude of perceived pain relative to the stimulus intensity (Raja et al., 1999). The result of this is that nociceptors of damaged tissue display an increased sensitivity to mechanical, thermal and chemical stimuli.

Hyperalgesia may be classified into two types, according to where it appears in relation to inflammation resulting from tissue damage. The first, referred to as the zone of primary hyperalgesia, is immediately around the area of damage. The second is the zone of secondary hyperalgesia and occurs in undamaged tissue surrounding the area of inflammation.

Hyperalgesia can also be classified as mechanical or thermal hyperalgesia, depending on the type of nociceptor that shows altered activity. Mechanical hyperalgesia may be further classified into allodynia (perceived pain due to a non-noxious stimulus) and punctate, pressure or impact hyperalgesia, depending on the type of stimulus used (Raja et al., 1999). Allodynia is

mediated by large myelinated A β fibres which, under normal conditions, are responsible for transmission of non-noxious “touch” sensation. In the event of tissue damage a change in sensory processing occurs in these fibres resulting in transmission of noxious sensations (Millan, 1999).

The mechanisms underlying mechanical or thermal hyperalgesia vary depending on whether the change is seen in the primary or secondary zone. Within the primary zone, both mechanical and thermal hyperalgesia are present. Hyperalgesia here can predominantly be explained by events at peripheral nociceptors, including changes in sensitivity, response and activity, and recruitment of silent nociceptors (Millan, 1999). Primary hyperalgesia is therefore regarded as a consequence of peripheral sensitisation.

Of the mechanical forms, only allodynia and punctate hyperalgesia exist in the zone of secondary hyperalgesia (Raja et al., 1999). Though both involve mechanisms of central sensitisation, their peripheral mechanisms appear to differ: both are induced by peripheral nociceptor input, but whereas sustained activity from these fibres is required to maintain allodynia, punctate hyperalgesia does not depend on this (Handwerker & Schmelz, 2004)

While thermal hyperalgesia in the primary zone has been clearly demonstrated (Zahn & Brennan, 1999), it is still unclear if thermal hyperalgesia exists in the secondary zone; some authors have demonstrated it whilst others have not (Sumikura et al., 2005). It is possible that two types mediated by different fibres may occur and this may explain the discrepancies between various studies (Sumikura et al., 2005). Further work investigating the relationship of stimulus duration and intensity, as well as the temporal and spatial development of secondary thermal hyperalgesia is required to clarify this phenomenon.

Referred hyperalgesia may also occur when increased sensitivity to painful stimuli is perceived not only in damaged tissue, but in uninjured tissue at a site remote to the injury (Sandkühler, 2009). The most common occurrence of this type of hyperalgesia is the referral of visceral pain to somatic structures that share the same sites of spinal neuronal input, for example bladder pain referring to the legs (Jaggar et al., 1999). Finally, it is also possible for a state of generalised hyperalgesia to exist if the level of central sensitisation is sufficient to trigger supra-spinal excitation (Wilder-Smith & Arendt-Nielsen, 2006)

2.2.3 The Dorsal Horn & Neuronal Plasticity

From the above overview of pain physiology, it is clear that the spinal cord is a key site of sensory processing. Thus, it can be concluded that the role of the DH is a dynamic one, which allows it to operate in three modes and exhibit neuronal plasticity (Doubell et al., 1999):

- 1) Normal transmission: Low intensity stimuli are interpreted as innocuous by A β fibres; high intensity stimuli activate high-threshold A δ - and C-fibres, producing a localised transient pain, usually without subsequent tissue damage.
- 2) Suppressed transmission: Descending inhibitory systems suppress the sensation of pain, despite nociceptor activation due to high intensity stimuli.
- 3) Sensitisation: An increase in DH excitability occurs, leading to facilitation of responses to sensory input.

With an understanding of the physiology of pain, attention is now turned towards the assessment of pain in cattle. An outline of the techniques used, and the information they have provided regarding the existence of pain in this species is given.

2.3 Assessing Pain in Cattle

Four main strategies have been used to assess pain in animals: behavioural observations; physiological parameters; nociceptive threshold testing (NTT); and assessing changes in these various parameters after administration of analgesia. It is unlikely that any of these methods alone will fully represent the pain experience of an animal; therefore assessing multiple parameters simultaneously is more likely to give a better understanding of pain. However, practicalities and costs limit the use of most of these tools to research; veterinary clinicians and owners/producers must rely on behavioural observation to inform them of pain in their charges. Therefore the research environment may also provide useful opportunities to explore correlations between changes in behaviour and the other parameters to gauge if the different methods give concurring indications of the presence of pain.

The above approaches have typically been adopted for the assessment of acute pain in cattle. An outline of the methods of NTT and behavioural observations is given below, along with an overview of how these have been used to evaluate acute pain in cattle. Physiological parameters are mentioned in brief. Most work has focused on the effects of routine husbandry procedures such as dehorning/disbudding, castration and branding, and these form the basis of most examples given. The effect of administration of analgesia on the pain response of cattle will be considered in the next section of this review.

2.3.1 Nociceptive Threshold Testing (Analgesiometry or Quantitative Sensory Testing)

Nociceptive threshold testing (NTT) is defined as “the determination of thresholds or stimulus response curves for sensory processing under normal and pathophysiological conditions” (Arendt-Nielsen & Yarnitsky, 2009). It involves the application of a painful stimulus (either thermal, mechanical, electrical or chemical) to a body area until a behavioural response is seen, at which time the stimulus is removed. Depending on the intensity of the stimulus, these responses may be spinally-mediated reflex behaviours (such as tail-flicking or leg/paw withdrawal), or more complex behaviours (such as licking) (Fan et al., 1995). Thus nociceptive thresholds (NTs) can be considered as an example of an “input-output” system (Le Bars et al., 2001), where, if optimal results are to be obtained, the characteristics of both the stimulus (input) and the response (output) need to be clearly defined according to species and individual variation (Love et al., 2011).

NTT can be used to indicate pain sensitivity in animals. It can be applied in one of three ways: to establish baseline NTs (i.e. the point at which a healthy uninjured animal demonstrates a behavioural response to a noxious stimulus); to identify hyper- or hypoalgesic states in painful or stressed animals; to demonstrate the effect of analgesics on both of the first 2 previous categories.

There are several advantages of using NTT to assess pain sensitivity under both normal and pathological conditions (Arendt-Nielsen & Yarnitsky, 2009):

- 1) The intensity and duration of the experimental stimulus can be controlled, so do not vary over time.
- 2) Responses can be differentially assessed, depending on the stimulus modality and site of application.
- 3) Quantitative assessment of the responses is possible and these can be compared over time (e.g., profiling new/existing drug efficacy over time).
- 4) Testing can be performed to quantitatively compare reactions between normal, affected, and treated regions of the same animal.
- 5) Experimental proxies of clinically occurring pathological conditions (e.g. hyperalgesia) can be used to evaluate the effects of drugs/procedures on particular mechanisms.

Experimental pain models using NTT are often based around examining specific pain fibre or receptor types, and this dictates the type of stimulus selected (Nielsen et al., 2009). However, clinical pain is much more complex than this, therefore it is unlikely that a single testing modality

can represent the complete experience of such pain (Nielsen et al., 2009), although use of more than one type of stimulus can give a more complete evaluation (Love et al., 2011)

Although initial efforts were made to develop NTT methods for farm animal species (Ley et al., 1996; Veissier et al., 2000; Welsh & Nolan, 1995), uptake of the technique for these species has not been rapid, even though the work done so far indicates that it is a useful tool (Viñuela-Fernández et al., 2007). The different types of NTT and the research that has occurred in cattle are outlined below.

2.3.1.1 Thermal NTT

Thermal NTT may use a variety of stimulus types. Typically this is either radiant heat (e.g. from a xenon lamp), contact thermodes, or a laser (usually carbon dioxide (CO₂) or argon). The measured variable may be skin temperature, although this is recognised as being difficult to assess (Le Bars et al., 2001). Instead, the time taken until a response is shown (often called “latency to respond”) is frequently measured. A description of how each stimulus type functions and how they have been used to assess pain in cattle is provided.

2.3.1.1.1 Radiant Heat Source

These are typically conducted by a person focusing a light beam of adjustable intensity onto the area of skin to be stimulated. The disadvantage of this technique is that some of the heat is reflected off the skin, causing variation in the amount of energy being delivered to the nociceptors (Arendt-Nielsen & Chen, 2003). The close proximity of people to the animal may also be disadvantageous, as this may increase stress in the subject and impact the test responses (Veissier et al., 2000). This method has been used to demonstrate opioid-mediated analgesia (both endogenous and exogenous) in cattle, with significant elevations in thermal thresholds occurring after opioid administration (Pineiro Machado et al., 1997; Pineiro Machado et al., 1998). Thus it is possible to demonstrate the effect of analgesic drugs in cattle using TNT testing

2.3.1.1.2 Contact Thermodes

These contain a heating probe and a temperature sensor, and are placed on the area of skin to be stimulated (Love et al., 2011). The probe can then be heated in a graded way, and the temperature at which the animal responds can be recorded from the thermometer. They are advantageous as the stimulus can be very precisely controlled; however the mechanical stimulation of the skin that occurs due to the contact is generally regarded as a significant

negative factor (Arendt-Nielsen & Chen, 2003). Use of this technique has been trialled in cattle: attempts to establish the presence of thermal hyperalgesia in lame heifers have been made, through the use of a contact thermode on the ear (Whay et al., 1997). However, there was a low success rate of stimulus application with this method and no significant relationship between lameness and TNT was found.

2.3.1.1.3 CO₂ Laser

This method uses an infrared laser beam to target the area of skin to be stimulated. Absorption of the heat occurs in the superficial skin layers, and is passively conducted to deeper layers where activation of the nociceptors occurs (Arendt-Nielsen & Chen, 2003). The advantages of this method of thermal stimulation are that very little energy is lost due to reflection off the skin, there is no mechanical stimulation of the skin and there is the ability to precisely control the intensity and timing of the stimulus (Herskin et al., 2003; Veissier et al., 2000). It also involves less handling of the animals being tested, as the stimulus is applied from a distance, without requiring physical contact with the subject. This technique has been favoured for use in cattle over the previously mentioned methods.

The validity and reliability of this method for assessing pain sensitivity in cattle has been shown. Baseline thresholds in healthy animals were ascertained in studies on adult cows and calves (Herskin et al., 2003; Veissier et al., 2000). The inverse relationship between laser power output and the latency to respond indicated that animals were responding to a progressive increase in skin temperatures. Thus it was concluded that this method is a valid measure of thermal nociception in cattle (Veissier et al., 2000). The repeatability of the method was also demonstrated, as latencies to respond were unaffected when repeat testing was conducted 60 minutes and 24 hours after initial testing (Veissier et al., 2000). The short-term repeatability was also found to be acceptable when repeat testing was conducted 15 minutes after the initial test (Herskin et al., 2003)

Subsequent use of this technique in cattle has primarily been to investigate the phenomenon of stress-induced hypoalgesia (SIH). Social stressors have been shown to increase laser-based TNTs in cattle (Herskin et al., 2007; Herskin et al., 2004; Rushen et al., 1999). In contrast, studies that have used TNT testing to look for SIH after physical stressors such as tissue damage have not identified its existence. A comparison of cattle that underwent either hot-iron or freeze branding on the ribs showed no demonstrable SIH up to 120 minutes after the procedure (Schwartzkopf-Genswein et al., 1997a). These authors expressed uncertainty as to whether this was a true reflection on the occurrence of SIH, or whether aspects of the methodology, such as the laser setting, did not allow them to detect subtle changes between

treatments. SIH was also not demonstrated in calves after Burdizzo castration (Ting et al., 2010). Although thermal thresholds tended to increase after the procedure, suggesting that SIH may occur, this effect was not significant.

Thermal thresholds were also not significantly altered in cows that had experimentally induced *Escherichia coli* mastitis when the latency to respond to the laser stimulus was compared in the days after disease induction (Rasmussen et al., 2011). The anticipated outcome was evidence of hyperalgesia due to the release of sensitising inflammatory mediators released after the induction of mastitis. However these authors suggested that changes in the behavioural responses to the stimulus may have indicated hypoalgesia after induction of mastitis. One day after mastitis was induced, an increased proportion of cows responded with stepping (the least forceful leg movement) after stimulation on the udder, and showed decreased leg movements between laser stimulations of the hind legs; these changes were considered to represent an increased nociceptive threshold which would support hypoalgesia. However, it is also possible these changes reflected a disease-induced decrease in responsiveness, rather than altered pain processing. Though other work shows that higher laser power settings resulted in a greater proportion of kicking response in calves (Veissier, et al., 2000), the relationship between the type of behavioural response shown and the intensity of pain experienced after laser stimulation has not been quantified. Thus the proposition that the altered behaviour of the mastitic cows may be due to hypoalgesia remains to be proven.

It is of interest that hyperalgesia to the laser stimulus was not seen after branding, castration or the occurrence of mastitis in the studies mentioned above. Although the branding and castration studies were looking specifically for SIH, decreases in TNTs that might indicate thermal hyperalgesia after these painful procedures were likewise not noted.

With the exception of the study by Whay et al. (1997), no work has been done in assessing hyperalgesic states in cattle using TNT testing. Likewise, investigating the effect of analgesic drugs in cattle with this tool has received very little attention.

2.3.1.2 Mechanical NTT

Of the studies using NTT that have been conducted in farm animal species, mechanical nociceptive threshold (MNT) testing has been the technique most commonly used. Methods of assessment may use a quantifiable stimulus, such as a pressure algometer (Chambers et al., 1995; Whay et al., 1998), or subjective assessments of responses to haemostat pressure,

pinpricks or pinch tests (Araújo et al., 2012; DeRossi et al., 2009; Prado et al., 1999). The measured variable is either the pressure or force required to elicit a behavioural response. This technique has proved to be useful for evaluating changes in pain sensitivity in cattle, further indicating that NTT may be successfully used in this species. Methods involving quantifiable stimuli usually require attachment of equipment to the animal, so as with other methods requiring physical intervention may cause increased stress to the subject.

The majority of work using MNTs in cattle has focused on changes in pain sensitivity due to conditions resulting in hyperalgesia. Acute lameness occurring for a variety of reasons consistently reduced mechanical thresholds of dairy cattle (Ley et al., 1996; Whay et al., 1997; Whay et al., 1998). MNT testing has also identified chronic hyperalgesia due to lameness (Laven et al., 2008; Whay et al., 1998).

MNT testing in cows with mastitis has yielded some contradictory results. Mastitic cows showed a significant hyperalgesia in the ipsilateral leg of the affected quarter when compared to normal non-mastitic cows (Fitzpatrick et al., 1998). In contrast with these findings, Kemp et al. (2008) found the only significant difference in MNTs was supposed hypoalgesia in the contralateral leg from the affected quarter. They suggested that this increased tolerance was due to an increased reluctance of mastitic cows to weight bear on the affected side.

The effect of some analgesic drugs on MNTs in cattle has been examined, with significant elevation occurring in subjectively assessed mechanical thresholds after the administration of alpha2-adrenoceptor agonists (DeRossi et al., 2009; Lin et al., 1998; Prado et al., 1999). This provides further evidence that NTT techniques are effective at demonstrating analgesic effects on pain sensitivity in cattle.

2.3.1.3 Electrical NTT

Electrical thresholds are assessed by placing electrodes on the skin and adjusting the current sent until a behavioural response is observed. This technique has similar drawbacks to mechanical threshold testing in that a higher level of physical contact with the animal is required. It has received very little use in cattle. The effect of some analgesics on the electrical thresholds of cattle has been assessed (Baldrige et al., 2011; Prado et al., 1999), with some significant elevations of thresholds occurring. An attempt to investigate post-operative hyperalgesia has also been made in calves after dehorning and castration (Baldrige et al., 2011), however no significant changes in thresholds was found.

2.3.1.4 Research Opportunities Using NTT

Use of a CO₂ laser has proved to be effective for determining baseline pain sensitivity in cattle, and this method confers a number of advantages that make it a suitable option for investigating NTs in cattle. Therefore opportunities exist to use this technique to examine other aspects of pain physiology in cattle that have so far not received attention. These would include examining the effect of analgesic drugs on baseline thresholds, identifying hyperalgesic states after painful procedures, and determining how analgesics might moderate such hyperalgesia. This may assist in further quantifying the effect that many routine husbandry procedures have on the pain processing systems in animals subjected to them, and to what extent analgesic drugs might alleviate such pain.

2.3.2 Quantifying Pain-Related Behaviour

Observations of behaviour are advantageous because they are non-invasive and can be seen immediately as they develop, thereby allowing rapid evaluation (Mellor et al., 2000). They also have relatively little requirement for equipment so are economical and practical to perform. People who are familiar with the animals in question are best placed to assess pain-related behaviour, as experienced observers can identify deviations from normal behaviour patterns (Livingston, 2010). The accuracy of assessment is also improved when observers have knowledge of what behaviours animals in pain might exhibit (Anil et al., 2005).

Methods of behavioural assessment may be broadly categorised as either subjective or objective. Subjective methods range from personal judgement through to the use of descriptive scoring systems (see below), whilst objective measures focus on quantifying defined behaviours in more detail (Rutherford, 2002). Three main methods of behaviour-based assessment have been used to evaluate pain in farm animals (Weary et al., 2006):

1. Identifying and quantifying pain specific behaviours, such as calves showing increased head-shaking and ear-flicking after dehorning (Sylvester et al., 2004). Such behaviours are usually seen only when the animal has experienced an event presumed to be painful, and are rarely seen in animals not exposed to the painful event.
2. Quantifying changes in normal behaviour, such as cows spending an increased amount of time upright after liver biopsy (Mølgaard et al., 2012). The presence of pain can be inferred by comparing the frequency or duration of normally occurring behaviours (e.g. grazing or rumination) between animals subjected to a presumed painful event and those that were not.

3. Measuring preference or avoidance behaviour, such as sheep learning to associate movement along a race with the expectation of electro-immobilisation and thus becoming increasingly harder to move (Weary et al., 2006). Such avoidance learning may be used to infer the relative level of aversion different treatments pose to animals.

A further method of behaviour-based assessment is the use of pain scoring systems derived from human medicine, such as the visual analogue scale (VAS) or numerical rating scale (NRS). These systems require the observer to assign a subjective pain rating (e.g., between “no pain” and “worst possible pain” for VAS, or from 0-10 for NRS) according to the degree of pain they believe the animal to be in.

In general, systems such as the VAS and NRS show poorer reliability than those methods that quantify behavioural parameters, because of the subjective interpretation of behaviour (Weary et al., 2006). Such interpretations may be influenced by observer factors such as age and sex and therefore lead to possible under or over diagnosis of pain (Laven et al., 2009). However, when such scoring systems are used in conjunction with more objective measures, good correlations between the two methods can provide greater confidence that the inference of pain is true. An example of this is the dose-dependent decrease in both subjective pain scores and objectively quantified pain-related behaviours seen in rats that were administered analgesia after abdominal surgery (Weary et al., 2006).

Objective quantification of behaviours may be useful for assessing the relative noxiousness of different methods of the same procedure. For instance, when compared with caustic paste disbudding, hot-iron dehorning resulted in a nine-fold increase in head-shaking in the four hours post-treatment (Vickers et al., 2005), suggesting hot-iron dehorning leads to greater levels of pain-induced distress. However, behaviour alone cannot always be used in this way, as different methods of the same procedure (e.g. surgery vs. Burdizzo castration) or different procedures (e.g. dehorning vs. castration), may elicit specific behavioural responses (Mellor et al., 2000). For example, in the initial period after castration, lambs castrated with a rubber ring show increased activity levels whereas those castrated surgically show decreased activity (Mellor et al., 2000). In such instances it may be necessary to use other methods of pain assessment to determine the relative noxiousness of such treatments.

2.3.2.1 Behaviour as an Indicator of Pain in Cattle

Behaviour-based pain assessment in cattle has predominantly focused on quantifying pain-specific behaviours and alterations in normally occurring behaviours. This work has provided a

solid body of evidence that demonstrates the presence of pain after routine husbandry procedures such as castration and dehorning. In some instances it has also identified behaviours that are specific to a particular procedure and site of injury. Examples of this are presented below.

General behavioural signs of pain in cattle include inactivity and decreases in appetite and rumination (Stafford, 2012). Amputation dehorning without analgesia resulted in almost non-existent levels of rumination in calves in the 6 hours immediately after the procedure (Sylvester et al., 2004). In other studies, decreased rumination, grazing and grooming and increased levels of lying down were seen in dehorned calves, when compared with calves that were not dehorned (McMeekan et al., 1999; Stafford et al., 2000).

Castrated calves showed an increased time spent standing vs. lying when compared with their non-castrated cohorts, had shortened stride lengths, and took fewer steps (Coetzee, 2011). In addition, differences in behaviour were identified between calves subjected to different methods of castration: in the 2 hours after castration, surgically castrated calves moved less than chemically castrated or Burdizzo castrated calves, while 2 days later both surgically- and Burdizzo castrates moved less than chemically castrated calves (Stafford & Mellor, 2005b). This reduced movement of the surgically- and Burdizzo castrated calves suggest these procedures to be more painful compared to chemical castration. These dissimilar behaviours are likely due to the different tissue damage caused by differing methods of castration (Stafford & Mellor, 2005b).

Alterations to behaviour indicate that branding is also a painful experience. Significant differences in rates of tail-flicking, kicking, falling and vocalisation were found between branded and sham-branded animals (Schwartzkopf-Genswein et al., 1997b), and this is supported by the findings of other studies where increased frequencies of some of these behaviours were seen in branded versus non-branded animals (Lay et al., 1992a; Lay et al., 1992b)

Preliminary evidence of pain after liver biopsy has been found in behavioural observations of cattle. In the four hours after biopsy cows that were sham-biopsied looked at the wound site less than their biopsied cohorts. Proportions of time spent ruminating also differed between the treatment groups: sham-biopsied cows ruminated significantly more than did cows that had received only local anaesthetic at the time of biopsy, whereas biopsied cows that received additional analgesia spent a similar proportion of time ruminating as the sham-biopsied cows (Beausoleil & Stafford, 2012). Increased tail-pressing, time spent standing abnormally and

restlessness were also seen in cows after liver biopsy when compared with their behaviour during a control treatment (Mølgaard et al., 2012).

2.3.2.2 Research Opportunities Using Quantified Behavioural Observation

From these examples, it is clear that behaviour-based assessment of pain is a useful tool for examining changes induced by painful procedures. Though it has been most extensively used for assessing castration and dehorning, it has the potential to be applied to numerous other conditions or procedures presumed to be painful. Liver biopsy is a procedure that is commonly performed on cattle in New Zealand (Grace et al., 2012; Smith et al., 2010), but only two studies have addressed whether post-biopsy pain is experienced in cattle. Cattle are also subject to many more common conditions or procedures that are likely to be painful, such as dystocia or caesarean section, yet no information exists on how this pain might impact on their welfare. Therefore, further behavioural evaluation of these procedures may assist with identifying the presence and significance of pain in affected animals.

2.3.3 Physiological Parameters

A full examination of how physiological parameters have been used for assessing pain in cattle is beyond the scope of this thesis. However, as the body of research using these techniques has contributed so much to our understanding of pain in this species, a very brief overview will be presented here. Examples of the use of plasma cortisol concentration, sympathoadrenal system (SAS) parameters and the electroencephalogram (EEG) for the assessment of pain in cattle are given.

Of all physiological variables, plasma cortisol (mediated by the hypothalamic-pituitary (HPA) axis) has been most commonly used to assess pain-induced distress because its response magnitude usually accords with the predicted noxiousness of different procedures (Mellor et al., 2000). SAS parameters such as heart rate (HR), respiratory rate (RR), temperature and humoral factors (such as adrenaline and noradrenaline) have also been used for the assessment of acute pain in cattle. However because these and HPA indicators may be influenced by factors other than pain (e.g. stress or fear), they may be unreliable when used alone (Dobromylskyj et al., 2000). The usefulness of these variables in the assessment of pain is therefore limited to being indicators of the overall noxiousness of the experience to the animal, rather than an absolute measure of pain specifically.

Elevations in plasma cortisol concentration after painful procedures in animals that have not received analgesia have been used as indicators of the presence of pain.

Dehorning/disbudding of calves results in a clear pattern of plasma cortisol elevation in the following hours, with peak plasma cortisol occurring about 30 minutes post-procedure, before returning to pre-treatment levels approximately 9 hours later (Stafford & Mellor, 2005a). It is thought that the acute plasma cortisol peak is associated with the initial nociceptor barrage, whilst the plateau and decline back to pre-treatment levels is probably associated with inflammatory pain (McMeekan et al., 1998).

Procedures such as castration and branding also result in significantly elevated plasma cortisol concentrations. This is seen after all commonly used methods of castration performed without local anaesthetic (rubber ring or latex band, surgical cut or pull, or Burdizzo clamp) (Stafford et al., 2002), and after branding using either hot-iron or freeze-branding methods (Lay et al., 1992a; Schwartzkopf-Genswein et al., 1997a). Comprehensive reviews of the research that has used plasma cortisol concentration to illustrate pain in cattle after castration and dehorning/disbudding can be referred to for additional information (Coetzee, 2011; Stafford & Mellor, 2005a, 2006, 2011).

Elevations in heart rate (HR) have been seen in calves that underwent dehorning, disbudding or castration without any analgesia (Gibson et al., 2007; Schwartzkopf-Genswein et al., 2005; Stewart et al., 2009; Stewart et al., 2010). Significant elevations in HR have also been seen after both hot-iron and freeze branding (Lay et al., 1992a). These same procedures also resulted in significant elevations in plasma epinephrine and/or norepinephrine (Lay et al., 1992b; Lay et al., 1992c; Mellor et al., 2002; Stewart et al., 2010).

Measurement of brain activity in response to stimuli presumed to be painful is another method that may be used for the assessment of pain in animals. For example, analysis of the electroencephalogram (EEG), which reflects the activity of the cerebral cortex, is a technique that has gained importance in pain research in recent years. Cortical activity shows an altered pattern of activation when a painful stimulus is applied to an animal (Gibson et al., 2007). The use of EEG under minimal anaesthesia provided further evidence for the acute noxiousness of dehorning in calves (Gibson et al., 2007), and demonstrated that slaughter without stunning in calves using a ventral neck incision is likely to be perceived as painful by these animals prior to loss of consciousness (Gibson et al., 2009). Alterations in EEG recordings of conscious calves undergoing castration also provided further evidence of the noxiousness of this procedure (Bergamasco et al., 2011).

The above evidence clearly indicates that cattle experience pain as a consequence of routine husbandry procedures. Increasing awareness of this has led to much research being undertaken to investigate how this pain might be alleviated. The main classes of analgesics that have been used in cattle and their effects on the acute pain response of cattle after these husbandry procedures are outlined in the following section.

2.4 The Effect of Analgesia on the Acute Pain Response in Cattle.

The presence of animal pain can be assessed by administering analgesic agents and observing changes in behavioural and/or physiological variables (Livingston, 2010). Examining responses of animals with or without pain, and with or without analgesia means it is possible to determine if the changes in the measured variable are due to the painful condition or to other associated factors (e.g. sedative properties of some analgesic drugs) (Weary et al, 2006). The validity of this strategy also relies on knowing that a particular analgesic will be effective in relieving a specific type of pain, (Weary & Fraser, 2008)

The efficacy of several analgesics on ameliorating acute pain in cattle has been demonstrated through the quantification of behavioural observations and physiological parameters. These analgesics include local anaesthetics, nonsteroidal anti-inflammatory drugs, and α_2 -adrenoreceptor agonists. General information on the classes of drugs and an outline of their effect on the pain response in cattle is given below.

2.4.1 Local Anaesthetics

Local anaesthetics (LA) are applied to areas of intended injury in order to block the nociceptive barrage that would otherwise occur upon incision of the tissue. The molecules penetrate the neuronal cell membrane, binding to sodium channels which prevents nociceptive input in the sensory afferent nerves being transmitted (Skarda & Tranquilli, 2007b).

These drugs can be used to provide regional analgesia through a variety of techniques, the most common of which are topical application, local infiltration, nerve-blocks, epidurals and regional intravenous (IV) blocks (Skarda & Tranquilli, 2007a). They are beneficial as the reduction in acute pain at the time of a procedure minimises escape and avoidance reactions (Stafford et al., 2006), meaning the procedure can be performed with greater accuracy and safety.

Lignocaine (lidocaine) is most commonly used in cattle and is preferred for its intermediate duration of action (approximately 2 hours) and low cost (Skarda & Tranquilli, 2007a). It is the only LA licenced for use in cattle in New Zealand (Nottingham, 2012).

2.4.1.1 The Effect of LA on the Acute Pain Response of Cattle

The behaviour of calves receiving LA for painful procedures is similar to control animals (those not undergoing the procedure) than those that have the procedure performed without LA. Application of LA to the scrotum and spermatic cords significantly reduced the behavioural responses to cutting of the scrotum and manipulation of the testes when compared with animals not receiving LA (Stafford et al., 2002). Behaviour patterns in calves that received LA prior to amputation dehorning were similar to those of calves in the control group for the first 2 hours after the procedure (Sylvester et al., 2004). However, once the anaesthetic effect had worn off, increases in restlessness and decreases in rumination of calves were noted, suggesting the presence of pain

Local anaesthesia has also been shown to significantly affect the magnitude of the cortisol response when given prior to painful procedures. LA injected into the testes and scrotum prior to rubber ring or latex band castration resulted in almost total elimination of the cortisol response in calves (Stafford et al., 2002). When administered before dehorning, the associated cortisol response was suppressed for a period of time that mirrored the duration of action (DoA) of the LA used. However, the overall cortisol response remained the same, indicating that LA only delays the onset of pain and does not result in a reduction in the magnitude of the pain response (Stafford & Mellor, 2005a).

Changes to the EEG indicative of pain were seen in calves that did not receive LA prior to dehorning. In comparison, these changes were not seen in calves that had received LA before the procedure (Gibson et al., 2007). In the same study, tachycardia during the dehorning procedure was seen only in calves without LA.

The above evidence indicates that use of LA can successfully attenuate the acute pain experienced by cattle subjected to painful procedures. However, evidence from behavioural and physiological variables indicates that LA alone only reduces pain for the duration of its activity. It does not fully ameliorate the accompanying inflammatory pain that develops subsequent to these routinely performed procedures.

2.4.2 Non-Steroidal Anti-Inflammatory Drugs (NSAIDs)

The NSAIDs are weak organic acids with analgesic, anti-inflammatory and antipyretic properties. They are well absorbed after all routes of administration and are highly bound by plasma protein, which accounts for their extended duration of action in inflamed tissues relative to their plasma elimination half-life (Nolan, 2000). Peripherally, they inhibit the activity of the enzyme cyclooxygenase (COX) thus preventing conversion of arachidonic acid to the eicosanoids which lead to inflammation. COX occurs in 3 isomers, and different NSAIDs show preferential inhibition of one or more forms, depending on the agent. This inhibition prevents both sensitisation of the nociceptors and activation of the silent C-fibres (Ochroch et al., 2003). Peripheral antinociception is also determined by their penetration into inflamed tissues, thus giving a local effect (Lamont & Mathews, 2007)

Historically, NSAIDs were thought to reduce pain perception only via these peripheral mechanisms. It is now recognised that they also have centrally mediated analgesic effects acting via spinal and supraspinal pathways (McCormack, 1994). This has led to the understanding that NSAID analgesia can exist without prior inflammation being present (Pinaridi et al., 2003). Much of this research has been based on rodent or human models, though studies on sheep have been published (Chambers et al., 1995; Lizarraga & Chambers, 2006).

The development of NSAIDs in the 1980s revolutionised pain management in veterinary practice, as prior to this few efficacious analgesic agents were available for use in farm animal species (Stafford, 2012). Their duration of action and analgesic efficacy making them ideal for the treatment of both acute and chronic pain in veterinary species (Lamont & Mathews, 2007). Currently there are five available NSAIDs for use in cattle in New Zealand: carprofen, flunixin meglumine, ketoprofen, meloxicam and tolfenamic acid (Nottingham, 2012).

2.4.2.1 The Effect of NSAIDs on the Acute Pain Response of Cattle

NSAIDs administered alone have shown varying effects on pain-related behaviour after dehorning. Calves dehorned with ketoprofen only showed significantly more pain-related behaviour compared to control calves in the 6 hours immediately after dehorning (McMeekan et al., 1999). This was seen as increased incidences of tail-shaking and ear flicking, and decreases in rumination. This suggests that NSAIDs given alone do not prevent the acute nociceptor barrage at the time of the procedure, or decrease inflammatory pain in the hours after. However, calves that received meloxicam immediately after dehorning spent more time walking, lying and feeding than their untreated cohorts in the days after (Theurer et al., 2012),

suggesting their level of comfort was greater than untreated calves. This indicates that NSAIDs can be effective at moderating the inflammatory pain that develops subsequent to dehorning.

The impact of NSAIDs given alone on the plasma cortisol response also provides evidence that these drugs are effective at decreasing acute pain in the hours after painful procedures. Although ketoprofen did not significantly alter the initial peak plasma cortisol response after dehorning, establishment of the plateau was prevented and plasma cortisol returned to pre-treatment values much sooner than in animals without NSAID treatment (McMeekan et al., 1998). This adds weight to the supposition that the acute cortisol peak is due to the acute nociceptor barrage, and the latter part of the cortisol response is primarily associated with inflammatory pain, as the rapid return to pre-treatment levels was likely due to the anti-inflammatory effect of ketoprofen.

Administration of the NSAIDs ketoprofen and salicylate prior to surgical castration has been shown to reduce the cortisol response in calves of varying ages (Baldrige et al., 2011; Coetzee et al., 2007; Stafford & Mellor, 2005b). Salicylate treatment prior to castration did not prevent pain-related changes occurring in the EEG response (Bergamasco et al., 2011), further suggesting that these drugs do not prevent acute pain at the time of the procedure.

2.4.3 Local Anaesthetic plus NSAIDs

As indicated above, LA or NSAIDs used alone may provide inadequate analgesia for the duration of the pain response seen in cattle after routinely performed painful procedures. Findings suggest that an analgesic protocol of NSAIDs plus locally infiltrated LA provide superior alleviation of pain.

Positive effects of NSAIDs and LA together are seen in the reduced pain-related behaviour after painful procedures. Calves that received LA with ketoprofen, flunixin meglumine or meloxicam prior to dehorning or disbudding had a decreased prevalence of pain behaviours when compared with calves that receive LA only (Heinrich et al., 2010; McMeekan et al., 1999; Stilwell et al., 2009). Additionally, tolerance to MNT testing over the site of cautery dehorning was significantly increased in calves that received meloxicam plus LA compared to those that received LA only (Heinrich et al., 2010).

Meloxicam had a minor effect on pain behaviours exhibited by cows after liver biopsy. Those that had received meloxicam in addition to LA did not differ from sham-biopsied cows in proportion of time spent ruminating post-biopsy. In comparison, cows that received LA only, spent significantly less time ruminating than the control group (Beausoleil & Stafford, 2012)

The combination of NSAIDs and LA are also more effective at decreasing physiological indicators of pain in cattle than LA or NSAIDs alone. The acute plasma cortisol peak was significantly lower in calves subjected to dehorning or disbudding when LA was given in conjunction with ketoprofen, meloxicam, or flunixin meglumine, compared with calves given LA only (Heinrich et al., 2009; McMeekan et al., 1998; Milligan et al., 2004; Stilwell et al., 2009). Meloxicam plus LA also significantly lowered HR and RR in response to disbudding (Heinrich et al., 2009).

Use of NSAIDs and LA together also caused significant reductions in the plasma cortisol response seen in calves after castration. When ketoprofen and LA were given prior to castration, plasma cortisol did not differ significantly from uncastrated control calves, irrespective of the castration method used (Stafford et al., 2002). Epidurally administered LA used with either carprofen or flunixin meglumine showed a similar effect (Stilwell et al., 2008).

2.4.4 Alpha2-Adrenoceptor Agonists

These drugs are potent analgesics; however their usefulness is restricted by the accompanying dose-dependent sedation and cardio-vascular depression (Nolan, 2000). Analgesia is produced by the binding of molecules to specific alpha2 receptors in the central nervous system, these being located in brain and spinal cord structures that are closely related to pain processing (Nolan, 2000). These receptors activate potassium channels in the post-synaptic neurons, rendering them less responsive to excitation (Greene, 2003).

Use of these drugs for caudal epidural analgesia in cattle produces effective analgesia with minimal proprioceptive deficits when compared to local anaesthetic; xylazine, detomidine, medetomidine and clonidine have all been successfully used for this purpose (Skarda & Tranquilli, 2007a). Of these, only xylazine and detomidine are licenced for use in cattle in New Zealand (Nottingham, 2012).

Alpha2-agonists have been assessed for their effect on acute pain responses of healthy cattle to noxious mechanical, thermal and electrical stimuli. Some subjective mechanical measures (e.g. pinpricks or pedal withdrawal reflexes) were increased after administration of the alpha2-agonists clonidine, detomidine or medetomidine (DeRossi et al., 2009; Lin et al., 1998; Prado et al., 1999). Electrical thresholds (expressed as millivolts required to elicit a behavioural response) were also significantly elevated by detomidine (Prado et al., 1999).

Epidural use of alpha2 agonists has been effective in decreasing pain-related variables in cattle. Xylazine plus LA epidural reduced the integrated cortisol response in Burdizzo-castrated calves compared to untreated calves, while epidural xylazine plus parenteral flunixin resulted in significantly decreased salivary cortisol in treated versus untreated animals after band castration (Coetzee, 2011). IV xylazine given alone or in combination with ketamine reduced peak plasma cortisol in surgically castrated calves compared to untreated castrated controls, but the integrated cortisol response was greater in treated animals, suggesting a rebound effect of cortisol once the drugs have worn off (Coetzee, 2011). This suggests that these drugs also do not control the development of inflammatory pain subsequent to their duration of action.

2.4.5 Opioids

Opioid drugs exert their effects by interacting with opioid receptors, thus mimicking the endogenous opioid peptides' inhibition of neuron activation. The use of opioids has been considered by some to be "the cornerstone of effective pain treatment in veterinary medicine" (Lamont & Matthews, 2007). However, although they have been used in ruminants, there is large variability in the analgesic and behavioural responses after administration (Greene, 2003). Generally, their use in ruminants is considered clinically ineffective and is not recommended in cattle (Stafford et al., 2006). Experimental use has shown that morphine and endogenous opioids significantly increase TNTs in cattle (Pinheiro Machado et al., 1997; Pinheiro Machado et al., 1998)

As can be seen from the evidence above, several options exist for ameliorating pain after routine husbandry procedures in cattle. Most of the main classes of drugs are effective in cattle, though a multi-modal approach is the most effective strategy for reducing both the acute nociceptive barrage and the longer-term inflammatory pain.

2.5 Conclusions

The experience of pain is one that is shared across mammalian species, as indicated by the presence of homologous structures associated with the perception and interpretation of noxious stimuli. The positive adaptive value of pain occurs through its functions of warning of impending tissue damage, eliciting protective behaviours, and the associated learning from painful events that alter future behaviours. However, pain also imparts a negative effect on an individual's welfare which, if unabated, may lead to distress.

As our awareness of pain in other species grows, so too does our obligation to alleviate it. It is no longer acceptable to ignore the impact pain may have on these animals because they cannot or do not communicate its presence to us. A significant body of research makes evident the pain-induced distress in cattle after husbandry procedures. It is also clear that the provision of effective analgesia significantly improves the welfare of animals subjected to these procedures.

Review of the current literature pertaining to pain in cattle has identified numerous areas for further research. The use of NTT, and specifically laser-based TNT, has potential to provide further information on several aspects of pain physiology in cattle. These include the effects of analgesic drugs on baseline thresholds, the development of hyperalgesia after painful procedures, and the extent to which analgesia might reduce this hyperalgesia

The vast majority of research has focused on castration and dehorning, however cattle may experience pain after many other routine procedures. As liver biopsy is a common procedure in New Zealand, further attention should be directed toward assessing the impact it has on the welfare of cattle.

The following research used laser-based TNT testing to explore two aspects of pain physiology in cattle. Firstly, the central analgesic effect of drugs on baseline pain sensitivity of cows without known pain or inflammation was investigated. Secondly, liver biopsy was performed on cows and their pain sensitivity and paddock behaviour were examined for indications of post-biopsy pain. Analgesic protocols that included NSAIDs were used to determine if these drugs reduced any changes in pain sensitivity or behaviour.

3 The Effect of Ketoprofen and Medetomidine on the Thermal Nociceptive Threshold of Dairy Cattle: A Pilot Study



Laser-Based Thermal Nociceptive Threshold Testing

3.1 Introduction

Increased understanding of pain in ruminant species and how this affects their welfare has resulted in efforts to further identify, quantify and alleviate pain in these animals. Even so, pain management in farm animals has not progressed as rapidly as in companion animals (Short, 2003) and painful procedures are still widely performed on cattle without any or inadequate analgesia.

Non-steroidal anti-inflammatory drugs (NSAIDs), such as ketoprofen, are the most commonly used drugs for pain relief in ruminants. The primary action of NSAIDs was traditionally thought to be peripheral inhibition of cyclooxygenase (COX) during the development of inflammatory conditions, resulting in a decreased production of the inflammatory eicosanoids. However, a number of studies have since provided evidence that these drugs exert central mechanisms of analgesia in the absence of inflammation (McCormack, 1994). Alpha2-adrenoceptor agonists, such as medetomidine, also have central analgesic properties. They are used in cattle to provide short-term analgesia and sedation for a variety of painful procedures (Scott, 2004; Stafford & Mellor, 2005a). The analgesia is mediated at the cerebral and spinal levels and unlike the NSAIDs there are no peripheral anti-inflammatory properties directed towards COX inhibition (Hellyer et al., 2007).

Central analgesic effects of ketoprofen have been suggested to exist in cattle, as no cortisol response was seen in calves castrated via the surgery-pull method (where stretching and tearing of the spermatic cords occurs after incision of the locally anaesthetised scrotum). The absence of a cortisol response following the presumed nociceptor barrage accompanying the stretching and tearing of the cords indicated that its central analgesic properties were likely significant at the time of castration (Stafford & Mellor, 2005b). Medetomidine has been shown to provide centrally-mediated analgesia sufficient to perform surgical procedures in cattle (Lin et al., 1998).

Nociceptive threshold testing (NTT) can be used to assess pain sensitivity in animals. A painful stimulus is applied to a body area until a behavioural response is seen, at which point the stimulus is stopped. Stimuli may be thermal, mechanical, electrical or chemical in type. NTT using healthy uninjured subjects can be used to investigate analgesic properties of drugs. Examining responses of pain-free animals that have received either analgesic or placebo treatment helps to determine the presence of any analgesic effects on behaviour (Weary et al., 2006). The analgesic action over time may also be better shown in healthy subjects, as in post-operative subjects pain intensity may decline within hours; the concurrent change in pain perception during this time may represent this changing intensity, rather than analgesic activity (Arendt-Nielsen et al., 1991).

Laser-based thermal nociceptive threshold (TNT) testing is an NTT method that has many advantages over other thermal and mechanical modalities. It requires less interference with test subjects, as attachment of equipment (such as pressure algometers for mechanical testing or contact thermodes for thermal testing) is not required. This reduces the amount of stress experienced by the animal due to handling and restraint, which might otherwise affect the test results (Veissier et al., 2000). Laser-based TNT also results in specific activation of thermoceptors without the concurrent stimulation of mechanoreceptors that contact thermodes result in (Arendt-Nielsen & Chen, 2003).

TNT testing using a carbon dioxide (CO₂) laser has been used to ascertain baseline thresholds in cattle (Herskin et al., 2003; Veissier et al., 2000). A clear relationship between increasing laser power output and decreased latencies to respond was found in both studies. This graded response, due to increasing skin temperature, indicates that this method is a valid measure of thermal nociception in both calves and adult dairy cows. The method was also shown to be repeatable, as latencies to respond were unaffected when repeat testing was conducted 60 minutes and 24 hours after initial testing (Veissier et al., 2000), and the short-term repeatability was also found to be acceptable when repeat testing was conducted 15 minutes after the initial test (Herskin et al., 2003)

TNTs have also been used in cattle to investigate changes in pain sensitivity, including thermal hyperalgesia as a result of painful conditions such as mastitis or lameness (Rasmussen et al., 2011; Whay et al., 1998) and stress-induced hypoalgesia (Herskin et al., 2007; Herskin et al., 2004; Rushen et al., 1999; Schwartzkopf-Genswein et al., 1997a; Ting et al., 2010).

NTT has been used to demonstrate the central analgesic effects of NSAIDs and alpha₂-agonists in various mammalian species. For example, ketoprofen has significantly increased NTs in healthy subjects of several species including electrical thresholds in humans (Willer et al., 1989), thermal thresholds in mice (Pinardi et al., 2003) and mechanical thresholds in sheep (Lizarraga & Chambers, 2006). In all cases, the effect of ketoprofen cannot be explained by its peripheral COX inhibition, as there was no pre-existing inflammation. Administration of medetomidine significantly elevated the NTs in several species: thermal and mechanical thresholds in rats (Pertovaara et al., 1991), thermal thresholds in cats (Slingsby & Taylor, 2008), electrical thresholds in dogs (Vainio et al., 1989), and mechanical thresholds in cows (Lin et al., 1998) and sheep (Kästner, 2006).

There are currently no studies that have used TNTs to demonstrate central analgesic effects of any NSAIDs or α_2 -adrenergic agonists in healthy cattle without existing inflammation. Such information would enhance our knowledge of these drugs' activity in cattle, and may result in

more appropriate use of them in pain management strategies. The aim of this pilot study was to ascertain whether TNT testing could be used to assess the central analgesic effect of ketoprofen and medetomidine on the acute pain response of cattle without known pain or inflammation. It was hypothesised that the central analgesic properties of these drugs would result in a decreased sensitivity to a noxious thermal laser stimulus, which would manifest as an increased latency to show a behavioural response to the stimulus.

3.2 Materials and Methods

The procedures and experimental design were approved by the Massey University Animal Ethics Committee (Protocol 11/48)

3.2.1 Animals

Eighteen cows from the resident herd at the Massey University Veterinary and Large Animal Teaching Unit were used. The cows were non-pregnant, non-lactating and of mixed ages and breeds (predominantly Friesian, Jersey or Friesian x Jersey cross). Cows were kept as part of their usual larger herd for the duration of the study period, and were maintained in their normal outdoor pasture-based grazing system with occasional hay supplementation. Individual weights ranged from 333-642 kilograms (kg), with the average weight being 444 kg. Cows were visually assessed for pre-existing conditions that might have associated inflammation (e.g. lameness or skin disease) prior to the study commencing, and were excluded from selection if any such conditions were identified.

An additional four cows were used for preliminary testing to determine the laser power setting that would be used in the experiment. One week before the experiment commenced these cows received several single laser exposures of varying intensities for no more than 20 seconds duration, and the time taken for them to show a behavioural response was recorded. These cows were not included in the final 18 used for the experiment.

3.2.2 Experimental Design

The effects of two different analgesic drugs on TNTs were investigated in a blinded cross-over trial conducted on three study days (A,B,C) over three weeks. Treatments were given in a pre-determined order (Table 3.1). Administration of treatments and testing of TNTs occurred on the same day of the week for each animal, with a wash-out period of 1 week between study days.

Each cow received each of the three following treatments once:

- Saline placebo, 0.9% sodium chloride (Baxter Healthcare, Auckland, NZ), 5ml intramuscularly (IM).
- Ketoprofen (Ketofen 10%, Merial, Auckland, NZ), 3mg/kg IM (as per manufacturer recommendations).
- Medetomidine hydrochloride (Domitor, Pfizer, Auckland, NZ), 10 µg/kg IM. Due to a lack of published data on the dose-response effects of medetomidine in cattle at sub-anaesthetic doses, a dose range was determined after consultation with a veterinary pharmacologist (P. Chambers, personal communication, June 2011). Cows allocated to receive medetomidine in mobs 1 & 2 on study day A (4 cows in total) received 10 µg/kg IM. However 2 cows given this dose showed heavy sedation and went into sternal recumbency 20-30 minutes after administration and could not be roused back to standing position. Recumbency was likely to influence the cow's ability to demonstrate a clear response to the thermal stimulus; therefore the dose rate was halved to 5µg/kg IM for the remainder of the study.

All treatments were administered into the neck muscle via an 18 gauge 1 ½ inch needle by an experienced technician.

Table 3.1: Order of treatments for 6 cows within each of 3 mobs (total n=18). All 3 mobs followed the same order as that outlined here. KET = ketoprofen, MED = medetomidine, SAL = saline

	WEEK 1	WEEK 2	WEEK 3
COW	STUDY DAY A	STUDY DAY B	STUDY DAY C
1	KET	MED	SAL
2	KET	SAL	MED
3	MED	SAL	KET
4	MED	KET	SAL
5	SAL	KET	MED
6	SAL	MED	KET

3.2.3 Experimental Procedure

The study was conducted over three weeks from early July 2011 in Palmerston North, New Zealand. One week prior to the first day of testing, cows were individually weighed (Tru-test Cattle Scales, Auckland, NZ). Individual doses for medetomidine and ketoprofen were then calculated based on these weights.

All testing occurred between 10.00 and 16.30 hrs on each of the 3 study days. Ambient temperatures ranged between 9°C and 12.5°C across the 3 days. Animals were assigned into one of three mobs of six at the beginning of the trial, based upon their order of arrival into the yards on the first study day. Mobs were tested in the same order on each study day, but the order of animal testing within each mob was not necessarily the same.

On each study day all cows were brought from the paddock into covered yards with access to fresh water. Each mob of six cows was drafted from the main herd and moved into the stocks area for testing. During testing, each cow was individually restrained in stocks adjacent to her mob-mates. Once restrained, an area approximately 10cm wide x 20cm long on the caudal thigh was shaved with electric clippers, beginning just below the level of the vulva and extending distally. The cow was then allowed 15-20 minutes to settle. A pre-treatment (time - 20) TNT was then measured. The assigned treatment was injected into the neck muscle 20

minutes later (time 0). Repeated measures of TNT were then recorded at 20, 40 and 60 minutes after injection

After each measure of TNT a subjective assessment of sedation was made based on the two behaviours of head position and menace response (Table 3.2). The same person performed all assessments and was blinded to the treatment each cow had received. Menace response refers to the test of reflex closure of the eyelids following rapid movement of assessor's fingers towards the cow's eye. Combined scores ranged from 0/0 for no signs of sedation through to a maximum score of 2/2 for heavily sedated cows. After the final TNT test and sedation score, the mob was returned to the paddock.

Table 3.2: Subjective Assessment of Level of Sedation Post-Treatment.

Sedation Score	Head Position	Sedation Score	Menace Response
0	Normal – nose held at or above shoulder height	0	Normal
1	Low – nose dropping between shoulder and elbow, some neck flexion present	1	Sluggish
2	Dropped – nose is dropped past the level of the elbow towards the ground, significant neck flexion present	2	Absent/ Eye Closed

3.2.4 Laser Equipment and TNT Testing

TNTs were determined by measuring the cow's latency to respond to a thermal laser beam applied to a shaved area of the caudal thigh (photo, page 31). A CO₂ laser (116 Mk3 Manfrotto, MPB Technologies, Dorval, Canada; wavelength 10.60 µm, maximum power 10W) was used as the thermal stimulus. One person operated the laser and another timed and recorded the cow's latency to respond to each exposure of the laser. The laser operator and time-keeper were the same throughout the study, and were blinded to the treatment each cow received.

Due to technical difficulties, the power output of the laser was not quantified; however the setting remained constant for all tests on all individuals, as did the distance between the laser and the subject (approximately 2 metres). The power setting used was chosen on the basis of previous research using a similar laser to measure TNTs of cattle, where most latencies to first

response occurred between 5 and 10 seconds after exposure commenced (Herskin et al., 2003; Veissier et al., 2000). Preliminary testing using a range of power outputs indicated that the selected setting resulted in the most number of cows responding within these guidelines. Testing of the laser at this setting and distance on volunteers did not result in tissue redness, swelling or damage when the exposed area was removed immediately upon perception of pain.

A cow was deemed to have reached her TNT when she expressed one of the end-point response behaviours described in Table 3.3. Although previous authors (Herskin et al., 2003; Veissier et al., 2000) have not considered the tail-flick as an end-point response, preliminary testing showed this to be a purposeful movement that was consistently correlated to the occurrence of the laser stimulus. Thus it was felt that including it as a response behaviour was warranted.

The laser was immediately turned off when one of these responses was observed, or after a duration of 20 seconds if no response occurred (Herskin et al 2003; Veissier et al, 2000). The threshold was measured as the latency between activating the laser and observation of the response and was recorded to the nearest second. Cows that did not respond within 20 seconds were monitored for a further 5 seconds after stimulus removal for any response behaviours, and if none occurred the TNT was recorded as 25 seconds (Herskin et al., 2003; Veissier et al., 2000).

At each time point (-20, 20, 40, 60 minutes) three laser exposures were applied to the cow 30 seconds apart (Veissier et al., 2000). Each laser application was focused on a different area of shaved skin to avoid tissue sensitisation or damage. The site of testing was altered between hind legs each day (study day A = left hind, study B = right hind, study day C = left hind but slightly more distal location to A). If, during laser exposure, the cow urinated, defaecated or performed any movement obviously related to other disturbances, the laser was stopped and the test repeated after 30 seconds had elapsed.

Table 3.3: Ethogram of end-point response behaviours performed by cows exposed to a laser thermal stimulus (derived from Herskin et al., 2003)

Behaviour	Definition
Tail Flick	One rapid movement of the tail directed from the midline towards the site of exposure
Lifts Leg	The hoof of the exposed leg is lifted clear of the floor and replaced in the same position.
Kicks Leg	The hoof of the exposed leg is rapidly withdrawn from the floor and thrust away from body.
Step	The hoof of the exposed leg is lifted from the floor and replaced in a different position
Weight Shift	The area of exposure is moved by horizontal weight transfer from the exposed leg to contralateral leg; the exposed leg does not leave the floor.
No Response	The exposed leg is not moved within 20 seconds of laser exposure

3.2.5 Statistical Analysis

Analysis of data was conducted using the statistical software R (version 2.14, R Development Core Team, 2011). All three TNTs from each time point on each study day were included for analysis.

Cows that received the higher dose of medetomidine on study day A (two each in mobs 1 and 2), post-treatment repeated measures of TNT (20, 40, 60 minutes) were removed from the analysis due to the different dose rate they received and their subsequent sedation.

3.2.5.1 Exploratory Data Analysis

Data were initially examined for indications of trends within variables (treatment, study day, repeated measures and mob) and interactions between variables. Visualisation of data was assisted graphically. Information gathered through this process was then used to inform subsequent modelling.

3.2.5.2 Construction of Model

Investigation of the effect of individual variables (treatment, study day, mob and time) on the TNT was performed using linear modelling. The outcome variable data (latency to respond) were log-transformed to satisfy the requirement of normality. Individual explanatory variables (treatment, study day, group and repeated measures) were regressed in simple linear models to determine if they were significantly associated with the outcome variable (TNT). Interactions between the main effect explanatory variable (treatment) and the others were tested by the inclusion of an interaction term. A liberal p-value of 0.20 was chosen as the cut-off at this stage of the modelling process. Variables significant at this cut-off were carried forward for inclusion in the final model. A correlation adjustment was introduced to account for the non-independence of the data. This was achieved by using random effect terms for time and study day, the latter being nested within cow.

Model fit was assessed by examining residuals and the choice of correlation structure was assessed using the log likelihood test statistic. Observations with the largest and smallest residuals ($\pm 0.5\%$, $n = 7$ in each direction) were investigated to determine if they were overly influential in the model. Any apparently influential observations were removed individually from the data set and the model was rerun each time on the reduced data set to determine the effect of the removal on the magnitude and direction of the regression coefficients.

3.3 Results

3.3.1 Exploratory Data Analysis

The raw mean TNT for all cows to thermal laser stimulation according to treatment is shown in Figure 3.1. Medetomidine appears to increase the TNT as time progresses. The range of TNTs within treatment groups across time is shown in Figure A.1 (Appendix A). The raw mean TNT of all cows over time according study days is presented in Figure 3.2. Here it can be seen that TNTs progressively decreased with each study day. The range of TNTs across time according to study day is presented in Figure A.2 (Appendix A).

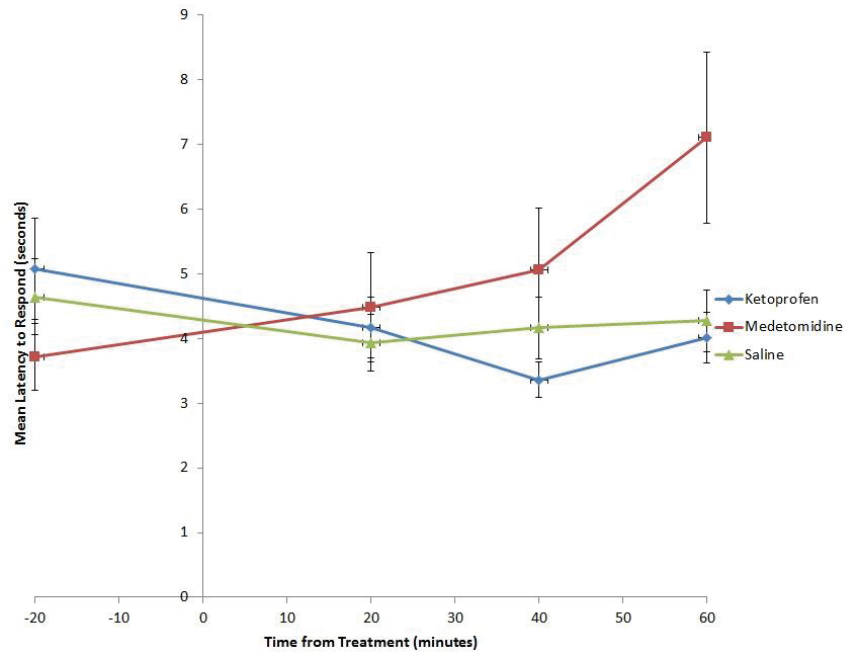


Figure 3.1: Plot of the raw mean TNT (expressed as latency to respond) as a function of time after treatment. Vertical lines represent standard error of the mean. n=18 for all data points except medetomidine at 20, 40 & 60 minutes, where n=14.

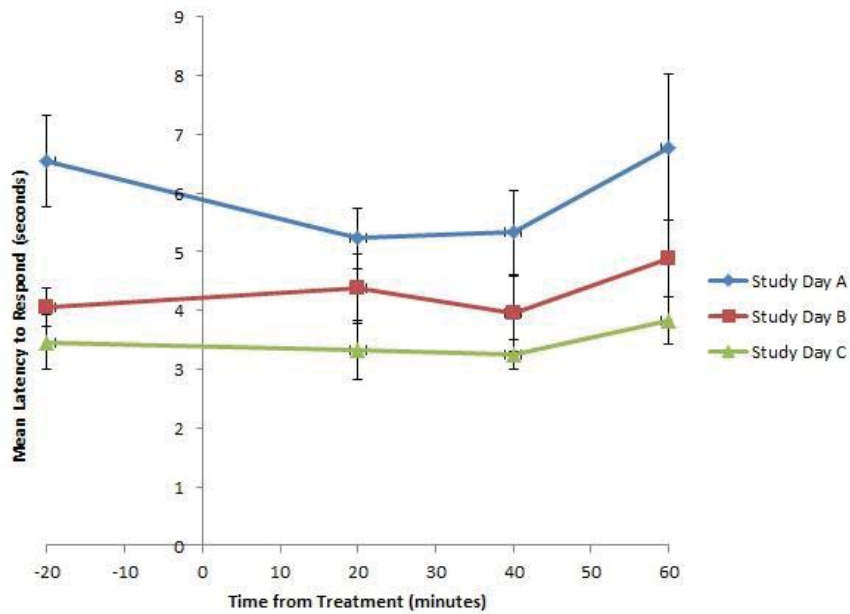


Figure 3.2: Plot of the raw mean TNT (expressed as latency to respond) as a function of time and study day. Vertical lines represent standard error of the mean. n=18 for all data points except medetomidine at 20, 40 & 60 minutes, where n=14.

3.3.2 Construction of Model

When examined individually, the effect of treatment and study day were found to be significant. Cows took significantly longer to respond after receiving medetomidine compared with saline ($p < 0.001$), and latencies to respond were significantly lower on study day B compared with A, and study day C compared with A ($p < 0.001$). An interaction between treatment (medetomidine) and time (60 minutes) compared with saline and -20 minutes was also significant ($p < 0.01$). On the basis of these findings, the final model included an interaction term between treatment and time, and random effects for time and study day nested within cow.

The residuals showed an approximately normal distribution. When observations with extreme residuals were removed from the data set, the magnitude and direction of the regression coefficients did not change, indicating these were not overly influential on the outcome. Therefore all observations were retained in the final model. Regression coefficients for the effect of treatment, time and the interaction of treatment with time are presented in Table 3.4

3.3.3 Model Output

A significant treatment x time effect was found sixty minutes after treatment with medetomidine, (1.539 seconds, $p < 0.01$, 95% CI = 0.107 – 2.972s) (Table 3.4). A trend of increased response times was also seen with increasing time at 20 and 40 minutes after medetomidine, though this was not significant. Once adjustment was made for the other variables in the model, there was no significant effect of treatment alone on TNTs. No significant differences were found at any time point between the TNTs of cows after treatment with ketoprofen compared with treatment of saline (Table 3.4). Variance estimates of the random effects indicated that most of the variance existed at the level of study day nested within cow.

Table 3.4: Point estimates of regression coefficients with standard errors (SE), log transformed estimates and their 95% confidence intervals from mixed effects model of thermal nociceptive thresholds of 18 cows. Values in bold-type are significantly different at $p < 0.05$.

Variable	Level	Estimate. (log seconds)	SE	Transformed Estimate (seconds) (95% CI)	p value
Treatment	Saline (Sal)	Reference			
	Medetomidine (Med)	-0.06	0.17	0.94 (-0.44-2.32)	0.74
	Ketoprofen (Ket)	0.10	0.17	1.11 (-0.27-2.48)	0.55
Time	-20 min	Reference			
	20 min	-0.15	0.12	0.86 (-0.40-2.12)	0.90
	40 min	-0.07	0.12	0.93 (-0.34-2.20)	0.55
	60 min	-0.04	0.13	0.96 (-0.32-2.24)	0.73
Treatment x Time	Sal x -20	Reference			
	Med x 20 min	0.25	0.17	1.28 (-0.36-2.45)	0.16
	Ket x 20 min	0.01	0.16	1.02 (-0.41-2.35)	0.93
	Med x 40 min	0.22	0.18	1.25 (-0.32-2.51)	0.21
	Ket x 40 min	-0.24	0.17	0.79 (-0.59-2.20)	0.15
	Med x 60 min	0.53	0.18	1.70 (0.11-2.97)	<0.01
	Ket x 60 min	-0.16	0.18	0.85 (-0.51-2.31)	0.36
Variance estimate of random effects	Study day: Cow ID	0.20			
	Cow ID	0.002			

Model Statistics: Intercept= 1.285; Likelihood ratio test= -413.564 on 33 df

3.3.4 Behavioural Responses

The tail-flick was the most common first response to the thermal stimulus (see Fig 3.3), this being the observed response in 69.1% of all measures of TNT performed across the 18 cows over the three study days. No behavioural response to the thermal laser stimulus was noted for 0.82% of the total exposures. On this basis it was considered unnecessary to account for these few right censored data in the statistical analysis as their effect on the results would be minimal.

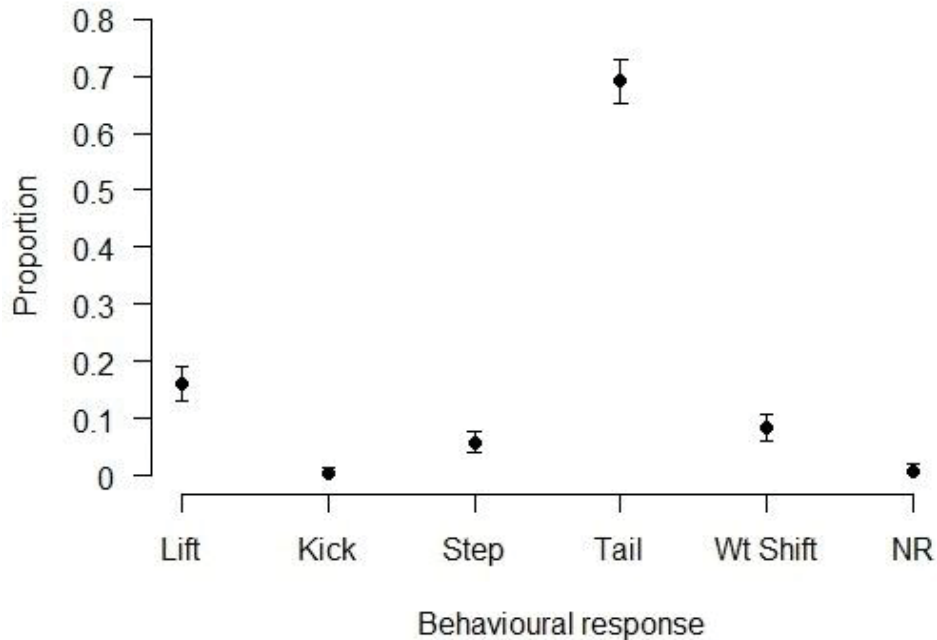


Figure 3.3: Total proportion (+/- 95% confidence interval) of behavioural response types shown by cows to TNT testing across all three study days. Lift = lifts leg, Kick = kicks leg, Step = step, Tail = tail flick, WtShift = weight shift, NR = no response.

3.3.5 Sedation Scores

All 14 cows given the lower dose of medetomidine (5 µg/kg) remained standing for the entire duration of post-treatment assessment. Medetomidine altered the sedation score of 5 of the 14 cows included in the final analysis to 0/1 at 40 minutes (1 cow, 7.1%) or 0/1 at 60 minutes (4 cows, 29%). Sedation scores remained at 0/0 at all time points for all cows that received either ketoprofen or saline.

3.3.6 Thermal Burn Injury

During preliminary testing no evidence of thermal burn injury was seen on animals exposed to the laser setting used for the trial. However, on each study day many individuals developed small welts after laser exposure, irrespective of treatment group. These had visually resolved by the following week, but in most cases some superficial skin sloughing of small (approximately 1 cm diameter) circular areas occurred 3-4 weeks after the initial exposure, indicating damage of skin tissue. No complications (e.g. infection, lameness, loss of condition) were associated with these areas of skin damage.

3.4 Discussion

The aim of this study was to investigate whether laser-based TNT testing could demonstrate the central analgesic properties of ketoprofen and medetomidine in cattle without pre-existing inflammation. It was hypothesised that the latency to respond to the thermal stimulus would be increased in cows that received these drugs. Treatment with ketoprofen did not significantly affect the TNT of cattle at any time point when compared with saline. Thus, no evidence of a central analgesic effect of ketoprofen in cattle was found using this method.

In contrast, 60 minutes after treatment with medetomidine cows took significantly longer to respond to the laser stimulus than they did after treatment with saline. This result indicates that some properties of medetomidine affected the acute pain response of cattle. This increased TNT may be due to central analgesia. However, it is also possible other properties of medetomidine have led to this result:

- *Sedation*

Medetomidine exerts a sedative effect, resulting in decreased vigilance and impaired ability to react to stimuli (Stenberg, 1989). In cattle an IM dose of 30-40 µg/kg results in recumbency and heavy sedation (Ranheim et al., 1999; Rioja et al., 2008). As these were not desired effects in this study a lower dose was selected. Of the 14 cows that received the lower dose (5 µg/kg) of medetomidine, subjective observational assessment of sedation at 60 minutes post-treatment found no change in the majority of subjects (10 cows) and only a small increase in the degree of sedation in the remaining 4 cows. This was seen as a sluggish menace response, but with no changes in head position. Other clinical parameters (e.g. heart rate, respiratory rate, rectal temperature) were not assessed, so as to reduce the amount of physical interference. It is therefore possible that a level of sedation existed which affected TNTs 60 minutes post-treatment but was not evident based on observation of gross behaviour alone.

- *Motivation to Respond to Pain*

The use of a sub-anaesthetic medetomidine dose may have only affected one dimension of the pain response in these cows. It has been suggested that full analgesia is only induced by anaesthetic doses of medetomidine (Pertovaara et al., 1991). Subanaesthetic doses in humans only attenuated the unpleasantness of pain (the affective-motivational component), not the intensity of pain (the sensory-discriminative aspect), even in the presence of sedation (Angst et al., 2004; Kauppila et al., 1991). Thus the increased TNT 60 minutes after medetomidine treatment may not be due to decreased perception of pain but rather by a decreased motivation of the cattle to respond to the thermal stimulus.

- *Thermoregulatory Changes*

The increased TNT seen 60 minutes after medetomidine administration could also be due to drug effects on thermoregulation. Medetomidine is known to induce hypothermia in some species (MacDonald et al., 1989; Vainio et al., 1989) resulting in cooling of the skin due to peripheral vasoconstriction. A consequence of this decreased skin surface temperature may be an increased TNT (Le Bars et al., 2001), as response latency is influenced by initial skin temperature (Pertovaara et al., 1996). This occurs because when initial skin temperature is low more heat energy is required to reach the temperature for nociceptor activation (Pertovaara et al., 1996). The degree of hypothermia associated with medetomidine is dose-dependent and appears not to be significant at the dose used here (MacDonald et al., 1989). Additionally, studies of other alpha2-agonists have shown these drugs cause hyperthermia in cattle (DeRossi et al., 2009; Ranheim et al., 1999). Given this evidence, it is unlikely that inhibited thermoregulation explains the elevated TNT at 60 minutes. However, skin and core temperature measurements should be taken to definitively eliminate this possibility.

Consideration must be given as to why no significant effect of ketoprofen and only a limited effect of medetomidine were seen in this study. The possible explanations will be considered in the following sections.

3.4.1 Ketoprofen & Medetomidine Do Not Act Centrally in Cattle

The main assumption of this study was that both ketoprofen and medetomidine have central analgesic activity in cattle without existing inflammation. The central analgesic effects of both ketoprofen and medetomidine have been demonstrated for various mammalian species.

Ketoprofen exerted a centrally mediated analgesia in sheep (Lizarraga & Chambers, 2006) mice (Miranda et al., 2001) and humans (Willer et al., 1989). It is believed that ketoprofen also had a central effect in calves when administered before castration (Stafford & Mellor, 2005b). Given

this, and that ketoprofen is centrally active in other ruminant species without inflammation, the assumption in this study that ketoprofen has central analgesic activity in cattle appears valid.

Medetomidine, like all alpha2-agonists, induces central analgesia by stimulating spinal and supra-spinal receptors in the pain pathway. This effect has been shown through NTT in rats, cats and dogs (Pertovaara, 1993). In cattle, medetomidine induced significant analgesia, as indicated by elevated thresholds to an electrical stimulus (Lin et al., 1998). Therefore the assumption that medetomidine has central analgesic effects in cattle also appears valid.

3.4.2 Are TNTs Suitable for Assessing Central Analgesia?

Assuming that these drugs do have central analgesic effect in cattle, the failure to see significant changes in TNTs may be because this method is not effective for measuring central analgesia. Thermal threshold testing, like all forms of NTT, has limitations that may decrease its validity for this purpose.

The primary assumption is that the observed behavioural response occurs because of a stimulus, and that this stimulus was sufficient to trigger the nociceptive pathways (Le Bars et al., 2001). Interpretation of the results is based on acceptance that the stimulus-response relationship is true, though this may not be verified. Furthermore, even assuming that the stimulus will incite a behavioural response, the variable measured (i.e. latency to respond) may not be linearly related to the physiological variable that produces the observed pain response. With respect to TNT testing, the physiological parameter producing the response is a change in skin temperature. If the stimulus-response relationship is curvilinear, the measured variable may no longer represent the increasing noxiousness of the stimulus, as the rate at which an animal can perform a response behaviour will have a maximum limit based on the speed at which pain signals can physically be processed (Le Bars, 2001). Such a curvilinear pattern has been seen in TNT studies of cattle and rats (Fan et al., 1995; Veissier et al., 2000). Recognition of this is important when testing baseline thresholds or attempting to identify changes in threshold due to analgesia, as they may be open to misinterpretation that no further effect of stimulus intensity or analgesia occurs.

A further limitation is that the analgesic effect of some drugs may not be detected by TNT testing. It is likely that most acute experimental pain responses to thermal stimuli are those resulting from the activation of A δ -fibres and the “first, sharp” pain they induce (Le Bars et al., 2001). The slower conducting velocity of the C-fibres means that they may have no impact on

the measured response; however it is the activity in these which is more reliably attenuated by analgesics (Khambam et al., 2012) Therefore, the analgesic effect may not be quantified fully with acute pain models that preferentially result in A δ -fibre activation. It has been stated that acutely induced thermal pain is not a reliable indicator of centrally mediated NSAID analgesia (Dobromylskyj et al., 2000; McCormack, 1994), a conclusion which may be due to this issue.

However, central analgesic properties have been successfully demonstrated with the use of TNT testing. This effect of medetomidine and other alpha2-agonists has been shown in a number of species (Nolan et al., 1987; Pertovaara, 1993). The use of TNTs to assess NSAID analgesia in healthy subjects with no inflammation has produced mixed results. Though some authors have demonstrated an analgesic effect of NSAIDs with TNT techniques (Hunnskaar et al., 1986; Nielsen et al., 1990, 1992), others have failed to show any differences between NSAID and placebo treatments (Steagall et al., 2007; Taylor et al., 2003), as was the case in this study.

Despite such limitations, these methods are generally regarded as useful tools for investigating the effects of analgesics on pain sensitivity (Le Bars et al., 2001). Given this, specific examination of the TNT method used here may improve understanding of why only a limited effect on TNTs after treatment was seen in these cows.

3.4.3 Current Methodology May Have Affected the Experimental

Outcome

Several aspects of the specific method used in this experiment may have affected the data collected, and therefore the experimental outcome. These include the anatomical location chosen for the thermal threshold testing, the nature of the laser stimulus, and the chosen post-treatment assessment period. Further discussion of these is given.

3.4.3.1 Location of Stimulus Application

This trial differs from previous work on TNTs in cattle in that the region of testing is one that has not previously been described. Most authors have used the caudal metatarsal region and this is the only site for which validation studies have been done (Herskin et al., 2003; Veissier et al., 2000). Others have targeted the dorsal skin of the middle phalanx of the forelimb (Pinheiro Machado et al., 1997; Pinheiro Machado et al., 1998).

The original intent was to use the caudal metatarsal region, but preliminary testing showed that the cows did not respond when this area was targeted with a range of power settings, so an alternative location was sought. The front legs were considered unsuitable in this instance, both because of the desire to reduce levels of arousal (which may have increased with equipment and operators in sight) and the safety risks associated with the laser being directed in the vicinity of the eyes. It was found that the caudal thigh area gave the most repeatable response pattern, had the most convenient access, and still gave the same range of response types reported in previous literature. However, use of this location for assessing pain sensitivity in cattle has yet to be validated.

3.4.3.2 Stimulus Intensity May Affect Results

Although it was not possible to quantify the laser power output in this study, the occurrence of thermal burn injury suggests that the laser stimulus was of a high intensity, as does the high number of response times occurring in under 5 seconds (66% of 612 responses, see Figures A.1 & A.2). NSAIDs have typically only caused significant elevations of TNTs when the intensity of the stimulus is low (Ankier, 1974; Hunskaar et al., 1986; Nielsen et al., 1990, 1992). This is likely due to analgesics preferentially affecting the slower conducting C-fibres (see Section 3.4.2). The presumed high intensity in this study and the associated short latencies to respond could have impaired the ability to discriminate any treatment effect on TNTs due to ketoprofen or any subtler effects due to medetomidine. It would be of future interest to see if a central analgesic effect of ketoprofen and medetomidine can be identified using a graded range of thermal stimulus intensities.

3.4.3.3 Onset of Analgesia and Post-Treatment Assessment Period

3.4.3.3.1 Medetomidine

In this study, the only significant effect of medetomidine on TNTs occurred at 60 minutes post-treatment. As far as is possible to determine, no studies have been done showing the time of onset of analgesic effect of IM medetomidine in cattle. Detomidine (another alpha₂-agonist) administered IM produced analgesia in the flank and perineum 5 minutes after administration in cattle (Prado et al., 1999). Given that medetomidine and detomidine share similar pharmacokinetic properties (Lin et al., 1998), a similar time to onset of analgesia after IM administration could be expected. The different results in Prado et al.'s study may be due to use of an electrical rather than a thermal stimulus. The use of mechanical stimuli has shown onset of analgesia due to alpha-agonists begins between 30-60 minutes post-injection (DeRossi et al., 2009; Prado et al., 1999). Thus results from this study, although using a

thermal stimulus, would seem to be in line with some previous findings of onset of analgesia of other alpha₂- agonists. However, it would be of future interest to evaluate nociception at times beyond 60 minutes, in order to determine when the peak effect of medetomidine on TNTs occurs, and when this effect begins to decline.

Peak analgesia of medetomidine appears to be related to plasma drug concentration. In dogs and cats IM dosing resulted in peak plasma concentrations after 30 and 15 minutes respectively (Salonen, 1989), whilst the peak analgesic effect occurred at approximately 30 minutes post-IM injection (Ansah et al., 1998; Vainio et al., 1989). It is not clear if the changes in analgesia seen in the current study correlate to the plasma concentration of medetomidine, as there is no published data regarding pharmacokinetics (PK) after IM administration in cattle. In sheep, peak plasma concentrations of medetomidine occurred within 30 minutes of IM administration (Kästner et al., 2003). Though it seems reasonable that IM medetomidine PK in cattle would be similar to other ruminants, further investigation is warranted to gauge the relationship between plasma drug concentration and analgesic effect in this species.

3.4.3.3.2 Ketoprofen

The final testing time of 60 minutes post-injection used in this study may have been insufficient to demonstrate any central analgesic effect of ketoprofen on TNTs. Ketoprofen is highly lipophilic and undergoes rapid absorption after administration (Netter et al., 1985), however mechanisms of NSAID analgesia not involving local prostaglandin synthesis may take longer to develop than this rapid absorption would suggest (Machin & Livingston, 2002). Studies in healthy humans using brief painful argon laser stimuli have shown that the peak analgesic effect of oral paracetamol and ibuprofen occur approximately 2-3 hours post-treatment (Nielsen et al., 1990, 1992). Development of this analgesia lagged peak plasma concentrations (T_{max}), which occurred 1-2 hours after ingestion.

If it is assumed that, like the NSAIDs above, ketoprofen analgesia occurs well after T_{max} is achieved, then changes to TNTs may not be detectable before 60 minutes post-administration. No published data exists for time to T_{max} of ketoprofen after IM injection in cattle, although evidence suggests that the onset of analgesia should be similar between oral and IM routes: ketoprofen shows similar levels of therapeutic activity in cattle whether given IM or orally (Banting et al., 2008) and these routes also have similar PK profiles in children (Kokki et al., 2001). Studies of IM ketoprofen in other species produced a range of values for T_{max} : 0.25-0.68 hours in piglets (Fosse et al., 2011); 0.5 hours in children (Kokki et al., 2001); 0.31 hours in rabbits (Wong & Wang, 1994); 1.5 hours in camels (Alkatheeri et al., 1999). If T_{max} of IM

ketoprofen in cattle falls within the range above, this might explain why no effect on TNTs was seen here, as onset of analgesia would occur outside the 60 minute cut-off. Examining changes in TNTs over longer durations after IM ketoprofen would be of future interest.

3.4.3.4 Effect of Ambient Temperature

The relatively low ambient temperatures (9-12.5°C) for this study may have affected TNTs as in cooler environments peripheral vasoconstriction can lead to increased TNTs due to lower initial skin surface temperatures (Section 3.4 – Thermoregulatory Changes). This could lead to increased thresholds being incorrectly interpreted as an effect of treatment. Previous work in calves and sheep suggests that ambient temperatures below 7-8°C decreased sensitivity to thermal and mechanical stimuli (Chambers et al., 1994; Veissier et al., 2000), whilst decreased thermal sensitivity in horses has been seen at temperatures below 10°C (Love et al., 2011).

However, it seems unlikely that ambient temperature would explain the effect of medetomidine at 60 minutes post-treatment seen here. Although the temperature was relatively low, it was stable over the entire testing period, so would not lead to any further decreases in skin temperature that would explain changes in TNTs relative to the pre-treatment test. If this had occurred, it would be expected that such changes should have been seen in all treatment groups, as all cows were exposed to the same conditions on each study day. Thus it seems improbable that ambient temperature has influenced the outcome of this study.

3.4.3.5 Effect of Study Day

There was a significant effect of study day on TNTs across all treatments, with response times progressively decreasing with each study day. The decreasing thresholds may have resulted from sensitisation of the spinal dorsal horn subsequent to the thermal burn injury caused to some animals by the laser. Because of altered testing sites between weeks the decreased TNTs seen on day B (one week after the first test day) could not be due to peripheral sensitisation of previously exposed tissue. Care was also taken on day C (week 3) not to repeat exposure on areas used in day A. On every study day care was taken not to apply the laser to an area that had already been exposed. Thus it seems more likely that central rather than peripheral sensitisation was the cause for a reduction in TNTs across study days.

A second possibility for decreasing TNTs may be due to decreases in stress-induced hypoalgesia (SIH). SIH refers to the reduced sensitivity (manifested as increased NTs)

occurring in response to stressful situations (Herskin et al., 2007). The animals used in this study formed part of a teaching mob for veterinary students and were regularly used for a variety of manipulations (e.g. blood sampling, rectal examination). It is possible that at the start of the trial they associated new people and the yards/stocks with the occurrence of potentially aversive experiences and that the expectation of these induced SIH. Acclimation to the researchers and trial procedures over the three weeks of testing may have reduced SIH resulting in the progressive decrease in TNTs over study days.

It is unlikely that the effect of study day had a significant influence on the results between treatments, as the distribution of cows receiving the 3 treatments was even across all study days. However, it is possible that the decreasing TNTs meant that subtle changes within treatments were masked.

3.5 Conclusions

While it is likely that ketoprofen and medetomidine have central analgesic effects in cattle, little evidence was found here that TNTs are useful for detecting these. A limited effect of medetomidine on TNTs was found, however, it is possible that this change was due to properties other than central analgesia.

This study should be repeated using a range of quantified laser stimulus intensities in order to determine if the effects of these analgesic drugs on the TNTs of cattle can be better discriminated. Examining dose-response changes in TNTs over longer durations of time after treatment with both drugs may provide further information on the development and time course of analgesia.

Refinement of the methodology is required in order to reduce the occurrence of thermal burn injury and improve animal welfare outcomes. Further studies using a range of stimulus intensities to validate the use of this location for TNT testing in cattle would assist with this.

4 Assessment of NSAID Analgesia on the Pain Response of Dairy Cattle after Liver Biopsy



“Number 12” After Liver Biopsy

4.1 Introduction

Liver biopsy is a routine veterinary procedure for ascertaining mineral status in New Zealand dairy cattle (Grace et al., 2012; Hittmann et al., 2012). The procedure involves making a skin incision in the animal's right side, then inserting a biopsy trocar through the skin, intercostal muscle, pleura, diaphragm and liver capsule to collect a sample of liver tissue. Local anaesthetic (LA) is infiltrated into the skin and muscle. This prevents pain due to the initial nociceptor barrage, reduces behavioural responses to incision (Stafford et al., 2006), and provides analgesia in these tissues for approximately 2 hours.

Pain may still occur as a result of the biopsy procedure. Nociceptors will be activated as the biopsy trocar pierces the unanaesthetised tissues, which can lead to sensitisation of these neurons (Ochroch et al., 2003). In addition, the development of inflammation at the incision site can cause pain which outlasts the duration of LA (Stafford & Mellor, 2005a). Together, these may induce sensitisation of the spinal cord, leading to hyperalgesia. However, no other form of analgesia is routinely administered to diminish inflammation or sensitisation after liver biopsy.

Non-steroidal anti-inflammatory drugs (NSAIDs) are effective at decreasing inflammatory pain in cattle after painful procedures, particularly when used in conjunction with LA (Stafford & Mellor, 2005a, 2005b). This is due to their peripheral inhibition of cyclooxygenases (COX), resulting in decreased production of the prostaglandins (PG) that lead to nociceptor sensitisation (Ochroch et al., 2003). In addition, many NSAIDs exert a central inhibitory effect on spinal PG production which decreases central sensitisation (Ochroch et al., 2003).

Nociceptive threshold testing (NTT) can be used to assess pain sensitivity in cattle. Animals with pre-existing pain may develop central sensitisation and show hyperalgesia to a painful stimulus by reacting quicker than those without pain (Whay et al., 1998). In cattle, thermal and mechanical stimuli have been used to try to identify central sensitisation resulting from painful conditions (Fitzpatrick et al., 1998; Kemp et al., 2008; Laven et al., 2008; Rasmussen et al., 2011; Whay et al., 1997; Whay et al., 2005). Thermal nociceptive (TNT) testing has identified central sensitisation in both rodent and human pain models (De Tommaso et al., 2004; Jaggar et al., 1999)

Alterations in the frequency and/or types of behaviour seen after a painful procedure have been used to infer the presence of pain in cattle. For instance, decreases in activity, eating and ruminating are often associated with pain (Anderson & Muir, 2005a). Pain-related behaviours may increase, such as ear-flicking after dehorning or abnormal gaits/postures after castration (Coetzee, 2011; Stafford & Mellor, 2005a). Recent studies showed behavioural changes

indicative of pain occurred in cattle in the hours immediately after liver biopsy (Beausoleil & Stafford, 2012; Mølgaard et al., 2012).

Use of NSAIDs in conjunction with LA has been shown to decrease pain-related behaviour in cattle (McMeekan et al., 1999; Stilwell et al., 2008), as these animals suffer less peripheral inflammation and central sensitisation. The use of meloxicam with LA decreased some pain behaviour in cattle after liver biopsy, when compared with those that received LA only (Beausoleil & Stafford, 2012).

Previous studies have investigated the effect of NSAIDs on mechanical nociceptive thresholds (MNTs) of lame cattle with central sensitisation (Laven et al., 2008; Whay et al, 2005). However, as far as it is possible to determine, no studies exist that examine the effect of NSAIDs on TNTs in cattle with pre-existing pain. In addition, we still do not fully understand the nature and duration of pain experienced by cattle after liver biopsy once the LA has waned. Post-biopsy pain may negatively affect animal welfare, and cattle subject to this procedure might benefit from the concurrent use of NSAID analgesia at the time of biopsy. Therefore, the aims of this study were:

- To use TNT testing to determine if central sensitisation occurs in cattle in the days after liver biopsy.
- To use TNT testing to examine whether the inclusion of NSAIDs in the analgesic protocol reduced post-biopsy central sensitisation.
- To use complex behaviour to assess whether NSAIDs decrease the expression of post-biopsy inflammatory pain in the days following liver biopsy.

It was hypothesised that A) liver biopsy would result in central sensitisation, resulting in decreased TNTs due to hyperalgesia; B) The degree of this hyperalgesia would be reduced in those cows that had received NSAID analgesia at the time of biopsy; C) The behaviour of biopsied cows that received NSAIDs at the time of biopsy would be more like that of sham-biopsied cows.

4.2 Materials and Methods

The procedures and experimental design were approved by the Massey University Animal Ethics Committee (Protocol 11/74). For reasons of animal welfare, the inclusion of a positive control group (liver biopsy performed without local anaesthetic) was not requested in the submission.

4.2.1 Animals

Twenty four cows from the Massey University Veterinary Large Animal Teaching Unit (VLATU) were randomly selected from a teaching herd. The cows were non-lactating and of mixed ages and breeds (predominantly Friesian, Jersey or Friesian x Jersey cross). Cows were individually weighed (Tru-test Cattle Scales, Auckland, NZ) five days prior to commencing the trial, with the average weight being 518.5 kg (range 386-626 kg). They were divided into two mobs of twelve based on their order of arrival into the stocks on the day of weighing, and were maintained within these mobs in a pasture grazing system for the duration of the trial period.

An additional 5 cows were used in preliminary tests to determine the laser power setting used. Each cow was subject to no more than 6 laser exposures of varying power and maximum durations of 20 to 30 seconds. These cows were not included in the experimental mobs.

4.2.2 Experimental Design and Procedure

A blinded prospective study was undertaken to assess the efficacy of three different analgesic protocols on the pain response of dairy cows after liver biopsy. The study consisted of two five-day replicates, which proceeded as outlined in Table 4.1. Each mob was assigned to only one replicate. The study was conducted over two consecutive weeks from mid to late February 2012, in Palmerston North, New Zealand.

Each morning the 12 cows were brought in from the paddock and contained in covered yards with access to fresh water. Each mob was divided into smaller groups of 6 and these were moved indoors for individual restraint by the head in cattle stocks for liver biopsy or TNT testing. All morning procedures were completed on one sub-group before the next was brought to the stocks. After completion of liver biopsy and TNT testing, cows were released back to pasture for field observations.

Table 4.1 Schedule for assessing pain-response to liver biopsy in dairy cows.

	DAY 0	DAY 1	DAY 2	DAY 3	DAY 4
A.M	Baseline TNT testing	Analgesics administered, liver biopsy performed	24hr Post-biopsy TNT testing	48hr Post-biopsy TNT testing	72hr Post-biopsy TNT testing
P.M	---	Post-biopsy behavioural observations	24hr Post-biopsy behavioural observations	48hr Post-biopsy behavioural observations	72hr Post-biopsy behavioural observations

4.2.3 Treatment Groups

On Day 1, the cows were semi-randomly allocated a treatment group, with one cow in each sub-group being assigned to one of the four following treatments:

- Control group (C, n = 6): received LA at the biopsy site; sham liver biopsy performed.
- LA only (LA, n = 6): received LA at the biopsy site; liver biopsy performed
- LA plus meloxicam (LAM, n = 6): received LA at biopsy site and systemic meloxicam; liver biopsy performed.
- LA plus ketoprofen (LAK, n = 6): received LA at biopsy site and systemic ketoprofen; liver biopsy performed.

Drugs were administered at the following doses:

- Lignocaine local anaesthetic (Nopaine 2%, Phoenix Pharm, Auckland, NZ): 10ml into the skin and intercostal muscle of biopsy site (see below), at least 10 minutes prior to actual or sham biopsy being performed.
- Meloxicam (Metacam, Boehringer Ingelheim, Auckland, NZ): 0.5mg/kg intravenously (IV), at least 15 minutes prior to biopsy.
- Ketoprofen (Ketofen, Merial, Auckland, NZ): 3mg/kg IV, at least 15 minutes prior to biopsy.

All drugs were administered by a veterinarian. LA was delivered via a 20 gauge 1 inch hypodermic needle after preparation of the biopsy site (see below). Individual doses for meloxicam and ketoprofen were calculated based on the pre-trial weights. These were given via a 16 gauge 1 inch hypodermic needle into the jugular vein, using a nose twitch to restrain the cow's head to one side.

4.2.4 Liver and Sham Biopsy

All liver and sham biopsies were performed by a veterinarian. The site for biopsy was located on the right side in the 11th intercostal space (photo, page 53), approximately 300mm ventral to the vertebral transverse process. The hair in the surrounding area was clipped and the site was surgically cleaned. LA was infiltrated at the biopsy site as described above. The efficacy of the LA was checked 10 minutes after administration by scratching a scalpel blade on the skin at the intended site of incision.

For liver biopsy, once the LA had taken effect a stab incision was made in the skin with a size 20 scalpel blade. A 5mm liver biopsy trocar (Shoof International, Cambridge, NZ) was then passed through the skin incision, intercostal muscle, pleura and diaphragm into the liver. A 5ml syringe was attached to the trocar and suction was applied to collect liver tissue. After removal of the trocar, a Michelle clip was placed to close the incision. The entire biopsy procedure lasted approximately 2 minutes/cow.

Sham biopsies were performed on animals assigned to the control group. All procedures except skin incision, trocar insertion and Michelle clip placement were performed. Once the LA had taken effect the actions of biopsy were simulated for approximately 30 seconds by the veterinarian.

Assessment of post-biopsy pain was made using two methods: thermal nociceptive threshold (TNT) testing; and behavioural observations

4.2.5 Laser and Nociceptive Threshold Testing

All TNT testing was conducted between 8.00 and 11.00 h each day. Ambient temperatures were in the range of 14-22°C across all test periods. TNTs were determined by measuring the cow's latency to respond to a thermal laser beam applied to a shaved area of the caudal thigh. An adjustable CO₂ laser (Model 48-1, Synrad, Mulkioteo, WA; max power 10W, wavelength 10.60 µm, beam diameter 3.5mm) with power output setting of 1.5W placed approximately 2 metres behind the cow was used as the thermal stimulus. A visible laser (JG-4A Class IIIA, wavelength 532nm) mounted to the infrared laser was used to aim the infrared beam. One person operated the laser and another timed and recorded the cow's latency to respond to each exposure of the laser. The laser operator and time-keeper were blinded to the treatment each cow received.

A cow was deemed to have reached her TNT when she performed one of the end-point response behaviours previously described in Table 3.3. The laser was immediately turned off when one of these responses was observed, or after a duration of 30 seconds if no response occurred. Cows that did not respond within 30 seconds were monitored for a further 5 seconds after stimulus removal for any response behaviours, and if none occurred the TNT was recorded as 35 seconds.

For each test of nociception on each day, three laser exposures were applied to the cow 30 seconds apart (Veissier et al., 2000). Each laser application was focused on a different area of shaved skin to avoid tissue sensitisation or damage. The site of testing was alternated between hind legs each day (e.g. day 0 = left hind, day 2 = right hind, day 3 = left hind, day 4 = right hind). If, during laser exposure, the cow urinated, defaecated or performed any movement obviously related to other disturbances, the laser was stopped and the test repeated after 30 seconds had elapsed.

The laser power setting and duration of exposure were determined based on the results of previous studies and the preliminary testing. A power setting of 1.3W for a maximum of 20 seconds had resulted in 45% incidence of non-response to the laser, while at 2.2W for 20 seconds the non-response incidence was 8% (Herskin et al., 2003). In order to achieve a low incidence of non-response but maximise the ability to discriminate between differences in treatment groups (as indicated in chapter 3), it was thought an intermediate power output with a slightly longer duration of exposure would be appropriate. However, longer durations would increase the risk of thermal burn injury, as skin temperature increases with the square root of time when exposed to a constant heat (Le Bars et al., 2001); therefore a power setting in the lower range was desirable. In preliminary testing, a power output of 1.5W with a maximum exposure of 30 seconds resulted in all cows exhibiting a behavioural response, while single exposures of 30 seconds maximum duration on 2 cows did not result in evidence of thermal burn injury. Thus a final setting of 1.5W for 30 seconds was selected for the experiment.

4.2.6 Behavioural Observations

Post-biopsy pain was assessed by observing the behavioural responses of all cows in the field. Behaviour during the four hours immediately after biopsy on day 1 was observed, to assess acute changes as the LA wore off. Behaviour during days 2-4 was observed for two hours at approximately the same time on each day, in order to assess the presence of subsequent inflammatory pain. The behaviours that were recorded are shown in Table 4.2. All observations for both mobs took place in the same paddock between the hours of 10.30 a.m.

and 4pm on day 1, and between 11.30 a.m. and 2.30 p.m. on days 2-4. Observations for each subgroup on day 1 began at staggered time points according to the time they arrived back into the paddock after release from the stocks (approximately 20 minutes between subgroups). Observations on days 2-4 were performed within the same time period for the entire mob.

Table 4.2: Ethogram of cow behaviour measured in the paddock after actual or sham liver biopsy (derived from Beausoleil and Stafford, 2012).

Behaviour	Description
Event Behaviours	
Tail Swishes	One movement of tail from side-to-side across midline of body
Looks at Wound	Gaze directed at the incision site on the right flank
Transitions	Number of transitions between standing and lying or vice versa
State Behaviours	
Lying or Standing	The proportion of total time spent lying or standing (these being mutually exclusive behaviours)
Walking	The proportion of total time spent walking
Feeding	The proportion of total time spent eating pasture
Ruminating	The proportion of total time spent ruminating

Event behaviours for each cow were observed for a 30 second period every 10 minutes using a focal sampling technique (Stamp-Dawkins, 2007). The number of times each behaviour was performed during that period was recorded. Event behaviours were observed by the same person for the entire study period.

The state behaviour of each cow in the mob was noted every five minutes using a scan sampling technique (Stamp-Dawkins, 2007). Both posture (lying, standing or walking) and feeding (feeding or ruminating) states were noted for each cow at each occasion. State behaviours were observed by one of two people for the entire study period.

4.2.7 Statistical Analysis

Analysis of data was conducted using the statistical software R (version 2.14; R. Development Core Team, 2011). All three TNTs from each cow on each study day were included for analysis.

4.2.7.1 TNT Testing

4.2.7.1.1 Exploratory Data Analysis

Data were visualised graphically for indications of trends within and between variables (treatment, day, cow, and mob). Information gathered through this process was used to inform subsequent modelling.

4.2.7.1.2 Construction of Model

Due to the potential truncation of time in the TNT testing, data was handled as right-censored. Of all observed responses, 137 out of 288 (47.6%) were censored data. Accelerated failure-time modelling was considered the most appropriate choice for analysis of these types of data (Collet, 2003). Data were log-transformed to satisfy the requirement of normality.

Individual explanatory variables (treatment, day, and mob) were screened for their association with the outcome variable (TNT). An interaction term was included to test the interaction between the main effect explanatory variable (treatment) and the others. A liberal p-value of 0.20 was selected as the cut-off, and variables with significance at this stage were included in the final model.

The non-independence of the data was adjusted by fitting a clustering term for cow. Model fit was then assessed by examining the AIC and residuals. Standardised residuals were first visualised graphically. Plots of influence statistics were then generated to determine the effect of any observations on the regression coefficient.

4.2.7.2 Behavioural Observations

For each event behaviour, the total number of events occurring for each cow on each day was calculated. In addition, for day 1 the total number of events was calculated at 30 minute and 60 minute intervals, in order to evaluate acute changes over the 4 hour observation period. To satisfy the requirement of normality, data were square-root transformed after the addition of a constant.

For each state behaviour, the proportion of time spent by each cow in that state each day was calculated by summing the number of periods spent in that state, then dividing by the total number of periods. For day 1, these proportions were also calculated at 30 minute and 60 minute intervals for each state. Arcsine transformation of the proportions was performed to normalise the data distribution.

4.2.7.2.1 Exploratory Data Analysis

Data were visualised graphically. Indications of trends between each behaviour and the variables of treatment, time (day 1), day (days 2-4) or mob were noted.

4.2.7.2.2 Analysis of Variance (ANOVA)

Because of the different time periods used to collect data on day 1 and days 2-4, behavioural observations for day 1 were analysed separately to those of days 2-4. For day 1 each behaviour at each level of time (30 minutes, 60 minutes, 4 hours) had individual one-way ANOVA performed for treatment, mob and time. These were then combined in a two-way ANOVA (treatment and mob) for the 4-hourly totals or factorial ANOVA (treatment, mob and time) for the 30 and 60 minute totals. An error term for cow was included in the factorial analysis to account for the non-independence of the data due to repeated measures of behaviour on the same cow. Interactions between treatment and time, and treatment and mob were also performed.

For days 2-4, each behaviour was assessed by performing individual one-way ANOVA of treatment, mob and day. These were then combined in a factorial ANOVA, and an error term for cow was again included. Interactions between treatment and day, and treatment and mob were also performed.

As treatment was the main effects variable, it was decided a priori to investigate this and its interaction terms further if significance was found in the ANOVA. Other explanatory variables (time, day and mob) were considered as potential confounders and were not investigated further beyond the ANOVA.

4.3 Results

4.3.1 TNTs

4.3.1.1 Exploratory Data Analysis

The mean TNTs within each treatment group across the study days are presented in Figure 4.1. The range of TNTs for each treatment group across the study days are shown in Figure 4.2. These figures indicate the wide range of TNTs within group, with the effect of truncation being prominent in Figure 4.2, as indicated by the high medians.

4.3.1.2 Construction of Model

When examined individually, the effect of treatment and day were found to have a significant effect on TNTs and were therefore carried forward to the final model. The interaction term was not significant. The residuals showed a relatively normal distribution, and plots of influence statistics showed tight clustering around zero, indicating an absence of overly influential observations. Therefore all observations were retained in the final model. Regression coefficients for the effect of treatment and day on TNT are presented in Table 4.3.

4.3.1.3 Model Output

The effect of treatment and day on TNTs of cows undergoing liver biopsy is presented in Table 4.3. No significant differences in TNTs were seen between treatment groups. A significant effect of day was found on day 4 (1.690 seconds, $p < 0.01$, 95%CI 1.318-2.062) when adjusted for the effect of treatment.

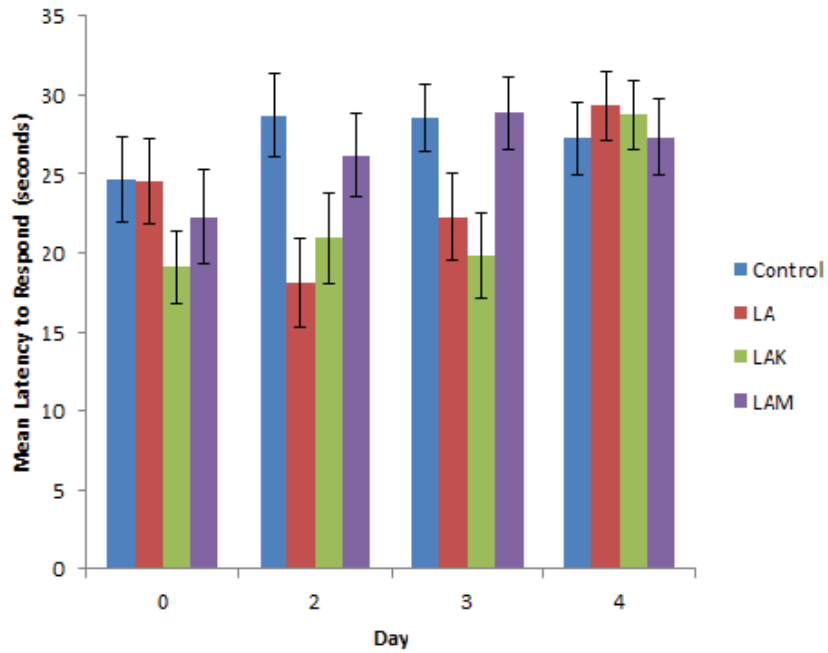


Figure 4.1: Histogram of the mean TNT (expressed as latency to respond) of treatment groups across study days. Vertical lines are the standard error of the mean. C = control group, LA = LA + biopsy, LAK = LA + ketoprofen + biopsy, LAM = LA + meloxicam + biopsy.

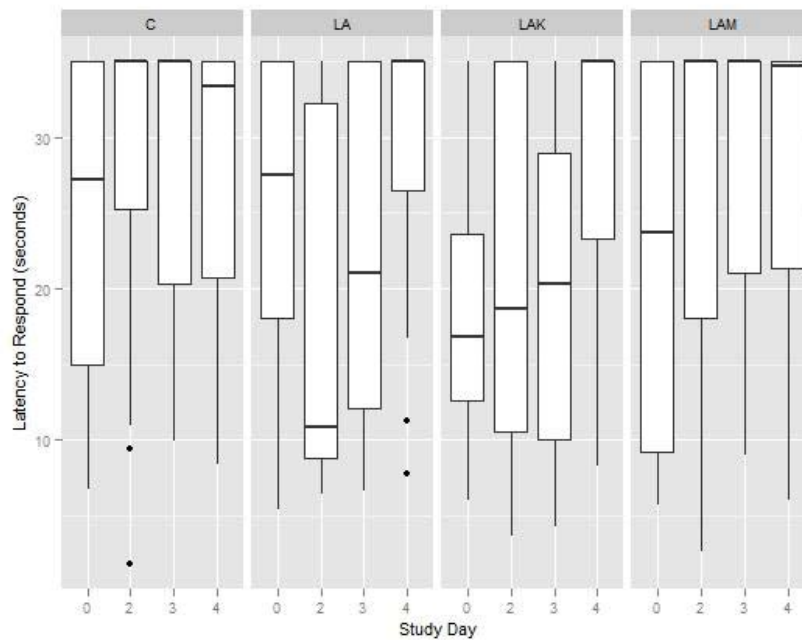


Figure 4.2: Boxplots of raw TNTs (expressed as latency to respond) as a function of study day, stratified by treatment. C= control, LA= local anaesthetic (LA), LAK= LA + ketoprofen, LAM = LA + meloxicam.

Table 4.3: Point estimates of regression coefficients with standard errors, log transformed estimates and their 95% confidence intervals from an accelerated failure time model of thermal nociceptive thresholds of 24 cows. Values in bold-type are significantly different at $p < 0.05$. LA = local anaesthetic; LAK = LA + ketoprofen; LAM = LA + meloxicam

Variable	Level	Estimate (log seconds)	Standard Error	Transformed (seconds)	Estimate (95% CI)	p-value
Treatment	Control	Reference				
	LA	-0.34	0.28	0.71	(0.17-1.25)	0.21
	LAK	-0.49	0.26	0.61	(0.11-1.11)	0.06
	LAM	-0.11	0.27	0.90	(0.38-1.42)	0.69
Day	Day 0	Reference				
	DAY 2	0.08	0.27	1.08	(0.67-1.53)	0.73
	DAY 3	0.22	0.21	1.25	(0.84-1.66)	0.29
	DAY 4	0.53	0.19	1.69	(1.32-2.06)	<0.01

Model Statistics: Intercept = 3.46; AIC = 1430.03

4.3.1.4 Behavioural Responses to TNT Testing

No response to the laser stimulus occurred in 47.6% of exposures, and this was the most common outcome of TNT testing in this study (Figure 4.3). The tail flick and lifting of leg were the next most common response types, occurring 19% and 18% of the time respectively.

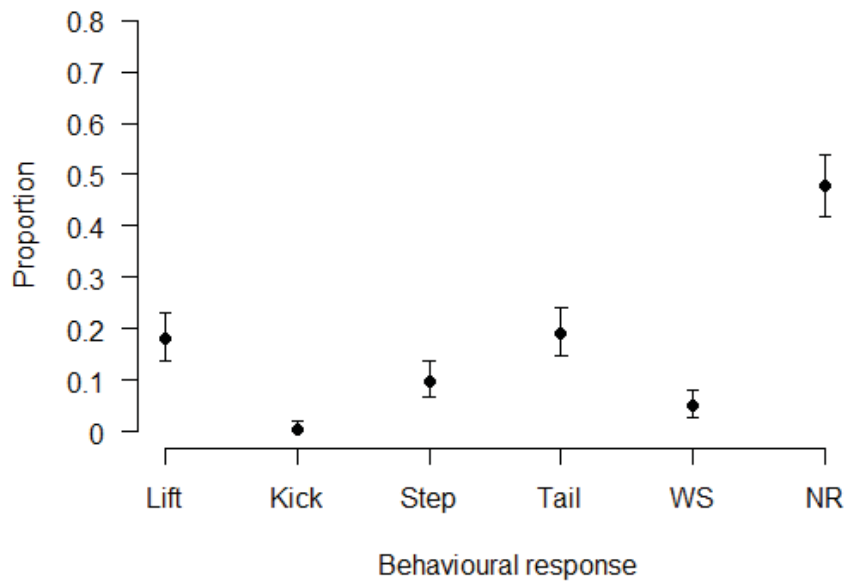


Figure 4.3: Total proportion (+/- 95% confidence interval) of types of behavioural response shown by cows to laser stimulus across all days. WS = weight shift, NR = no response to laser.

4.3.2 Behavioural Observations

4.3.2.1 Exploratory Data Analysis

Counts of the number of times each event behaviour occurred on each day are presented in Figures B.1-B.3 (Appendix B). Day 1 is shown in hourly totals, whilst days 2-4 are shown as totals for the daily 2-hour period. Proportions of time that cattle spent in any one state are shown in Figures B.4-B.9 (Appendix B). Here again day 1 is presented as the hourly totals while days 2-4 are the daily totals.

4.3.2.2 Analysis of Variance (ANOVA)

Some data was deemed unsuitable for further analysis with ANOVA due to difficulties with normalising the distribution. Excluded data sets were: all 30 minute totals of all behaviours from day 1; transition event behaviour on all days; looks at wound event behaviour for day 1 hourly totals and day 2-4 totals.

Test statistics for the effect of treatment, time or day and mob are shown in Tables 4.4 and 4.5. It can be seen that no significant differences were observed in behaviour between treatment groups at any point in the study. No significant treatment x mob, treatment x time (day 1) or treatment x day (days 2-4) interactions were found.

Significant differences were found between mobs of tail-flicking and walking when the 4-hour totals for day 1 were examined (Table 4.4). Mob differences were also found in the hourly totals of day 1 results for tail-flicking and standing. A significant effect of time on all analysed behaviours was also demonstrated for day (Table 4.4). Analysis of behaviour across days 2-4 found an effect of day on tail-flicking, standing and lying, and an effect of mob on walking, feeding and rumination (Table 4.5). As no effect of treatment was found in the ANOVA, no further post-hoc testing was undertaken.

Table 4.4: Results of statistical analysis of transformed cow behaviour data for the total 4 hour observation period and hourly aggregates on day 1. Values in bold-type are significantly different at $p < 0.05$

	DAY 1 4-HOUR TOTALS				DAY 1 HOURLY TOTALS					
	TREATMENT		MOB		TREATMENT		TIME		MOB	
	F (3,18)	P	F (1,18)	P	F (3, 87)	P	F (3, 87)	P	F (1,87)	P
TAIL FLICKS	0.62	0.61	19.73	<0.01	0.46	0.71	5.20	<0.01	12.24	<0.01
LOOKS AT WOUND	2.22	0.12	0.19	0.67	-	-	-	-	-	-
STANDING	0.88	0.47	2.83	0.11	0.89	0.45	3.76	0.01	4.37	0.04
LYING	0.91	0.46	0.02	0.90	0.98	0.40	18.51	<0.01	0.38	0.53
WALKING	0.82	0.50	19.02	<0.01	0.50	0.68	9.55	<0.01	2.69	0.12
FEEDING	0.71	0.56	0.38	0.54	0.33	0.80	34.43	<0.01	0.11	0.74
RUMINATING	0.13	0.94	0.37	0.55	0.14	0.94	31.18	<0.01	0.12	0.73
NO FEED/RUM	0.56	0.64	0.02	0.90	0.52	0.67	4.14	<0.01	0.11	0.74

Table 4.5: Results of statistical analysis of transformed cow behaviours data for the daily 2 hour observation periods on days 2-4. Values in bold-type are significantly different at $p < 0.05$

	TREATMENT		DAY		MOB	
	F (3,64)	P	F (2, 64)	P	F (1, 64)	P
TAIL FLICKS	1.99	0.13	16.70	<0.01	3.60	0.06
STANDING	0.10	0.96	3.56	0.03	0.37	0.55
LYING	0.046	0.987	3.732	0.03	2.91	0.09
WALKING	0.25	0.86	2.60	0.08	13.26	<0.01
FEEDING	0.37	0.78	0.13	0.88	6.87	0.01
RUMINATING	0.75	0.53	0.76	0.47	7.02	0.01
NO FEED/RUM	0.20	0.70	0.30	0.74	2.35	0.13

4.4 Discussion.

The aims of this study were three-fold: firstly, to determine if TNT testing can be used to find central sensitisation after liver biopsy in cattle; secondly, to use TNTs to see if NSAIDs reduce the level of this sensitisation; and thirdly to investigate if NSAIDs decrease the expression of pain-related behaviour after liver biopsy.

This study found no evidence of post-biopsy pain in the results of either TNT testing or behavioural observations. It was hypothesised that biopsied cows would have decreased TNTs due to hyperalgesia from central sensitisation. Furthermore, it was thought that biopsied cows that received NSAIDs would show less reduction in TNTs compared to LA-only cows, due to less sensitisation occurring. No indication of central sensitisation was found between LA-biopsied and sham-biopsied cows. Therefore it was not possible to determine the relative effect of NSAIDs on central sensitisation. It was also postulated that biopsied cows would show post-biopsy changes in behaviour that were indicative of pain, and these changes would be minimised by NSAIDs. No differences in behaviour between any treatment groups were found.

TNTs were significantly elevated on day 4 compared to day 0. This is likely due to the highest incidence of non-response to the laser occurring in all treatment groups on this day (Figure 4.2). There were significant effects of time and mob on some post-biopsy behaviour throughout the study. As these did not involve the main variable of interest (treatment), they will not be considered further in this discussion. Primary focus instead will be on considering why no evidence of post-biopsy pain or central sensitisation was detected. Possible explanations for this are considered in the following sections.

4.4.1 Is Liver Biopsy a Painful Procedure?

Pain during the biopsy may arise from direct activation of the nociceptors in unanaesthetised tissues, as well as subsequent development of inflammatory pain at the incision site. In humans, liver biopsy is recognised as invasive and painful (Castera et al., 1999). Most patients reported experiencing pain immediately after biopsy had been performed under LA and sedation, with 28% stating pain of at least moderate severity was present 24 hours post-operatively (Eisenberg et al., 2003).

However, it is possible that liver biopsy does not result in significant inflammatory pain in cattle. In cows, multiple liver biopsies did not result in any change to dry matter intake, daily milk yield, body temperature or white blood cell counts (Vels et al., 2009). Liver biopsy did not reduce feed

intake or weight in sheep (Ferreira et al., 1996), or alter the subjectively assessed attitude or appetite in camelids (Anderson & Silveira, 1999). Although changes to the gross parameters used above may be taken as general indicators of pain in ruminant species, they are not specific to pain alone, and may not be sensitive enough to detect pain in stoic species such as cattle. Additionally, as it was not a primary aim of these studies to evaluate post-biopsy pain, it is possible that other signs of pain may have gone unnoticed.

Behaviour can be a more sensitive indicator of pain when objective quantification of specific parameters (e.g. frequency of occurrence or duration) is undertaken (Rushen et al., 2008). Using such principles, behavioural evidence of inflammatory pain in cattle in the hours after liver biopsy has been found (Beausoleil & Stafford, 2012; Mølgaard et al., 2012). Cows that were biopsied after receiving only LA ruminated significantly less in the four hours following biopsy than did sham-biopsied cows (Beausoleil & Stafford, 2012). Decreased rumination is regarded as a general indicator of pain in cattle (Stafford, 2012) and has been associated with other painful procedures (McMeekan et al., 1999; Sylvester et al., 2004). Other indicators that cattle experience pain as a consequence of liver biopsy include a significant increase in tail pressing and a tendency for increased “perching” (adopting a stance where the front legs are elevated and the body stretched) in the 4¹/₂ hours immediately after biopsy (Mølgaard et al., 2012). In addition, significant elevations in the amount of time spent standing and the number of leg movements were found in the overnight period after biopsy. These authors also found that, despite administration of LA prior to biopsy, an increase in restless behaviour and decrease in rumination occurred during the biopsy procedure when compared to restraint alone. This further suggests that pain does occur at the time of biopsy.

The available evidence indicates that liver biopsy causes pain in cattle. Therefore the absence of post-biopsy pain is unlikely to explain the failure to find differences in pain-related behaviour and TNTs between treatment groups in this study. Given that some degree of post-biopsy pain was likely to have occurred in this study, consideration must be given as to why it was not identified.

4.4.1.1 Pain was Too Mild to Detect Using Behavioural Observations

The main assumption of this study was that liver biopsy would induce a degree of pain that would be detected in at least the first 24 hours post-procedure. The lack of difference in behaviours between the sham-biopsied and LA-biopsied animals could indicate that post-biopsy pain was mild in this study. If only mild pain was present it may not have been significant enough to the cattle to alter normal behaviour, and they were able to successfully mask its presence. This is due to the behavioural evolution of prey species not overtly displaying

abnormal behaviour patterns (Livingston, 2010). However, as noted above, recent studies report significant pain-related changes in behaviour in the hours after liver biopsy (Beausoleil & Stafford, 2012; Mølgaard et al., 2012)

Although the biopsy technique used in the present study was the same as that used in Beausoleil & Stafford (2012), those cows had previously been biopsied. It may be that multiple biopsies were the trigger for the altered patterns of rumination and looking at the wound. Although this would contradict the findings of Vels et al (2009), who found no apparent indications of a negative welfare impact after multiple biopsies, it must be remembered that those authors did not seek to specifically assess pain behaviour.

There are several important differences with the current study and that of Mølgaard et al. (2012) which may explain the contrasting results. In that study, procaine was the LA used to anaesthetise the biopsy site, whereas lignocaine was used here. Procaine has a slower onset of analgesia and a shorter duration of action than lignocaine (Anonymous, 2000). Additionally lignocaine is regarded as more efficacious than procaine as it disseminates through tissues faster and produces a larger area of anaesthesia (Nottingham, 2012). Given that the cattle administered procaine showed behavioural responses during the biopsy itself (which was not noted in the study by Beausoleil & Stafford (2012) or this study), it is possible that the 10 minute waiting time after application was insufficient and that full analgesia of the skin and muscle layers was not achieved. In addition, multiple passes through the incision site and into the liver were required in order to gain sufficient liver tissue (range of 13-32 per cow), whereas only one pass was required for an adequate sample in this study. These two factors together may have resulted in a greater noxious stimulus and subsequently increased intensity of pain which was more detectable than any pain that was present in the current study.

If the pattern of development of post-biopsy pain in cattle is similar to humans it is unsurprising that changes in behaviour were not detected on days 2-4. The intensity of pain in people wanes markedly by 6 hours post-biopsy (Eisenberg et al., 2003; Kramskay et al., 2011). Thus, if only mild pain occurred on day 1 and was not significant enough to alter behaviour, it would not be expected to see changes on days 2-4, as resolution would presumably have occurred in this time.

4.4.1.2 Pain was Too Mild to Cause Central Sensitisation

Hyperalgesia is a result of central sensitisation, and the likelihood of inducing this in the spinal cord depends on both the duration and intensity of the noxious surgical stimulus (Ochroch et al., 2003). As nociceptive input to the DH from the skin and muscle layers would be blocked with

the LA, and the biopsy itself was brief (approximately 2 minutes/cow), it is possible that there was not a sufficient barrage of afferent input to the DH to induce central sensitisation. In addition, as noted above, significant inflammatory pain may not have developed after liver biopsy. The lack of difference in TNTs between sham-biopsied and LA-biopsied animals supports this theory. These factors may explain why no evidence of hyperalgesia was found in the TNT testing on days 2-4. As TNT testing was not conducted in the immediate post-biopsy period on day 1, it is not known whether central sensitisation was present during this time but had receded by day 2, or if it did not develop at all.

4.4.2 The Use of TNTs to Evaluate Central Sensitisation

In this study, TNT testing was being used to investigate whether central sensitisation developed after liver biopsy. Studies using human and animal models of pain have found central sensitisation using TNTs (Fernández-de-las-Peñas et al., 2010; Jaggar et al., 1999; McMahon & Abel, 1987), including use of a CO₂ laser as the thermal stimulus (De Tommaso et al., 2004). Based on this, TNT testing was an appropriate means to identify thermal hyperalgesia resulting from central sensitisation if it existed after liver biopsy. However, specific aspects of the testing method used in this study may have affected the outcome. These are considered below.

4.4.2.1 The Location of TNT Testing

The location of TNT testing (on the caudal thigh) may not have been suitable for determining post-biopsy pain. The use of TNT testing in this study was chosen in an effort to identify the occurrence of central sensitisation manifested as thermal hyperalgesia. Given that the site of biopsy and the site of TNT testing are distant from each other, this would require development of a generalised hyperalgesia in order for changes to occur in TNTs on the hind leg. However, as noted above, it appears that any post-biopsy pain that existed in this study was relatively mild. Therefore, it may be that the level of central sensitisation required to elicit general hyperalgesia did not occur in this study. Initially it was intended that assessment of localised hyperalgesia would also be done using TNTs around the biopsy site. However logistic difficulties and the heightened anxiety of the cattle due to having people and equipment within their sight meant this was not successful. With refinement, it may be that use of this location, or the contralateral side (which would share the same sites of neuronal input into the spinal cord and therefore likely be subject to the same central processing changes) would be a better indicator of central sensitisation after liver biopsy.

4.4.2.2 Laser Settings

It is possible that the power setting used in this study was too low, and this may have affected the outcome of the TNT testing as the assignment of a 35 second cut-off for observation of non-responders may have masked differences in pain sensitivity between the treatment groups. Previous work has stated that a power output of 4.5W is required to attain a reliable measure of TNTs in cattle (Veissier, et al., 2000). However, the mean latency to respond to this power output was approximately 6 seconds. It was felt that this short response time may not be adequate to discriminate between the effects of the differing analgesic protocols used (as indicated in chapter 3), as rapid response latencies are likely mediated by A δ -fibres (Le Bars et al., 2001). C-fibres are more sensitive to the effects of analgesics (Khambam et al., 2012) but have a lower activation temperature than A δ -fibres (Le Bars et al, 2001). Therefore a lower power setting was required to provide a flatter gradient of skin heating in order to preferentially activate C-fibres.

A study using a power setting of 1.3 W for a maximum of 20 seconds had a non-response incidence of 45% with a median response latency of 9 seconds (Herskin et al., 2003). This decreased to 8% non-response and a median response latency of 5 seconds when the power was 2.2W. Disadvantages of both these settings exist, in that 1.3W resulted in a high proportion of non-response, whilst 2.2W elicited a response time similar to 4.5W. It was thought that increasing the duration of exposure to a lower power output may provide the gradual heating slope required to preferentially activate C-fibres whilst decreasing the percentage of non-responses to the laser stimulus by providing more time for responses to occur. Based on preliminary testing, the choice of 1.5W for maximum exposure duration of 30 seconds used here was considered to be a suitable compromise, although this was only determined on a small number of cows which may have led to unrepresentative results.

A further study indicated that 1.8W for 25 seconds duration averaged 21% incidence of non-response (Herskin et al., 2007). It may be that a power setting in this region with duration of 30 seconds would have provided more accurate data on pain sensitivity after liver biopsy.

4.4.2.3 Stress-Induced Hypoalgesia

It is possible that stress-induced hypoalgesia (SIH) occurred in this study, due to the biopsy and TNT testing procedure, and influenced the results of the TNT testing. SIH has previously been documented in cattle subject to social stressors (Herskin et al., 2007; Herskin et al., 2004). The cattle used in the present study were new to the farm, and had no experience of the yards and shed or the research team prior to commencement of the experiment. It is possible that these

factors were sufficient to induce SIH, and this may be why such a high number of non-responses were recorded during the TNT testing, potentially obscuring any effects of treatment on TNTs. Unfortunately this was an unavoidable situation, as time constraints on the availability of animals and facilities meant that there was little opportunity to familiarise the cattle with the experimental set-up and procedures. This is therefore a consideration that should be addressed in future work of this nature.

4.4.3 The Use of Behaviour to Evaluate Post-Biopsy Pain

4.4.3.1 Suitability of Behaviours Chosen

The selection of both the observed behaviours and the methods of data collection were based on prior research that had demonstrated the presence of pain-related behaviour in cattle. The state behaviours selected were considered suitable because previous studies have reported that decreases in activity, feeding and rumination occur after various painful procedures and are therefore considered to be generic indicators of pain in cattle (Stafford, 2012). As noted previously, changes in rumination and posture have been evident in previous studies of pain after liver biopsy in cattle (Beausoleil & Stafford, 2012; Mølgaard et al., 2012).

Event behaviours such as tail-flicking and transitions have also been used in previous pain behaviour studies in cattle (Stilwell et al., 2009; Stilwell et al., 2012; Sylvester et al., 2004), and it was thought that changes in frequency of transitions may support any noted changes in activity found in the state behaviours. The selection of the more specific behaviour of looking at the biopsy site was chosen as behaviours directed towards wound sites have been used as an indicator of pain in cattle (Stilwell et al., 2008), with Beausoleil & Stafford (2012) finding a significant difference between the number of times biopsied and sham-biopsied cows looked at their biopsy site.

4.4.3.2 Other Factors

It may be that no significant differences in behaviour were found because of the relatively small number of cows in each treatment group; increasing the group size may have reduced Type II error in the statistical analysis and revealed an effect of treatment. However, Beausoleil & Stafford (2012) used the same number of animals in each treatment group as that used here (six) and still found significant differences in behavioural patterns. Additionally, the power analysis performed as a requirement for approval by the Massey University animal ethics committee indicated that 24 subjects would be sufficient to show statistically significant differences between treatment groups. Thus, the sample size for this study seems appropriate.

The duration and frequency of the focal sampling used for the chosen event behaviours may have yielded non-representative results and contributed to the finding of no significant differences in post-biopsy behaviour between treatment groups. It is possible that the relatively short sampling periods of 30 seconds may have meant that some event behaviours were not seen within that time, and so differences that may have existed between groups were not identified. Previous studies of painful procedures often use durations of 1 minute (McMeekan et al., 1999), or on occasion 15 minutes (Stilwell et al., 2008). However, these studies used longer durations but less frequent observations. Here, a much greater frequency of observation was undertaken, which would help to offset the effect of short duration in elucidating any differences between groups. Given that Beausoleil and Stafford (2012) used this same method and were able to find an effect of treatment on post-biopsy pain based on observation periods of only 20 second duration, a period of 30 seconds was an appropriate choice for recording of event behaviours.

The chosen state behaviours are activities that are typically engaged in for long durations at a time, and are often inter-related, such that standing is likely to be more associated with feeding, and lying with rumination (Fraser & Broom, 1990). Intervals of 5 minutes to assess these behaviours should therefore have been adequate to detect any differences between treatment groups, as it is unlikely that transitions between these states would occur so frequently as to be missed between observation periods.

4.4.4 The Effect of NSAID Analgesia

Based on the results of this study, it could be concluded that LA provides adequate pain relief for the procedure of liver biopsy, and that NSAID analgesia is not required. The mild nature of the pain caused by liver biopsy is suggested by the lack of significant differences occurring between the control and the LA groups. This is in contrast with previous findings which suggest some pain of significance occurs after liver biopsy. Biopsied cows looked at the wound site significantly more than sham-biopsied cows, irrespective of whether they had received NSAIDs in addition to LA (Beausoleil & Stafford, 2012). However, the authors suggested that some analgesic effect of NSAIDs was evident from the fact that rumination was significantly less in LA-only cows compared to controls, but no difference was seen between controls and meloxicam-treated cows. Further work is required before definitive conclusions can be made regarding the requirement for additional analgesia during liver biopsy.

4.5 Conclusions

No evidence of significant pain occurring as a result of liver biopsy was found in this study. However, it cannot be concluded that pain did not exist, as previous studies provide evidence of post-biopsy pain occurring in cattle. The lack of change in observed behaviours suggests that any pain that was present here was mild, and not of enough significance to alter normal behaviour in the cattle. There was also no evidence that liver biopsy resulted in altered pain processing in cattle, as indicated by the lack of significant difference in TNTs. This may be because the procedure is not noxious enough to induce central sensitisation, or because the sensitisation was not generalised enough to be detected with the TNT method used here. Both rationales support the behavioural evidence that any post-biopsy pain that existed was mild in nature.

Without identifying the presence of post-biopsy pain, it is not possible to infer what effect additional NSAID analgesia would have on alleviating it. When the results of the behavioural observations and TNT testing are considered together, the indication is that liver biopsy as it was performed here does not result in significant pain in cattle. However, given that this procedure is performed so commonly and that previous evidence suggests some pain is a likely outcome, refinement of this experimental model is warranted. Future studies should include use of more appropriate locations for TNT testing and earlier measures of nociception after liver biopsy, to better identify the temporal development of hyperalgesia. Investigation of how single pass versus multiple-pass techniques or solitary biopsies versus repeated biopsies over time affect the development of post-biopsy pain should also be examined, along with the effect that NSAIDs may have on any such pain resulting from these different methods.

5 General Discussion



Chewing the Cud

The overall objective of this research was to evaluate if TNT testing was useful for assessing the effect of several analgesic drugs on different types of pain in cattle. In chapter 3, the central analgesic effect of medetomidine and ketoprofen on TNTs of healthy cows was examined. This was done to explore how these drugs affect the acute pain response to a noxious thermal stimulus in animals without pre-existing pain. In chapter 4, the effect of different analgesic protocols on the pain response of cattle after liver biopsy was assessed through use of TNT testing and behavioural observations. Here, TNT testing was used to determine if a painful procedure would incite central sensitisation of the spinal cord, and if NSAIDs moderated that sensitisation. Behavioural observations were used in addition to confirm the presence of post-biopsy pain, and to evaluate the effects of NSAIDs on mitigating such pain.

TNT testing revealed an effect of medetomidine 60 minutes after administration on the acute pain response of healthy cows. However, it is not definitive that this limited change was due to the central analgesic properties of this drug. It is possible that the changes seen were due to sedation, which was observed in a third of the cows at this time, rather than analgesia. No central analgesic effect of ketoprofen was found using TNTs. This was most likely due to the laser stimulus intensity being too high to be able to discriminate subtle effects of NSAID analgesia on C-fibre responses to noxious stimulation.

Liver biopsy did not induce significant differences in TNTs or behavioural observations in any of the treatment groups. The lack of difference in TNTs is most likely due either to liver biopsy not being painful enough to cause central sensitisation, or the sensitisation not being generalised enough to be detected at a site distant from biopsy. The lack of difference in behavioural observations between treatments also suggests that liver biopsy as performed here did not result in significant post-biopsy inflammatory pain.

Based on these results, the general conclusions of this research are that TNT testing may be useful to investigate some effects of some analgesics on the acute pain response of healthy cattle. However, the method used here was not useful for demonstrating either central analgesic effects of an NSAID, or central sensitisation resulting from liver biopsy.

Modification of the methods used here may yet prove that TNT testing in cattle is an effective tool. The location used for TNT testing in this research has not previously been reported. The position on the caudal thigh was found to be much easier to access than the previously reported distal hind leg, both for clipping of the hair and for siting the laser beam. Although it was assumed here that this site was equally appropriate for TNT testing, it would be of value to

perform a full range of baseline TNT testing at various laser power outputs (as per Veissier et al., 2000) to determine this conclusively.

It is clear from the findings of this research that the laser stimulus intensity can affect the experimental outcome. The incidence of thermal burn injury in chapter 3 was an unexpected and unwanted result, and clearly indicates that the laser power output used was too high. The associated fast latencies to respond may have hindered the ability to discriminate subtle effects between treatment groups, due to preferential activation of A δ -fibres over C-fibres. In contrast, the large number of non-responses to the laser stimulus in chapter 4 suggests that the power output may have been too low to reliably initiate a response to the laser stimulus, and this may have masked any differences that existed between treatment groups. Although no subsequent complications of the burn injury were evident in the cattle, its occurrence likely caused some degree of pain beyond the acute response being sought by TNT testing. Thus, refinement of the methods is required in order to improve animal welfare outcomes. Performing validation studies at this location, as suggested above, would also help identify a range of laser power outputs for this location that result in a low percentage of non-response to the stimulus, while still avoiding the occurrence of thermal burn injury.

TNT testing on the caudal thigh is a convenient location for studies of analgesic effect; however for studies of central sensitisation, it may be more appropriate to begin investigations at location close to the site of injury, in order to identify localised central changes initially. It is probable that one of the reasons for no differences in TNTs of the different treatment groups in chapter 4 is due to TNT testing being conducted too far away from where central sensitisation was likely to develop.

The issue of stress-induced hypoalgesia (SIH) confounding the experimental outcome has been raised in both chapters 3 and 4. The decreasing TNTs over study days in chapter 3 may be a result of a reduction in SIH, as cows became increasingly accustomed to the experimental set-up. However, it is more likely to have been of significance in chapter 4, where the animals used were very new to the farm and had no previous experience of the facilities. This may have contributed to the high number of non-responses to the laser stimulus. Future work that uses this method to investigate aspects of pain other than SIH therefore needs to minimise stressors by allowing time for familiarisation of the subjects with the research facilities and team, so that the results of testing are not unduly affected.

Extending the time course of TNT testing in healthy cattle after treatment with analgesics may provide more information regarding the onset and duration of the central analgesic effects of different classes of drugs. It appears that the post-treatment assessment period in chapter 3 was too short, thus it is possible that further significant changes in TNTs after medetomidine were missed. Although some work has been done on the pharmacokinetics (PK) of many analgesic drugs administered intravenously in cattle, the PK after other routes of administration have not been investigated. Performing such work could better inform future decisions on appropriate post-treatment assessment periods, as there appears to be some relationship between PK and onset/duration of analgesia.

It is not clear why behavioural observations did not provide evidence of pain occurring after liver biopsy, as the methods used in chapter 4 have previously indicated that post-biopsy pain occurs (Beausoleil & Stafford, 2012). One possibility is that cows undergoing a single biopsy with only one pass into the liver experience less pain than those subject to multiple single-pass biopsies over time (as in Beausoleil & Stafford, 2012) or a single biopsy with multiple passes (as in Mølgaard et al., 2012). The failure to find evidence of significant post-biopsy pain in this study suggests that local anaesthetic provides sufficient pain-relief when liver biopsy is performed as it was here. Further work examining the differences in the way liver biopsy is performed would help to clarify this. Future investigations into different biopsy techniques should also address whether the addition of NSAIDs is beneficial in reducing post-biopsy after such methods.

In summary, this thesis has shown that TNT testing can be used to investigate some aspects of alpha2-agonist activity in cattle. Future work will require further development of the methodology if it is to be used in models of NSAID analgesia. Similarly, for models of hyperalgesia, some refinement of the methods is required. Based on the findings here, liver biopsy does not result in significant pain and therefore may not be a suitable procedure to use in pain models, as any changes in behaviour or central pain processing may also be mild and difficult to detect.



Summer Storm Brewing

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Appendix A

Extended graphs relating to Chapter 3:

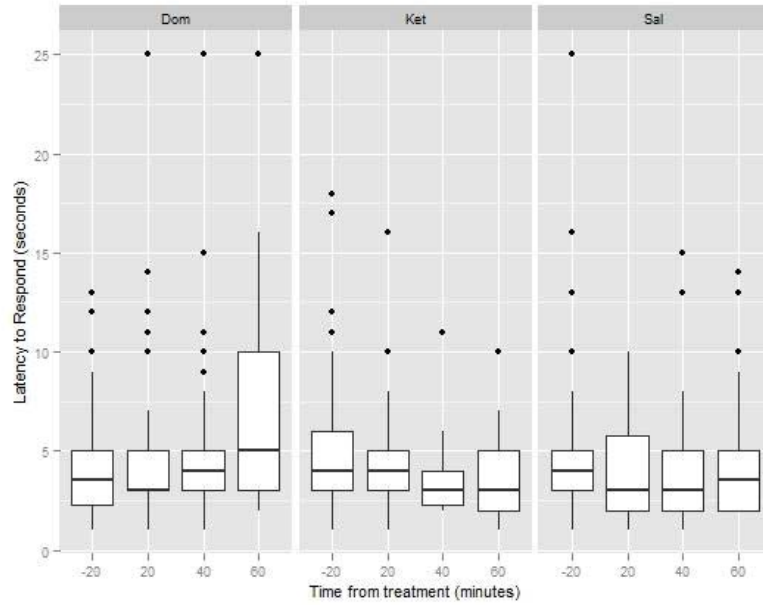


Figure A.1: Box-plots of TNTs (expressed as latency to respond) as a function of time from treatment, stratified by treatment. Dom = medetomidine, Ket = ketoprofen, Sal = saline.

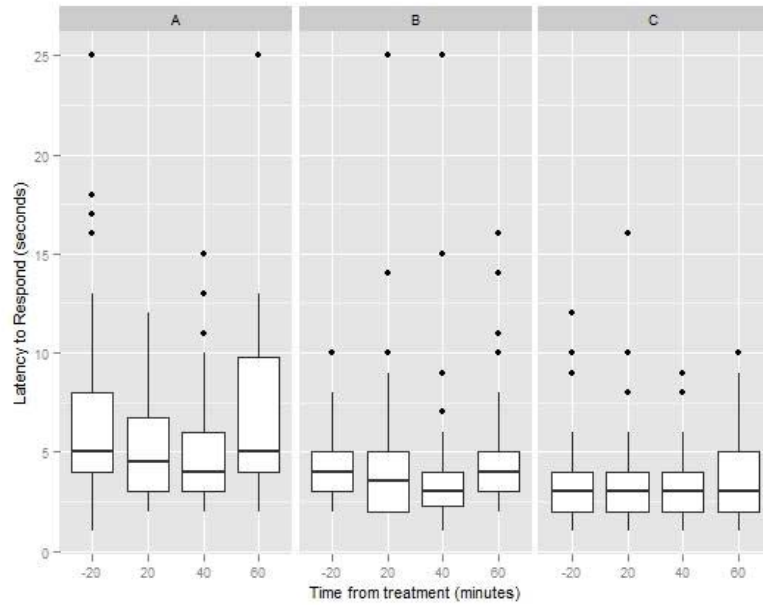
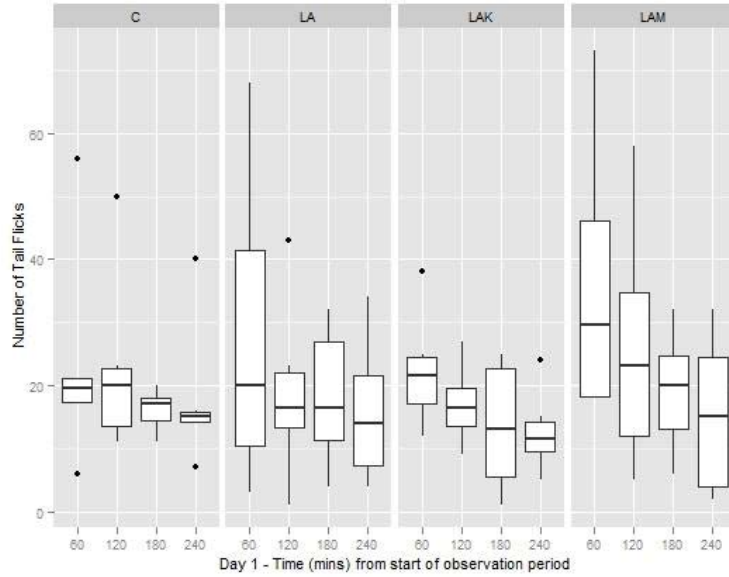


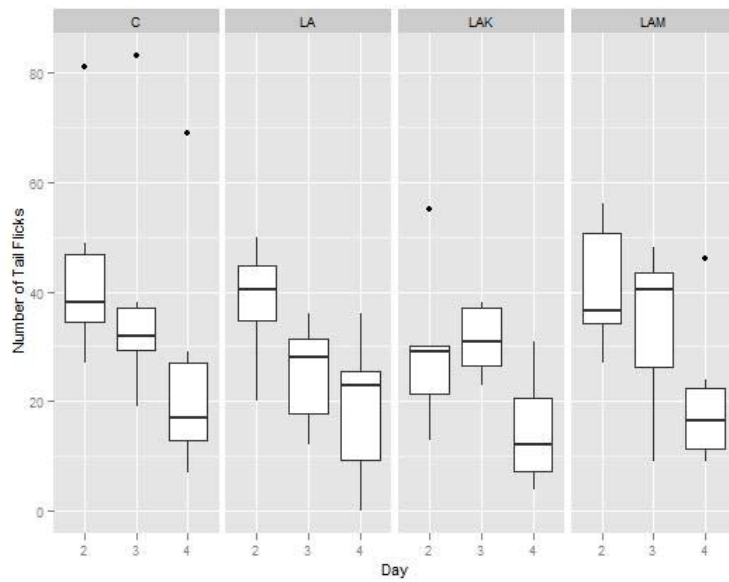
Figure A.2: Box-plots of TNTs (expressed as latency to respond) as a function of time from treatment, stratified by study day (A, B, C).

Appendix B

Extended graphs relating to Chapter 4:

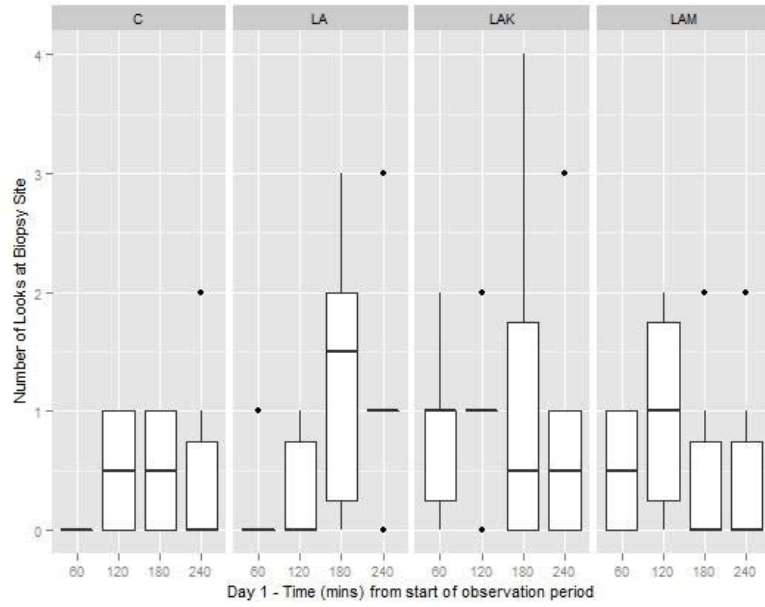


A

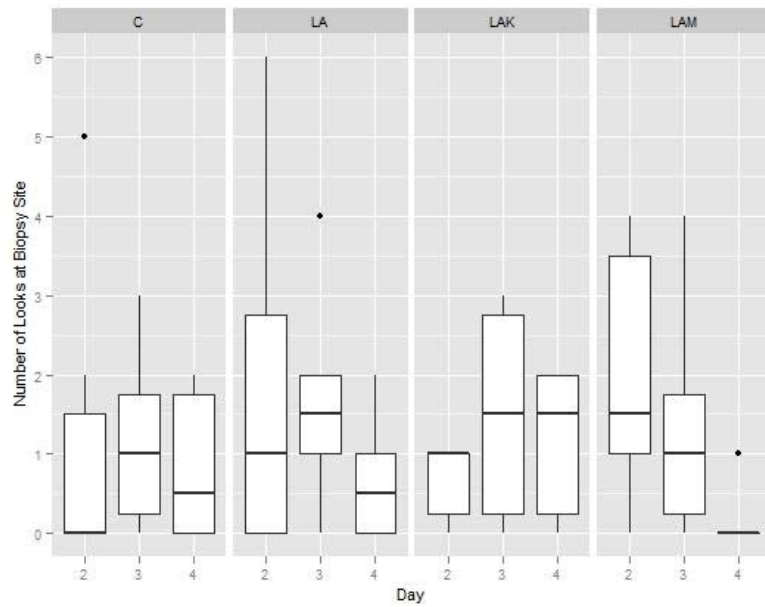


B

Figure B.1: Box plots of number of tail-flicks performed by cattle in the post-biopsy observation period, stratified by treatment group. A is the hourly total from day 1. B is the daily total for days 2-4. C= control, LA= local anaesthetic (LA), LAK= LA + ketoprofen, LAM = LA + meloxicam.

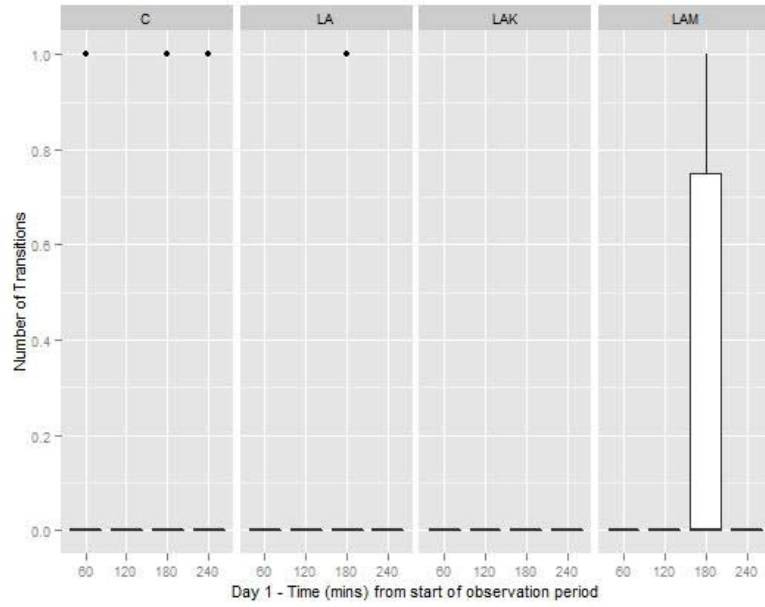


A

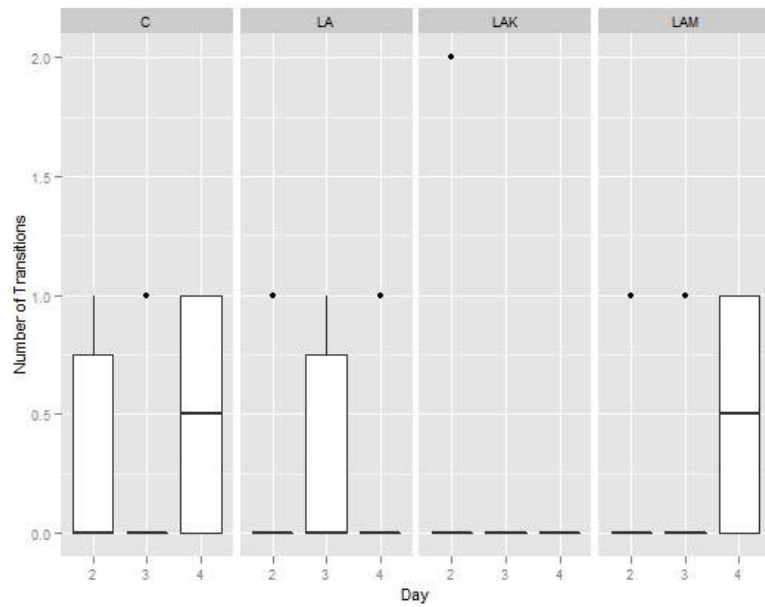


B

Figure B.2: Box plot of number of looks at biopsy site performed by cattle in the post-biopsy observation period, stratified by treatment group. A is the hourly total from day 1. B is the daily total for days 2-4. C= control, LA= local anaesthetic (LA), LAK= LA + ketoprofen, LAM = LA + meloxicam. .

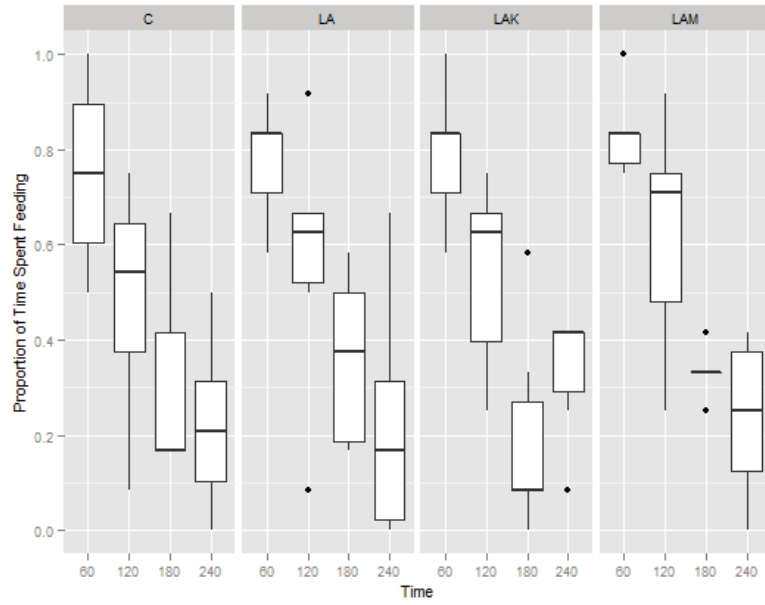


A

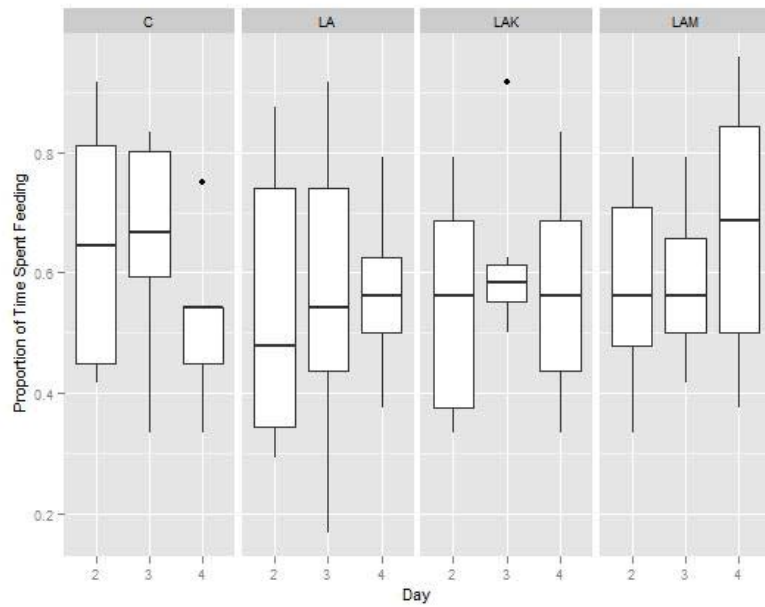


B

Figure B.3: Box plot of numbers of transitions performed by cattle in the post-biopsy observation period, stratified by treatment group. A is the hourly total from day 1. B is the daily total for days 2-4. C= control, LA= local anaesthetic (LA), LAK= LA + ketoprofen, LAM = LA + meloxicam.

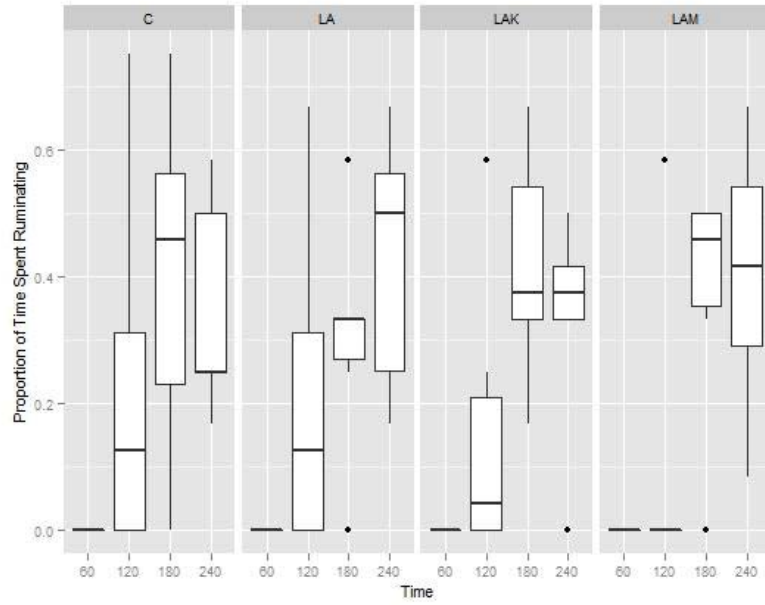


A

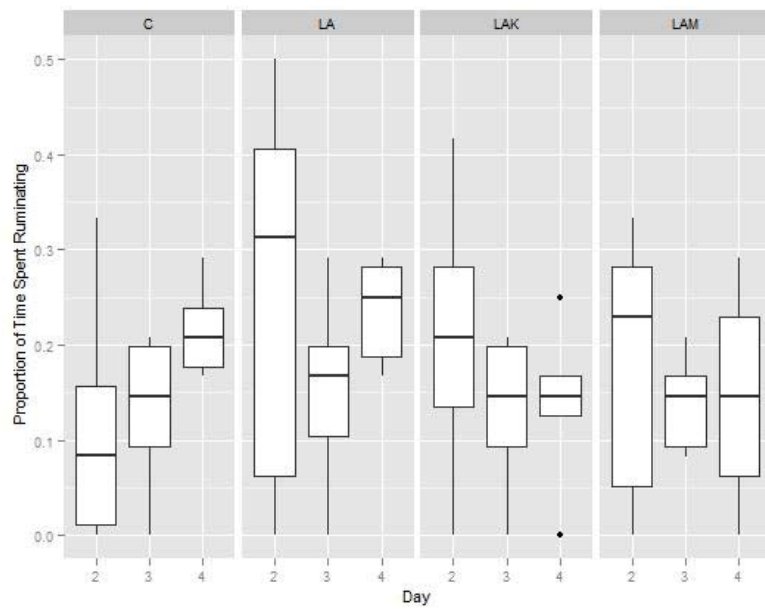


B

Figure B.4: Box plot of proportion of time spent feeding by cattle in the post-biopsy observation period, stratified by treatment group. A is the hourly aggregates from day 1. B is the daily totals for days 2-4 C= control, LA= local anaesthetic (LA), LAK= LA + ketoprofen, LAM = LA + meloxicam.

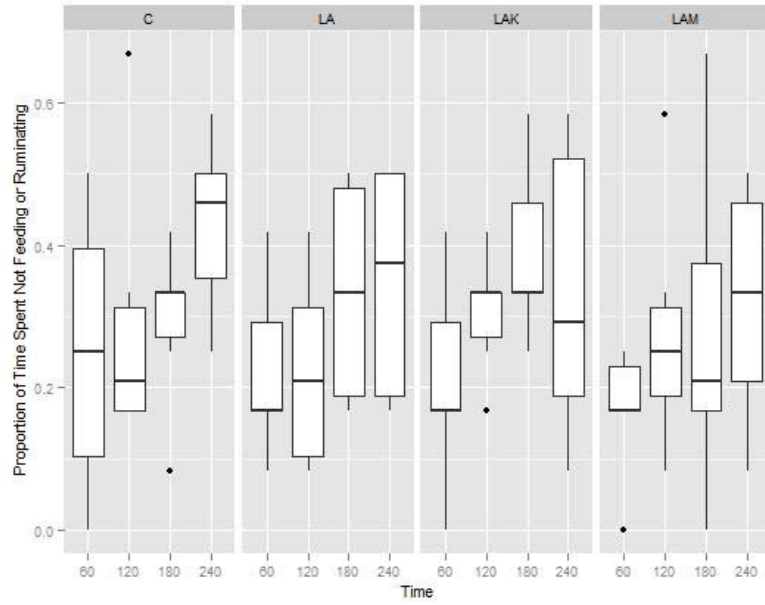


A

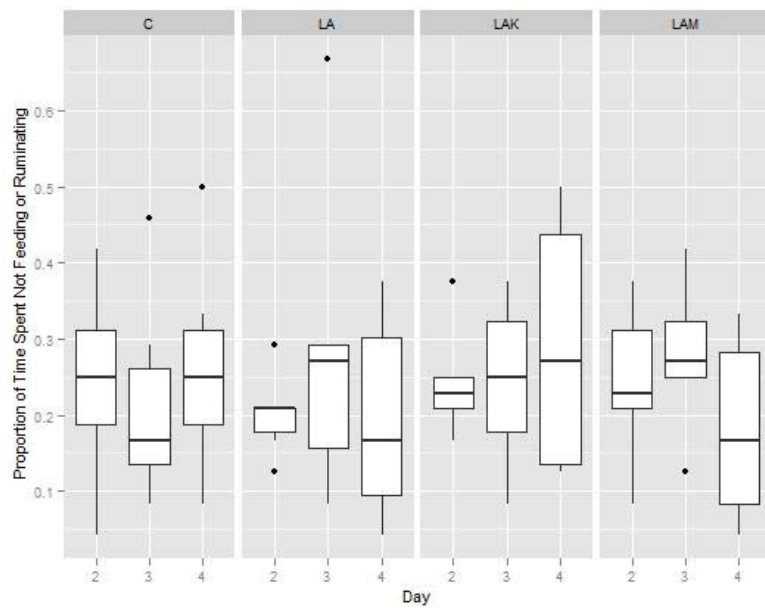


B

Figure B.5: Box plot of proportion of time spent ruminating by cattle in the post-biopsy observation period, stratified by treatment group. A is the hourly total from day 1. B is the daily total for days 2-4. C= control, LA= local anaesthetic (LA), LAK= LA + ketoprofen, LAM = LA + meloxicam.

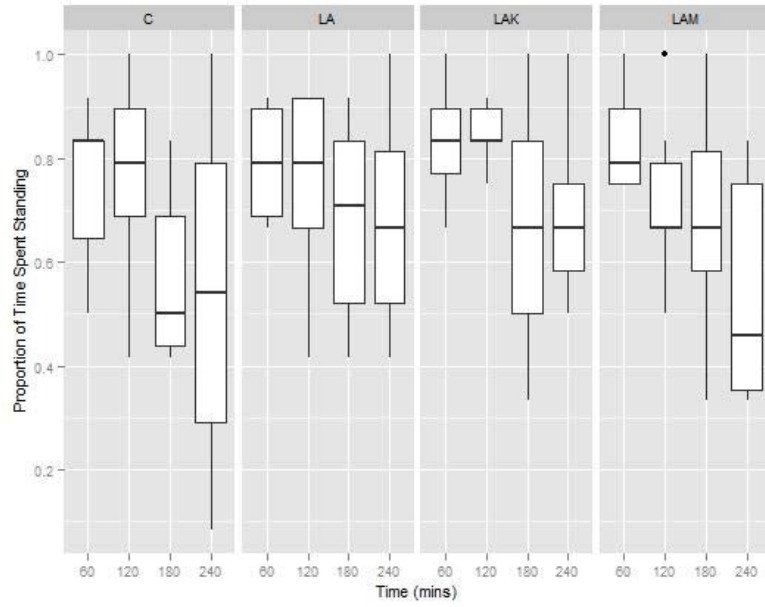


A

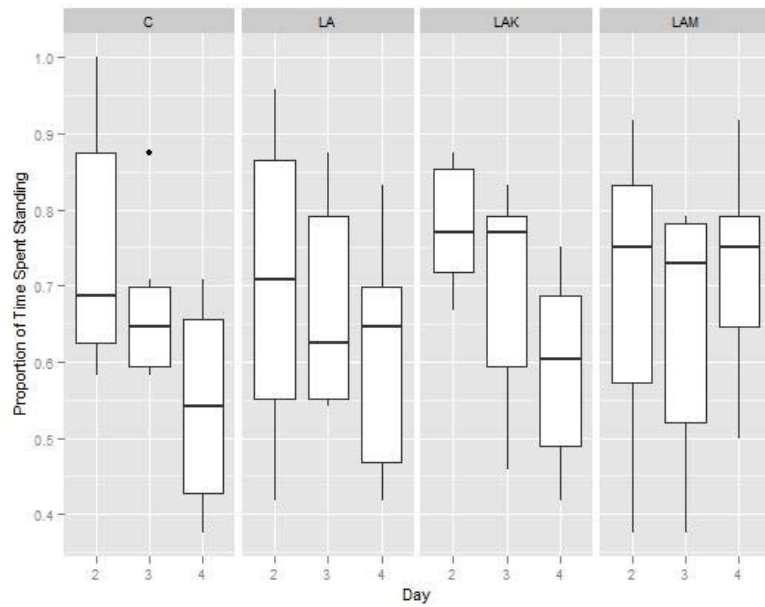


B

Figure B.6: Box plot of proportion of time without feeding or rumination occurring in the post-biopsy observation period, stratified by treatment group. A is the hourly total from day 1. B is the daily total for days 2-4. C= control, LA= local anaesthetic (LA), LAK= LA + ketoprofen, LAM = LA + meloxicam.

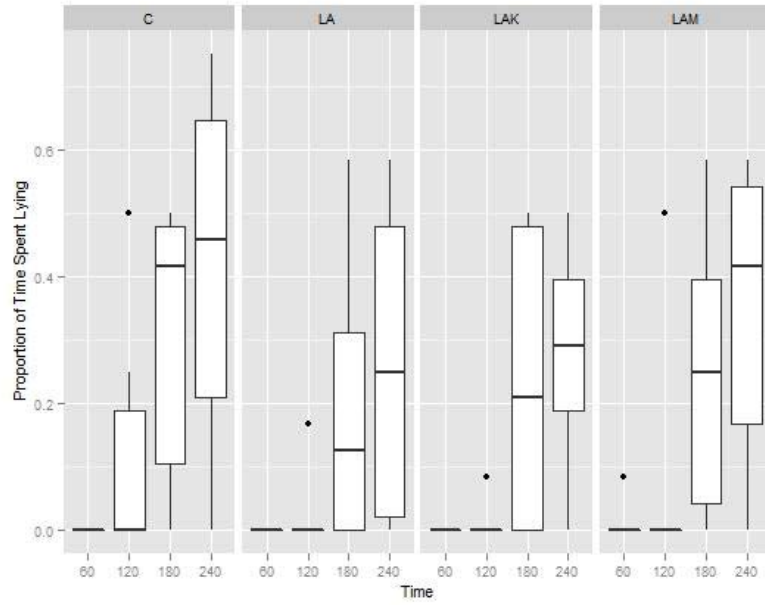


A

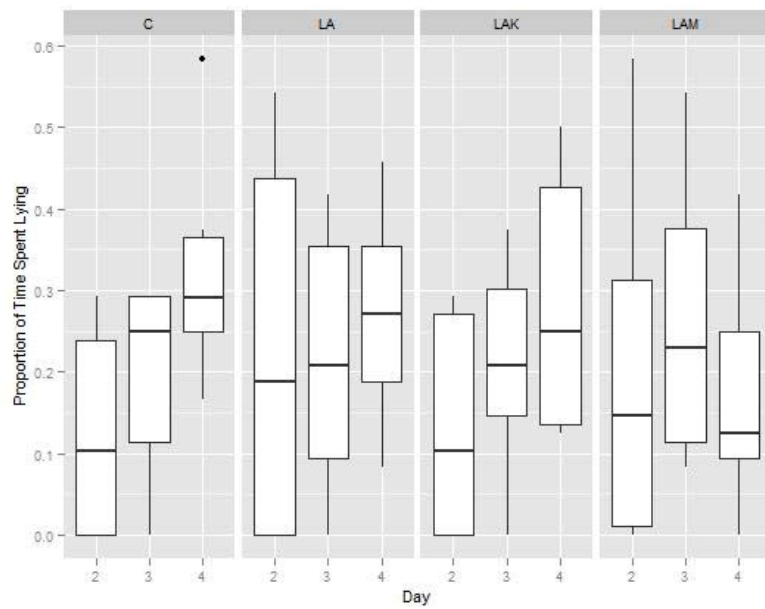


B

Figure B.7: Box plot of proportion of time spent standing by cattle in the post-biopsy observation period, stratified by treatment group. A is the hourly total from day 1. B is the daily total for days 2-4. C= control, LA= local anaesthetic (LA), LAK= LA + ketoprofen, LAM = LA + meloxicam.

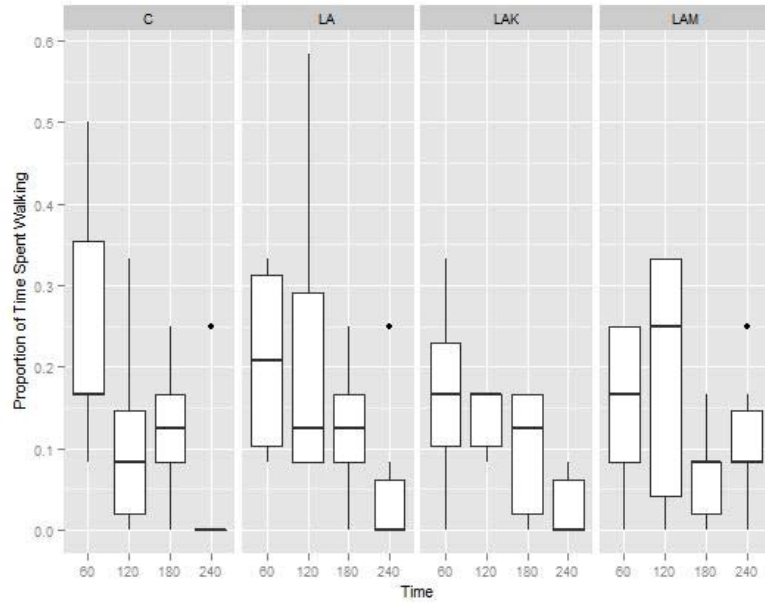


A

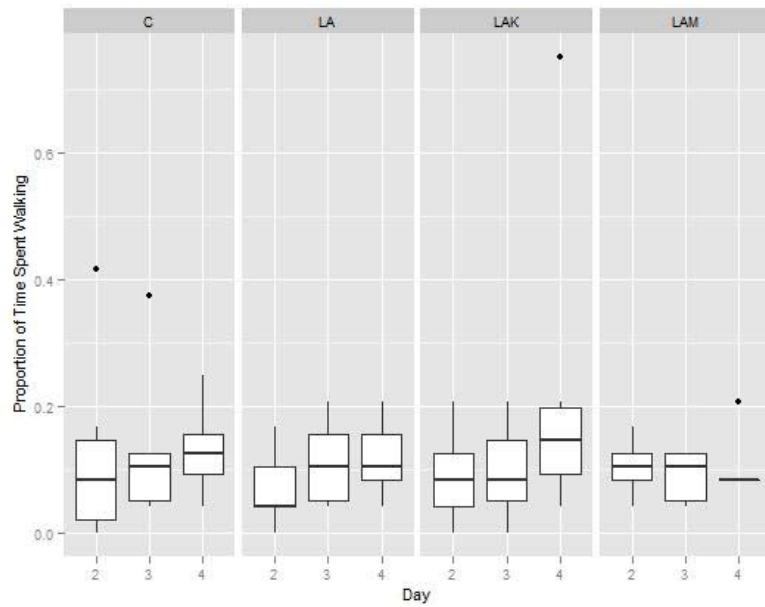


B

Figure B.8: Box plot of proportion of time spent lying by cattle in the post-biopsy observation period, stratified by treatment group. A is the hourly total from day 1. B is the daily total for days 2-4. C= control, LA= local anaesthetic (LA), LAK= LA + ketoprofen, LAM = LA + meloxicam.



A



B

Figure B.9: Box plot of proportion of time spent walking by cattle in the post-biopsy observation period, stratified by treatment group. A is the hourly total from day 1. B is the daily total for days 2-4. C= control, LA= local anaesthetic (LA), LAK= LA + ketoprofen, LAM = LA + meloxicam.