Refining a ‘toolkit’ for objective assessment of pain and stress in ruminants

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Abstract

Pain is a complex phenomenon, and to address pain management in animals, the presence of pain needs to be determined. Most studies assess the presence of pain based on the appraisal of either a single biomarker or a combination of a few parameters, which were often not examined simultaneously. In ruminants, measurements of cortisol and haptoglobin (Hp), and the assessment of behavioural changes have been the standard practice for this purpose. Nevertheless, there are still limitations in using these parameters, as they are also indicators of psychological stress, and can be misinterpreted as to indicate the presence of pain. Assessment of behavioural changes in ruminants can be difficult as prey animals conceal their experience of pain to avoid predators’ attention. Therefore it is recommended that a reliable assessment tool be devised that includes a combination of various parameters to determine the presence of pain in ruminants in order to address pain management effectively.

The primary aims of this study were to evaluate the conventional and contemporary biomarkers to determine the response of an animal to a noxious experience, and to refine a multi-parameter ‘toolkit’ that can be applied in assessing pain in ruminants. For these purposes, castration was used as a model of a common painful husbandry procedure that presumably evokes pain associated with the tissue injury.

Merino cross lambs were assigned to two groups that were either surgically castrated or subjected to restraint stress (control). Castration was an open surgical technique without the provision of anaesthesia or analgesia. The conventional biomarkers estimated were plasma cortisol, Hp and beta-endorphin (β-EP). The contemporary biomarkers measured were interleukin-6 (IL-6), substance P (SP), and prostaglandin E$_2$ (PGE$_2$). The peripheral leukocytes expression of IL-6 and proopiomelanocortin (POMC), the precursor of β-EP were also estimated. Behavioural changes were recorded to assess pain-related behaviour caused by the surgical procedure.

Following treatment, the cortisol concentration in the castrated lambs was higher than control animals from ten minutes to two hours post-castration, indicative of the acute noxious experience associated with the surgical procedure. Surgical castration also caused a response in SP indicative of nociception and neurogenic inflammation, which was increased from 30 minutes and became significant eight hours following castration. The behavioural assessment showed that the castrated lambs demonstrated statue standing, a pain-like behaviour, from three to five hours post-castration, which were associated with the increase in SP, indicative of the pain sensitisation caused by the tissue injury. Increase in the systemic inflammatory mediators, the Hp and IL-6 were observed from
day two, in which Hp remained significant until day four and resolved by day five. The IL-6 continued to increase over time, suggesting of an ongoing inflammatory response and inflammatory pain possibly until wound healing had occurred. No significant changes of β-EP and PGE2 were found, suggesting a local response. The quantification of IL-6 and POMC gene expressions by the circulating leukocytes showed a depletion of response compared with the plasma levels, which indicated that the cells might have migrated to the injury area. This pattern of changes supports the assumption of a local tissue response and the lack of detectable plasma IL-6 in the initial phase of a post-tissue insult.

The response of plasma IL-6 and (interleukin-1) IL-1 were also measured in samples of a study in cattle that were castrated surgically and using tension banding in two age groups (weaner and mature bulls). Interleukin-1 was below detection level and IL-6 did not change in the surgically castrated bulls. Nonetheless, the IL-6 increased significantly in the bulls that were castrated using banding from as early as three days following castration, more evidently in the mature bulls indicative of a chronic inflammatory pain. Some variability was detected, which was also observed in the surgically castrated lambs suggesting that breed background could influence the variation in the immune response in both species.

In conclusion, the studies conducted in this thesis were aimed at understanding the underlying relationships of the various biomarkers in the assessment of pain in ruminants. It was found that these results support the hypothesis that more than one parameter is required to assess pain in ruminants due to tissue injury caused by a common husbandry procedure. Substance P and IL-6 are recommended as potential good markers in the assessment of nociception and inflammatory pain. The trends and magnitude of response of these markers until point of tissue healing will be more informative of the pain induced and the implications of the procedure on the animal. A local insult such as castration may have evoked a local response in β-EP, PGE2, and IL-1 that could not be detected in plasma. Cortisol and Hp should still be measured together with these parameters until assessment of pain can be refined further on other forms of pain models in ruminants.
Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Publications during candidature

Conference proceedings


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Publications included in this thesis

No publications included.
Contributions by others to the thesis

The majority of the work integrated into this thesis was conducted by Azalea Hani Othman. Dr Steven Kopp, Dr. Stephen Anderson, Dr. Carol Petherick and Dr. Rachel Allavena contributed in assisting in the data interpretation and revision of the written material. Dr. John Alawneh assisted in the statistical work described in Chapter 3. Professor Alison Johnston and Angela Lees assisted with the proofreading of this thesis.

The study described in Chapter 4 was designed and conducted by the team led by Dr. Carol Petherick. The archived plasma samples from this study were obtained and analysed with the permission of the Meat and Livestock Australia (MLA). The study had been published in the Applied Animal Behavioural Science Journal (Petherick et al., 2014a, 2014b), in which the results described in this thesis were not included in the publications.

Statement of parts of the thesis submitted to qualify for the award of another degree

None.
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lambs, cattle, ruminants, castration (surgical, banding), pain, inflammation, biomarkers, stress, animal welfare

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ANZSRC code: 839901, Animal Welfare, 40%

ANZSRC code: 070702, Veterinary Anatomy and Physiology, 20%

Fields of Research (FoR) Classification

FoR code: 0707, Veterinary Sciences, 80%

FoR code: 0702, Animal Production, 20%
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<tr>
<td>15-PGDH</td>
<td>15-hydroxyprostaglandin dehydrogenase</td>
</tr>
<tr>
<td>β-EP</td>
<td>beta-endorphin</td>
</tr>
<tr>
<td>°C</td>
<td>degree Celsius</td>
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<tr>
<td>µg</td>
<td>microgram</td>
</tr>
<tr>
<td>µg/mL</td>
<td>microgram / millilitre</td>
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<tr>
<td>µL</td>
<td>microlitre</td>
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<tr>
<td>ACTH</td>
<td>adrenocorticotrophin hormone</td>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>APP</td>
<td>acute phase protein</td>
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<tr>
<td>APR</td>
<td>acute phase response</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CGRP</td>
<td>calcitonin gene-related peptide</td>
</tr>
<tr>
<td>COX-2</td>
<td>cyclooxygenase-2</td>
</tr>
<tr>
<td>CRF</td>
<td>corticotrophin releasing factor</td>
</tr>
<tr>
<td>CV</td>
<td>coefficient of variation</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EEJ</td>
<td>electroejaculation</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>g</td>
<td>gravitational acceleration</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>Hp</td>
<td>haptoglobin</td>
</tr>
<tr>
<td>HPA</td>
<td>Hypothalamic-Pituitary-Adrenal</td>
</tr>
<tr>
<td>IL-1</td>
<td>interleukin-1</td>
</tr>
<tr>
<td>IL-6</td>
<td>interleukin-6</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>mg/mL</td>
<td>milligram / millilitre</td>
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<tr>
<td>MgCl₂</td>
<td>magnesium chloride</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>ng/mL</td>
<td>nanogram / millilitre</td>
</tr>
<tr>
<td>NSAID</td>
<td>non-steroidal anti-inflammatory drug</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>pg/mL</td>
<td>picogram / millilitre</td>
</tr>
<tr>
<td>PGE₂</td>
<td>prostaglandin E₂</td>
</tr>
<tr>
<td>pNpp</td>
<td>p-nitrophenyl phosphate</td>
</tr>
<tr>
<td>POMC</td>
<td>proopiomelanocortin</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative PCR</td>
</tr>
<tr>
<td>RIA</td>
<td>radioimmunoassay</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
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<tr>
<td>RT</td>
<td>reverse transcriptase</td>
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<tr>
<td>s</td>
<td>second</td>
</tr>
<tr>
<td>SDHA</td>
<td>succinate dehydrogenase complex subunit A</td>
</tr>
<tr>
<td>SE</td>
<td>standard error</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
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<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>sIL-6R</td>
<td>soluble interleukin-6 receptor</td>
</tr>
<tr>
<td>SP</td>
<td>substance P</td>
</tr>
<tr>
<td>TIU/mL</td>
<td>trypsin inhibitor unit / millilitre</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumour necrosis factor alpha</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>U/µL</td>
<td>units / microlitre</td>
</tr>
<tr>
<td>YWHAZ</td>
<td>tyrosine 3-monoxygenase/tryptophan 5-monoxygenase activation protein zeta polypeptide</td>
</tr>
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Chapter 1

Literature review
1.1 Introduction

Pain is a complex phenomenon that results in unpleasant sensation and emotional experiences which are difficult to assess and quantify. Pain as experienced by humans cannot be used as a reference for animals as some validated biomarkers for pain have been shown to be species-specific (Le Bars et al., 2001; Allen, 2005). However, it has been established from behavioural observations that animals do experience pain based on their aversive response to painful stimuli (Hellebrekers, 2000; Sneddon et al., 2014). In ruminants, routine husbandry procedures that involve tissue injury such as castration (Rault et al., 2011), dehorning (Mellor and Stafford, 1999) and tail docking (Sutherland and Tucker, 2011) are painful to the animals and have raised animal welfare concerns (Rollin, 2004). This has led to National advisory bodies in various countries and regions, including Australia, New Zealand and the European Union to recommend that steps are taken to avoid or manage animal pain in order to minimise the suffering it would otherwise cause. Due to these concerns and increased public awareness of animal welfare, the last few decades has seen much research and numerous publications emerge regarding the assessment of pain in animals (Jelena and Slavoljub, 2010; Reid et al., 2013; Prunier and Leterrier, 2014).

The French National Institute for Agricultural Research has established the ‘3S’ steps, “Suppress, Substitute and Soothe”, for approaching pain in farm animals (Guatteo et al., 2012). The first step, suppress, advocates avoidance of the use of painful procedures when and where possible, the second step, substitute, recommends choosing the least painful technique, and the last step, soothe, advocates use of analgesia to alleviate any pain that may have been incurred. According to Woolf and Max (2001), pain management requires the identification of the underlying cause of pain and to treat pain adequately and appropriately. The current pain treatment regime is mostly based on the symptoms and signs generated from the underlying cause or mechanism of pain. In production animal industries, economical constraints limit the availability of pain relief options that are commercially available and practical to use (Bayley et al., 2003; Lomax et al., 2009). Thus there is a need to search for a reliable and specific pain assessment tool to ensure appropriate pain treatment is administered in such circumstances.

To evaluate the response of an animal to a noxious stimulus, it is also important to incorporate the affective components of pain responses, which have been proven by scientific evidence to be associated with pain in animals. It was stated by Thorpe in the seminal Brambell report (1965) that the fundamental aspects of scientific evidence must involve both physiological and ethological (behavioural) evaluations because both parameters act interdependently of one another. Mellor et al. (2000) mentioned that the conclusions made on the subjective content of painful experiences cannot
be regarded as statements of fact. Furthermore, pain assessment in prey animals such as ruminants can be complicated as the behavioural demonstration of pain is often concealed to avoid predators’ attention (Underwood, 2002). Due to its subjective nature, it is evident that one parameter cannot independently define the noxious experience. Particularly in ruminants, there is a need to develop tools for identifying pain and evaluating its intensity (Gigliuto et al., 2014). This also emphasises the need to have a panel of robust biomarkers for the assessment of pain in animals.

This thesis reports the investigation into various circulating physiological variables that can enhance the evaluation of pain and stress in ruminants caused by routine husbandry procedures. The studies conducted in this thesis aim to establish a reliable pain and stress assessment ‘toolkit’ in ruminants, which can potentially assist in the development of efficient pain management.

### 1.2 Stress response in animals

Broom and Johnson (1993, p. 58) refer to ‘stress’ as “an exposure to unpleasant conditions with adverse effects”. Moberg (2000, p. 1) defined stress as “the biological response elicited when an individual perceives a threat to its homeostasis” and the threat is called a stressor. In the context of ‘stress’ caused by a noxious experience, Mellor et al. (2000, p. 172-173) used the word ‘distress’ to describe “the emotional content of noxious experiences that elicit physiological stress responses in animals, whether that noxiousness is predominantly emotional (e.g. fear), predominantly physical (e.g. vigorous exercise) or a combination of both (e.g. pain)”. In addition, ‘pain-induced stress’ elicits physiological responses that reflect the interaction between the emotional and physical components of a noxious experience (Mellor et al., 2000).

Stress responses require an interaction between various systems which fall into four broad categories: behaviour, the autonomic nervous system, the neuroendocrine system, and immune system (Moberg, 1999). The complexity of the systems, the individual variation and species-specific variability make it essential to understand the well-coordinated regulation of stress response when selecting physiological measures for stress assessment (Pekow, 2005). Moberg (2000) suggested a model that outlines the three stages of the stress response in animals, which includes the recognition of the stressor, the animal’s biological defence against the stressor, and the consequences of the stress response on the animal (Figure 1.1). An animal can perceive a situation as stressful and this can be influenced by prior experiences and developmental history such as exposure to regular handling (Burdick et al., 2011). Rushen et al. (2008) suggested that it is the animal’s perception of a threat that is important for producing the stress response. The response towards a stressor can be in the form of behavioural, neurophysiological and peripheral
physiological changes that reflect how the animal attempts to deal with or avoid the stressor (Rushen et al., 2008). Together with prior experiences, the stressful situation will sensitise the animal to a particular challenge and subsequently protect the animal from certain challenges in the future (McEwen et al., 1997).

**Figure 1.1** A model for the biological response of animals to stress. Adapted from Moberg (2000).
An acute stress response is interpreted as a brief exposure to a stressor, that is sufficient to shift biological functions and induce distress (Moberg, 2000). According to Pekow (2005), the acute stress response is indicated by an initial rapid increase in neurally-derived substances which act within a short time frame, followed by a slower increase in endocrine derived substances which have a longer duration of action. The sympathoadrenal (‘fight or flight’) and Hypothalamic-Pituitary-Adrenal (HPA) axis responses are the main mechanisms that are involved in acute stress (Chrousos, 1998). The sympathoadrenal system involves a rapid-onset, short-lived catecholamine (adrenaline and noradrenaline) response that helps activate the ‘fight or flight’ reactions. The HPA axis has a slower onset and longer duration of glucocorticoids response (Mellor et al., 2002). However, prolonged elevation of glucocorticoids can cause protein catabolism, immune suppression, increase susceptibility to infection and depression (Munck et al., 1984; Matteri et al., 2000), and impair wound healing (Christian et al., 2006). These effects can be observed in chronic stress. Chronic stress is described as a long-term stress that can occur due to exposure to a prolonged unrelenting stressor, a series of acute stressors or due to repeated exposure to the same acute stressor (Moberg, 2000). Chronic stress not only causes distress, but also can cause accumulation of biological cost to the animal without any opportunity to replenish its biological resources, and possibly leads to a pathological condition (Moberg, 1985; Chrousos, 1998; Moberg, 2000).

1.3 The concept of pain perception

Pain is described as a perception of the output of a nociceptive system, which forms a component of the overall set of controls responsible for homeostasis (Le Bars et al., 2001). Pain has been recognised as a complex phenomenon that involves sensory, cognitive and affective components (Melzack and Katz, 2013). The International Association for the Study of Pain has established the definition of pain in general as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or is describable in terms of such damage” (IASP, 1979, p. 249). It also arises when the skin or tissue are subjected to sufficient intensity of stimuli to cause or threaten damage (Kitchell and Erickson, 1983). Tissue injury that induces pain also disrupts the regulation of homeostasis by the brain, which contributes to a stress response, leading to the complex mechanism to re-establish homeostasis (Melzack and Katz, 2013). Woolf and Max (2001) also described that pain can be translated as an unpleasant and stressful experience. Since pain contributes to the alteration of body homeostasis or stress response, pain is also considered as a stressor.
According to Beecher (1957) the general classification of reactions towards painful stimuli are 1) skeletal muscle responses, 2) reactions mediated by the autonomic nervous system, and 3) pain perception responses. Specific pain receptors (nociceptors) detect harmful or potentially harmful stimuli and send impulses via the nerve pathways in the spinal cord which the lower and higher centres of the brain convert into perceived pain (Stein and Lang, 2009). The nociceptors are mainly divided into two types of fibres. The myelinated A\(\delta\) fibres are described as the afferent fibres that mediate the acute and localised pain, and the unmyelinated C fibres which signal a more poorly localised, secondary pain are slower compared with A\(\delta\) fibres (Ringkamp et al., 2013). The A\(\delta\) pathways convey sharp pain, whereas C fibers transmit aching pain (Gregory, 2004). In rats, it has been described that 50% of C fibres contain peptides substance P (SP), calcitonin gene-related peptide (CGRP) and somatostatin, and only 20% of A\(\delta\) fibres contain these peptides (McCarthy and Lawson, 1989; Lawson et al., 1996).

Tissue injury initiates pain by the activation of nociceptors, which are also activated by a local inflammatory response sustained by multiple mediators and immune cells in the event of inflammation (Carr and Goudas, 1999), causing inflammatory pain. Following tissue injury, inflammatory mediators are released from the local and migrating inflammatory cells which result in an ‘inflammatory soup’, rich in substances such as cytokines, growth factors, kinins, prostanoids and neuropeptides causing peripheral sensitisation (Hellebrekers, 2000; Woolf and Ma, 2007). The inflammatory mediators sensitise the nociceptors causing the latter to decrease their stimulus threshold, and together, the nociceptive input in the peripheral area is sustained and amplified (Hellebrekers, 2000). Tissue injury and inflammation can cause hyperalgesia, which is enhanced sensitivity to painful stimuli (Gregory, 2004). Primary hyperalgesia develops at the site of injury, while secondary hyperalgesia develops in the surrounding injured area (Gregory, 2004). If pain stimulation persists, a wide dynamic range of neurons become sensitised, central sensitisation would occur involving the central nervous system and the non-painful stimuli would be perceived as painful (Hellebrekers, 2000). Tissue injury also causes a spontaneous neuropathic pain which is contributed by the damage of the nervous system, such as injury of the peripheral nerves and spinal cord (Woolf, 2004). Examples of types of pain mediated by neurotransmitters or substances have been summarised by Gregory (2004) as described in Table 1.1.
Table 1.1 Examples of types of pain mediated by neurotransmitters or substances.

<table>
<thead>
<tr>
<th>Neurotransmitters/substances</th>
<th>Type of pain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylcholine</td>
<td>Acute pain in tissue damage</td>
</tr>
<tr>
<td>Bradykinin</td>
<td>Hyperalgesia, inflammatory pain, skin pain</td>
</tr>
<tr>
<td>CGRP</td>
<td>Chemical stimuli, joint pain, mechanical stimuli, sensitisation to inflammatory pain, skin pain, thermal pain</td>
</tr>
<tr>
<td>Cytokines</td>
<td>Hyperalgesia, inflammatory pain</td>
</tr>
<tr>
<td>Histamine</td>
<td>Hyperalgesia, tissue swelling</td>
</tr>
<tr>
<td>Prostaglandins</td>
<td>Hyperalgesia, inflammatory pain</td>
</tr>
<tr>
<td>Substance P</td>
<td>Arthritic pain, back pain, cardiac pain, hyperalgesia, joint pain, skin pain</td>
</tr>
</tbody>
</table>

CGRP = calcitonin gene-related peptide. Adapted from Gregory (2004).

There are different ways in describing the various categories of pain. Acute pain is defined as “the normal, predicted physiological response to an adverse chemical, thermal or mechanical stimulus associated with surgery, trauma and acute illness” (Federation of State Medical Boards of the United States, 2005). Acute pain causes minimal damage compared with pathological pain and usually terminates when the stimulus ends (Livingston, 1994). The time period of an acute pain is also relatively short (Brearley and Brearley, 2000). Acute pain has been described by some as lasting from the onset of injury until pain is resolved by healing of the injury (Molony and Kent, 1997; Anil et al., 2002).

If mobilisation of acute pain is not suppressed and consequently leads to amplification of pain, then chronic pain will occur (Carr and Goudas, 1999). Chronic pain can last for weeks or months and it can be prolonged to beyond expected healing time or, as with pain that involves an alteration in the nervous system, remained painful after the removal of the stimulus and the injury is healed (Cheng and Morton, 2010). This is also referred to as phantom pain. Chronic pain can also be that which does not respond to treatments directed to the source of pain (Anil et al., 2002). In animals, the acute pain response may be obvious as manifested by overt changes in behaviour, however, in animals experiencing chronic pain, behavioural changes may not be expressed (Anil et al., 2002).

1.4 Pain assessment in animals

According to Molony and Kent (1997, p. 226), animal pain is defined as “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 damage, to
reduce the likelihood of recurrence and to promote recovery”. Rutherford (2002) suggested that, in general, pain assessment in animals relies on the following approaches: general body functioning (e.g. food and water intake, weight gain), physiological responses (e.g. cortisol concentration), and behaviour (e.g. vocalization). However, these changes are also found when an animal is experiencing stress, anxiety or discomfort, which may not necessarily implicate the nociceptive component (Prunier and Leterrier, 2014). Hence, the interpretation of behavioural responses may not be a true interpretation of a response to pain. Although, various nociceptor inputs (site, duration, intensity) influence the characteristic of pain perception, and it is also influenced by factors such as the emotional state and individual variation (Mellor et al., 2000). Variability between individuals within and between species that exist with respect to pain tolerance thresholds also makes pain assessment difficult (Dobromylskyj et al., 2000; Mellor et al., 2000; Anil et al., 2002). Furthermore, pain is a complex experience in animals as it is not only depending on the severity of the insult and tissue damage, but also contributed by previous experiences and social status in the flock or herd (Fitzpatrick et al., 2006). Reviews on animal pain had recommended that one parameter alone could not be the golden rule in assessing pain (Anil et al., 2002; Prunier et al., 2013). Thus, the assessment may be more meaningful if various indicators are assessed together.

1.5 Pain and stress in ruminants caused by routine husbandry procedures

The practice of animal husbandry is to provide food and shelter, control of reproduction, and promote animal health (Stafford and Mellor, 2010). It also involves the use of handling and restraint methods to minimise the risk of injury to the animals and their handlers, in which these procedures are painful to the animals (Stafford and Mellor, 2010). Even though most of these procedures were developed years ago, the scientific understanding on their implication to animal pain and stress has expanded. Moreover, the public awareness on the ethical treatment of production animals had also become more widespread (Rollin, 2004). This has led to the need to develop efficient pain relief management for painful husbandry procedures.

Routine husbandry procedures in ruminant animals maintained for farming purposes have been described by Stafford and Mellor (2010) and are presented in Table 1.2. Some of the procedures such as dehorning and branding are only practiced in cattle farming, while mulesing is only practiced in sheep farming, more commonly in Australia. In general, pain due to tissue damage associated with husbandry procedures is divided into three categories which are acute, chronic and pathological pain (Flecknell and Waterman-Pearson, 2000; Gregory, 2004).
Table 1.2 Routine husbandry management procedures practiced in cattle, sheep and goat farming systems.

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Cattle</th>
<th>Sheep</th>
<th>Goats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Castration</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Docking</td>
<td>+</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>Dehorning</td>
<td>+++</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Disbudding</td>
<td>+++</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Spaying</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ear notching</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Ear tagging</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Branding</td>
<td>++</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mulesing</td>
<td>+</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

+ in some management systems; ++ in many management systems; +++ in all management systems. Adapted from Stafford and Mellor (2010).

According to the summary by Mellor and Stafford (1999), routine husbandry procedures such as tail docking, dehorning and castration using surgical technique are perceived as the most pain-induced distress compared with other bloodless techniques such as the application of ring and tension banding. Other studies on lambs using surgical techniques compared with non-surgical techniques (Shutt et al., 1988a; Paull et al., 2009) are in agreement with the observations of Mellor and Stafford (1999). Shutt et al. (1988a) and Paull et al. (2009) evaluated the cortisol response and the presence of pain-related behaviour in animals which was marked within hours up to 1-2 days of post-treatment. However, studies in cattle castration comparing surgical and tension banding techniques had found that surgical castration caused acute pain, whereas banding caused delay in the pain response, which may be detected weeks after castration (Moya et al., 2014; Petherick et al., 2014b). Gregory (2004) described the application of tourniquet on appendages such as castration and tailing by rubber rings causes an immediate pain sensation following the application, and progresses into an inflammatory pain, which is also described as ischaemic pain. It involves the compression of the nerves together with occlusion of blood vessels to the distal tissue leading to hypoxia and ischemia of the tissue (Gregory, 2004). Ischaemic pain is also described as chronic pain because the inflammation may take a long time to resolve, sometimes up to weeks until the scrotum is detached (Stafford and Mellor, 2010). Hence based on the different inflammatory processes elicited by different techniques of routine husbandry procedures, it is important to understand further the underlying mechanisms involved, so that pain alleviation can be best addressed.
Whilst the provision of analgesics is recommended as part of the standard husbandry practice, it incurs cost particularly to farmers with large numbers of animals as extra labour is also needed to administer the drugs effectively (Stewart et al., 2014). Husbandry procedures involving surgical technique that include mulesing, castration, tail docking and dehorning are traditionally performed without provision of analgesia (Sheil, 2008). Treatments for pain relief that are commercially available and practical to use in farm animals are still limited (Bayley et al., 2003; Lomax et al., 2009). Although there have been studies investigating practical alternatives to administer pain relief on farm animals such as via topical (Paull et al., 2009; Lomax et al., 2010) or oral administrations (Faulkner and Weary, 2000), there is still a demand in research to devise reliable quantitative measurements of pain, and to determine optimal doses of pain relief required (Stewart et al., 2014). The provision of pain relief may be impractical unless the presence of pain in animals is detected accurately.

1.6 Objective indicators of pain and stress in animals

It is important in ruminants that the pain and stress assessment tools are easily used in field conditions, particularly on commercial farms (Prunier et al., 2013). Some of the physiological and behavioural indicators of distress in ruminants and other species from various literature are presented in Table 1.3. For the purpose of this thesis, the parameters used for the assessment of pain and stress are divided into two broad categories, which are conventional and contemporary parameters. The conventional parameters are well established in identifying the presence of pain and stress in ruminants and have been used for decades. The contemporary markers on the other hand are more commonly used in humans and other animal species studies, but are still not well documented in ruminants.
Table 1.3 Examples of physiological and behavioural indices of distress responses to noxious stimuli in ruminants and other species.

<table>
<thead>
<tr>
<th>Physiological indices</th>
<th>Blood hormones</th>
<th>Adrenaline</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Noradrenaline</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Corticotrophin-releasing factor</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glucocorticoid (e.g. cortisol)</td>
</tr>
<tr>
<td>Blood metabolites</td>
<td>Glucose</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lactic acid</td>
<td></td>
</tr>
<tr>
<td>Inflammatory</td>
<td>Haptoglobin, fibrinogen</td>
<td></td>
</tr>
<tr>
<td>response (in blood)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other variables</td>
<td>Heart rate</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Packed cell volume</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Skin, eye or internal body temperature</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Electroencephalogram (EEG)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Behavioural indices</th>
<th>Vocalisation</th>
<th>Number and duration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intensity</td>
<td></td>
</tr>
<tr>
<td>Posture</td>
<td>Crouches, hiding, lying (legs extended, all or some legs tucked in), standing</td>
<td></td>
</tr>
<tr>
<td>Locomotion</td>
<td>Reluctant to move, stagsfers, falls, stands up/lies down repeatedly, pacing, restless, escape and avoidance</td>
<td></td>
</tr>
<tr>
<td>Temperament</td>
<td>Withdrawn, depressed, aggressive, docile</td>
<td></td>
</tr>
</tbody>
</table>

Adapted from Mellor et al. (2000) and Prunier and Leterrier (2014).

1.6.1 Conventional indicators of pain and stress in ruminants

1.6.1.1 Recording of behavioural changes

Recording of behavioural changes as a tool in assessing pain has its advantages. The collection of data is immediate, regardless of whether the manifestation of pain is rapid or of slow onset (Mellor et al., 2000). According to Morton and Griffiths (1985), the study of behavioural changes and patterns in animals should be a part of pain assessment. It was suggested that behavioural changes in response to pain and stress are more readily assessed in field conditions, and are considered as closely related to the welfare of the animal (Fell and Shutt, 1989; Prunier et al., 2013).

According to Molony and Kent (1997), there are several types of pain-like behavioural response that can be recognised in animals: “1) those that modify the animal’s behaviour that enables the
animal to avoid recurrence of the experience; 2) automatic responses that protect the parts or the whole body (e.g. withdrawal reflexes); 3) those that minimise pain and assist in healing (e.g. lying or standing still); and 4) those that elicit help or to stop another animal (including humans) from inflicting more pain (e.g. communication via vocalisation)”. Animals express their experience of pain in different forms of behaviour such as escape reactions, vocalisation, aggression, restlessness, abnormal gait or posture, and reluctance to move (Gregory, 2004). These behavioural changes have been used as indicators in assessing animal pain in farm animal welfare (Mellor et al., 2000; Taylor and Weary, 2000; Grant, 2004). Assessing these behavioural changes is still considered reliable because they are overt responses to pain and are easily recognised (Seksel, 2007).

Behavioural recording may have been a popular pain assessment method in animals but it has its limitations. It must be carefully interpreted to avoid misinterpreting the behavioural changes from signs of fear and/or stress, which are not specifically a pain response (Anil et al., 2002; Mellor et al., 2008). Since behavioural recording can be anthropomorphic, it also depends on the experience of the assessors who may underestimate or overestimate the pain-like behaviour observed (Stafford and Mellor, 2007; Tuyttens et al., 2014). It has been reported that factors such as the gender and age of the assessor also influence the accuracy of the behavioural assessment of pain as different assessors may described the behaviour as manifestations of different degrees of pain (Stafford and Mellor, 2007; Laven et al., 2009; Millman, 2013).

It is also worth considering that most domesticated livestock animal species such as ruminants are prey animals. It is a survival advantage for the prey animal to not display any abnormal behaviour, which might attract a predator’s attention, hence adding difficulties when assessing pain response in these animals (Dobromylskyy et al., 2000; Fitzpatrick et al., 2006). Sheep for example, are gregarious animals (Arnold, 1985), and it is a common behaviour that they mimic the behaviour of their fellow herd or pen mates, therefore making it challenging to identify pain-related behaviour in individual animals (Guesgen et al., 2014). This behaviour is also observed in cattle, in which calves that were castrated by rubber bands displayed pain-related behaviour in the absence of humans, but would run to their herd mates when a human walked up to their pen (Grandin and Johnson, 2005). Lack of acclimatisation of animals with the handler also leads to difficulties in differentiating the behaviour represents pain or fear (Millman, 2013). Ruminants may also perceive their owners or the farmers as predators, and therefore may not manifest their experience of pain in their behaviour in the presence of humans (Federation of Animal Science Societies, 2010). Hence, behavioural changes may be considered as a substantial obstacle for pain assessment, as the absence of visual signs of pain does not indicate that the animal is not experiencing pain (Anil et al., 2002;
Underwood, 2002). Therefore these findings have led to the demand in developing other objective tools of pain assessment that include biomarker indices, particularly in livestock animals.

1.6.1.2 Cortisol

According to Mellor et al. (2000), the changes in plasma cortisol are a good indicator for acute distress. However, it does not specifically represent whether the distress is pain-induced because the activation of HPA axis can be an overall response to both emotional and physical noxious experiences (Mellor et al., 2000). A study by King et al. (1991) showed that the cortisol response of 78 day-old calves weaned from their dams and castrated using Burdizzo or surgery was similar to control calves. The cortisol concentration peaked at 3 hours (h) and declined by 12 h post-castration, however there was no significant difference between treatments. A similar finding was also observed in another surgical castration study in cattle, in which the elevation in cortisol concentrations did not differ from the control animals, which were only handled (Coetzee et al., 2008). Increases in cortisol are also associated with psychological stressors such as weaning (Kim et al., 2011), isolation (Rivalland et al., 2007; Tilbrook et al., 2008) and transportation (Qiu et al., 2007; Kostro et al., 2009; Giannetto et al., 2011). It has been suggested by Stafford and Mellor (2005) that isolation and restraint are emotionally noxious experiences to the animals. Even though cortisol has been used extensively as an assessment parameter for distress in husbandry and clinical practices, the lack of specificity in response makes it debatable to use cortisol as a singular parameter to assess pain.

1.6.1.3 Catecholamines

The response of the autonomic nervous system (ANS) has been used to assess farm animal pain. Parrot et al. (1994) and Lefcourt and Elsasser (1995) recommended that assessment of physiological changes due to activation of the sympathetic-adrenomedullary system, such as heart rate and catecholamine plasma concentrations (adrenaline and noradrenaline), are suitable in assessing the response at the early stages of distress. Catecholamines regulate the ‘flight and fight’ response which offers more information on fear and immediate pain compared with the HPA axis hormones, which give a much slower response (Stewart et al., 2010).

In cattle studies, increases in adrenaline concentrations were observed following branding (Lay Jr et al., 1992), weaning isolation (Lefcourt and Elsasser, 1995), ring castration and dehorning (Mellor et al., 2002). Rialland et al. (2014) examined the cerebrospinal fluid noradrenaline levels in dairy cows subjected to chronic pain caused by surgically induced traumatic reticuloperitonitis and no significant changes were observed. Plasma catecholamine concentrations are difficult to measure
because of the rapid change in response and its short plasma half-life (1 to 2 minutes) (Hjemdahl, 1993). Therefore, autonomic activity consequential to catecholamine release is measured instead, such as heart rate (HR), HR variability, eye pupil diameter, skin resistance, and peripheral blood flow (Stewart et al., 2010). Changes in eye temperature using an infrared thermography (IRT) device have also been used to assess stress and pain in cattle (Stewart et al., 2008). Changes in eye temperature are due to vasoconstriction of the capillaries mediated by sympathetic nervous system tone, which causes a decrease in temperature. However, a study by Church et al. (2014) found that environmental factors such as wind speed, distance of camera to subject and solar loading influenced the IRT measurement in cattle. Hence the applicability of using IRT in field conditions still requires validation. It was suggested by Stubjsjoen et al. (2009) that the technique has less significance in sheep in response to pain caused by ischaemic stimulus of forelimb tourniquet. However, they claimed that their study was the first to use IRT in sheep to monitor the presence of pain. Although IRT is non-invasive to the animals, further studies on the usage of this technique as an alternative pain assessment tool are still required.

1.6.1.4 Acute phase proteins

According to Gruys et al. (1999), the acute phase response (APR) refers to a systemic response of an organism towards infection, tissue injury, trauma, neoplastic growth or immunological disorders that intrudes its homeostasis. The systemic response is most often characterised by alterations in the levels of various serum proteins commonly referred as acute phase proteins (APPs) (Heinrich et al., 1990). The common major acute phase proteins in ruminants are haptoglobin (Hp) and serum amyloid A (SAA) which have been used as diagnostic tools in various pathological conditions (Eckersall and Bell, 2010; Ceciliani et al., 2012).

The early signs of clinical inflammation are commonly associated with the appearance of APPs in blood and tissue fluids, which only start to increase 4 h after injury (Cheville, 1999; Carroll et al., 2009). These changes in the blood remain until the underlying inflammatory process starts to resolve (Toussaint et al., 1995). According to Heinrich (1990) and Gabay and Kushner (1999), the serum levels of the APPs rise markedly within the 24 to 48 h period after the initial injury and the level is dependent on the severity of the injury. In a less complicated injury, the serum levels will normally decline in the following 48 h and return to baseline level, 72 to 96 h after injury. However, in cases of chronic diseases or sepsis, the serum level of the APPs remains elevated. Under these circumstances, the serum level of these proteins are said to be of diagnostic or prognostic value (Thompson et al., 1992). In surgical trauma, the APR is expected to control the tissue damage, kill the infective organisms, and induce repair processes to restore the normal body function (Sheeran
and Hall, 1997). In an experimental surgery in sheep, Hp increased rapidly within 24 h post-surgery and returned to pre-surgical concentrations within 7 days (Abu-Serriah et al., 2007). Changes in Hp was also measured to monitor the inflammatory response in castration (Paull et al., 2009) or tail docking in lambs (Price and Nolan, 2001) and castration in cattle (Ballou et al., 2013; Petherick et al., 2014b). Inflammatory states are important in generating pain, hence it has been suggested that the elevation of APP can be used as an indirect indicator of pain (Prunier et al., 2013).

Increase in Hp and SAA due to weaning had also been observed in Holstein calves (Kim et al., 2011). A report by Giannetto et al. (2011) demonstrated that road transportation had significantly increased Hp and SAA in cattle. Murata (2007) proposed a hypothesis of the stress-APP linkage which is based on the concept of the neuroendocrine-immune network (Figure 1.2). Interleukin-6 in particular, is a potent stimulating cytokine that induces the expression of APP in hepatocytes and also, by working with other cytokines, enhances the expression of APP which increase the peripheral APP levels in stressed animals (Kurash et al., 2004). Glucocorticoids and growth factors can mediate the APP release from hepatocytes by modulating the activity of the cytokines (Ceciliani et al., 2002). Glucocorticoids can also potentially induce the expression of APP directly from the hepatocytes (Kurash et al., 2004). Therefore it is important to note that a rise in APP is also indicative of psychological stress, and evaluation of this biomarker must be carefully interpreted, as it may be deduced as an indicator of pain, although inflammation might be absent.
Figure 1.2 Hypothesis for the induction of acute phase protein response in stressed animals. Adapted from Murata (2007).
1.6.1.5 Beta-endorphin

Beta-endorphin (β-EP) is a hormonal-like neuropeptide synthesised by its precursor proopiomelanocortin (POMC) and released during pathological conditions (Heijnen et al., 1991; Przewlocki et al., 1992; Sprouse-Blum et al., 2010). Proopiomelanocortin is mainly located within the anterior pituitary gland, primarily regulated by corticotrophin-releasing factor (CRF) (Heijnen et al., 1991) and cleaved β-EP and adrenocorticotrophin hormone (ACTH) after being stimulated. Beta-endorphin and ACTH are also found within the cells of the immune system such as lymphocytes, monocytes and macrophages (Smith et al., 1990; Rittner et al., 2005).

Beta-endorphin is considered as one of the opioid peptides and is substantially involved in pain (Stein et al., 1990; Schäfer et al., 1994). There have been several reviews on peripheral opioids in animals and humans contributing to pain inhibition following tissue injury (Khoury et al., 1992; Stein and Lang, 2009). Opioid neuropeptides affect pain regulation by binding to the Delta- and Mu-opioid receptors (Herz, 1997). The peptides activate the receptors on the peripheral terminal of sensory neurons causing a decrease in the neurons’ excitability and release of pro-inflammatory neuropeptides followed by pain inhibition (Yaksh, 1988; Aimone and Yaksh, 1989; Stein, 1995).

Several reports addressing animal welfare concerns in husbandry procedures have used changes in the β-EP response as one of their assessment tools (Smith et al., 1986b; Jephcott et al., 1987; Shutt et al., 1987; Shutt et al., 1988b; Anil et al., 1990; Mears and Brown, 1997). It has been demonstrated that tissue injury caused by painful surgical husbandry procedures, such as castration and tail docking, exert acute increase in β-EP in lambs (Shutt et al., 1987; Mears and Brown, 1997). According to Mears and Brown (1997) psychological stressors such as isolation and weaning have limited effects on β-EP. Hashizume et al. (1994) reported that 1 h of isolation stress transiently increased the β-EP response in adult sheep. However in another study, it was found that shearing and electroimmobilisation caused significant increase in β-EP in sheep (Jephcott et al., 1987). Amir et al. (1980) discussed the role of β-EP in stress in humans and it was proposed that β-EP modulates the neural systems that elaborate and express the emotional components of stress. It was suggested that β-EP indirectly regulates the stress-induced production of adrenal steroids by manipulation the ACTH release from the pituitary gland. Notwithstanding there are studies that have measured β-EP response in sheep as reported by Mears and Brown (1997) and Shutt et al. (1987). However, within the past two decades, there are no recent studies that look further into the response in β-EP as an indicator of pain caused by routine husbandry procedures.
1.6.2 Contemporary quantitative markers of pain and stress

1.6.2.1 Cytokines

Cytokines are a group of low-molecular-weight proteins produced from activated leukocytes, fibroblasts and endothelial cells (Zellweger et al., 2003). Cytokines bind to their target cell receptors and exert their actions through autocrine, paracrine and endocrine systems, which also cause the release of other cytokines from the cells (Cunha and Ferreira, 2003). The release of cytokines indicates an early response to tissue injury, and plays a significant role in initiating and mediating the immune response and inflammation (Desborough, 2000; Gragnani et al., 2013). Cytokines modulate local inflammation through cell-to-cell communication and also cause systemic inflammation by gaining access to the circulation (van Miert, 1995).

Cytokines have been used in assessing disease and stress responses in humans, dogs and mice, especially in the alteration of T-helper (Th) cell differentiation and activity (Fonfara et al., 2007). Following tissue injury, Th 1 cells produce pro-inflammatory cytokines such as interleukin (IL) -1, IL-2, interferon gamma and IL-12, which promote cell-mediated immunity. Th 2 cells, which include IL-4, IL-5, IL-10 and IL-13, produce anti-inflammatory cytokines that inhibit cell-mediated responses and promote humoral immunity (Elenkov and Chrousos, 1999; Fonfara et al., 2007). Interleukin-6 is released by both Th1 and Th2 cells (Lin et al., 2000). Infiltrating leukocytes that produce cytokines which contribute to inflammation and hyperalgesia are also a part of pain manifestation in inflammation (Cunha and Ferreira, 2003). Following an inflammation, local cells first release cytokines and produce the initial stage of acute hyperalgesia, and migrating cells (neutrophils, lymphocytes) further add to the intensity and extend the duration of the hyperalgesia (Cunha and Ferreira, 2003). The release of cytokines leads to the induction of inflammatory mediators (e.g. prostaglandins, neuropeptides SP and CGRP), and together cause sensitisation of the primary afferent neurons which result in inflammatory pain (Cunha and Ferreira, 2003).

Cytokines that participate primarily in acute inflammation are tumour necrosis factor alpha (TNF-α), IL-1, IL-6, and interferon-gamma (IFN-γ) which induce and sustain inflammation (Cheville, 1999). The IL-6 and IL-1 cytokines are considered as the major regulators of the hepatic release of acute phase proteins during inflammation (Bode et al., 2012). In a study on knockout mice, it was demonstrated that APP production relies on multiple players, in which the IL-6 and IL-1 type cytokines are said to be the chief regulators (Quinton et al., 2009). It was suggested that the regulation of APP production as well as the cytokines involved depends mostly on the nature of the inflammatory stimulus and species (Bode et al., 2012). A study by Baigrie et al (1992) showed that
IL-1β always precedes the IL-6 response in a major surgical injury (elective aortic surgery in humans), providing evidence that IL-1β induces IL-6 synthesis and release (Shalaby et al., 1989; Tosato and Jones, 1990).

1.6.2.1.1 Interleukin-1

The IL-1 family originally consisted of three ligands, which are IL-1α and IL-1β as well as IL-1 receptor antagonist (IL-1Ra), and has now expanded to 11 members (Dinarello, 2011). Its actions include induction of T-cell activation, B-cell proliferation, as well as alterations in APP production (Dinarello, 1984; Bode et al., 2012). Interleukin-1β in particular has been studied mostly, due to its active role in inflammatory diseases in humans (Dinarello, 2009, 2011) and induction of cyclooxygenase-2 (COX-2) and prostaglandin E2 (PGE2) synthesis (Schweizer et al., 1988; Cunha and Ferreira, 2003). Interleukin-1 is also one of the immune system substances that activate the HPA system through the stimulation of CRF (Berkenbosch et al., 1987; Uehara et al., 1987; Suda et al., 1990). Interleukin-1β-immunoreactive neurons have been reported to exist in the hippocampus and hypothalamus (Breder et al., 1988; Lechan et al., 1990), regions that play important roles in releasing ACTH and CRF hormones, suggesting that IL-1β-containing neurons are involved in stress response (Minami et al., 1991).

In pain perception, IL-1β is produced by the glial cells located in the dorsal horn of the spinal cord as the afferent point of hyperalgesia (Watkins and Maier, 2000; Cunha and Ferreira, 2003). Administration of IL-1 or TNF-α antagonists prevent hyperalgesia induced by either cell walls of bacteria following infection or the pro-inflammatory cytokines themselves (Watkins et al., 1995). In burn injuries in human patients, it was found that IL-1 increased in response to thermal injury (Gragnani et al., 2013). Microglia were responsible for producing IL-1β as well as IL-6 in spinal cord injury in humans as early as 30 minutes following injury (Yang et al., 2004).

In ruminants, IL-1 has been mostly studied in relation to infection. Enhanced expression of IL-1β had been reported in studies of pneumonia in cattle following *Manhaemia haemolytica* infection (Yoo et al., 1995; Morsey et al., 1999; Ackermann and Brogden, 2000) and in goat *in vivo* epithelial cells due to mastitis (Ru et al., 2015). Islam et al. (2013) had measured serum levels of IL-1 as a diagnostic tool for endometritis and postpartum reproduction diseases in cows. The expression of IL-1 and other inflammatory cytokines from circulating leukocytes (Pang et al., 2011) and local scrotal tissues, the epididymis, testis and scrotal skin (Pang et al., 2009) has been studied in banding and Burdizzo castration in cattle. In these studies, there were no significant expression changes observed in the peripheral leukocytes compared with the local tissues indicating that both castration
techniques did not induce severe systemic inflammation but a localised inflammatory response. Some studies have found strong correlation between circulating concentration of cytokines detected using ELISA with quantification of their mRNA expression using real-time PCR (Blaschke et al., 2000; Hein et al., 2001). To date, no other studies have been conducted to determine the plasma levels of IL-1 following husbandry procedures that involved tissue injury.

1.6.2.1.2 Interleukin-6

Interleukin-6 is produced by immune and immune accessory cells (e.g. monocytes, macrophages, lymphocytes, endothelial cells, astrocytes) and also non-immune cells and organs (e.g. osteoblasts, keratinocytes, chondrocytes) (Papanicolaou et al., 1998). In certain situations IL-6 may act as a hormone, inducing production of APPs from hepatocytes (Liuzzi et al., 2005), and regulating secretion of hormones from the hypothalamus, pituitary and adrenal glands (Salas et al., 1990; Yasin et al., 1994). According to Papanicolaou (1996), IL-6 production and/or secretion is also stimulated by the sympathetic nervous system through two possible ways. The first is in a paracrine manner, which is through noradrenaline release at the sympathetic nerve terminals that innervate the immune organs containing IL-6 production cells. The second is in an endocrine manner, through the adrenal medulla as well as distant effects of this hormone on IL-6 producing cells.

Interleukin-6 concentrations in the circulation are normally low and undetectable. However within 30-60 minutes after surgery in humans, the concentration of IL-6 in the blood starts to increase and becomes significant after 2 to 4 h (Desborough, 2000). As mentioned earlier, cytokine production reflects the degree or severity of tissue trauma, and it was observed that the least invasive surgery such as laparoscopic surgery in humans caused minimal release of cytokines (Cruickshank et al., 1990; Gebhard et al., 2000). Other major surgeries such as joint replacements cause the highest increase in IL-6 which reaches maximal concentration in the circulation at about 24 h, and remains elevated for 48 to 72 h post-operatively (Desborough, 2000). This suggests that the magnitude of the IL-6 response reflects the degree of tissue damage which has occurred (Burton et al., 2004). A study by Baigrie et al. (1992) on elective aortic surgery in humans showed that an increase in IL-6 correlated with post-operative complications (minor complications: atelectasis, atrial fibrillation, mild congestive cardiac failure; severe complications: chronic obstructive airway disease, hypotensive shock, renal failure). It was concluded that the IL-6 response not only preceded the clinical signs by 12-36 h, but also showed a significantly greater and longer response in patients with post-operative complications compared with the group with lesser complications (Baigrie et al., 1992; Roumen et al., 1993; Svoboda et al., 1994). It was suggested that IL-6 plays a crucial role in regulating leukocyte infiltration, angiogenesis and collagen accumulation, which are important in
wound healing (Mast and Schultz, 1996; Behm et al., 2012). A study on skin wounds in mice showed that animals with a lack of IL-6 gene expression displayed a delay in wound healing even as long as 10 days after injury (Lin et al., 2003).

Due to the active role of IL-6 in inflammation, IL-6 had also been associated with the modulation of pain (De Jongh et al., 2003). Besides IL-1, peripheral hyperalgesia can also be induced by IL-6 (Cunha and Ferreira, 2003). Study on rats observed that injection of IL-6 in hind paws caused hyperalgesia in both of the hind paws, and local pre-treatment with indomethacin, a non-steroidal drug (NSAID) reduced the hyperalgesia (Cunha et al., 1992). Localised pain in tissue injury caused by surgery is presumably correlated with the increase in IL-6 in the wound area, which may have sustained the pain sensitisation (Holzheimer and Steinmetz, 2000). The correlation of tissue injury severity with plasma IL-6 had also been associated with the intensity of postoperative pain (Cruickshank et al., 1990). It was mentioned that IL-6 is actively involved in causing chronic pain such as rheumatoid arthritis in humans (De Jongh et al., 2003; Scheller et al., 2011). It was shown that IL-6 that binds to soluble IL-6 receptor (sIL-6R) is involved in modulating chronic pain that leads to the activation of immune system by recruiting monocytes to the inflamed site (Scheller et al., 2011).

Increased IL-6 concentration has been correlated with viral and bacterial infections in ruminants (van Miert, 1995; Coussens, 2004; Vordermeier et al., 2012) and used as a prognostic tool for postpartum reproductive diseases in cattle (Ishikawa et al., 2004). A study by Pang et al. (2011) had shown that expression of IL-6 mRNA from leukocytes was upregulated in cattle that were castrated using Burdizzo compared with banding at 24 h following castration. However, they did not examine the plasma protein concentrations of the IL-6. No other studies have looked further into measuring IL-6 in correlation with pain in ruminants.

1.6.2.2 Substance P

Substance P is a neuropeptide and a member of the tachykinin family (Nakanishi, 1987). Substance P consists of 11 amino acids and is a prototypic neuropeptide for more than 50 neuroactive molecules, which include CGRP, neuropeptide Y, and endothelin (Onuoha and Alpar, 1999; Carrasco and Van de Kar, 2003). The preferential receptor for SP is the neurokinin 1 (NK1) receptor (Caberlotto et al., 2003) and both SP and NK1 can be found in various regions of the peripheral and central nervous systems (Devane, 2001). Substance P can be detected in areas that are involved in the integration of pain, stress and anxiety within the central neuro-axis (Devane, 2001; Ebner and Singewald, 2006). In the pain pathway, SP acts as a neurotransmitter from the
primary afferent neurons that mediate pain signals (Otsuka, 1977; Szreniawski et al., 1980). Following tissue injury, the afferent neurons produce SP along with CGRP, which then mediates neurogenic inflammation (Lagerström et al., 2011; Wall et al., 2013). Substance P causes increased capillary permeability while CGRP causes vasodilation, hence contributing to plasma extravasation and edema (Wall et al., 2013). It can persist in the extracellular fluid for a relatively long period of time, which enables it to diffuse into areas and act on targets which are distant from the release site (Landgraf and Neumann, 2004). A study in humans had found that SP is not only produced and released by the neural system, but also from cells such as neutrophils and macrophages (Ho et al., 1997).

Plasma concentration of SP had been used in human cases to evaluate muscle pain, spinal cord disease, soft tissue injury, and the efficacy of therapeutic agents for cases of osteoarthritis, headache and fibromyalgia (Onuoha and Alpar, 2001; Shen et al., 2006). Coetzee et al. (2008) conducted a study to evaluate the response of SP and cortisol following castration in cattle. It was found that non-castrated calves had a more uniform SP response and it was suggested that this is because the animals were not exposed to a nociceptive stimulus. The response to the castration procedure involved the determination of vocalisation and behaviour scores of 0 to 3, with score of 3 representing the most painful response. The cortisol response did not correlate with the vocalisation score, whereas SP concentrations were significantly higher for a vocalisation score of 3 compared with a score of 0. The authors suggested that the significant increase in plasma SP concentration might be associated with nociception. A study by Whitlock et al. (2012) reported that following electroejaculation (EEJ) in Angus bulls, plasma SP was not found to be different from the control animals, while marked cortisol response and vocalisation intensity were evident. It was interpreted that there was a lack of pain response related to nociception caused by the EEJ procedure, therefore concluding it to be an acute stressful event for the bulls. Plasma SP may have the potential in assessing pain accurately in ruminants, however the role of SP in sheep in relation with pain has yet to be examined.

1.6.2.3 Prostaglandins

Prostaglandins are prostanoids, which are formed when arachidonic acids are released from the plasma membrane by phospholipases, and metabolised mainly by the actions of the enzyme cyclooxygenase (Murakami et al., 2000; Ricciotti and FitzGerald, 2011). Cyclooxygenase-2 is expressed from macrophages, fibroblasts, vascular endothelial and smooth muscle cells by cytokines, endotoxins, growth factors or tumour promoters, and the expression corresponds with the production of the prostaglandin E₂ (PGE₂) (Smith and Langenbach, 2001). The COX-2 enzyme is
upregulated at the site of injury and in the spinal cord in inflammatory pain (Hay et al., 1997; Samad et al., 2001) and also in damaged nerves in neuropathic pain (Murakami et al., 2000; Sommer and Kress, 2004). Prostaglandin E$_2$ is a potent pain and inflammatory mediator (Trebin et al., 2003) by causing increase in vascular permeability, vasodilation and the formation of wheals, sensitivity to pain receptors and increase chemotaxis (Higgs et al., 1975; Funk, 2001). It also has a rapid turnover, in which it is degraded and eliminated from the tissues and the circulation by 15-hydroxyprostaglandin dehydrogenase (15-PGDH) (Tai et al., 2002).

Measurement of PGE$_2$ had been well documented in human traumatic and surgical injuries. It has been demonstrated that thermal injuries cause the release of large amount of prostaglandins (Artuson, 1977, 1979; Heggers et al., 1980). A report on hip replacement surgery showed an upregulation of PGE$_2$ and IL-6 in the cerebrospinal fluid and in surgical site drainage fluid post-operation (Buvanendran et al., 2006). In a caesarean study, it was observed that plasma PGE$_2$ level remained high until 6 h following delivery (Carvalho et al., 2008).

The roles of anti-inflammatory agents such as aspirin and NSAIDs are to block the actions of these COX enzymes on the prostanoids, preventing PGE from forming an inflammatory response (Yuan et al., 2009). Reports have shown that COX inhibitors reduce pain-associated behaviours in rats (Ma and Eisenach, 2003a, 2003b). The use of NSAIDs in castration has also been studied in cattle (Ting et al., 2003; Petherick et al., 2014b) and sheep (Price and Nolan, 2001; Paull et al., 2012). Some of these reports in ruminant castration studies mainly observed the reduction of pain response following NSAID administration as evidenced by reduced in cortisol concentrations and pain-related behaviours, but did not observe the changes in PGE$_2$. A recent study on cattle undergoing surgical dehorning had demonstrated an increase in plasma PGE$_2$ which was reduced significantly after treatment with flunixin (Fraccaro et al., 2013). Therefore, measurement of PGE$_2$ has the potential to be applied as one of the parameters in the assessment of pain caused by routine husbandry procedures.

1.7 Thesis hypotheses and aims

1.7.1 Summary

Certain husbandry procedures are important for efficient livestock management. However it is acknowledged that some of these procedures are painful to the animals. These procedures have become a concern for animal welfare organisations and the broader community. Numerous studies have been conducted addressing these welfare concerns and involve the use and validation of
various objective measures that could be applied to determine the noxiousness of the procedures for the animals.

Husbandry procedures involving physical insults, such as castration and tail docking, involve multiple responses, such as inflammatory, nociception and HPA axis responses, to maintain homeostasis. Cortisol and behavioural changes are common examples of objective assessments that had been used to evaluate noxiousness due to painful husbandry procedures. These measures have also been used to determine the efficacy of anaesthetics and analgesics to ameliorate the painful experience. However, there are still controversies and conflicting opinions on whether the changes in the parameters observed are a direct reflection of pain. Some studies have shown that there were no significant differences in the cortisol response observed between animals experiencing painful physical insults and those that were only handled and restrained. Behavioural changes are immediate responses that are readily assessable, however the assessment of pain in prey animals such as ruminants can be difficult, as they would not display abnormal behaviour in the presence of predators. Furthermore, behavioural assessment requires skilled and knowledgeable observers to evaluate the various forms of abnormal behaviours that are indicative of pain.

Most researchers working in the area of livestock pain agree that the assessment of pain and stress in animals requires the use of several parameters to define the presence and degree of noxiousness experienced. However there are still difficulties in developing a suitable assessment tool that can be used with confidence, taking into consideration each parameters’ specificities as well as practicality and feasibility of use in field conditions. Hence, based on the literature review, this research aims to formulate and develop a reliable ‘toolkit’ to assess the implications of husbandry procedures on the welfare of animals. This ‘toolkit’ comprises of a combination of objective markers from various biosystems, including those that are conventionally used in ruminants, and contemporarily used in various species.

1.7.2 General aims of the investigation

This study was generated to achieve two general aims:

1. To critically evaluate the biomarkers conventionally used to quantify the response of an animal to a noxious experience, particularly those markers used to evaluate painful husbandry procedures in production animals.
2. To refine a multi-parameter ‘toolkit’ that enhances the ability to measure ruminants’ responses to purportedly painful husbandry procedures.
To accomplish these general aims, there were three specific aims:

1. To refine and formulate a multi-parameter ‘toolkit’ combining the conventional and contemporary circulating markers, including behavioural changes, using a sheep surgical castration model.
2. To determine the cellular expression of selected markers in circulating leukocytes and to compare their responses with the circulating plasma levels.
3. To examine the role of systemic pro-inflammatory cytokines in relation with inflammation that contributes to pain and stress and the progress of wound healing in a cattle castration model, and comparing the outcomes in two different castration methods, surgical and tension banding.

1.7.3 General hypotheses

Based on the aims stated, the hypotheses generated were that:

A standard practice to evaluate animal responses to painful husbandry procedures should incorporate one or more parameters representative of the physiological and behavioural responses to the noxious experience elicited. Critical evaluation of conventional and contemporary parameters, and proof-of-concept studies to assess novel parameters will allow the production of a multi-parameter system for reliable assessment of the responses of a ruminant to painful stimuli of routine husbandry procedures.

1.7.4 Structure of thesis

To address the aims described above, this thesis is structured as follows:

Husbandry procedures that are reported as being painful generally involve direct physical insults such as in castration, dehorning and tail docking. Among the various methods used in husbandry practices, surgical castration has been viewed as one of the most painful procedure as this procedure causes the most acute response compared with other castration techniques. Aim 1 is designed to formulate a reliable multi-parameter ‘toolkit’ by analysing biomarkers that have been used conventionally as well as contemporary biomarkers to assess pain caused by a routine husbandry procedure. It is hypothesised that surgical castration would be a suitable model that generates a substantial and immediate response of the biomarkers of interest. Chapter 2 addresses Aim 1 by measuring various plasma biomarkers determined from the literature review i.e. cortisol, Hp, IL-6, SP, β-EP and PGE₂ as well as behavioural recording in a sheep surgical castration model.
Pang et al. (2011) investigated the expression of mRNA of various cytokines from circulating leukocytes in response to different cattle castration techniques (banding and Burdizzo). It was found that there was significant difference in IL-6 response between the techniques, however overall, the techniques used did not significantly affect the peripheral leukocyte inflammatory cytokine gene expression levels. Nevertheless the surgical technique was not used in their study, and it is hypothesised that the technique would produce a pronounced acute inflammatory response compared with banding or Burdizzo. Furthermore POMC, the precursor of β-EP, is also expressed from the leukocytes in the immune response of inflammation as an endogenous analgesia to inhibit pain. **Chapter 3** investigates the expression of IL-6 and POMC mRNA from the leukocytes of lambs that were surgically castrated in the same experiment described in Chapter 2. This investigation is to address **Aim 2** by examining the relationship between the expression of IL-6 and β-EP at the cellular level with their proteins synthesised in the circulation to determine whether the plasma levels measured were produced by the circulating leukocytes.

In a cattle castration study conducted by Petherick et al. (2014a, 2014b), it was found that castration using tension banding caused a delayed response in Hp compared with surgical castration and the responses were also correlated with the progression of wound healing. Review of the literature reveals that IL-1 and IL-6 stimulates the release of APP from the liver to restore the normal body functions following tissue injury. **Chapter 4** addresses **Aim 3** by measuring the circulating IL-1 and IL-6 in cattle castration using different methods, and to correlate the plasma level of these cytokines in relation with acute and chronic inflammation that may contribute to the progression of pain in the animals.
Chapter 2

Assessment of pain, stress and inflammatory plasma biomarkers in lambs in response to surgical castration
2.1 Introduction

Numerous studies have been conducted to evaluate painful routine animal husbandry procedures. These often compare surgical versus non-surgical procedures (Fell et al., 1986; Shutt et al., 1988a; Cohen et al., 1990; Robertson et al., 1994; Prunier et al., 2006). In general, there is an agreement that surgical methods are more painful, with this conclusion being based on the acute and overt changes in both behavioural and physiological responses. Surgical castration in lambs for example, has been suggested to be more painful than other methods such as rubber ring application (Mellor and Stafford, 2000; Paull et al., 2009). Due to the welfare concerns that surgical castration causes greater pain compared with other methods of castration, the Farm Animal Welfare Council (FAWC) of the United Kingdom has suggested that surgical castration in sheep should be banned, unless it is conducted by a registered veterinarian who provides pharmacological pain relief (FAWC, 2008).

Pain caused by routine husbandry procedures must be adequately assessed before appropriate pain management can be applied (Grant, 2004). A standard method of assessing pain in animals is behavioural recording. The main advantage of behavioural recording is the immediacy of assessment, especially in field conditions (Fell and Shutt, 1989), compared with physiological markers which are usually measured and quantified after the event (Mellor et al., 2000). Behaviour such as statue standing, abnormal ventral lying, vocalisation and restlessness have all been associated with pain from surgical castration in sheep (Lomax et al., 2010). However, whilst different behaviours might infer differences in pain intensity, overall behavioural assessment does not accurately discriminate the degree of pain experienced (Stafford, 2013). The lack of homogeneity and reproducibility in behaviour across animals are most likely due to individual animal variability in pain tolerance and/or the inability of the observer to discriminate pain-related behaviour accurately (Mellor et al., 2000; Prunier et al., 2013). Therefore behavioural recording, although informative and necessary, needs to be complemented by other more specific pain assessments.

Tissue injury in surgical procedures causes changes in neuronal, endocrinological, immunological and haematological parameters (Scholl et al., 2012). The integration of such physiological responses is often called the ‘surgical stress response’ and is thought to be proportional to the degree of the injury (Desborough, 2000). Surgical castration in ruminants is often associated with significant increases in the stress hormone cortisol (Melches et al., 2007; Bonelli et al., 2008; Paull et al., 2009), and haptoglobin (Hp) as the major acute phase protein in ruminants (Paull et al., 2009; Petherick et al., 2014b). However despite robust cortisol and Hp responses to surgical trauma, significant changes in these biomarkers can also be associated with psychological stressors, such as
weaning (Kim et al., 2011), isolation (Rivalland et al., 2007) and transportation (Qiu et al., 2007; Kostro et al., 2009; Giannetto et al., 2011). Thus it is often difficult to clarify whether cortisol and Hp responses are specifically related to the pain of surgical castration per se, or possibly more generalised stress responses. Consequently, the measurement and validation of more specific pain and inflammatory biomarkers arising from surgical procedures is needed.

Pain caused by tissue injury is initiated by the activation of afferent sensory neurons, signalling via the dorsal root ganglion of the spinal cord, through the brain stem and thalamus to the cerebral cortex where the stimulus is perceived as ‘pain’ (Price and Géranton, 2009). The activated afferent sensory neurons also release neuropeptides locally, like substance P (SP) and calcitonin gene-related peptide that generate neurogenic inflammation within the injured area (Lembeck and Holzer, 1979; Stanisz, 2001; Lagerström et al., 2011; Wall et al., 2013). Increased local and circulating SP concentrations have been observed in human patients undergoing surgery (Sjöström et al., 1988; Onuoha and Alpar, 2001; Carvalho et al., 2008). Coetzee et al. (2008) observed an increase in plasma SP concentrations in surgically castrated cattle, suggesting SP may be a potential biomarker of pain in routine ruminant husbandry procedures. Tissue injury also enhances the production of endogenous opioid neuropeptides that bind to the opioid receptors on activated nerve terminals, to attenuate the pain sensation (Machelska et al., 2003; Stein and Lang, 2009). One of the opioid neuropeptides, beta-endorphin (β-EP) is derived from the precursor polypeptide proopiomelanocortin (POMC), that also yields adrenocorticotropic hormone (ACTH) (Sprouse-Blum et al., 2010). Beta-endorphin is mainly synthesised in the anterior pituitary gland (Guillemin et al., 1977; Heijnen et al., 1991) although it is also expressed in immune cells such as lymphocytes, monocytes and macrophages (Jessop, 1998; Rittner et al., 2005). Both β-EP and ACTH release are stimulated by hypothalamic corticotrophin-releasing factor in response to physiological and psychological stressors (Lariviere and Melzack, 2000; Slominski et al., 2000; Yao and Denver, 2007; Sprouse-Blum et al., 2010). An acute increase in circulating β-EP has been observed in sheep that were castrated and tail docked surgically (Shutt et al., 1987). Therefore, measurement of circulating neuropeptides such as SP and β-EP may reflect pain responses in routine animal husbandry procedures. However, since tissue damage results in an inflammatory response, which also contributes to neuropeptide responses (Stein and Machelska, 2011), assessment of pain sensitisation and inflammation would seem appropriate for the assessment of the overall pain response.

In tissue injury, cytokines and prostaglandins are important mediators of both local and systemic inflammatory responses (Desborough, 2000). Pro-inflammatory cytokines such as interleukin-1 (IL-
1), tumour necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) are released locally from resident immune cells such as macrophages and monocytes primarily to initiate the inflammation, and to recruit other circulating immune cells to the injury site. These cytokines also exert systemic effects, in particular stimulating acute phase protein responses (Baigrie et al., 1992; Sheeran and Hall, 1997). In humans, an increase in circulating IL-6 levels correlates with the severity of tissue damage during surgical procedures and traumatic injury (Cruickshank et al., 1990; Maruszynski and Pojda, 1995; Gebhard et al., 2000; Carvalho et al., 2008). Further complications such as the development of sepsis are associated with increased IL-6 levels, suggesting IL-6 is a good prognostic indicator of inflammation (Biffl et al., 1996; Kimura et al., 2006). Although numerous studies have been done in humans, those investigating the use of IL-6 as an inflammatory marker in animals, specifically in ruminants, are limited. Tissue injury also causes the release of prostaglandins, which mediate inflammatory processes such as vasodilatation and erythema (Funk, 2001; Cunha and Ferreira, 2003; Trebino et al., 2003). Prostaglandins are generated from arachidonic acid, by the action of cyclooxygenase (COX) enzymes during inflammation (Ricciotti and FitzGerald, 2011). In humans, patients undergoing hip surgery exhibit increased levels of prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) and these are reduced by the administration of non-steroidal anti-inflammatory drugs (NSAID), which are COX-2 inhibitors (Buvanendran et al., 2006). Cyclooxygenase-2 inhibitors have also been used in ruminants undergoing surgical castration and animals receiving these inhibitors generally show a reduction in pain-related behaviours (Price and Nolan, 2001; Ting et al., 2003; Paull et al., 2012). However to date, no ruminant study has measured a direct effect of surgical pain on PGE\textsubscript{2} responses.

From a welfare perspective, it is necessary to define an objective, specific and reproducible marker of pain in animals caused by routine surgical husbandry procedures. This is required to determine appropriate pain management during these procedures. The objective of the present study was to verify and validate a combination of circulating biomarkers, namely plasma cortisol, Hp, β-EP, IL-6, SP and PGE\textsubscript{2} concentrations, by measuring these biomarkers in conjunction with behavioural assessment in lambs following surgical castration. In addition to the expected increases in the classical biomarkers, cortisol and Hp, it was also hypothesised that the changes in the other circulating biomarkers may also occur as a result of them being more specific to the pain and inflammation associated with surgical castration.
2.2 Materials and methods

2.2.1 Animals and treatments

The use of animals in this study was approved by The University of Queensland Production and Companion Animals Ethics Committee (AEC approval number SVS/507/12/ACA). Twelve merino-cross male lambs aged between 10 and 12 weeks and weighing 26.6 ± 4.5 kg (mean ± SE) were used and had been weaned at 8 weeks of age. The lambs were housed in the University of Queensland School of Veterinary Science Clinical Studies Centre (CSC) and were kept in 16m² pens with hay bedding. The lambs were grouped into three per pen to minimize isolation stress, and they were allocated into each pen according to their body weight. All animals were vaccinated upon arrival at CSC against Cheesy Gland (caseous lymphadenitis) and clostridial diseases (tetanus, blackleg, black disease, malignant oedema, and pulpy kidney) using a 6-in-1 vaccine (Glanvac® 6, Pfizer, Australia).

2.2.2 Acclimatisation

Lambs were initially grazed on pasture but were acclimatised to the CSC environment for one week prior to the commencement of the study. During this acclimatisation period and until completion of the experiment, the animals were fed a standard diet of 50% cottonseed meal with 50% lucerne chaff. Both feed and water were provided ad libitum. Lambs were handled on a daily basis in order to familiarise them to close human contact. Lambs were randomly assigned to either surgical castration (n=6) or sham castration, i.e. ‘restraint’ as control (n=6).

At 48 hours (h) prior to treatment, each lamb was catheterised via the jugular vein to facilitate the intensive blood sampling. The lamb was gently restrained on a sheep-restraining cradle, whilst the head was held up and away from the vein to be used. The wool over the jugular area was clipped and the skin was prepared aseptically with a chlorhexidine-soaked swab. A small amount of topical anaesthetic cream containing 2.5% lidocaine and 2.5% prilocaine (EMLA®, AstraZeneca, Wilmington, Delaware, USA) was then applied to the skin. After 10 minutes (min) of applying the cream, a small bleb of lignocaine hydrochloride (2%) was introduced into the subcutis overlying the vein. The catheter (14 gauge, 3.25 inch BD Angiocath™, BD Australia) was placed into the jugular vein and sampling port with tap was then attached. The catheter and sampling port were sutured to the skin using 2-0 non-absorbable suture material. The placement of the catheter procedure took approximately 20 to 25 min for each lamb.
2.2.3 Surgical castration and sham restraint treatment

Surgical castration was conducted by two operators. One operator gently restrained the lamb in a sheep-restraining cradle, while the other prepared the scrotal skin aseptically with a chlorhexidine-soaked swab and then, using a surgical knife, incised the left lateral distal one-third of the scrotum, exposing the left testis. The connective tissue between the spermatic cords was separated by blunt dissection. Then, an emasculator (Hauptner, Solingen, Germany) was placed around the spermatic cord proximal to the testis, and the spermatic cord distal to the emasculator was resected and the testis was removed. After approximately one min of application, the emasculator was released. Another incision was then made on the right lateral scrotum, and the same technique was applied to remove the other testis. Sham castration was conducted by restraining the lamb on the same cradle whilst the testes and scrotum were manipulated and spermatic cord massaged within the scrotum to emulate the ‘handling’ of the surgical castration. Both surgical and sham castration techniques were conducted within three to five min, once the lambs were restrained on the cradle. Following the procedures, lambs were placed in a trolley and returned to their pen within five min.

2.2.4 Blood sampling

Blood samples were taken at 24 h prior to (-24 h) and immediately before treatment (time 0), and 10 min, 20 min, 30 min, 45 min, 1 h, 2 h, 8 h and 12 h post-treatment via the jugular catheter. The catheter was removed after sampling on day 1 post-treatment. Blood samples were further taken daily on days 2, 3, 4, 5, 6 and 7 post-treatment via venepuncture (23 gauge, 1¼ inch, BD Australia).

Blood samples were collected in EDTA vacutainers containing 10.6 TIU/mL blood aprotinin (A1153, Sigma-Aldrich, Australia) and lithium heparin vacutainers. The vacutainers were immediately centrifuged at 2,140 x g for 10 min at 4°C for plasma separation. After centrifugation, plasma samples from EDTA vacutainers were kept at -80°C for SP, PGE₂ and β-EP assays, while plasma from lithium heparin vacutainers were kept at -20°C for cortisol, Hp and IL-6 assays. Whole blood was also collected into acid citrate dextrose anticoagulant for leukocyte expression of IL-6 and β-EP mRNA, which are described in Chapter 3.

2.2.5 Laboratory analysis

Samples for cortisol and β-EP were assayed using RIA; IL-6, SP and PGE₂ using ELISA; and Hp using calorimetric assay. Samples that were collected in EDTA were extracted using a solid phase extraction method before they were assayed for β-EP, SP and PGE₂.
2.2.5.1 Sample extraction using solid phase extraction method

Samples for SP, β-EP, and PGE$_2$ assay were firstly extracted using a solid phase extraction method to yield purified analytes. The solid phase extraction method used was modified and optimised based on the combination of procedures recommended in SP ELISA kit (ADI-901-018 Ann Arbor, Michigan, USA) and β-EP RIA kit (RK-022-06, Phoenix Pharmaceuticals, Burlingame, California, USA) for maximum assay measurements according to sample volume and laboratory conditions.

Plasma samples (volumes 75 to 200 µL) were transferred to 1.5 mL microcentrifuge tubes and acidified by addition of 500 µL volume of 1.0% trifluoroacetic acid (TFA) (302031, Sigma-Aldrich, Australia) in distilled water. The acidified samples were then vortexed and centrifuged at 13,100 x g for 20 min at 4°C. Concurrently, a 24-port vacuum manifold was used as a platform for the solid-phase extraction Strata C-18E columns (RK-Sepcol-1, Phenomenex®, Torrance, California, USA). The columns were equilibrated with 1 mL of high-performance liquid chromatography-grade acetonitrile and 1% aqueous TFA (60:40), followed by washing with 10 mL of 1% aqueous TFA under slight pressure. The columns were then loaded with the acidified samples, and washed with 3 mL of 1% aqueous TFA without pressure. The peptide-containing fraction was collected by elution with 2 or 3 mL of acetonitrile in 1% of aqueous TFA (60:40). The eluents were then evaporated to dryness using a vacuum centrifuge at 48°C for 15-17 h. The dried extracted samples were then rehydrated and dissolved appropriately in assay buffer from each assay kit. After addition of the assay buffer, the samples were left at room temperature for 1-2 h with occasional vortexing. Samples that were not immediately used for the assay were stored at -20°C.

2.2.5.2 Cortisol

Plasma cortisol concentrations were measured using a commercial RIA (IM1841, Beckman Coulter, Roissy CDG, France) according to the manufacturer’s protocol. Briefly, 50 µL of standards, quality control or test samples were added to assay tubes coated with an anti-cortisol monoclonal antibody. Then, 500 µL of $^{125}$I-labeled cortisol tracer in buffer with bovine serum albumin (0.1%) and sodium azide (<0.1%) was added to each tube. The assay tubes were vortexed before incubation at room temperature for 30 min. Tubes were decanted to remove all liquid and then counted for 1 min on a 2470 WIZARD® automatic gamma-counter (PerkinElmer, Massachusetts, USA). The antibody used is highly specific for cortisol with low cross reactivities to naturally occurring steroids such as aldosterone (<0.1%), cortisone (1.5%), progesterone (1.8%) and corticosterone (8.4%). The sensitivity of the kit was 0.8 ng/mL. For a quality control of 51.3 ng/mL, the intra-assay and inter-assay coefficient of variations (CV) were 3.4 and 6.3% respectively.
2.2.5.3 Haptoglobin

Plasma Haptoglobin (Hp) was measured using a bovine specific colorimetric assay (PHASE Haptoglobin assay, Tridelta Development Ltd, Maynooth, Co. Kildare, Ireland) on 96-well ELISA plates (224-0096, Bio-Rad Laboratories, Gladesville, New South Wales, Australia). Briefly, 7.5 µL of the standards, quality control or test samples were added to a blank microplate. Then, 100 µL of Reagent 1, stabilised haemoglobin, was added and the plates were tapped to ensure mixing before adding Reagent 2, chromogen reagent, to each well. After incubating for 5 min at room temperature, the absorbance was immediately read at 630 nm using an absorbance microplate reader (Tecan Sunrise™ Absorbance Reader). The sensitivity of the kit was 0.01 mg/mL. The intra-assay and inter-assay coefficient variations (CVs) of sample containing 0.96 mg/mL Hp was 3.0% and 7.0% respectively.

2.2.5.4 Interleukin-6

Plasma IL-6 concentrations were determined using a commercial ELISA kit (ESS0029, Thermo Scientific, Rockford, Illinois, USA) on 96-well ELISA plates (224-0096, Bio-Rad Laboratories, Gladesville, New South Wales, Australia), according to the manufacturer’s protocol. Briefly, the wells were first coated with 100 µL of anti-bovine IL-6 coating antibody in carbonate-bicarbonate buffer, pH 9.4 (1:100) into each well and incubated overnight (12-16 h) at room temperature before being aspirated. Then, 300 µL of the blocking buffer, containing 4% bovine serum albumin (BSA) and 5% sucrose in Dulbecco’s phosphate buffered saline (D-PBS) was added and was incubated for 1 h. After the aspiration of the blocking buffer, 100 µL of standards, quality control or test samples were added to the wells. The plate was incubated for 90 min on a plate shaker at 750 rpm, and was then aspirated and washed three times with 0.05% Tween-20 detergent in D-PBS (pH 7.4). Then, 100 µL of anti-bovine IL-6 detection antibody in 4% of BSA in D-PBS (1:100) was then added and the plates were left to incubate for another 1 h. The detection antibody was aspirated and the plates were washed three times before adding streptavidin-HRP to each well. The plates were incubated for 30 min, aspirated and washed with wash buffer. Substrate solution (3,3’5,5’-tetramethylbenzidine) was then added and incubated in the dark for 20 min. The reactions were stopped by adding 0.16 M sulfuric acid to each well. All incubation steps were done at room temperature. The absorbance was measured at A450 minus A550. The assay sensitivity was 10 pg/mL. The intra-assay and inter-assay CVs using sample containing 523.7 pg/mL of IL-6 were 10.7% and 9.8% respectively.
2.2.5.5 Substance P

Plasma SP concentrations were determined using a commercial ELISA (ADI-901-018, Assay Designs Inc, Ann Arbor, Michigan, USA) according to the manufacturer’s specifications. Briefly, 50 µL of standards, quality control or appropriate rehydrated test samples were added into wells coated with goat anti-rabbit IgG. Then 50 µL of alkaline phosphatase conjugated with SP, followed by 50 µL of antibody solution (rabbit polyclonal antibody to SP) were added to the wells. After 2 h of incubation at room temperature with shaking at 500 rpm, the contents were discarded and the wells were washed 3 times with 400 µL of wash buffer (Tris buffered saline containing 0.6% Tris hydrochloride and 0.1% of Tris base) for each wash. Then, 200 µL of p-nitrophenyl phosphate (pNpp) substrate was added into every well. Following a further 1 h incubation, the reaction was stopped by adding 50 µL of stop solution (10% of trisodium phosphate in water). The absorbance was measured immediately at A405 minus A580 using a microplate absorbance reader (Tecan Sunrise, Tecan Inc, Mannedorf, Switzerland). The antibody in this ELISA is reported to be highly specific for SP, with low cross-reactivity to other related compounds such as somatostatin (<0.001%), α-Neurokinin (0.8%) and β-Neurokinin (0.2%). The assay sensitivity was 10 pg/mL. For a quality control of 191 pg/mL, the intra-assay and inter-assay CVs were 14.7% and 34% respectively.

2.2.5.6 Prostaglandin E2

Plasma PGE2 concentrations were determined using a commercial ELISA (ADI-901-001, Assay Designs Inc, Ann Arbor, Michigan, USA) according to the manufacturer’s protocol. Briefly, 100 µL of standards, quality control or appropriate rehydrated samples were added into wells coated with goat anti-mouse IgG. After adding 50 µL of the conjugate solution (alkaline phosphatase conjugated with PGE2), 50 µL of the antibody solution containing a monoclonal antibody to PGE2 was added into each well, and was incubated for 2 h at room temperature on a plate shaker (500 rpm). The contents were then discarded, and the plate was washed 3 times with 400 µL of wash buffer for each wash, followed by addition of 200 µL of the pNpp solution to every well. After incubation for 1 h at room temperature, the reaction was stopped using the stop solution, and the absorbance was measured immediately at A405 minus A580 using the microplate absorbance reader. The content of the wash buffer, pNpp and stop solutions were the same as described for SP assay. The antibody used in this ELISA is highly specific for PGE2 with low cross-reactivity to other related eicosanoid compounds such as arachidonic acid (<0.1%) and prostaglandin A2 (0.2%). The assay sensitivity was 10 pg/mL. For a quality control of 282.5 pg/mL, the inter-assay and intra-assay CV were 11.5% and 13.3% respectively.
2.2.5.7 Beta-endorphin

Plasma concentrations of \( \beta \)-EP were measured using a commercial RIA (RK-022-06, Phoenix Pharmaceuticals, Burlingame, California, USA) according to the manufacturer’s protocol. Briefly, 100 \( \mu \)L of standards, positive control or appropriate rehydrated test samples was added into polystyrene tubes. Then 100 \( \mu \)L of the primary antibody (rabbit polyclonal anti-\( \beta \)-EP serum) was added into each tube. The contents were then vortexed and sealed for incubation at 4\(^{\circ}\)C for 16-24 h. After incubation, 100 \( \mu \)L of \( ^{125} \)I tracer solution was added and vortexed. Following a further 16-24 h incubation at 4\(^{\circ}\)C, 100 \( \mu \)L of the secondary antibody (goat polyclonal anti-rabbit IgG serum) and 100 \( \mu \)L of normal rabbit serum were both added. Tubes were then vortexed and incubated at room temperature for 90 min. Finally, 500 \( \mu \)L of the assay buffer was added to the tubes, followed by centrifugation at 1,700 x g for 40 min at 4\(^{\circ}\)C. Tubes were decanted to remove all liquid and were counted for 1 min using a 2470 WIZARD\(^{\text{2}}\) automatic gamma-counter. The antibody in this RIA is highly specific for \( \beta \)-EP, with no cross-reactivity to other related peptides such as met-enkephalin and human ACTH. The sensitivity of the kit was 10 pg/mL. For a quality control of 205.9 pg/mL, the inter-assay and intra-assay CV were 18.3\% and 12.2\% respectively.

2.2.6 Behavioural observations

The behaviours of the lambs were recorded continuously using video cameras that were positioned above each pen. Within the first 2 h of treatment, the lambs displayed fear behaviour by huddling at one corner of the pen due to the presence of humans taking blood samples. Therefore, behavioural recordings that were used for analysis began 3 h post-castration. Two broad categories of postures were recorded, which were standing or recumbent. The postures within each category were further classified into postures based on the common and successful criteria used by other studies of acute pain in lambs during husbandry procedures, as described by Dinniss et al. (1999); Molony et al. (2002) and Grant (2004). The postures were recorded for 180 seconds at an interval of 10 min for a total of 120 min period. Each animal was observed twelve times (i.e. twelve time points), and the amount of time spent by an animal in each posture was recorded. The total numbers of animals that displayed each posture for each group were determined. The sampling time points for each individual animal were randomly selected before the observer begins to record for postures. The camera that was used to record the behavioural activities in one pen malfunctioned during the day of treatment, therefore the animals (two surgically castrated and one sham castrated) were not included for analysis. No observations were taken when humans were present in the pen at any time point of data collection. Further evaluation of the video revealed that there were a number of missed behavioural recordings for analysis as some of the animals were outside of the camera viewing area.
during the observation time points. Therefore, the number of animals and the time spent for each posture from all sampling time points within each treatment group were summed, and the proportion of animals and the time spent for each posture in each group was calculated. The description of the postures recorded is described in **Table 2.1**.
Table 2.1 Descriptions of postures recorded.

<table>
<thead>
<tr>
<th>Category</th>
<th>Posture</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standing</td>
<td>Normal standing (S)</td>
<td>Lamb was standing, eating or walking and was still alert and responsive to surrounding stimuli such as sound and interaction with other lambs within the same pen.</td>
</tr>
<tr>
<td></td>
<td>Statue standing (SS1)</td>
<td>Lamb was standing motionlessly and unresponsive to its surrounding stimuli for more than 10 s. The head was up or immediately in front of the body, and the hind limbs were splayed apart.</td>
</tr>
<tr>
<td></td>
<td>Statue standing (SS2)</td>
<td>Similar to SS1 but with the head down and obvious arching of the back, hind limbs splayed apart and extended out posteriorly such that the root of the tail was anterior to the hind hooves.</td>
</tr>
<tr>
<td>Recumbent</td>
<td>Ventral recumbency (V)</td>
<td>Lamb was lying on its sternum and abdomen with both front and rear legs tucked in with no visibility of the limbs, and the head was positioned in front of the body or down.</td>
</tr>
<tr>
<td></td>
<td>Ventro-lateral recumbency (VL)</td>
<td>Lamb was lying with its front limbs tucked under the sternum but one or both of its hind limbs were extended to one side. The head was positioned in front of the body or down.</td>
</tr>
</tbody>
</table>

Adapted from Dinniss et al. (1999), Molony et al. (2002) and Grant (2004).
2.2.7 Statistical analysis

Data were analysed using statistical software GraphPad Prism (Version 6.0, GraphPad Software Inc., San Diego, California, USA). Two-way analysis of variance (ANOVA) with repeated-measures design was used to observe for treatment, time and treatment by time interaction effects. Where significant time and/or treatment by time interaction were observed, two further analyses were used. An unpaired t-test was used to examine the effect of treatment at each time point, and a one-way ANOVA with a post-hoc Dunnett’s multiple comparison test was used to examine the effect of time within a treatment. Results are expressed as mean ± standard error of the mean (± SEM) concentrations. Due to high variability of values observed prior to treatment in IL-6, PGE$_2$ and SP, the results for these biomarkers are expressed as mean percentage change concentrations from pre-treatment values. The data for SP were log transformed to remove the heterogeneity of variance.

For behavioural recordings, a nonparametric chi-square test was used to compare the difference of proportion of time spent in the described postures, between treatment groups.

For both biomarkers and behavioural data analysis, P values of less than 0.05 were considered as statistically significant.
2.3 Results

2.3.1 Blood parameters

2.3.1.1 Cortisol

Prior to treatment, mean plasma cortisol concentrations in all animals were 17.9 ± 0.3 ng/mL. Mean cortisol concentrations in surgically castrated animals were significantly ($F_{15, 150} = 3.027; P<0.01$) increased after castration, peaked at 10-20 min (approximately 3-fold increase) and were significantly ($P<0.05$) higher than control animals from 10 min to 2 h (Figure 2.1). Cortisol concentrations in castrated animals returned to pre-treatment values by 8 h and remained low through to day 7. Although there was approximately 2-fold increase in control animals at 10-20 min, this elevation was not significant.

![Cortisol concentrations graph](image)

**Figure 2.1** Plasma cortisol concentrations (mean ± SEM) in lambs (n=6 lambs/group) in surgically castrated (black circles) or sham castrated (white triangles) group. The asterisk denotes significant ($P<0.05$) difference between groups at each time point. Different superscripts denote a significant (a $P<0.01$, b $P<0.05$) difference in concentrations compared with pre-treatment values (time 0) within the surgically castrated group.
2.3.1.2 Haptoglobin

The mean pre-treatment Hp concentrations in all animals were 0.9 ± 0.1 mg/mL. The Hp concentrations increased significantly ($F_{8,80} = 3.292; P<0.01$) in surgically castrated animals starting one day after castration, and were 5-fold higher than pre-treatment values by day 2 (Figure 2.2). Mean concentrations were significantly ($P<0.05$) greater in castrated than the control sheep from day 2 to day 4. The Hp concentrations in control animals also increased, however this elevation was not significant.

![Figure 2.2](image)

**Figure 2.2** Plasma haptoglobin concentrations (mean ± SEM) in lambs (n=6 lambs/group) in surgically castrated (black circles) or sham castration (white triangles) group. The asterisk denotes significant ($P<0.05$) difference between groups at each time point. Different superscripts denote a significant (a $P<0.01$, b $P<0.05$) difference in concentrations compared with pre-treatment values (time 0) within the surgically castrated group.
2.3.1.3 Interleukin-6

Prior to treatment, the plasma IL-6 concentrations of most animals ranged from 20 to 657 pg/mL, although two animals had higher baseline values of 1856 pg/mL (control group) and 20,168 pg/mL (surgically castrated group). Further diagnosis revealed that the outlier animal in the surgically castrated group was positive for regenerative anaemia. Therefore, the two animals were identified as outliers and were omitted from the final analysis.

The mean IL-6 concentrations increased significantly ($F_{9, 72} = 3.445; P<0.01$) in surgically castrated animals over time, peaked at 5 days after castration (Figure 2.3) and were significantly ($P<0.05$) higher than control animals at day 2, and day 4 onwards, albeit there was high variability between animals in the castrated group.

![Figure 2.3](image)

**Figure 2.3** Percentage change of plasma interleukin-6 concentrations from pre-treatment values (mean ± SEM) in lambs (n=5 lambs/group) in surgically castrated (black circles) or sham castrated (white triangles) group, after excluding two animals with high concentrations prior to treatment. The asterisk denotes significant ($P<0.05$) difference of mean percentage change between treatments at each time point. Superscript denotes a significant ($b P<0.05$) difference of percentage change compared with pre-treatment values (time 0) within surgically castrated group.
2.3.1.4 Substance P

Overall, plasma SP concentrations in surgically castrated animals increased significantly ($F_{7, 70} = 2.645; P<0.05$) over time, starting from 30 min post-castration (Figure 2.4). However, due to high variability in SP responses in the castrated group, concentrations were only significantly (P<0.05) higher compared with control animals at 8h post-castration after the data were log transformed.

Figure 2.4 Percentage change of plasma substance P concentrations from pre-treatment values (mean ± SEM) in lambs (n=6 lambs/group) in surgically castrated (black circles) or sham castration (white triangles) group. The asterisk denotes significant (P<0.05) difference of mean percentage change between treatments at each time point.
2.3.1.5 Prostaglandin E$_2$

No significant treatment or time by treatment interaction in PGE$_2$ concentrations was noted (Figure 2.5).

**Figure 2.5** Percentage change of plasma prostaglandin E$_2$ concentrations from pre-treatment values (mean ± SEM) in lambs (n=6 lambs/group) in surgically castrated (black circles) or sham castration (white triangles) group. Percentage change of prostaglandin E$_2$ concentrations did not differ significantly between treatments.
2.3.1.6 Beta-endorphin

Overall, there were no significant treatment effects or changes over time in plasma β-EP concentrations between surgically castrated and sham castrated animals (Figure 2.6). Although there was a slight elevation of β-EP at 10 min and at day 2 in castrated animals, these observations were not significant.

Figure 2.6 Plasma beta-endorphin concentrations (mean ± SEM) in lambs (n=6 lambs/group) in surgically castrated (black circles) or sham castration (white triangles) group.

2.3.2 Behavioural Observations

The proportion of time spent for SS1 and SS2 were more significant (P<0.01) in surgically castrated animals, whereas V was more significant (P<0.01) in the sham castrated animals (Table 2.2). Normal standing S and VL were not significantly different between the groups.
Table 2.2 Number of lambs and the total time spent (seconds) that displayed each posture observed, and their proportion (%) to the total observation within each group. The mean and the median of each posture observed during sampling time points for both groups were also recorded.

<table>
<thead>
<tr>
<th>Posture</th>
<th>Sham castrated</th>
<th></th>
<th></th>
<th></th>
<th>Surgically castrated</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of animals (%)</td>
<td>Time</td>
<td>Median (range)</td>
<td>Number of animals (%)</td>
<td>Time</td>
<td>Median (range)</td>
<td>*P value</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Seconds (%)</td>
<td>Mean (SEM)</td>
<td></td>
<td>Seconds (%)</td>
<td>Mean (SEM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>22 (42)</td>
<td>3021 (137)</td>
<td>180</td>
<td>21 (42)</td>
<td>2450 (117)</td>
<td>140</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>SS1</td>
<td>6 (11)</td>
<td>688 (115)</td>
<td>120</td>
<td>16 (32)</td>
<td>1639 (102)</td>
<td>91</td>
<td>P&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>SS2</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0</td>
<td>4 (8)</td>
<td>660 (165)</td>
<td>180</td>
<td>P&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>18 (35)</td>
<td>2291 (127)</td>
<td>180</td>
<td>5 (10)</td>
<td>861 (172)</td>
<td>180</td>
<td>P&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>VL</td>
<td>6 (12)</td>
<td>960 (160)</td>
<td>180</td>
<td>4 (8)</td>
<td>630 (158)</td>
<td>180</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>52 (100)</td>
<td>6960 (12.7)</td>
<td>(120-180)</td>
<td>50 (100)</td>
<td>6240 (22.5)</td>
<td>(90-180)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

S = normal standing; SS1 = statue standing with head up or immediately in front of the body and hind limbs were splayed apart; SS2 = statue standing with head down, obvious arching of the back, hind limbs splayed apart and extended out posteriorly; V = ventral recumbency with all limbs tucked in; VL = ventro-lateral recumbency with at least one of the hind limbs extended to one side; SEM = standard error of the mean; NS = non significant.

*The difference of the proportion time spent between treatment groups of each posture (%) was tested using a non-parametric chi-square test. A significance of P<0.05 indicates that the proportion time spent of the posture between the groups differed significantly.
2.4 Discussion

This study was conducted to evaluate the response of pain, stress and inflammation in lambs following surgical castration. To achieve this, the changes in the concentration of selected biomarkers namely cortisol, Hp, β-EP, SP, PGE$_2$ and IL-6 over time were measured. On the first day of treatment, there was an acute increase in cortisol observed within the first 2 h following castration. The neuropeptide, SP, increased over time from 30 min and became significant at 8 h. Surgically castrated animals demonstrated a significant pain-related behaviour characterised by statue standing, SS1 and SS2, rather than ventral recumbency which was more evident in sham castrated animals. Increases in the systemic inflammatory mediators, the Hp and IL-6 were observed from day 2, in which Hp remained significant until day 4 and resolved by day 5, while IL-6 continued to increase over time. The opioid neuropeptide β-EP appeared slightly increased at 10 min, although this observation was not significant compared with the control animals. No changes in plasma PGE$_2$ concentration were detected following castration. Different systems are involved in the response of the body towards an injury, which include the immune, endocrine, and nervous systems. The integration of these systems and their actions exert both local and systemic responses. Some of these were expressed and might have exerted their actions locally, and possibly due to their short half-lives such as with β-EP and PGE$_2$, the responses were not apparent in the circulation. However, surgical castration in lambs evidently caused marked response of cortisol, SP, IL-6, and Hp indicating the stress, pain and inflammatory responses towards the painful surgical procedure.

In comparison to other studies of sheep surgical castration (Thornton and Waterman-Pearson, 1999; Bonelli et al., 2008), similar immediate cortisol responses were also observed in the current study. In the studies by Thornton and Waterman-Pearson (1999) and Bonelli et al. (2008) however, the cortisol responses were approximately twofold increased from pre-treatment values. It is well accepted that a surge in cortisol production is a sensitive indicator of the acute pain induced by surgical castration (Thornton and Waterman-Pearson, 1999; Stafford et al., 2002; Bonelli et al., 2008). The immediate cortisol increase indicated that the activation of the HPA axis was due to the rapid transmission of the afferent neurons from the site of injury to the higher centres of the nervous system (Desborough, 2000; Jan and Lowry, 2010; Scholl et al., 2012). Furthermore, repeated handling did not cause further elevation of the cortisol response, suggesting that the surgical procedure may have caused a maximum stimulation of the HPA axis. This finding is supported by the surgical castration study conducted by Lester et al. (1991) in which the cortisol levels in all lambs were the same during the acute period, although the animals were subjected to different frequency of sampling. The cortisol level would then decline, and this downregulation of cortisol
that was observed immediately after the peak concentration was the result of the effective negative feedback of cortisol on the HPA axis following an acute stress caused by the surgical tissue insult (Sapolsky et al., 1986; Matteri et al., 2000). Although the slight elevation of cortisol in the sham castrated animals might not be significant from the pre-treatment values, the concentrations were within an acute stress level, as reported by Shutt et al. (1988b), following an acute restraint stressor. Nevertheless, the marked elevation of cortisol observed in the surgically castrated lambs compared with the control was highly indicative of a real response towards the surgical procedure. Hence, it can be concluded that the cortisol response observed in the current study was an indirect indicator of the acute pain caused by the surgical castration.

The acute phase increase in Hp and IL-6 concentrations observed in the current study indicated that the surgical procedure caused an acute systemic inflammatory response. These findings were supported by Horadagoda et al. (1999) and Ceciliani et al. (2002) that Hp in ruminants is a good indicator of inflammation, and the pro-inflammatory cytokines released following a tissue insult are responsible for causing Hp release from the hepatocytes. It has also been shown that a general stress event that leads to the elevation of cortisol, could also contribute to the secretion of APP (Kurash et al., 2004). This was observed in transportation stress in sheep (Murata and Miyamoto, 1993; Kostro et al., 2009). Hence, the elevation of Hp observed could be due to the stimulation of both IL-6 and cortisol (Murata, 2007), indicating that multiple factors could have caused the increase in Hp following tissue injury. Furthermore, Hp has also been used as an indirect indicator of pain caused by inflammation (Prunier et al., 2013). Nevertheless, in the current study, Hp was only elevated until day 4, while the IL-6 levels in the castrated animals still remained elevated. In a surgical study in humans conducted by Sakamoto et al. (2003), it was suggested that the IL-6 released in the tissue injury area enters the circulation to induce the increase of systemic IL-6 level. This elevation of IL-6 could also possibly be due to the anti-inflammatory role of the cytokine in the promotion of tissue healing (Lin et al., 2003). Inflammatory pain typically decreased when the inflammation had resolved, and the wound had fully healed (Woolf, 2004). Therefore, IL-6 could be a better indicator of inflammatory pain caused by a surgical procedure compared with Hp, possibly until wound healing has resolved.

Surgical castration was enough to cause a gradual increase in SP that began at 30 min, indicating that the procedure had caused the sensitisation of a pain response. A cattle castration study conducted by Coetzee et al. (2008) also observed significant increase in SP absolute values at 45 min, compared with their control animals. The significant SP increase in the castrated animals in the present study was only observed hours later, and this significance was not detected until after the absolute values were transformed. The gradual increase in SP in the castrated lambs could have
been a result of the accumulation of SP release from the central nervous system that processed the pain perception, and also from the local wound following neurogenic inflammation (Stanisz, 2001). In tissue injury, the activation of the nociceptive sensory neurons will cause SP release within the local tissue area, leading to vasodilatation and extravasation of plasma proteins, causing oedema (Lembeck and Holzer, 1979; Louis et al., 1989). The SP released from the sensory neurons causes local pain sensitisation (Üçeyler et al., 2009). It also amplifies the inflammatory response of neighbouring innocuous tissue and neurons causing them to produce more inflammatory mediators and nociceptive neuropeptides, with the additional sympathetic nerve terminals releasing catecholamines, creating a chemical ‘sensitising soup’ of proalgesic mediators near the wound area (Kessler et al., 1992; Woolf and Ma, 2007). Therefore, the gradual increase in circulating SP could have been a result of both pain sensitisation and the inflammatory response, indicating the inflammatory pain caused by the surgical procedure.

The lack of significant response of β-EP in this study was not in agreement with other studies of lamb surgical castration by Mears and Brown (1997), and surgical castration plus tail-docking by Shutt et al. (1987) which observed a significant response of β-EP that peaked at 15 min post-castration. Although within the present study, β-EP levels of some of the castrated animals increased at 10 min, this observation was not statistically significant compared with the control animals. In the study conducted by Mears and Brown (1997), 3-week-old Suffolk lambs were used, and the animals were not weaned from their dams. Possibly being younger age, non-weaned and lack of human interaction and handling may have incremented their β-EP responses compared with the animals in the present study. Furthermore, the time of marked elevation of β-EP in all castrated animals might have been missed during the sampling period, due to its short half-life in the circulation. It has been reported that half-life values of circulating β-EP in rats and rabbits range between 2-10 min (Houghten et al., 1980). Moreover, in a study on antinociceptive actions of β-EP in rats, it was found that the leukocytes were more involved in peripheral pain inhibition compared with other sources such as pituitary and adrenal glands (Parsons et al., 1990). Thus, the possibility that the local cells and leukocytes that might have migrated to the injury area and produced endogenous opioids to inhibit the pain sensitisation and inflammation caused by the surgical castration could not be dismissed, even though signs of spill-over into circulation were not evident.

Although PGE₂ is one of the key inflammatory modulators following tissue injury, there was no difference in the plasma levels of PGE₂ detected. In a study of acute incisional pain, Carvalho et al. (2008) also did not observe any changes of circulating PGE₂ levels following caesarean delivery in humans, but found more significant levels in the surgical site exudates indicating a site-specific response. A human hip surgery study conducted by Buvanendran et al. (2006) observed significant
PGE\textsubscript{2} elevation at 1 h post-surgical incision. In a cattle dehorning study by Fraccaro et al. (2013), it was observed that the cattle that were dehorned had a gradual increase in plasma PGE\textsubscript{2} which was more evident 24 h following the painful procedure. Nevertheless, these trends of plasma PGE\textsubscript{2} response might not occur similarly in sheep following a surgical procedure. The peak level of PGE\textsubscript{2} response following castration might have been missed during sampling and therefore could have led to a negative observation. The PGE\textsubscript{2} responses could have been degraded by the enzyme 15-hydroxyprostaglandin dehydrogenase, through the normal catabolic pathway (Tai et al., 2002; Backlund et al., 2005). It has also been suggested that the physiological pH of sheep is ideal for the PGE\textsubscript{2} degradation to occur (Hensby, 1974). Nevertheless, more evidence is required to determine the reliability of measuring PGE\textsubscript{2} in sheep following a local inflammatory response caused by a tissue injury.

It was evident that surgical castration in the present study caused pain-related behaviour in lambs from the observation of statue standing. It has been established that statue standing indicates that the animal is avoiding movement to minimise stimulating the source of pain from the surgical site (Molony and Kent, 1997; Molony et al., 2002). This was supported by the increase SP levels, which could have contributed to the increase in sensitivity of the wound area, due to neurogenic inflammation. Notwithstanding the fact that all animals in the castrated group were subjected to the same procedure, some animals showed an indication of a more severe response than others, as observed in those displaying SS2, which could indicate the different pain tolerance thresholds of each animal. The V posture which is known as a normal lying behaviour (Molony et al., 2002) was more evident in the control animals, indicating that these animals were not experiencing pain. Nevertheless, the VL was not significantly different between the groups, although according to Dinniss et al. (1999), ventral recumbency with hindlimbs partially or fully extended is an indicator of an abnormal behaviour. Furthermore, Molony et al. (1993) reported that in sheep, “extension, rather than flexion, of the hind limb indicates more pain”. However, in the present study, the observation of the VL posture in the sham castrated animals suggests that the extended limbs could also indicate that the animals were stretching their limbs in their comfortable position. Although the lambs displayed fear behaviour within the first 2 h of treatment, it was not evident in the cortisol responses that the animals were stressed. Sheep are known to be gregarious animals (Arnold, 1985) and it is a common observation that the behavioural expression of pain can be buffered by the behaviour of other pen mates (Guesgen et al., 2014). This indicates that behaviour assessment can be easily confounded and is more of a subjective assessment, which could contribute to a false stress response. Hence, this further supports the justification that physiological responses must also be measured together to be more conclusive.
It is also noteworthy that the lack of marked IL-6 increase during the first day of treatment in the present study was due to the high variability of the absolute values, and the treatment effect was only observed 48 h post-castration. The evidence of high baseline levels of Hp also supports these findings, suggesting that the immune system of the lambs were already challenged prior to treatment, possibly due to the vaccination given upon about a week before the conduct of the experiment (Yun et al., 2014). Nevertheless, it is noted that this observation was not consistent in all lambs, possibly indicating the different immune status between animals. This finding is in agreement with the proposal by Gånheim et al. (2007) that Hp can be a good biomarker for herd health screening. From the findings in the current study, IL-6 could also be a potential biomarker for this purpose.

In the event of surgical castration in lambs, the pain and inflammatory responses were postulated based on the pathophysiology and biochemical reactions of the biomarkers measured. The overall outcomes of this study elucidate the importance of measuring multiple parameters representing the stress, pain and inflammatory responses, in the assessment of a painful surgical husbandry procedure. The SP response observed suggests that this biomarker is potentially more reliable than cortisol to indicate pain caused by the acute surgical insult. It is possible that from the trend of IL-6 changes observed in this study, IL-6 could be used as a better inflammatory indicator than Hp to assess the status of inflammation until wound resolution. Nevertheless, further validations are still required to support these observations, as well as the reliability in measuring biomarkers of short circulating half-life, such as β-EP and PGE$_2$.

2.5 Conclusion

Within the current study, surgical castration caused significant increase in cortisol, Hp, IL-6 and SP in lambs. Substance P being a neuropeptide responded in the early stage, and its elevation was an indicator of nociception. The IL-6 and Hp can be recommended as potential biomarkers in assessing inflammation and the associated pain caused by the surgical husbandry procedure. Although a previous study on surgical castration in lambs revealed a significant β-EP response, a similar response was not observed in the current study. There were no changes of PGE$_2$ that could be detected following surgical castration. More extensive studies are still required to refine and validate further the reliability of using these biomarkers as the assessment of pain experience caused by surgical castration.
Chapter 3

The effect of surgical castration on the expression of interleukin-6 and proopiomelanocortin mRNA by circulating leukocytes in lambs
3.1 Introduction

As previously discussed in Chapter 2, tissue insults trigger a complex interaction between the immune, neuronal and endocrine systems. Immune mediators, such as interleukin-6 (IL-6) are produced from various cells including leukocytes (e.g. neutrophils, monocytes and macrophages) to mediate the local and systemic inflammatory responses (Sakamoto et al., 1994; Petersen et al., 2004), by facilitating local hyperalgesia (Cunha and Ferreira, 2003; Schäfer, 2003; Üçeyler et al., 2009) and also enter the bloodstream and other remote tissues such as the brain causing central stimulation of stress and pain responses (Samad et al., 2001; Banks, 2005; Jawa et al., 2011). The initial increase of pro-inflammatory cytokines causes further stimulation through their paracrine actions, which in turn result in the systemic release of cytokines. The increase of cytokines in the circulation was suggested to cause the stimulation of an acute phase response from the liver (Moshage, 1997). Tissue injury also leads to the production of beta-endorphin (β-EP) from the pituitary gland and leukocytes, inducing endogenous analgesia (Stein et al., 2003; Rittner et al., 2005). Leukocytes are known to be the source of opioids that interact with the peripheral opioid receptors (Machelska and Stein, 2002). Hence, it is understood that these mediators in the circulation play an important role in the regulation of stress, inflammation and pain following tissue insults (Rittner et al., 2001; Scholz et al., 2002).

Significant levels of IL-6 (Cruickshank et al., 1990; Cuschieri et al., 2010; Jawa et al., 2011) and β-EP (Sprouse-Blum et al., 2010; Stein and Machelska, 2011) in circulation have been described in human studies using surgical models to determine the severity of the inflammation and pain associated with tissue injury. As described in Chapter 2, plasma concentrations of IL-6 and β-EP were measured to determine the associated pattern of reactions and changes, in their roles in tissue injury caused by surgical castration in lambs. From the results obtained it was observed that overall, the percentage change of plasma IL-6 significantly increased over time, and was markedly higher in the surgically castrated lambs than in the sham castrated lambs 48 h following castration, although some degree of variability was also present. The haptoglobin (Hp) levels in the castrated animals were also elevated, as IL-6 is a major stimulant of acute phase protein release. This observation was due to the stimulation by IL-6 from the leukocytes. Beta-endorphin was also measured. The surgically castrated animals had slight increases at 10 min, and at 2 h post-castration, but these were not significant. It is not possible to determine whether the source of β-EP was from the leukocytes or from other sources, in particular the anterior pituitary gland. It has been described that endogenous pain modulation correlates with the degree of immune cell recruitment from the circulation (Rittner et al., 2001; Rittner et al., 2005; Sitte et al., 2007). Hence, investigation of
leukocyte expression of such mediators by the immune cells may be examined to determine the
degree of tissue injury caused by a surgical insult.

The communication between migrating leukocytes and resident cells at the site of injury also affects
the outcome of the inflammatory episode. According to Griffis et al. (2007), the infiltration of
leukocytes is affected by the pathophysiological changes caused by nociceptive stressors. This
highlights that leukocyte trafficking is one of the important links between the local and systemic
response of target tissues during acute inflammation. In light of the above, the leukocyte expression
of key mediators, IL-6 and proopiomelanocortin (POMC), precursor of β-EP, were examined to
determine whether their expressions were involved in the response to tissue injury caused by
surgical castration in lambs. This study also aims to further validate whether the acute response of
these mediators in the plasma as observed in Chapter 2 were sourced by the peripheral leukocytes.

3.2 Materials and methods

3.2.1 The study

This study has been described in Chapter 2. Briefly, 12 merino-cross male lambs aged between 10
and 12 weeks were used in this trial and were housed in University of Queensland School of
Veterinary Science Clinical Studies Centre (CSC) with food and water provided ad libitum. The
animals were grouped into three per pen to minimize isolation stress and were handled on a daily
basis to familiarise them with close human contact. Lambs were randomly assigned to either
surgical castration (n=6) or, sham castration, i.e. ‘restraint’ only (n=6) treatment groups. At 48
hours (h) prior to treatment, a catheter was placed in the jugular vein of each animal for blood
sampling.

To perform the castration, each lamb was gently restrained in a sheep-restraining cradle. The
surgical castration was conducted by incising the distal one-third of the scrotum, exposing the testes
and incising the spermatic cords. The sham castration was conducted by restraining the lamb on the
same cradle whilst the testes and scrotum were manipulated and spermatic cord massaged within
the scrotum, to emulate the ‘handling’ that takes place during surgical castration. Both surgical and
sham castration techniques were conducted over a period not exceeding five minutes (min).

Blood samples were taken at 24 h prior to (-24 h) and immediately before treatment (time 0), and at
10 min, 20 min, 30 min, 45 min, 1 h, 2 h, 8 h, and 12 h post-treatment via the jugular catheter. The
catheter was removed after the 24 h sampling point. Further blood samples were taken daily until
day 7 following treatment via venepuncture. Whole blood was collected into acid citrate dextrose
anticoagulant at time -24h, time 0, 2 h, 8 h, day 1, day 2, day 3 and day 4 for leukocyte mRNA expression of IL-6 and POMC genes.

3.2.2 Laboratory analysis

Total blood leukocytes were isolated by hypotonic lysis and isotonicity restoration. The blood was centrifuged at 600 x g for 5 min to generate a relatively clear supernatant with a large cell pellet. The supernatant was removed and 1 mL of Red Blood Cell Lysing Buffer Hybri-Max™ containing 8.3 g/L ammonium chloride in 0.01 M Tris-Hydrochloride buffer (R7757, Sigma-Aldrich, Australia) was added and the samples were re-suspended before centrifugation for another 5 min at 600 x g, for 4-5 times. Once the white blood cell pellet was observed with a clear supernatant, the supernatant was then removed except for 100 µL containing the pellet, which was left in the tube. The separated leukocytes were then re-suspended and 1 mL of GIBCO® Hank’s Balanced Salt Solution (HBSS, 14025092, Life Technologies Australia Pty Ltd., Victoria, Australia) was added followed by centrifugation at 600 x g for 5 min. Supernatant was removed except for 100 µL containing pellet, and 500 µL of RNA later® solution (AM7021, Ambion) was then added. The samples were stored at -20°C until ready to be used for RNA extraction.

Due to the limited amount of leukocyte material purified, the RNA extraction protocol was modified and optimised according to laboratory conditions for maximum recovery for the polymerase chain reaction (PCR) analysis. Total RNA was extracted using TRIzol® Reagent (15596-026, Ambion). The samples were firstly thawed in a water bath at 37°C for 15-30 min to dissolve the RNA later® salt. An equal volume of ice cold HBSS was then added to dilute the RNA later® and centrifuged for 5 min at 4,300 x g. The supernatant layer was removed as much as possible leaving out the floating and sedimented cells in the tubes. Three hundred µL of TRIzol® Reagent was then added and was incubated at room temperature for 5 min for cell lysis to take place. The samples were then centrifuged at 14,100 x g for 10 min at 4°C. Sixty µL of chloroform was added and the sample tubes were shaken vigorously for 15 seconds (s). After incubation at room temperature for 5 min, the samples were centrifuged at 4°C for 10 min at 14,100 x g to cause phase separation. The top layer of clear aqueous phase was then carefully removed and transferred into a new 1.5 mL microcentrifuge tube. An equal volume of cold isopropanol (I0516, Sigma-Aldrich, Australia) was then added to the tube, shaken vigorously for 15 s and incubated for 10 min on ice to precipitate the RNA. The sample was centrifuged at 4°C for 30 min at full speed and the supernatant was removed carefully without touching the pellet. Then, 300 µL of 75% ethanol was added to wash the pellet removing the remaining phenol, and vortexed before centrifuged again at 14,100 x g for 15 min at 4°C. As much as possible of the supernatant was removed, leaving the
RNA pellet to be air-dried. The pellet was reconstituted with 20 µL RNase-free water (10977-015, Invitrogen™), and left to incubate for 10 min at room temperature. It was then vortexed for 1 min and followed by RNA clean up.

The RNA clean-up was done using the RNeasy® Mini Kit, following the manufacturer’s instructions with some modifications (74106, QIAGEN Pty Ltd, Chadstone, Victoria, Australia). The samples were adjusted to a volume of 100 µL by adding RNase-free water followed by 300 µL of RLT lysing buffer and 250 µL of 100% ethanol. After re-suspending a few times using the pipette, the samples were then transferred into the RNeasy® spin columns and placed in 2 mL microcentrifuge tube. The tubes were briefly centrifuged for 15 s at 8,000 x g. After discarding the flow-through, 700 µL of the RW1 wash buffer was added to the spin column, briefly centrifuged, followed by the addition of 500 µL of the RPE buffer to wash the membrane-bound RNA. The tubes were then centrifuged at 8,000 x g for 15 s, followed by the addition of another 500 µL RPE buffer with centrifugation for 2 min at 8,000 x g. The spin columns were transferred into new 2 mL tubes, and were centrifuged at 13,100 x g for 1 min to remove the ethanol residual on the column membrane. Then, 30 µL of the RNase-free water was added directly to the column membrane and was left on the bench for 10 min before collecting the eluent after centrifugation for 1 min at 8,000 x g.

Following isolation and purification, both RNA concentration and purity were determined using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA) at 260 and 280 nm. The purified RNA was then converted to first-strand complementary DNA (cDNA) using SuperScript™ III First-Strand Synthesis System (18080-051, Invitrogen™) following the manufacturer’s instructions. Each component of the kit was mixed and centrifuged briefly before use. The primer mix was made by mixing the purified RNA (up to 5 µg of total RNA), with 1 µL of 50 µM oligo(dT)20, 1 µL of deoxynucleotide triphosphates (dNTP) mix and with DEPC-treated water to make a total mix volume of 10 µL. The primer mix was vortexed before incubation for 5 min at 65°C. After incubation, the tubes containing primer mix and RNA were placed on ice for 1 min while the cDNA synthesis mix was prepared. The cDNA mix containing 2 µL of 10X Reverse Transcriptase (RT) buffer, 4 µL of 25 mM MgCl₂, 2 µL of 0.1 M dithiothreitol (DTT), 1 µL of 40 U/µL recombinant ribonuclease inhibitor (RNaseOUT™) and 1 µL of 200 U/µL SuperScript® III RT per reaction was then added to the primer mix followed by brief centrifugation. The samples were incubated for 50 min at 50°C, and 5 min at 85°C for termination and were then chilled for 1 min at 4°C. Ribonuclease H (Rnase H) was added to each reaction followed by another incubation at 37°C for 20 min. The final reaction was used for PCR or stored at -20°C until ready to use.
Quantitative real-time PCR was performed using the Rotor-Gene SYBR® Green RT-PCR kit (204074, QIAGEN Pty Ltd, Chadstone, Victoria, Australia). Published primer pairs sequence for ovine IL-6, POMC, tyrosine 3-monoxygenase/tryptophan 5-monoxygenase activation protein zeta polypeptide (YWHAZ) and succinate dehydrogenase complex subunit A (SDHA) genes were used (Table 3.1) and were synthesised by GeneWorks (Australia). The YWHAZ and SDHA genes had been described as good reference genes that are not affected by disease or heat stress in sheep blood (Peletto et al., 2011; Vorachek et al., 2013), and therefore the genes were included in all real-time PCR analyses as housekeeping genes for data normalisation. Samples containing 2 µL cDNA, 1 µM of each forward and reverse primers, and SYBR® Green PCR master mix, were added into a 100-well gene disc in triplicate using the Corbett CAS-1200 automated workstation (QIAGEN Pty Ltd, Chadstone, Victoria, Australia). The real-time PCR was performed using Rotor Gene 6000 Real-Time PCR cycler (Corbett, Sydney, Australia). Real-time PCR conditions were as follows: stage 1 of 95°C for 5 min and followed by stage 2 of 40 repeats of 95°C for 5 s and 60°C for 10 s. The quantification of the gene amplifications was determined using the Rotor-Gene 6000 Series Software 1.7. The target gene expression changes were normalised to the average of YWHAZ and SDHA genes expression. Relative gene expression was then calculated using the average Ct of animals within respective groups at -24 h as calibrator, following the $2^{-\Delta\Delta Ct}$ method described by Livak and Schmittgen (2001).
<table>
<thead>
<tr>
<th>Primer</th>
<th>5’-3’ Primer sequence</th>
<th>Reference</th>
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<tr>
<td></td>
<td>Forward</td>
<td>Reverse</td>
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<td></td>
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<tr>
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<tr>
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<td>CATCCTACATGACGGAGCA</td>
<td>ATCTTGCCATCTTCAGTTCTGCTA</td>
</tr>
<tr>
<td>IL-6</td>
<td>AGCAGACTACTTCTGACCACCTCA</td>
<td>TTTTCACACTCGTCATTCTTCTCAC</td>
</tr>
<tr>
<td>POMC</td>
<td>CCGGCAACTGCGATGAG</td>
<td>GGAAATGGCCCATGACGTACT</td>
</tr>
</tbody>
</table>
3.2.3 Statistical analysis

A total of 84 relative gene expression measurements were recorded at seven fixed time points: immediately prior to treatment (time 0), and 2 h, 8 h, day 1, day 2, day 3 and day 4 post-treatment. The data were analysed such that each time point relative to time 0 for the surgically and sham castrated groups was considered to be a single observation. Each observation comprised details of the lamb’s identity and treatment group, as well as pen allocation. The mRNA expression of each gene in question was analysed using a mixed linear model using each lamb and pen as random effects. This model was used to estimate the gene mRNA expression accounting for the repeated measurements within lambs.

Explanatory variables were initially screened using a univariable analysis and were candidates to be included in the final multivariable model if the likelihood ratio was $P \leq 0.05$. A forward and backward model building procedure was used to build the multivariable model. Candidate explanatory variables were introduced individually into the multivariable model and were retained if the likelihood ratio was $P \leq 0.05$. Excluded variables were then introduced in the final model and were retained if they were significant at an alpha level of 0.05, or if they influenced other explanatory variables estimates by a factor of 20% or greater (declared as confounders). Treatment and interaction with time of measurement were forced into the final model. An autoregressive correlation structure was applied to account for the correlation of gene mRNA expression that was measured within individual animals (Diggle et al., 2002). Model fit was assessed using Akaike’s Information Criterion (AIC). The mixed model was fitted using the restricted maximum likelihood (REML) procedure within the NLME package (Pinheiro et al., 2014) in R (R Development Core Team, 2014).

The results were described as the estimated fold change relative to the reference values. However, due to lack of homogeneity, the data were log transformed and reanalysed into the model. Initial analyses of IL-6 and POMC expression were calculated for the effect of time, treatment, and treatment by time interaction as the explanatory variables across all time points. There was no significant effect of time by treatment interaction for IL-6 expression in the surgically and sham castrated animals. To observe whether there was an effect of time if the replicates were presented at equal time intervals across all time points, the replicates for day of castration were pooled (the average of replicates for time 0, and 2 h and 8 h post-castration) within each animal were presented as day of treatment or day 0. Subsequently, the data were reanalysed again using replicates for day of treatment, and day 1, day 2, day 3 and day 4 following castration. The statistical model became more stable (from log likelihood of -118.5 to -78.3), and therefore the results were described using
this analysis for better determination effect of treatment over time on IL-6 expression. The initial analyses of the effects of time and time by treatment interaction for the expression of POMC mRNA were significant within day of treatment, in particular at 2 h post-castration. To ensure that the model was analysed using equal time intervals (subsequent observations compared with the time points analysed at day of treatment), the subsequent replicates for day 1, day 2, day 3 and day 4 post-castration were pooled and were presented as replicates greater than 8 h starting from day 1. The data were reanalysed again, and the model using the pooled data of time points greater than 8 h had higher log likelihood (from log likelihood of -74.4 to -41.1) indicating that the model was more stable compared with the initial analyses. Therefore the results were described following the analyses using time points immediately before treatment, and 2 h, 8 h and greater than 8 h post-castration.

Raw and normalised residuals were evaluated graphically against predicted values, and were presented as log10 values with 95% confidence interval (CI) to test the assumption of homogeneity of variance of the error terms, and P values of less than 0.05 were considered as statistically significant.
3.3 Results

3.3.1 Expression of interleukin-6 mRNA by circulating leukocytes

There was a significant ($F_{3,45} = 5.767; P=0.002$) effect of treatment by time interaction of surgical castration upon leukocyte expression of IL-6 (Figure 3.1). Although there were no treatment or time effects observed at time points within 24 h of treatment, high variability in IL-6 mRNA expression was observed in both groups prior to treatment (95% CIs [-0.8, 2.2] in control, and [0.2, 1.8] in castrated animals) and at 2 h (95% CIs [0.4, 2.5] in control and [0.3, 2.8] in castrated animals) following castration. This variability diminished by 8 h (95% CIs [1.7, 2.2] in control and [1.1, 2.3] in castrated animals). The overall expression in both groups then began to decline from day 1 post-treatment, in which on days 2 and 4, expression of IL-6 mRNA was significantly lower in the castrated group compared with the control group ($P=0.006$ and $P<0.001$ respectively). However, some degree of variability was also observed in both groups at day 2, and castrated group at day 4.
Figure 3.1 Best linear unbiased predictions with 95% confidence interval of log10 relative expression of interleukin-6 (IL-6) mRNA by circulating leukocytes in lambs (n=6 lambs/group) that were surgically (black circles) and sham (white triangles) castrated. The statistical significance was tested after time 0, 2 and 8 hour (h) and pooled as day 0. The asterisk denoted a significant (P<0.05) difference between treatments at each day time point, relative to pooled t=0 time point.
3.3.2 Expression of proopiomelanocortin mRNA by circulating leukocytes

There was a significant ($F_{3,33} = 4.937; P=0.006$) effect of treatment by time interaction of surgical castration upon leukocyte expression of POMC mRNA (Figure 3.2). In the surgically castrated group, the POMC expression increased slightly post-castration, however it was not significant. In contrast, it was found that POMC expression in the control animals increased significantly ($P<0.0001$) at 2 h following treatment, in which it was significantly ($P<0.01$) higher than surgically castrated animals. No further changes in both groups were detected after 8 h.

**Figure 3.2** Best linear unbiased predictions with 95% confidence interval of log10 relative expression of proopiomelanocortin (POMC) mRNA by circulating leukocytes in lambs (n=6 lambs/group) that were surgically (black circles) and sham (white triangles) castrated. The asterisk denotes significant ($P<0.05$) difference between treatments at each time point.
3.4 Discussion

In this study, the effect of surgical castration in lambs on the expression of IL-6 and POMC mRNA by circulating leukocytes was investigated over a period of 4 days. It was observed that the expression of both IL-6 and POMC mRNA changed over time. Interleukin-6 expression gradually increased within the first 8 h following treatment in both groups. However, there was no difference between the control and castrated groups, suggesting that the inflammatory response due to surgical castration did not differ significantly from handling stress. Nevertheless, the decline of IL-6 expression observed 24 h later could indicate the phase of downregulation of IL-6 mRNA. The expression of POMC mRNA started to elevate following treatment in both groups, however this elevation was more prominent in the control animals with no further changes being detected after this. Therefore, the surgical procedure might not have affected the expression of POMC mRNA by circulating leukocytes.

Surgical castration is considered to cause a higher magnitude in physiological response and is purported to be more painful than other techniques such as rubber ring application (Mellor and Stafford, 2000; Melches et al., 2007; Paull et al., 2009). However in the present study, no significant changes were observed compared with the sham castrated animals within the first 8 h of castration with respect to IL-6 expression. In a cattle castration study conducted by Pang et al. (2011), the expression of IL-6 from the circulating leukocytes in both banding and Burdizzo castrated animals at 6 h did not differ from the control animals. Although a different castration method and animal species were used in the present study, the lack of expression of IL-6 mRNA from leukocytes in surgically castrated lambs might suggest that the effect of the surgical technique on IL-6 expression was no different from the bloodless castration methods used in Pang’s study, within this time period. Furthermore, being a surgical procedure that involved immediate inflammatory response and blood loss, it was presumed that the onset of IL-6 response could have occurred earlier compared with banding or Burdizzo castration, which may involve a later onset of inflammatory response.

There have been many human studies that showed the pattern or factors that influence the IL-6 changes. Lin et al. (2000) suggested that circulating IL-6 could be detected as early as 60 min and peaking between 4 to 6 h following surgical injury. A study by Ohzato et al. (1992) found that serum IL-6 started to increase within 3 h post-abdominal surgery and peaked at 24 h. It had been suggested that the levels of IL-6 correlated with the length of time of the operation and the amount of blood loss (Ohzato et al., 1992; Sakamoto et al., 1994; Hisano et al., 1997; Sakamoto et al., 2003). Lin et al. (2000) also suggested that IL-6 correlated with the extent of tissue injury more than
the duration of the procedure. In the present study, there was a high variability in IL-6 mRNA expression found at 2 h post-treatment in both castrated and control animals, although it was more consistent by 8 h. This finding could possibly indicate that a maximal response could have been reached by this time (Lin et al., 2000). On the contrary, the circulating plasma concentration of the IL-6 protein that was described in Chapter 2 did not change within this time period. The procedure conducted in the present study was over a very short duration of approximately 5 min, and only the scrotum, testes and spermatic cords were manipulated during the procedure, compared with the human surgical studies conducted by Ohzato et al. (1992) and Sakamoto et al. (1994). Their surgical procedures involved the manipulation of vital abdominal organs and the patients would be subjected to a minimum of 1 h on the operating table. Hence the surgical stress caused by castration in lambs might not be severe enough to evoke a response in the systemic concentrations of IL-6. Psychological stress could also initiate a peripheral immune response, in particular circulating IL-6 cytokine, as observed in mice following restraint (Nukina et al., 1998) and social stress (Hodes et al., 2014). This could suggest that even though in both groups there was a gradual increase over time within at least 8 h after castration, the surgical stress inflicted was no different from the handling stress in lambs.

Following the 24 h post-injury period, the IL-6 mRNA expression in the surgically castrated animals was observed to be downregulated over time compared with the control animals with some degree of variability observed in both groups. In a normal physiological response, IL-6 exerts its action by promoting cellular responses through the signal transducers glycoprotein (gp) 130, and the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signalling pathways (Hodes et al., 2014). After binding to the IL-6 receptors (IL-6R) on the cell surface, the pathways are activated and the suppressors of cytokine signalling (SOCS) are then induced, which causes negative feedback to the STATs activation (Heinrich et al., 1998). In addition, the IL-6 together with the surface binding sites are also internalised through endocytosis upon binding to the receptors (Jones, 2005). Another possible explanation is that a depletion of circulating leukocyte numbers in the circulation had occurred, most probably through infiltration into the injured tissue, as part of the general inflammatory process (Gerhardt et al., 1994). Following inflammation, the transition of innate to acquired immunity, was also defined by the recruitment of leukocytes into the tissue (Crockett-Torabi and Ward, 1996; Lin et al., 2003). According to Topley et al. (1996), the mobilisation of neutrophils and leukocytes from the circulation into the local sites occurred within 12–24 h after the insult had taken place. In another study by Pang (2009) assessing the expression of inflammatory genes from scrotal tissues, the expression of IL-8 mRNA was higher in the epididymis and testis in Burdizzo castrated cattle compared with banding. This may suggest that
Burdizzo castration led to a greater acute inflammatory response than Band, because IL-8 enhances inflammation by enabling immune cells to migrate into tissues, and is a powerful inducer of chemotaxis for neutrophils (Baggiolini and Clark-Lewis, 1992; Pang et al., 2009). Therefore, the lack of IL-6 mRNA expression detected in the leukocytes could be due to migration of the immune cells into the local tissue during this time period. Total and differential counts of the leukocytes, if estimated in the present study, could be helpful to further validate this postulation. Overall, the normal physiological response following tissue injury could be explained by downregulation of the innate immunity and depletion of circulating leukocytes that migrated into the injury area.

Notwithstanding the leukocyte expression of IL-6 mRNA might be downregulated from 24 h post-castration, the level of plasma IL-6 was elevated over time as described in Chapter 2. This finding suggests that the production of the plasma IL-6 could have originated from other sources such as the local fibroblasts and macrophages (Marchand et al., 2005) and possibly by the leukocytes which had migrated to the injured tissue (Ueo et al., 1994; De Jongh et al., 2003; Sakamoto et al., 2003). In humans, IL-6 mRNA is constantly expressed in peripheral blood leukocytes and, at the same time, is also inducible in almost every tissue and cell type (Biffl et al., 1996; Lin et al., 2000). Another possibility is that the binding of IL-6 to the unbound soluble IL-6 receptors (sIL-6R) in the circulation could also sustain the IL-6 levels, which in turn leads to the increase in IL-6 half-life in the circulation (Biffl et al., 1996; Peters et al., 1996). Hence, in this study, the gradual increase in plasma IL-6 over time might be due to the production of IL-6 by the local cells in the damaged tissue, and the enhancement of plasma half-life by the unbound receptors in the circulation.

The expression of POMC by circulating leukocytes did not change in the surgically castrated group, which supported the minimal acute β-EP response observed in the plasma as described in Chapter 2. This also suggested that the opioid peptide could have been generated within the local tissue without further recruitment of circulating leukocytes to contain the inflammatory pain caused by the surgical castration. Besides IL-6, the neutrophils also expressed opioids to cause local antinociception (Brack and Stein, 2004). Although the expression of POMC was not quantified at earlier time points than 2 h, it was possible that the slight elevation of plasma β-EP peptides in the castrated lambs observed at 10 min was contributed by the anterior pituitary gland. In the early stages of inflammation, the β-EP produced was due to the binding of POMC to both peripheral (immune cells) and central (pituitary gland) opioid receptors (Stein et al., 1990; Machelska et al., 2003). Following inflammation, lymphocytes that were expressing β-EP migrated through the blood and lymph nodes, and were sequestered in the tissue where β-EP is subsequently secreted (Jessop, 1998). Nevertheless, in the sham castrated group, there was an increase in POMC expression over time compared with the castrated group. This was detected at 2 h post-treatment and could be the
result of the restraint stress. Some studies had found that the corticotrophin-releasing factor (CRF) could also cause the expression of POMC in human leukocytes (Smith et al., 1986a; Heijnen et al., 1991). This is supported further by the suggestion that CRF is a potent stress hormone which is produced not just in the hypothalamus, but also in immune cells (Jessop, 1998). Glucocorticoid, the end product of CRF stimulation would then cause negative feedback on CRF to reduce its response to stress (Aguilera et al., 2007). Hence, there was a possibility that cortisol could have caused the inhibition of POMC expression in the surgically castrated animals, since cortisol was significantly elevated up to 2 h following castration as described in Chapter 2. Nevertheless, the significant upregulation of POMC expression observed in the control animals and the lack of response in the castrated animals were still unexplainable.

It is also important to note that a wide variability was observed in both groups prior to treatment in the IL-6 and POMC expressions and 2 h post-treatment in the IL-6 mRNA expression. The fact that the animals were subjected to handling and restraint for at least 20 min during the jugular catheterisation 48 h prior to treatment could have contributed to the variability. Besides that, the immune status of the animals might differ and therefore they responded differently following an insult as observed at 2 h post-treatment. Breed variability might also have been a contributing factor, since Merino cross lambs were used in this study. Breeding selection is a common practice in the sheep industry, as part of developing the immune resistance towards diseases and the desired trait quality for better husbandry management such as the absence of horns (Kijas et al., 2012). It is known that genetic selection has been used in Merino breeding in Australia to achieve less wrinkled skin and to increase the bare area of the breech in Merino lambs to minimise the occurrence of flystrike (James, 2006). Furthermore, in comparison with the cattle castration study conducted by Pang et al. (2011) the significant findings of gene expressions that were observed in their Holstein-Friesian bulls were only one to threefold differences, as opposed to the present study, in which the variability was contributed by nearly 100 to 1000-fold differences between all lambs, if the data were back log-transformed. Therefore, this could indicate that the implications of individual immune background towards a stressor are more variable in ovine species, possibly due to the variation in genetic profile of the breed used.

The lack of correlation between gene expression in leukocytes and circulating protein levels could be due to the differences in the rate of expression and the post-translational process from the circulating leukocytes. Some studies have found strong correlation between circulating concentration of cytokines detected using ELISA with quantification of their mRNA expression using real-time PCR (Blaschke et al., 2000; Hein et al., 2001). However, there were still some inconsistencies in the expression of mRNA and the protein levels because of the post-transcriptional
and post-translational alteration, as well as the stability of genes used (Gygi et al., 1999; Bustin, 2002; Overbergh et al., 2003). The use of reference genes that are consistently stable in various biological conditions is one of the recommended strategies to help in minimising the discrepancies that might occur (Huggett et al., 2005). The relative expression of target genes IL-6 and POMC were calculated after their Ct threshold had been normalised to its reference genes within each sample. Although YWHAZ and SDHA are genes recommended for normalisation in sheep blood due to their stability in various diseased animals (Peletto et al., 2011; Vorachek et al., 2013), it has been reported that this may not be consistent in all conditions (Bustin, 2002). In the present study, the expression of SDHA and YWHAZ genes were low in some samples, which implied the lack of starting material or that RNA may have been lost during the RNA isolation and purification as well cDNA synthesis (Bustin, 2002). However, their target genes IL-6 and POMC were not consistently low in parallel to their reference genes, hence the accuracy in defining the upregulation or downregulation of target genes observed could have been impacted. Nevertheless, the small sample size could also have contributed to this inconsistency. Hence this in turn raised the question on the justification of determining the gene expression from leukocytes as compared with measuring their protein concentrations in assessing pain caused by surgical injury.

The findings of the present study on the expression of IL-6 and POMC mRNA could not, at this stage, offer a conclusive explanation of the roles of leukocytes in expressing these mediators following surgical castration in lambs. The lack of IL-6 and POMC expressions suggest that the tissue injury caused by the surgical procedure might not be severe enough to cause a significant response across all animals. However, the variability observed in both groups for IL-6 and POMC expressions made it difficult to make a conclusion about the lack or absence of treatment effect. Moreover, the time interval in which the peak response of mRNA expression occurred following surgical castration might have been missed in this study. In addition, the influence of breed variation on the immune response and the inclusion of only six animals per group added to the limitations of this study. Therefore, further work is still required to identify the significance of measuring IL-6 and POMC mRNA expression by leukocytes in lambs following a local surgical procedure. These outcomes in turn raised questions about whether measurements of the expression of these mediators from leukocytes would be more informative than measuring the plasma levels, particularly in the assessment of pain.
Chapter 4

The effects of surgical and banding castration on plasma interleukin-1 and interleukin-6 in relation with cortisol and haptoglobin in cattle
4.1 Introduction

The noxious experiences associated with painful husbandry procedures such as castration, tail docking and dehorning are primarily the emotional and physiological responses elicited by the tissue injuries incurred by these procedures (Mellor and Stafford, 1999). According to Kushner (1993) the initial physiological response associated with tissue injury involves the local inflammatory response and systemic metabolic changes, which are collectively termed as the ‘acute phase response’ (APR). Gruys et al. (2006) described the APR as an innate, non-specific systemic reaction to either local or systemic disturbances in homeostasis. The APR is also considered as a systemic reaction towards inflammation, and it can be associated with either acute or chronic inflammation (Ceciliani et al., 2002).

In humans, pro-inflammatory cytokines such as interleukin-1 (IL-1), tumour necrosis factor alpha (TNF-α) and interleukin-6 (IL-6), have been shown to be involved in the modulation of the inflammatory responses (Zhang and An, 2007). Local release of pro-inflammatory cytokines with other mediators (e.g. SP and bradykinin) also contributes to the sensitisation of the primary afferent neurons of the nervous system leading to inflammatory pain (Cunha and Ferreira, 2003; Zhang and An, 2007). Interleukin-1 and IL-6 are said to be the main regulators of acute phase proteins (APP) expression during inflammatory process, and IL-1 is responsible for the modification of IL-6 to induce the APP production by the liver (Bode et al., 2012). In addition, it has been reported that IL-6 is also responsible for determining the levels of plasma haptoglobin (Hp) (Gabay and Kushner, 1999). The release of APP is also mediated by glucocorticoids by modulating the activity of the cytokines (Ceciliani et al., 2002). At the site of injury, both IL-1 and IL-6 are actively involved in mediating the wound repair (Mast and Schultz, 1996; Behm et al., 2012). Interleukin-1 is important in inducing the corticotrophin releasing factor to promote the increase of leukocytes available during infection and tissue injury (Hopkins, 2003). These research findings and conclusions supported the role of IL-6 as a pleiotropic cytokine, as it produces both pro- and anti-inflammatory effects in tissue injury (Biffl et al., 1996; Diehl and Rincón, 2002). It is also responsible for transitioning the innate to adaptive immune response by recruiting the immune cells to the area of insult (Gabay, 2006).

Measurement of plasma Hp has been well established for the assessment of pain and stress in ruminants (Petersen et al., 2004; Yun et al., 2014). Increase in Hp, a major APP in ruminants (Eckersall and Bell, 2010) has consistently been associated with different methods of castration in cattle, as a measure of inflammatory response (Fisher et al., 2001; Earley and Crowe, 2002; Pang et al., 2006). Interleukin-6 has an important role in modulating the optimal acute phase response to
tissue damage or infection (Kopf et al., 1994). Increased IL-6 concentration has also been correlated with viral and bacterial infections in cattle (van Miert, 1995; Coussens, 2004; Vordermeier et al., 2012) and used as a prognostic tool for postpartum reproductive diseases (Ishikawa et al., 2004). Pang et al. (2011) found that cattle castrated using Burdizzo had higher IL-6 mRNA expression by leukocytes, and Burdizzo castrates administered with a non-steroidal anti-inflammatory drug (NSAID) had higher IL-6 expression. However, they did not examine the effect of the castration methods on the protein concentrations of these cytokines in the circulation. The relationship between plasma IL-1 and IL-6 in association with tissue injury and Hp response in cattle has yet to be determined.

In a study conducted by Petherick et al. (2014a, 2014b), the welfare impacts of banding and surgical castration in two different age groups of cattle were assessed by measuring several physiological parameters, including changes in cortisol and Hp in relation to pain, stress and wound healing. They concluded that banding castration caused more unfavourable consequences to the animals’ welfare compared with surgical castration. This conclusion was made based on the observation of chronic inflammation, which was possibly associated with the pain experienced by the banded cattle, in conjunction with delayed wound healing. In addition to the Hp response, these outcomes could be detected by monitoring the responses of IL-1 and IL-6 in their roles as the pro-inflammatory cytokines, being the immune response following tissue injury during the acute phase, as well as the progression of wound healing.

The current study was designed to measure IL-1 and IL-6 from the archived plasma samples from the Petherick et al. (2014a, 2014b) study (with permission from Meat and Livestock Australia, MLA), to determine their relationships with cortisol and Hp responses. This study aims to verify whether the plasma concentrations of these cytokines are better indicators of the pain and stress associated with inflammation in cattle castrated surgically and using tension banding.

4.2 Materials and methods

4.2.1 Animals and Treatments

The details of the animals, treatments and the procedures conducted were fully described by Petherick and colleagues in the articles that were published in the Applied Animal Behavioural Science Journal (Petherick et al., 2014a, 2014b). Briefly, the animals used were of two age groups of cattle, aged 7-10 months old (weaner bulls) and 22-25 months old (mature bulls). The bulls were assigned to four treatment combinations of castration method and pain management (n=8 per treatment) which were tension band castration with an intramuscular (IM) injection of saline (Band
tension band castration with an IM injection of non-steroidal anti-inflammatory drug (Band + NSAID); surgical castration and an IM injection of saline (Surgical + Saline); and surgical castration and an IM injection of non-steroidal anti-inflammatory drug (Surgical + NSAID). The NSAID used in their study was ketoprofen (Ilium Ketoprofen, Troy Laboratories Pty., NSW, Australia), which was administered immediately prior to castration (time 0).

The castration was conducted on two consecutive days. Two blood samples were taken for time 0, followed by NSAID or saline injection. The bulls were castrated according to the pre-assigned methods and were then moved into small yards. The bulls were moved into the chute again for subsequent blood sampling which were at 30 minutes (min) for weaners, 40 min for mature bulls, and 2 hour (h), 7 h, day 1, day 2, day 3, day 7 (week 1), day 14 (week 2), day 21 (week 3) and day 28 (week 4) in both age groups post-castration.

4.2.2 Laboratory analysis

For the present study, the remaining archived samples that had been collected into heparin tubes and had been stored at -20°C were used. The samples were assayed for IL-6 and IL-1 using ELISA, and the pre-treatment samples additionally measured for Hp concentration using calorimetric assay. The methods of the laboratory analyses for IL-6 and Hp assays were fully described in Chapter 2. For cortisol and the remainder of Hp concentrations, the data determined in the initial study were used with the permission of MLA and reanalysed for the purposes of the current study.

4.2.2.1 Interleukin-1β

Plasma IL-1β concentrations were determined using ELISA (ESS0027, Thermo Scientific, Rockford, Illinois, USA) on 96-well ELISA plates (224-0096, Bio-Rad Laboratories, Gladesville, New South Wales, Australia), according to the manufacturer’s protocol. The buffers used for this assay protocol were similar to the buffers used for the IL-6 ELISA described in Chapter 2. For the assay, 100 µL of anti-bovine IL-1β primary antibody in carbonated-bicarbonate buffer pH 9.4 (1:100) was added into each well and incubated overnight (12-16 h) at room temperature. After aspiration, 300 µL of the blocking buffer was added and was incubated for 1 h. Then, 100 µL of standards, quality control or test samples were added into the wells after the blocking buffer was aspirated. The plate was incubated for 90 min on a plate shaker at 750 rpm, and the content was aspirated followed by washing three times with the wash buffer. The anti-bovine IL-1β secondary antibody (100 µL) was added and the plates were left to incubate for another 1 h followed by aspiration and washed three times with wash buffer. Then, 100 µL Streptavidin-HRP was added, and the plate was incubated for 30 min. After another wash, substrate solution was then added and
the plate was incubated in the dark for 20 min. The reactions were stopped by adding 0.16 M sulfuric acid to each well. The absorbance was measured at $A_{450}$ minus $A_{550}$. The assay sensitivity was 8 pg/mL.

4.2.3 Statistical analysis

The data were analysed using statistical software Stata 13.0 (Stata Corp, TX, USA). Repeated-measures data were analysed using mixed-effects model incorporating treatment groups, time, and treatment by time interaction as fixed terms, and bulls as a random effect. Results are expressed as mean ± standard error of the mean (± SEM) concentrations.

Baseline levels of IL-6 concentration were observed to vary markedly across all animals in all treatment groups from as low as below detection level to as high as 50,000+ pg/mL, with those animals with high levels tending to maintain high levels throughout the study. Due to the wide variation observed in the pre-treatment values, plasma concentrations of IL-6 were presented as percentage change from baseline. The raw data for Hp and cortisol concentrations that were obtained from Petherick et al. (2014a, 2014b) were recalculated and presented as percentage change, instead of absolute values as reported by Petherick et al. (2014a, 2014b). This was performed to observe the changes of IL-6 and IL-1 in comparison with the relative changes of Hp and cortisol concentrations. P values of less than 0.05 were considered as statistically significant.

4.3 Results

4.3.1 Interleukin-6

Three weaner bulls within Surgical + Saline group showed marked increase in IL-6 levels (1000-2200 per cent) at week 3 and 4 post-castration, and the cause of these marked elevations were unknown and unable to be determined. Therefore these animals were considered as outliers and were omitted from the statistical analysis. Overall, there was only a significant time effect ($F_{10,289} = 2.61; P=0.005$) in IL-6 concentrations in weaner bulls (Figure 4.1). Band + Saline and Band + NSAID increased significantly ($P<0.05$) from pre-treatment values over time from week 1 and week 3 respectively, although there was high variability observed. There were no changes found in IL-6 in the surgically castrated bulls across all time points.
Figure 4.1 Percentage change of plasma interleukin-6 concentrations from pre-treatment values (mean ± SEM) in weaner bulls (n=8 bulls/group) that were castrated surgically (Surgical, red circles) and by tension banding (Band, blue squares), with administration of a non-steroidal anti-inflammatory drug (NSAID) (connected lines) or saline (dotted lines) immediately prior to castration. Three bulls in the surgically castrated group that were not treated with NSAID were omitted from analysis. Lowercase letters denote bulls castrated by banding with NSAID (bn) or saline (bs) administration differed significantly (P<0.05) from pre-treatment values (time 0) at each time point within each group.
In mature bulls, there were significant treatment by time \( (F_{30,319} = 1.54; P=0.04) \) and time effects \( (F_{10,319} = 3.52; P=0.0002) \) in IL-6 concentrations (Figure 4.2). Interleukin-6 increased significantly \( (P<0.05) \) from pre-treatment values at day 3, week 2 and week 4 in Band + Saline, and at week 2 and week 4 in Band + NSAID. At day 3 following castration, Band + Saline was higher than Band + NSAID \( (P<0.05) \) and Surgical + Saline \( (P<0.01) \). At week 3, Band + NSAID was significantly \( (P<0.05) \) higher than other treatment groups. At week 4, Band + Saline was significantly \( (P<0.01) \) higher than the surgically castrated groups, but was not significantly different from Band + NSAID.

Similar to the weaner bulls, there was wide variability observed in the IL-6 responses in banded bulls at these time points. It was found that the IL-6 concentrations in the surgically castrated bulls did not change from the pre-treatment concentrations.

**Figure 4.2** Percentage change of plasma interleukin-6 concentrations from pre-treatment values \( (\text{mean ±SEM}) \) in mature bulls \( (n=8 \text{ bulls/group}) \) that were castrated surgically (Surgical, red circles) and by tension banding (Band, blue squares), with administration of a non-steroidal anti-inflammatory drug (NSAID) (connected lines) or saline (dotted lines) immediately prior to castration. Superscripts denote significant differences between groups at each time point \( (q = \text{Band + Saline and Band + NSAID}, r = \text{Band + Saline and Surgical + Saline}, s = \text{Band + Saline and Surgical + NSAID}, t = \text{Band + Saline and Surgical + Saline}, u = \text{Band + NSAID and Surgical + NSAID}) \). Lowercase letters denote bulls castrated surgically with NSAID \( (\text{sn}) \) or saline \( (\text{ss}) \), administration and castrated by banding with NSAID \( (\text{bn}) \) or saline \( (\text{bs}) \) administration differed significantly \( (P<0.05) \) from pre-treatment values \( (\text{time 0}) \) at each time point within each group.
4.3.2 **Interleukin-1β**

Based on the results of IL-6 determinations, 15 animals with varying range of IL-6 concentrations (below detection limit, n=2; 10-100 pg/mL, n=2; 100-600 pg/mL, n=8; 600 to > 1000 pg/mL, n=3) were selected for the measurements of IL-1β. Eleven (73%) of those samples had plasma IL-1β below the detection level with no evidence of a treatment effect.

4.3.3 **Haptoglobin**

The pre-treatment values (time 0) of Hp for weaner and mature bulls were 0.5 ± 0.01 mg/mL and 0.8 ± 0.02 mg/mL respectively.

There were significant castration method by time interaction (F_{21, 223} =1.76; P=0.02) and time effects (F_{7, 223} = 4.51; P=0.0001) in Hp concentrations in weaner bulls (Figure 4.3). All groups increased significantly (P<0.05) from pre-treatment values over time from day 1 in Band + NSAID, day 2 in Surgical + NSAID, day 3 in Surgical + Saline and from week 2 in Band + Saline. Band + NSAID was significantly (P<0.01) higher than other groups at week 2. At week 3, Band + NSAID was higher than Surgical + Saline (P<0.01) and Surgical + NSAID (P<0.05), and higher than Surgical + Saline (P<0.05) at week 4. NSAID caused a slight shift in Hp in surgically castrated bulls, however the elevation was not significantly different from Hp in bulls that were not treated with NSAID.
Figure 4.3 Percentage change of plasma haptoglobin (Hp) concentrations from pre-treatment values (mean ± SEM) in weaner bulls (n=8 bulls/group) that were castrated surgically (Surgical, red circles) and by tension banding (Band, blue squares), with administration of a non-steroidal anti-inflammatory drug (NSAID) (connected lines) or saline (dotted lines) immediately prior to castration. Superscripts denote significant differences between groups at each time point (q = Band + Saline and Band + NSAID, t = Band + Saline and Surgical + Saline, u = Band + NSAID and Surgical + NSAID). Lowercase letters denote bulls castrated surgically with NSAID (sn) or saline (ss) administration, and castrated by banding with NSAID (bn) or saline (bs) administration differed significantly (P<0.05) from pre-treatment values (time 0) at each time point within each group.
There were significant castration method by time interaction ($F_{21, 223} = 4.48; P=0.0001$) and time effects ($F_{7, 223} = 4.51; P=0.0001$) in Hp concentrations in mature bulls (Figure 4.4). The surgically castrated bulls increased significantly ($P<0.01$) from day 1 until day 3 in Surgical + Saline, and until week 2 in Surgical + NSAID from pre-treatment values. At day 2, Surgical + NSAID was significantly ($P<0.01$) higher than Band + Saline and Band + NSAID, while Surgical + Saline was significantly ($P<0.05$) higher than Band + NSAID. At day 3 post-castration, Surgical + NSAID was higher than all treatment groups (Band + Saline, $P<0.01$, Band + NSAID, $P<0.01$, Surgical + Saline, $P<0.05$), and Surgical + Saline was higher than both of the band castrated groups (Band + Saline, $P<0.05$, Band + NSAID, $P<0.01$). By week 1, banded bulls increased significantly ($P<0.05$) from pre-treatment values, while surgically castrated bulls returned to baseline. At week 2 and week 3, Band + NSAID were significantly ($P<0.05$) higher than Surgical + Saline.
Figure 4.4 Percentage change of plasma haptoglobin concentrations from pre-treatment values (mean ± SEM) in mature bulls (n=8 bulls/group) that were castrated surgically (Surgical, red circles) and by tension banding (Band, blue squares), with administration of a non-steroidal anti-inflammatory drug (NSAID) (connected lines) or saline (dotted lines) immediately prior to castration. Superscripts denote significant differences between groups at each time point (r = Band + Saline and Surgical + Saline, s = Band + Saline and Surgical + NSAID, t = Band + Saline and Surgical + Saline, u = Band + NSAID and Surgical + NSAID, v = Surgical + Saline and Surgical + NSAID). Lowercase letters denote bulls castrated surgically with NSAID (sn) or saline (ss) administration, and by castrated banding with NSAID (bn) or saline (bs) administration differed significantly (P<0.05) from pre-treatment values (time 0) at each time point within each group.
4.3.4 Cortisol

Overall, there was a significant time effect ($F_{4,127} = 6.40; P=0.0001$) in cortisol concentrations in weaner bulls (Figure 4.5). Surgical + Saline, Surgical + NSAID and Band + NSAID were significantly ($P<0.05$) higher than pre-treatment values at 30 min and 2 h, and only at 2 h for Band + Saline. There was no treatment effect observed at these time points. All treatment groups returned to baseline by 7 h, and remained low at all time points until week 4 following castration (data not shown).

Figure 4.5 Percentage change of plasma cortisol concentrations from pre-treatment values (mean ± SEM) in weaner bulls (n=8 bulls/group) that were castrated (Surgical, red circles) and by tension banding (Band, blue squares), with administration of a non-steroidal anti-inflammatory drug (NSAID) (connected lines) or saline (dotted lines) immediately prior to castration. Lowercase letters denote bulls castrated surgically with NSAID (sn) or saline (ss) administration, and castrated by banding with NSAID (bn) or saline (bs) administration differed significantly ($P<0.05$) from pre-treatment values (time 0) at each time point within each group.
There were significant castration method by time interaction (F_{12, 125} = 3.66; P=0.0001) and time effects (F_{4, 125} = 6.41; P=0.0001) in cortisol concentrations in mature bulls (Figure 4.6). All groups were significantly elevated at 40 min following castration, in which the Surgical + Saline bulls were significantly (P<0.05) higher than other treatment groups. At 2 h, all groups remained elevated from pre-treatment concentrations except for Surgical + NSAID which was significantly (P<0.01) lower than other treatments. All groups returned to baseline by 7 h post-castration, and remained low at all time points until week 4 following castration (data not shown).

Figure 4.6 Percentage change of plasma cortisol concentrations from pre-treatment values (mean ± SEM) in mature bulls (n=8 bulls/group) that were castrated (Surgical, red circles) and by tension banding (Band, blue squares), with administration of a non-steroidal anti-inflammatory drug (NSAID) (connected lines) or saline (dotted lines) immediately prior to castration. Superscripts denote significant differences between groups at each time point (r = Band + Saline and Surgical + Saline, t = Band + Saline and Surgical + Saline, u = Band + NSAID and Surgical + NSAID, v = Surgical + Saline and Surgical + NSAID). Lowercase letters denote bulls castrated surgically with NSAID (sn) or saline (ss) administration, and castrated by banding with NSAID (bn) or saline (bs) administration differed significantly (P<0.05) from pre-treatment values (time 0) at each time point within each group.
4.4 Discussion

In this study, plasma IL-1 and IL-6 cytokines were measured as well as the pre-treatment concentrations in Hp that were not measured in the Petherick et al. (2014b) study. The focus of the current study was to determine the effects of banding and surgical castration on the cytokines of interest in two different age groups of bulls. Petherick et al. (2014a, 2014b) assessed the welfare impact of these castration methods in which several physiological indices, including the changes in cortisol and Hp were measured. The current study was to evaluate whether the responses of IL-1 and IL-6 were more informative than Hp and cortisol as indicators of inflammatory response that could contribute to pain, stress and wound healing caused by the castration methods.

Although surgical castration is considered to cause greater pain-induced distress during the acute phase compared with other bloodless castration methods in cattle (Mellor and Stafford, 1999), the procedure might have caused a local response of IL-1 and IL-6, at least within the first week of castration. The Hp response in mature bulls that were surgically castrated was higher than in the banded bulls from day 2 to day 3 post-castration, although their IL-6 and IL-1 concentrations did not change within this time period. This finding could indicate that the cells involved in expressing the cytokines might have released the cytokines locally close to the liver to induce Hp release, as well as the tissue injury area after the surgical insult. In a cattle castration study conducted by Pang et al. (2011), it was found that the expression of IL-1 mRNA in the circulating leukocytes did not differ between their Burdizzo and banded castrates. However, Pang et al. (2009) reported that the IL-1 expression in the castrated tissue (epididymis) was upregulated by 12 h and downregulated by 24 h in both banding and Burdizzo castrated cattle, although these observations were reported to be not significantly different from the control. It has been suggested that the circulating half-life of IL-1β is within minutes (Lin et al., 2000), which could be due to the effective negative feedback on the IL-1β binding to its receptors by IL-1 receptor antagonist (IL-1ra). Interleukin-1ra is also induced following inflammation (Arend et al., 1998) and is normally produced in greater amount compared with IL-1α and β (Hopkins, 2003). Interleukin-6, also being an anti-inflammatory cytokine could have caused the inhibition of IL-1β through the induction of IL-1ra release (Tilg et al., 1994). Although in the current study, plasma IL-6 did not change within this time period, it could have exerted its action locally at the injured tissue. Therefore, in line with Pang et al. (2009) and Pang et al. (2011) studies, it is possible that both banding and surgical castration might have caused a local response of IL-1β, hence its plasma concentrations were not detected in the circulation.

It has been known that glucocorticoid cause inhibition of pro-inflammatory cytokines within the early events of an insult (Petersen et al., 2004). In the current study, the weaner bulls had a
significant cortisol response within at least 2 h of castration regardless of treatment. Pang et al. (2011) found that their Burdizzo castrates had a higher expression of IL-6 mRNA in circulating leukocytes compared with the banding castrates at 24 h post-castration, and they suggested that the high cortisol response in banded bulls might have suppressed the IL-6 mRNA expression. Pang et al. (2011) also found that 48 h following castration, the Burdizzo castrated bulls that were administered with carprofen had a higher response in IL-6 mRNA compared with Burdizzo only. They suggested that intravenous (IV) injection of carprofen caused suppression in cortisol, hence the IL-6 expression was found to be higher in the Burdizzo castrates administered with the NSAID. In the current study, it was found that IM injection of ketoprofen only decreased cortisol response in the mature bulls that were castrated surgically within at least 2 h of castration, while the NSAID had no effect on cortisol in the banding castrates. By 7 h post-castration, the relative changes in cortisol had already returned to baseline in all groups. Within 24 h of castration, the IL-6 response in all groups did not change. This raised the question that the relationship of IL-6 and the effect of NSAID on cortisol response may not necessarily correlate, and may only be applicable in a delayed onset of response of cortisol and IL-6 as observed by Pang et al. (2011). Furthermore, the findings by Pang et al. (2011) might suggest that it only affected the leukocyte expression of IL-6 in bloodless castration methods, rather than the plasma levels which may have exerted its actions locally at this time.

The elevation in IL-6 in the banded bulls from day 2 in weaners and week 3 in mature castrates could indicate the pleiotropic role of the cytokine, from an acute phase response of innate immunity, into a promotion of adaptive immune response such as by transitioning the different types of immune cells recruitments to the area of inflammation (Jones, 2005; Gabay, 2006). Banding causes an ischaemic necrosis leading to testicular atrophy and sloughing of the scrotum which incites a chronic inflammation (Capucille et al., 2002). Chronic inflammation has been associated with elevation in IL-6 by eliciting the transition of immune cells recruitments from neutrophils to monocytes, as part of the protection against reinfection until the resolution of inflammation is completed (Gabay, 2006; Naugler and Karin, 2008). The elevation in IL-6 in the current study was also in concurrence with the Hp response, in which the mature banded bulls also started to show an increase in Hp by week 1. It was suggested by Petherick et al. (2014b) that the increase in absolute values of Hp concentrations in mature banded animals were indicative of chronic pain, as a consequence of the chronic inflammation caused by the tension banding. The delayed increase in Hp observed in banded bulls compared with bulls castrated surgically was also correlated with the progression of wound healing, which appeared to resolve at the earliest, by 6 weeks post-castration (wound healing scores are described in Appendix 1). It has been suggested
that IL-6 is an important mediator in chronic wound healing (Behm et al., 2012). In concurrence with the significant response in Hp, IL-6 response could also be used as another marker of chronic inflammation that contributes to chronic inflammatory pain. However, it was found in this study that the IL-6 concentrations in surgically castrated animals did not change across all time points, although their wounds showed signs of healing by at least week 3 post-castration. Mast and Schultz (1996) found that elevation in IL-6 in wound exudates was more significant in the chronic wounds compared with acute wounds in humans. In recurrent or prolonged tissue injury or inflammation, the pro-inflammatory cytokine cascade is amplified (Mast and Schultz, 1996). Thus, the gradual elevation in IL-6 in the banded bulls might be due to the prolonged inflammatory process. Therefore, this resulted in IL-6 being detected in the circulation in contrast with the surgical castration, in which it may have exerted its action at the local tissue.

The findings of the current study showed that IM injection of ketoprofen caused a shift in Hp, within the first 3 days in mature bulls that were surgically castrated, and from week 2 until at least week 3 in band castrates in both age groups. The Hp elevation in the NSAID treated bulls in the current study was in contrast with the findings in cattle surgical castration by Earley and Crowe (2002) and Ting et al. (2003), although in their studies, ketoprofen was administered via IV injection. Earley and Crowe (2002) found that the surgically castrated bulls that received the IV injection of ketoprofen had reduced Hp responses at day 1 and day 3 following castration. Ting et al. (2003) also found that ketoprofen administered via IV reduced the Hp response at day 3, and repeated administration had no additional effect. Petherick et al. (2014b) only assessed the effect of ketoprofen on cortisol within 24 h post-castration, since it was suggested that the effect of the NSAID only lasted for 24 h following administration (Landoni et al., 1995). Marti et al. (2010) found that IM injection of ketoprofen did not alter the Hp response in their Holstein calves following ring castration. A similar study on surgical and banded castration in Angus calves that used ketoprofen administered via IM also concluded that the NSAID has less impact in mitigating pain following castration, although they did not measure the Hp response (Moya et al., 2014). In the current study, the NSAID might not have exerted any effects on cortisol after 24 h post-castration, however the effect of IM injection of ketoprofen might have lasted longer, which inversely affected the systemic inflammatory responses. No other studies have observed the effect of a local injection of ketoprofen on systemic inflammation in cattle surgical and band castration.

The extreme baseline levels in IL-6 in some of the animals might suggest that there could be an underlying systemic inflammation or infection in those animals before treatment, or could be due to the individual variability in immune function. The accepted baseline level for plasma IL-6 concentration in healthy cattle is less than 84 pg/mL or below the detection level of 20 pg/mL.
The wide variation of cytokines response might be due to different immune, and possibly nutritional state, of each individual animal (Yun et al., 2014). It was suggested by Hughes et al. (2014) that the immune background of cattle can be correlated to breed, and that breed temperament can influence the stress and immune response. Purebred Brahmans were used in the study by Petherick et al. (2014a, 2014b), and this breed has a tendency to have an undesirable temperament when compared with *bos taurus* genotypes (Burrow, 2001). Hence, it is possible that the variability observed in IL-6 was influenced by various factors that may have affected the immune background.

Three animals in the Surgical + Saline mature bulls were excluded due to much higher relative change concentrations of IL-6 at week 3 onwards. Baseline levels in two of the animals were either below detection levels (less than 10 pg/mL) until at least until day 3, and were increased by 1000-2000% by week 2. This elevation might be due to a possible subclinical immune challenge between data collections at week 2 and week 3. It is interesting to note that this finding was only observed in Surgical + Saline mature bulls and was not observed in other cohorts. Nevertheless, the undetectable levels within the first week in these animals could also be due to sample degradation, or possibly the animals had low levels in IL-6 concentration. Furthermore, their surgical wound scores were indicative of wound healing resolution by week 3 although the IL-6 increased markedly at this time and did not correlate with Hp. Moreover, the trend in relative changes in Hp was more consistent within each treatment compared with IL-6. This observation, as well as the variability in the baseline values, might also be confounded by the possibility of sample degradation, since the same set of samples were used by Petherick et al. (2014b) for other parameters measured in their study.

Though Hp increased significantly in both castration methods, particularly in mature bulls, the plasma concentrations in IL-6 and IL-1 did not change within the early phase of castration. The cytokines might have exerted their pro-inflammatory actions locally, and their systemic response might have been suppressed by cortisol. Nevertheless, the gradual elevation in IL-6 in the banded bulls suggests that it is worthwhile to measure for IL-6 in relation to progression of tissue repair. It can also be considered as an indicator of inflammation that may contribute to the chronic inflammatory pain. However the variability in the IL-6 concentration makes it difficult to give a definitive conclusion on the IL-6 role in cattle castration.
4.5 Conclusion

In conclusion, both surgical and band castration methods had no effect on plasma concentrations of IL-1 and IL-6 within the first 24 h post-castration. It is recommended that the changes of IL-6 and Hp be measured until the point of total healing. This is to determine the relationship of these markers with the progress of the healing process of castration wounds and their contribution to inflammatory pain. In this study, the IL-6 concentrations in surgically castrated bulls did not contribute any additional information compared with Hp assessment. However, the rise in IL-6 may have a potential role in causing chronic pain, as seen in the bulls that were castrated using tension banding.
Chapter 5

General discussion
5.1 Introduction

In ruminants, routine husbandry procedures such as castration, dehorning and tail docking are considered painful to the animals and have raised animal welfare concerns (Rollin, 2004). While the provision of analgesics is recommended as part of the standard husbandry practice, such practice incurs cost to the farmers. The effectiveness of the pain treatment may not be appropriate in some situations and may not fully address the pain experienced. Hence the need to seek and identify the most appropriate and accurate evidence to indicate the presence and the degree of pain is required.

Reviews on pain assessment in animals had recommended that researchers should conduct studies to devise a reliable assessment tool that comprises of various parameters (Prunier and Leterrier, 2014). The availability of such reliable quantitative measurements of pain will be useful in determining the appropriate and the optimal dose of the analgesics required (Stewart et al., 2014). Most studies derived the conclusions of the presence of pain from the results of either a single or a combination of a few indicators that were not examined simultaneously as part of the pain profile. In ruminants, measurements of cortisol and Hp, and assessment of behavioural changes have been well established for this purpose, however there are still limitations in using these parameters, as they are also indicators of psychological stress (Rivalland et al., 2007; Kim et al., 2011). Moreover, assessing behaviour in prey animals like ruminants can be challenging as they are inclined to hide their pain in the presence of humans who are unfamiliar to them (Anil et al., 2002).

According to Prunier and Leterrier (2014), tissue injury is considered as a key factor in identifying the presence and source of pain in production animals. In pain caused by tissue injury, it is important to understand the underlying pathophysiology involved such as inflammation, stress and nociception that contributed to the changes in the parameters measured. For the purpose of this thesis, castration, a common painful routine husbandry procedure that causes tissue injury was chosen as the pain model.

The aims of this thesis were:

1. To refine a multi-parameter ‘toolkit’ that combines measurements of circulating markers used conventionally in the assessment of pain namely cortisol, haptoglobin (Hp), and beta-endorphin (β-EP); and contemporary markers in which evidence are still limited in ruminants namely interleukin-6 (IL-6), substance P (SP) and prostaglandin E₂ (PGE₂). These measurements were evaluated together with the recording of behavioural changes, using a sheep surgical castration model.
2. To examine the cellular expression of IL-6 and proopiomelanocortin (POMC), the precursor of β-EP in circulating leukocytes and to compare the responses of these genes with the circulating protein levels in plasma.

3. To examine the role of systemic pro-inflammatory cytokines, interleukin-1 (IL-1) and IL-6 in association with inflammation that contribute to pain, stress and wound healing and their relationships with cortisol and Hp, in a cattle castration model using two different methods: surgical and tension banding.

5.2 Main findings in this thesis

Chapter 2 involved the assessment of various systemic biomarkers following surgical castration in lambs, in which their changes over time were measured. Although there still exist some controversies regarding cortisol as a reliable indicator of pain, the immediate cortisol response found in the lambs castrated surgically was indicative of the treatment effect caused by the procedure. However, from the results obtained from Petherick et al. (2014a, 2014b) in Chapter 4 comparing the surgical and tension banding castration methods, it was found that the cortisol responses in both treatments were not different. For this cattle castration study, Petherick et al. (2014b) suggested that the cortisol response was an indication of stress caused by the procedures rather than the induced pain. Hence in agreement with Mellor et al. (2000), the changes in cortisol concentration observed may not be an indicator of pain, but was more likely a result of the overall noxious experience of pain, inclusive of both physical and emotional components.

The study conducted in lambs was a comparative study between tissue injury and restraint, whereby to ensure that the experiment was well controlled, all animals were subjected to the same handling experience prior to treatment. Thus in this study, the cortisol response observed in the castrated lambs was likely contributed by the tissue injury. Although Petherick et al. (2014a, 2014b) compared the findings between two castration methods, an additional cohort of animals as a control group in their cattle castration study was not included. Hence it was difficult to determine whether the cattle which were only subjected to repeated movement from the yard to the chute during blood sampling were not experiencing added stress that could contribute to the measured cortisol levels.

Increase in Hp in surgically castrated lambs as described in Chapter 2 and cattle in the study conducted by Petherick et al. (2014a, 2014b) in Chapter 4, was an indication of an acute inflammatory process caused by the procedure. These observations are in agreement with Ceciliani et al. (2002) that Hp can be considered as a good indicator of an inflammatory response. Interleukin-6 being a major stimulant of Hp was found to be elevated in surgically castrated lambs.
Interleukin-6 is produced by local cells such as the macrophages, monocytes and fibroblasts as part of the inflammatory cascade (Papanicolaou et al., 1998; Gabay and Kushner, 1999). This further supports that the rise in Hp observed was due to inflammation, rather than an overall noxious response caused by the procedure. However, it was also found that there was a non-significant rise of Hp in the sham castrated lambs at 24 h paralleled to the non-significant elevation of cortisol. It is known that glucocorticoid is one of the stimulants of acute phase protein production by the hepatocytes (Kurash et al., 2004). Thus the early increase in Hp concentration in the control lambs could be either due to the actions of the pro-inflammatory cytokines released by the local cells, or due to the stimulation by cortisol on the hepatocytes.

The expression of IL-6 mRNA by circulating leukocytes in surgically castrated lambs as described in Chapter 3 did not differ from the sham castrated animals within the first 8 h of treatment. It was possible that the surgical stress caused by the castration did not differ from the generalised stress response as seen in sham castration. However, the lack of IL-6 expression and protein responses within the first day of castration indicate that the surgical procedure could have caused a local response within this initial time frame, hence changes in the circulation were not detected. Although a different animal species was used in comparison with the castration study in cattle conducted by Pang et al. (2011), the surgical castration that was presumed to cause an acute inflammatory response may not differ from the banding and Burdizzo castrations. Notwithstanding Pang et al. (2011) did not measure the corresponding protein concentrations in the circulation, and studies conducted in this thesis are the first castration study that compared the expression of IL-6 mRNA in leukocytes with the plasma protein concentration in ruminants.

In the present study, it was found that plasma IL-6 was increased in the surgically castrated lambs as early as day 2 onwards. The prolonged half-life of IL-6 could be explained by the binding of IL-6 to the soluble receptors (sIL-6R) in the circulation (Peters et al., 1996; Sakamoto et al., 2003). Hence, the response in IL-6 measured for the subsequent days after the onset of tissue injury can be used as an indicator of an ongoing inflammatory pain, which may only subside when the wound has completely healed (Behm et al., 2012). Interestingly, the IL-6 response in surgically castrated bulls described in Chapter 4 did not change in both age groups, although their corresponding Hp levels increased significantly, more so in the mature bulls. Additionally, the increase in cortisol response in both surgical and tension banding castrates did not show any treatment effect (Petherick et al., 2014a, 2014b). The lack of IL-6 response could be due to the immunosuppression by cortisol (Petersen et al., 2004). Nonetheless, sample degradation could have also contributed to this negative finding.
On the contrary to the findings on plasma protein levels, the IL-6 mRNA expression showed a downregulation from 24 h onwards, which could indicate that the leukocytes might have migrated into the site of injury as part of a normal inflammatory response following a tissue injury (Lin et al., 2003). The measurement of plasma concentration of IL-6 may be a better indicator of inflammatory pain, compared with assessing mRNA expression from the circulating leukocytes. Pang et al. (2011) suggested that the high cortisol response in their banded bulls might have suppressed the IL-6 mRNA expression, although they only measured IL-6 mRNA expression up to day 2. Hence, as opposed to the suggestion by Pang et al. (2011) that the banding only caused a local response of cytokines, the gradual increase in plasma IL-6 as early as day 3 post-castration in the banded bulls (Chapter 4) could be associated with the significant role of IL-6 in chronic inflammatory response. It has been documented in human studies that a significant rise in IL-6 was observed in chronic inflammation such as rheumatoid arthritis, which contributed to the chronic pain (De Jongh et al., 2003; Scheller et al., 2011), in which it could also be a result of IL-6 binding to sIL-6R in the circulation. Thus, the hypoxia caused by the constriction of the band used (Gregory, 2004) has led to the later onset and continuous production of inflammatory mediators within the tissue that includes IL-6, and subsequently entered the circulation hence were detected systemically (De Jongh et al., 2003).

Pain-related behaviour, such as statue standing was observed in surgically castrated lambs 3-5 h post-castration, which correlated with the rise in SP concentration detected within this time interval (Chapter 2). These findings are in agreement with a study on surgical castration in cattle conducted by Coetzee et al. (2008), which also correlated with the intensity of vocalisation and behavioural changes. So far there are no other studies that had measured the response of SP in sheep following surgical castration. Compared with other biomarkers that were measured, only SP seemed to show a correlation with the pain-related behaviour observed. The gradual increase in SP found could be attributed to the cumulative effect of nociception and neurogenic inflammation, as well as the noxious experience caused by the tissue injury (Stanisz, 2001).

Within the first 3 h of castration, the animals displayed fear behaviour towards the handlers during blood sampling, in which an assessor may misinterpret that all animals were experiencing some levels of distress during this period. However their physiological responses such as cortisol and SP measured showed otherwise. This supports that difficulties would arise when assessing behavioural changes in prey animals like ruminants (Dobromylskyj et al., 2000; Fitzpatrick et al., 2006) whereby these animals perceived humans as predators (Grandin and Johnson, 2005). Being a social animal species, the behavioural expressions of pain within the first 3 h of treatment were buffered by their pen mates that exhibited fear behaviour (Guesgen et al., 2014). This also supports the
suggestions that the absence of visual signs of pain does not indicate that the animals are not experiencing pain (Anil et al., 2002; Underwood, 2002). Unless the animals are accustomed to the humans who are handling them, the applicability in assessing behaviour in field condition is still debatable and yet to be justified by strong evidence.

The lack of circulating levels of $\beta$-EP and PGE$_2$ described in Chapter 2, and IL-1 in Chapter 4 might indicate that the tissue insult of castration mainly induced a local response. The lack of their responses could also be due to their short half-lives and effective negative feedback control compared with the other biomarkers that were measured in the studies conducted in this thesis. It was also possible that their peak levels in the circulation may have been missed during sampling. The lack of $\beta$-EP response correlated with the lack of POMC mRNA expression in leukocytes described in Chapter 3. Although, it was not in agreement with the study conducted by Mears and Brown (1997) that found increase in $\beta$-EP 15 min post-surgical castration in 3-week-old Suffolk lambs. This could be due to their younger age and possibly were not as exposed to human handling as in the lambs in the present study. The undetectable level of plasma IL-1 in the castrated bulls is supported by the study conducted by Pang et al. (2011), which also did not find any changes in IL-1 mRNA expression in circulating leukocytes. Although Pang et al. (2009) found upregulation of IL-1 expression in the castrated tissues. No other studies had measured the response of plasma PGE$_2$ following castration, however a study by Fraccaro et al. (2013) found increases in plasma PGE$_2$ in calves undergoing dehorning, which were significantly decreased following intravenous injection of flunixin, a non-steroidal anti-inflammatory drug. Despite the lack of changes of IL-1, $\beta$-EP and PGE$_2$ that were not raised in the circulation, their actions are important in modulating the pain sensitisation, hyperalgesia and inflammation within the local inflamed tissue (Machelska and Stein, 2000; Cunha and Ferreira, 2003; Zhang and An, 2007). Their systemic roles might have less impact in ruminants following a local tissue injury of castration. The extent of tissue injury caused by castration and the level of responses of IL-1, $\beta$-EP and PGE$_2$ might be appropriate and sufficient for a local response of this severity.

5.3 Limitations

5.3.1 Castration as a pain model

Although routine husbandry procedures such as surgical castration are gaining attention as a cause of pain in ruminants, the physiological outcomes of the tissue insult might have been limited to a local response. The amount of tissue insult involved was not severe enough to induce a high degree of tissue injury for biomarkers to be detected systemically. This could be the explanation for the
lack of systemic responses and the variability observed as shown in some of the measured biomarkers as observed in IL-6 and SP responses. The variability observed in SP response could be due to the differences in individual pain tolerance threshold, as supported by Coetzee et al. (2008). The choices of contemporary markers such as IL-6, IL-1, PGE₂ and SP were mostly based on the findings in human pain models that assessed varying degrees of surgical pain (Cruickshank et al., 1990; Baigrie et al., 1992; Buvanendran et al., 2006; Carvalho et al., 2008). An increase in plasma PGE₂ was found in cattle that were dehorned (Fraccaro et al., 2013). Substance P had been used in assessing various routine procedures in cattle, such as castration (Coetzee et al., 2008; Dockweiler et al., 2013), electroejaculation (Whitlock et al., 2012), and dehorning (Coetzee et al., 2012) however these studies were limited in cattle, and no studies had observed SP response in sheep as an indicator of pain caused by routine husbandry procedures. Hence the lack of homogenous responses in SP and IL-6, and detectable responses in IL-1, β-EP and PGE₂ may not necessarily indicate that these biomarkers are unreliable to detect pain in ruminants if used in other pain models.

5.3.2 Acclimatisation

Although the physiological indices were not affected greatly to show a treatment response, the fear-related behaviour observed within the first 3 h of castration suggests that the animals were not properly acclimatised to human handling. Nevertheless, this also highlights the possibility of the lack of practicality and specificity in using this parameter in field conditions. Unless each animal was observed with scrutiny, the immediate behavioural changes observed might be misinterpreted.

5.3.3 Variability in immune response

There was some variability of pre-treatment IL-6 levels observed in the lamb castration study (Chapter 2 and 3), which could be the result of the immunological challenge due to vaccination. Vaccination could also cause the high concentrations in Hp detected prior to treatment since the lambs were immunised only one week prior to castration. Thus, this could contribute to the inflammatory response observed. According to Lepherd et al. (2009), the reference interval of Hp for weaned Merino lambs is 0.06-0.12 mg/mL. The mean pre-treatment Hp concentrations in all animals in the lamb castration study were 0.9 ± 0.1 mg/mL, hence the Hp concentrations were almost more than 10 folds from the reference value. The vaccine used for the lambs in this study could induce and modulate the immune response, which affected the measured concentrations of Hp. This was also observed in a study by Bastos et al. (2013) that found lambs that were immunised against caseous lymphadenitis had an increase in Hp between the first day till day eleven of
immunisation. Although there was a treatment effect of the surgical castration observed in the present study, the non-significant elevation of Hp in the control animals may have been contributed by the vaccination. In field conditions, it is a routine practice to give vaccination to animals close to the time of conducting the painful procedures due to labour costs and to minimise the stress of handling. Hence this shows the importance of the biomarkers used to assess pain to be robust and minimally affected by the vaccination to avoid misinterpreting the results obtained.

There is also a possibility that the genetic variation factor due to breed selection practice in the sheep industry (James, 2006; Kijas et al., 2012) contributed to the variability observed. It had been described by Jacob and Pethick (2014) that breeding selection had been well practiced in Australia, which was evident in the lamb meat quality. The variability observed was not only limited to lambs, but had also been documented in cattle (Hughes et al., 2014; Yun et al., 2014). The acute phase response (APR) in cattle influenced by the natural variations of gender, breed and temperament, also influenced the recovery of the animal from stress (Hughes et al., 2014). This may further support the observed variability in IL-6 response in cattle prior to treatment (Chapter 4).

It has also been recognised that in human studies, variability in pain response is an issue in developing a homogenous pain assessment. Barrett (2015) mentioned that even in humans, genetic variability also imposes some differences in response, making it difficult to address pain relief effectively. Therefore it is understandable that in animals, this could pose some problems when assessing markers that are especially dependent on genetic variability. In the current study, IL-6 still imposed as an ambiguous parameter in its interpretation as part of the pain assessment tool. Hence, to account for the variability observed, individual changes were measured instead, by measuring the percentage change from the baseline value. However, the high baseline values also imposed a concern especially when there was a possibility that the animals were already subjected to an underlying immune response due to either infection or chronic inflammation, although the symptoms were not evident clinically. Therefore, assessment of IL-6 can be used as a pre-screening tool in determining the health status of animals and used as a selection criterion before the animals are used as subjects for experimental work or research.

5.3.4 Limited sample size and volumes

The small sample size of only six animals per group in the lamb castration study and eight animals per group in the cattle study might have contributed as a limiting factor in determining the reliability of measuring the biomarkers. This could have contributed to the variability in absolute values of SP, PGE₂ and IL-6, although variability in IL-6 could also be due to the genetic
background or vaccination. The variability in the pre-treatment concentrations in SP could also be due to fear and anxiety (Devane, 2001; Ebner and Singewald, 2006). Factors contributing to the variability in PGE$_2$ were not able to be determined. Hence for the purpose of this research, relative percentage change within each individual animal appeared sufficient to indicate a response. Nevertheless, variability was still observed following castration, in which if adequate sample size were used, a reference range can possibly be established and consequently the significance of these variations can be determined.

Although the usage of lambs as the animal model may be more cost effective compared with cattle, the relative small size and low body weight of the species restricted the amount of blood volume that could be withdrawn without causing haemorrhagic stress. The lamb surgical castration study involved intense serial sampling within the first 2 h (Chapter 2), however some of the appropriate time points for the measurement of chosen biomarkers might have still been overlooked. As mentioned earlier, the peak values of some biomarkers such as β-EP and PGE$_2$ might have been missed during sampling. This might not affect other biomarkers such as IL-6, cortisol and Hp, since the expected peak values had been predetermined and established by other studies according to their expected responses. Furthermore, these markers have longer half-lives in the circulation compared with β-EP and PGE$_2$, hence their peak concentrations were easily detected and measured for analysis.

Biomarkers such as β-EP, PGE$_2$ and SP are required to be extracted prior to the assay using solid phase extraction method, and together with the additional steps that include lyophilisation could have contributed to the variation between samples, especially with the limited volume of the starting materials. In Chapter 3, it was assumed that the downregulation of IL-6 mRNA and POMC expressions by leukocytes were due to the migration of these leukocytes to the site of injury of the local tissue. Although determining the total and differential white blood cell counts in the samples could assist in validating this assumption, the small volume of blood sample might have also been a contributing factor. Furthermore, the variability in the gene expression was nearly 100 to 1000 fold different, if the data were back log-transformed. Quantification of the gene expression required several steps that could have added to the errors in the end results. The expression of the reference genes SDHA and YWHAZ were low in some samples, which might imply the lack of the starting material or that RNA might have been lost during the RNA isolation and purification as well cDNA synthesis (Bustin, 2002). Although, the target genes IL-6 and POMC were not consistently low parallel to their reference genes. Hence this could have caused implications for defining the response of target genes observed. Although the assessment of gene expression can be regarded as sensitive, this in turn raised the question of whether determining the gene expression in leukocytes
will add any additional value as compared with measuring the protein concentrations of IL-6 and β-EP, especially for the use in the assessment of pain.

The measurements of IL-1 and IL-6 in the cattle castration study samples in Chapter 4 were from archived samples that were used earlier by Petherick et al. (2014a, 2014b) for their study. Surgical castration may have affected sheep and cattle differently in their IL-6 responses, being of different species. Although, this finding might not be conclusive especially it is likely that this was due to potential sample degradation of the cattle samples.

5.4 Implications and recommendations for future studies

The studies conducted in this thesis were an attempt to devise a pain assessment ‘toolkit’ that is practical to use. By having a profile of tests that is sensitive, reliable and robust, a definitive diagnosis of the presence of pain can be made and the necessary measures taken to address them. Changes detected in the circulation can be considered as good indicators in the determination of a painful experience. Difficulties in assessing parameters that are impacted by individual variability such as IL-6, PGE$_2$ and SP can be overcome by measuring the relative percentage change from the pre-treatment values rather than absolute values. The absence or a negative outcome as observed in PGE$_2$, β-EP and IL-1 responses, may indicate a less severe tissue injury and presumably a less painful response. This could also indicate that the responses were limited to local actions hence were not detected in the circulation.

The declination of cortisol response after administration of analgesic following invasive procedures such as surgical castration (Paull et al., 2009; Petherick et al., 2014b) indicated that measurement of cortisol is still required as part of the pain assessment ‘toolkit’. Being one of the initial biomarkers expressed and readily assessable in the circulation, measurement of cortisol is useful as the early indicator following the onset of a painful stimulus, in which single measurement within 30 min or subsequently until 2 h is sufficient. Although a non-specific ‘ceiling effect’ can be expected, the response detected still has significant welfare concerns that requires the attention of the assessor (Mellor et al., 2000). Hence, it must be determined and interpreted together with more specific biomarkers to identify whether the responses documented were associated with pain.

Following 24 h of treatment, Hp and IL-6 are good indicators of inflammatory pain. In humans, the half-life of unbound Hp is only for 5 days (Murray, 2009), although the presence of free haemoglobin and the haemoglobin-haptoglobin complex formed would cause an earlier clearance from the circulation (Gupta et al., 2011). Therefore, assessment of IL-6 response can be a more reliable marker to identify the chronic pain caused by the inflammation. The assessment can be
performed possibly until wound healing is completed, as one of the main roles of IL-6 is to modulate immune cell recruitment to prevent reinfection while tissue resolution takes place (Gabay, 2006). It is important to address chronic pain in farm animals, especially as it can induce negative effects on the animal’s overall well-being thus affecting the animal production criteria such as growth and reproduction rates (Guatteo et al., 2012). Nonetheless, in ruminants, the measurements of IL-6 may not be as straightforward as assessment of IL-6 in humans and other species with known baseline values and reference range. Hence, it is recommended that the evaluation of the IL-6 response should be measured based on individual response. The applicability of this measurement in the field may require further work, and the variability may be expected unless more animals are used for such animal pain model studies using ruminants.

In addition to the cortisol response, SP can be a good early indicator of pain. The changes in plasma SP can be an indicator of nociceptive response caused by a painful stimulus, which in this study was the tissue injury. The SP elevation can be detected in the circulation as early as 30 min until at least 8 h post-tissue insult. The plasma SP response indicates the neurogenic inflammation, hyperalgesia and also the central sensitisation and modulation of the pain response caused by the tissue injury. This is also reflected by the pain-like behaviour observed which was statue standing, whereby the animal attempted to minimise unnecessary movements due to the hypersensitive sensation of the injured area (Molony et al., 2002). As mentioned by Coetzee et al. (2008), the variability in SP can be expected due to the differences in the individual pain tolerance threshold, and therefore it is recommended to measure the relative percentage change of each lamb.

Behavioural recordings may be the most non-invasive method, however assessment of lambs in field conditions may be misinterpreted as anxiety or fear, in which the physiological responses measured such as cortisol and SP indicate otherwise. Behavioural recording is best to be conducted remotely without human intervention. This further emphasises that, where and when possible, the assessment of behavioural changes should be supported by physiological tests.

Quantification of POMC and IL-6 gene expression in circulating leukocytes in this study did not add further information hence these measurements may not be necessary to assess for a pain response. Measurement of their protein levels, although lacking in sensitivity, is considered as sufficient. Nonetheless, other gene expression quantification techniques such as using a multiplex qPCR in which more markers can be measured simultaneously may be more informative and specific compared with their circulating protein concentration measurements. It is also possible that by increasing the sample size and repeating the tests in other forms of pain model may elucidate some significant trend of changes that might be useful in its application to assess pain.
From the overall findings, it is best to address and determine the trends and the expected time points when the systems involved would exert their responses towards the pain sensation. Blood sampling is considered as an invasive procedure. Therefore, the process of obtaining samples such as those that require multiple sampling over an extensive time period must be addressed to avoid confounding factors such as handling stress that can lead to the erroneous interpretation of the results. From the results obtained, it is recommended to measure the biomarkers according to the expected elevation and peak detectable in the circulation: 1) cortisol within first 30 minutes to 2 h after insult; 2) SP from 30 minutes until at least 8 h post-insult; and 3) Hp and IL-6 from day 2 onwards following insult. Measurements of IL-1, PGE$_2$ and β-EP as part of the panel still require more research, possibly in other forms of pain models. It is recommended to determine their responses within the immediate period, possibly 1-2 h post-insult.

5.5 Conclusion

Based on the aims outlined in this project it is concluded that:

1. Responses of cortisol might not be specific to pain, however immediate assessment of cortisol within 2 h of treatment can be applied as a sensitive and an early indicator to alert the assessor that the animal’s welfare requires attention.
2. Contemporary markers such as SP and IL-6 are potentially good indicators in assessing pain resulting from the tissue injury. Measurement of IL-6 can be a better indicator of inflammatory pain compared with Hp. Nevertheless, assessment of IL-6 and SP must be individually accounted for in each animal.
3. There was poor correlation of peripheral leukocytes expression of IL-6 and POMC genes with their corresponding plasma levels. Quantification of peripheral leukocytes did not add further information to the plasma concentrations measured to indicate presence of pain.
4. Assessment of IL-1 response in the circulation is less valuable in assessing pain compared with IL-6.

Overall, the studies conducted in this thesis aimed to form a baseline research in an attempt to understand the relationships of the various biomarkers in the assessment of pain in ruminants. The overall results support the hypothesis that more than one parameter, including cortisol, Hp and behavioural changes are required to assess presence of pain appropriately and accurately. There were still many factors and limitations that should be addressed and controlled before being recommended as a standard assessment tool in the diagnosis of pain in ruminants. It is recommended that these biomarkers be examined further using other forms of pain model and
pathological conditions for comparison. The outcomes will be used as evidence of the presence of pain, and the severity of the pain can be graded to become the basis of treatment intervention in the management of pain in ruminants.


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Appendix
Appendix 1

The following data represent the results obtained from Petherick et al. (2014a, 2014b) on wound healing scores for weaner (Figure A1.a) and mature bulls (Figure A1.b) for the study presented in Chapter 4. The surgically castrated bulls in both age groups had significantly (P<0.05) higher scores at week 1 post-castration. At week 2, only the banded bulls in weaner group had higher score than the bulls that were surgically castrated. By week 3, the scores in Surgical bulls started to decrease and the Band bulls were significantly (P<0.05) higher than Surgical until week 5. The wound scores in all animals were closed by week 6.

Figure A1.a Scores of castration wounds in weaner bulls castrated surgically (red circles) and by tension banding (blue squares) with (connected lines) or without (dotted lines) administration of a non-steroidal anti-inflammatory drug (NSAID) prior to castration. (1) Wound closed/scabbed, dry and no pus; (2) wound part-closed, dry and no pus; (3) wound part-closed, moist and pus present; (4) wound fully open, moist and no pus present; and (5) wound fully open, moist and pus present). The asterisk denotes significant (P<0.05) difference between treatment groups at each time point.
Figure A1.b Scores of castration wounds in mature bulls castrated surgically (red circles) and by tension banding (blue squares) with (connected lines) or without (dotted lines) administration of a non-steroidal anti-inflammatory drug (NSAID) prior to castration. ((1) Wound closed/scabbed, dry and no pus; (2) wound part-closed, dry and no pus; (3) wound part-closed, moist and pus present; (4) wound fully open, moist and no pus present; and (5) wound fully open, moist and pus. The asterisk denotes significant (P<0.05) difference between treatment groups at each time point.