THE EFFECT OF EARLY PAINFUL EXPERIENCES ON SUBSEQUENT PAIN SENSITIVITY IN LAMBS

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Seini Pifeleti

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Abstract

There is evidence that painful events in early life can alter subsequent pain processing and sensitivity, at least in altricial species. However, it is not known whether similar effects occur in precocial species, such as lambs, which are relatively mature at birth. Lambs in New Zealand are routinely exposed to painful procedures like castration and tail docking at a young age. The possibility that these early procedures result in hypersensitivity to subsequent painful events is a serious welfare issue. The aim of this study was to assess the effect of early castration on the behavioural responses of lambs to subsequent tail docking. The effect of age at first treatment (castration or control handling) and age at docking were also assessed.

Lambs were castrated (C) or handled (H) at either 1 (Group 1, N=21; Group 2, N=27; Group 3, N=23; Group 4, N=24) or 21 (Group 5, N=26; Group 6, N=24) days of age and their behavioural responses to docking measured 3-6 weeks later. Differences between C and H lambs were evident before docking had taken place; C lambs walked backwards less frequently and spent less time standing unsteadily than H lambs. After docking, C lambs stamped their feet more frequently and spent less time lying laterally than H lambs. Age at first treatment was not found to have a significant effect on behaviour in response to docking. Interestingly, lambs docked at 42 days of age differed from those docked at 21 days of age in their response. Twenty-one-day old lambs displayed significantly higher frequencies and durations of a number of pain-related behaviours when compared to their 42 day old counterparts. Only one behaviour, unsteady standing, was performed for longer durations by the older lambs.

It can be concluded that castration does affect the behavioural response of lambs to subsequent docking and that age at docking is also a significant factor in this response. Further research is required to further clarify the magnitude of these effects.

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1.0 Introduction

A number of human and animal studies have shown that exposure to pain in the neonatal period can result in long-term changes in neural circuitry and behaviour (Taddio *et al.*, 1997; Anand *et al.*, 1999). Short-term responses to pain in lambs in early life are well-documented (e.g. Peers *et al.*, 2002), but longer-term effects of such responses have not been thoroughly investigated.

The bulk of published material on the effect of pain stimuli in early life is based on evidence from rodent and human studies. For example, neonatal circumcision in male infants has been associated with an increased pain response to subsequent vaccination (Taddio *et al.*, 1997). Similarly, neonatal rodents exposed to repeated pain display decreased pain thresholds later in life (Anand *et al.*, 1999).

It is common practice in New Zealand to perform painful husbandry procedures such as castration and tail docking on lambs at an early age without anaesthetic or analgesia. Although the acute pain caused by such procedures is acknowledged, longer-term effects of this pain on pain sensitivity are as yet unknown. Recent research suggests that lambs castrated at one day of age are more sensitive to pain associated with subsequent tail docking at one month than lambs that are castrated at ten days of age (McCracken *et al.*, 2010). Painful procedures such as castration or tail docking in early life may result in hyper-sensitivity to subsequent painful events. This sensitisation could impact significantly on the welfare of farmed livestock in New Zealand.

This study assessed longer-term responses to early painful experiences by exploring whether castration alters pain sensitivity to a subsequent painful experience (tail docking). The effect of age, both at the initial (1 or 21 days) and subsequent (21 or 42 days) painful experience has also been investigated.

2.0 Literature review

Pain in early life: short- and long-term effects and their implications for New Zealand sheep

2.1 Introduction

Livestock species are commonly exposed to pain in early life as part of normal husbandry procedures. These painful events are known to cause short-term welfare compromise, but the possibility that there are long-term effects has yet to be investigated. The acute effects of painful procedures carried out in early life are reasonably well-understood for a number of species including rats, humans and sheep. There is some evidence that pain in early life may effect persistent changes in central nervous function leading to alteration in pain processing and sensitivity.

Lambs in New Zealand are exposed to a number of painful procedures in early life. Castration and tail docking are standard practices in sheep husbandry that are routinely performed without the use of analgesic or anaesthetic on lambs anywhere from 1 day to 6 weeks of age. From an animal welfare perspective, the possibility that such procedures might results in hyper-sensitivity to subsequent painful events is a significant issue. While this phenomenon has been documented for altricial species, including neonatal humans (e.g. Taddio *et al.*, 1997) and neonatal rats (e.g. Anand *et al.*, 1999), the long-term effects on pain sensitivity in precocial livestock animals such as sheep have not been documented. This review will outline the definition and measurement of pain, mammalian pain processing systems and their development, modulation of developmental processes and the potential implications of pain in early life for ovine welfare.

2.2 Definitions

Pain can be defined as an "unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage" (Merskey, 1994). This definition was formed based on the human experience of pain. Given that this review will ultimately focus on pain in lambs, a perspective on animal pain is required. While animal pain is likely to be similar in experience and shares the same purpose as human pain, the two may not be the same (Molony & Kent, 1997). It follows that a more specific definition be used for animals to mark this distinction. Molony & Kent (1997) used the following definition for pain in farm animals: "Animal pain is an aversive sensory and emotional experience representing an awareness by the animal of damage or threat to the integrity of its tissues; it changes the animal's physiology and behaviour to reduce or avoid damage, to reduce the likelihood of recurrence and to promote recovery; unnecessary pain occurs when the intensity or duration of the experience is inappropriate for the damage sustained or when the physiological and behavioural responses to it are unsuccessful at alleviating it." The inclusion of animal awareness in this definition is important to highlight the importance that recognition of pain has as an indicator of danger or actual injury. Physiology and behaviour must change in order to reduce or alleviate pain so that the experience is of benefit to the animal involved. Alterations in physiology and behaviour as components of the painful experience are also important from an assessment perspective; the ability to monitor changes via such responses allows for the assessment of pain in animals.

2.3 What is the function of pain?

Animal responses (primarily behavioural) to pain can tell us a lot about its basic function. For example, animals experiencing pain will often display automatic or reflex responses that are designed to protect the individual from further injury. These responses can include aggressive and defensive behaviours. Reductions in activity and the assumption of specific postures in response to pain are also common and may help to minimise pain and aid the healing process. While pain principally functions to protect the animal, the negative experience also have value as a means of teaching animals which situations are

potentially harmful and should be avoided in the future; learning the cues associated with impending pain allows for the anticipation and avoidance of future painful events (Ploghaus *et al.*, 2000).

2.4 Animal welfare and the experience of pain

An animal's experiences can be positive, neutral or negative. These experiences are the result of integrated sensory input, from both the environment and from within the animal. Pain is one of these experiences. It is important to note that the experience of pain can only be fulfilled in individuals that are both conscious and sentient. Consciousness refers to the state of being conscious. In order to be sentient, the nervous system of an individual must have reached a state of development advanced enough to support the relay of sensory inputs to the higher brain centres (Mellor & Diesch, 2006). The balance of animal experiences contributes to their current welfare status. Given the prerequisites for the experience of pain, it follows that welfare can only be compromised by noxious inputs when an animal is both conscious and sentient.

2.5 The physical component of pain

Having defined pain, a physical description is now required. As mentioned in the definitions above, pain is an unpleasant or aversive experience involving sensory and emotional components (Merskey, 1994). There are two main types of pain: nociceptive and neurogenic. Nociceptive pain is caused by actual tissue injury. A stimulus that damages, or threatens to damage tissue is known as a 'noxious stimulus.' Noxious stimuli can be mechanical, thermal or chemical in nature. When a tissue is exposed to a noxious stimulus, specific neurons that are preferentially sensitive to these stimuli respond (Marchand, 2008). These neurons are known as 'nociceptors,' and are free nerve endings (non-myelinated nerve terminals which contain synaptic vesicles). The detection of nociceptive stimuli by the nociceptors is termed 'nociception.' The nociceptors are linked to nerve fibres which then transmit the nociceptive signal to the spinal cord and

brain. The pathways by which these signals reach the brain will be discussed in more depth in a later section.

It is important to note that the process of nociception does not include the production of emotional responses to noxious stimuli (Marchand, 2008). Depending on the terminal site in the brain for the nociceptive signals and the level of consciousness of the individual, the sensation of pain and the concurrent experience will differ. As with the pain pathways, this will be discussed further in a later section.

The second type of pain is neurogenic pain. This pain is caused by damage or malfunction within the nervous system where incorrect nervous signals are sent to the brain and experienced as pain.

2.6 Types of pain

As mentioned previously, there are two categories of pain and, across the two divisions, there are five subcategories of pain: nociceptive (somatic, visceral and inflammatory) and neurogenic (causalgia and functional) pain (Marchand, 2008; refer to Table 1).

Table 1. Classification of pain types, subcategories and their classification (from Marchand, 2008).

Type of pain		Characteristics	Mechanisms	Example of pharmacologic treatments
Nociceptive	Somatic (tissue injury)	Superficial (skin) or deep pain (muscle, fascia, tendon)	Mechanical, thermal or chemical stimuli	Acetaminophen Na+ blockers NSAID, steroids, opioids
	Visceral (irritable bowel, cystitis)	Constant or cramping, poor localization. Autonomic responses	Visceral distension	NSAID, antispasmodics
	Inflammatory (musculoskeletal)	Localized or diffuse pain hyperalgesia, allodynia.	Associated with localized inflammation	NSAID, steroids
Neurogenic	Causalgia (neuralgia, radiculopathy, CNS lesions)	Spontaneous, paroxysmal pain. allodynia, hyperalgesia.	Peripheral or CNS lesions	Anticonvulsivants, opioids, antidepressants
	Functional (FM, thalamic syndromes, irritable bowel syndrome)	Diffuse deep pain, hyperalgesia, allodynia	Dysregulation of excitatory or inhibitory mechanisms in CNS	Antidepressants, anticonvulsivants, opioids, cannabinoids.

In addition to different types of pain, there are also different sensations that noxious stimuli can elicit. For example, the sharp pain of stubbing ones toe is distinctly different

from the agonising, itchy pain of chicken pox, or the immobilising, awkward pain of hitting ones 'funny-bone.' Gregory (2004) listed thirty different descriptors for the sensation of pain, including aching, throbbing, burning, shooting and cramping.

2.7 What is the experience of pain?

The experience of pain is subjective. Pain, even as a result of the same stimulus, will be experienced differently by different individuals. For example, person A may find the pain of a needle inserted for blood sampling much more painful than person B; there is a distinct lack of relationship between the noxious stimulus and the amount of pain that is felt (Hilgard, 1969). Pain is derived from a convolution of afferent information coming from nociceptors and cognitive information about the present context, past history and future implications of the painful stimulus. There is a wide range of inputs which the brain integrates to produce the painful experience. Factors such as age, gender and genetics interact dynamically to provide a unique and individual experience.

In an elegant study which sought to investigate human cerebral responses to experimental heat pain, it was found that pain-sensitive subjects displayed higher levels of activity in pain-related areas of the cortex than did pain-insensitive subjects (Coghill *et al.*, 2003). These findings suggest that differences in perceptual responses are linked to differences in the central processing of painful stimuli and are not simply the by-product of measurement error or response bias (Coghill *et al.*, 2003).

2.8 Components of pain processing systems

In order to experience pain, individuals (human and animal) must possess the functioning neural equipment necessary to support consciousness and sentience. In the most basic terms, the experience of pain is a result of exchanges between the peripheral nerves, spinal cord and the brain; these are all components of the nociceptive system. Nociceptive pain is caused by actual or potential tissue injury via a nociceptive stimulus. This stimulus acts on nociceptors in the peripheral tissues. The resultant signal is then carried to the spinal cord and brain where a response is then coordinated. In this section,

the major components of the nociceptive system will be detailed, then relationship between the components discussed and the integrative process outlined.

The peripheral nociceptors are the first major component in the pain process. Nociceptors are classified according to the properties of their associated axons. There are two main types of nociceptor axon: A-fibre, and C-fibre. A-fibre nociceptors (δ and ß) have wide diameter cell bodies and are myelinated. These nociceptive fibres conduct pain signals rapidly, mediating what is known as 'fast' or pricking pain that can be well-localised. In contrast, C-fibres are small in diameter and are unmyelinated. They conduct action potentials much more slowly and are responsible for burning pain sensations.

Within the group of C-fibres, a further distinction can be made. Two separate classes of C-fibre have been identified that may mediate different types of pain (Stucky *et al.*, 2001). One class of fibres contain neuropeptides and a receptor for nerve growth factor, trkA (Stucky *et al.*, 2001). These fibres terminate mostly on spinal neurons via lamina I and outer lamina II of the dorsal horn of the spinal cord (Stucky *et al.*, 2001). The second class of C-fibres also contain some neuropeptides, but their distinguishing factor is the presence of a carbohydrate group which selectively binds isolectin B_4 (IB_4). Additionally, these fibres project to a different segment of the spinal cord, inner lamina II. It has been suggested that these two different types of fibre may mediate different types of pain. For example, IB_4-positive nociceptors might mediate neuropathic pain while IB_4-negative nociceptors could have a function in inflammatory pain (Snider & McMahon, 1998).

Transduction is the first major process in nociception. Transduction involves the activation of nociceptors by noxious stimuli and the subsequent conversion of this information into electrical current. Nociceptors detect thermal stimuli (hot or cold), intense mechanical stimuli and chemical irritants. Detection is mediated by a number of specialised receptors like heat-sensitive ion channels and proton-sensitive channels (Woolf & Costigan, 1999). The specific mechanism by which transduction occurs differs

between types of nociceptor and between stimuli; further detail regarding these mechanisms is beyond the scope of this review.

The next process is conduction; depolarisation of the nociceptor membrane caused by the electrical current from the transduction phase produces action potential which are conducted down the nociceptor axons towards their cell bodies in the dorsal root ganglion (DRG). Voltage-gated sodium channels mediate nociceptive conduction, transferring signals from the peripheral nerves via the primary somatosensory neuron across to the secondary neurons of the spinal dorsal horn (Woolf & Costigan, 1999; Marchand, 2008). Within the spinal cord, there are two classes of secondary neuron: nociceptive specific neurons and wide dynamic range (WDR) neurons. Nociceptive specific neurons respond only to nociceptive stimuli while WDR neurons respond to both nociceptive and innocuous stimuli (Marchand, 2008). These differences are related to the axonal input they receive from the nociceptors: nociceptive specific neurons receive input from Aδ and C fibres, and WDR neurons receive additional input from Aß fibres. Again, further detail regarding nociceptive conduction is beyond the scope of this review as the primary interest is in the inputs from Aδ and C fibres.

From the DRG, action potentials travel to the central terminals in the dorsal horn of the spinal cord where they initiate the release of neurotransmitters which relay the signal across synapses to the neurons of the dorsal horn; the process is known as transmission. Finally, the signal is passed via two main pathways (the spinothalamic and spinoreticular tracts; see below for further detail) to higher centres, for example, the thalamus and brain stem. From the thalamus, tertiary neurons send afferents to the primary and secondary somatosensory cortices (S1 and S2); these areas are involved in translating the location, duration and intensity of pain (Marchand, 2008). Tertiary projections to limbic structures also occur. It should be noted that not all secondary neurons synapse in the thalamus; some may synapse with neurons in other nuclei of the brainstem such as the periaqueductal grey (PAG) which is involved in descending endogenous pain modulation (Marchand, 2008).

2.8.1 Ascending pathways

The ascending pathways are the main route to the brain for information about the interaction of the body with the external environment, the internal condition of the body and the position/movement of its parts. Anatomically, there are three distinct pathways which make up the ascending sensory systems: the anterolateral system, the dorsal column-medial lemniscal pathway, and the somatosensory pathways to the cerebellum. The neuron cell bodies from all three pathways reside in the DRG.

The anterolateral system is comprised of at least five ascending pathways which transmit nociceptive, thermal and non-discriminatory touch signals to higher brain centres. These are the spinothalamic pathway, the spinoreticular pathway, the spinotectal pathway, the spinomesencephalic pathway and the spinohypothalamic pathway.

In the spinothalamic pathway, nociceptive axons enter the spinal cord and ascend to the grey matter of the dorsal horn where they branch and make contact with neurons in several laminae, primarily the marginal zone (lamina I) and the substancia gelatinosa (SG; lamina II). Information from the SG is transmitted to second order projection neurons in laminae IV, V and VI (collectively known as the nucleus proprius). The axons of these secondary neurons cross the cord and ascend to the brainstem and thalamus in the anterolateral quadrant of the spinal cord. This discriminative pathway provides information about the location of pain (Ranney, 1996).

In the spinoreticular pathway, nociceptive axons enter the spinal cord and ascend on both sides to thalamic nuclei from where information is passed to many areas of the brain including the cingulate gyrus, the amygdala and the hypothalamus.

The spinotectal, spinomesencephalic and spinohypothalamic pathways are not of direct relevance to this discussion, so their description will not be given in this review.

The second pathway in the ascending sensory system is the dorsal column-medial lemniscal pathway. Nociceptive signals travel along this pathway from the viscera to the thalamus. Comprised of two anatomical tracts (fasciculus gracilis and fasciculus cuneatus), this pathways also communicates signals regarding discriminative touch, vibratory sense and position sense (Hirshberg *et al.*, 1996).

The final anatomical division of the ascending pathway system concerns the somatosensory pathways to the cerebellum. Further detail is not required for the understanding of this review.

2.8.2 Descending pathways

Descending pathways have an important role in the modulation of pain. Fibres pass from three main areas: the cortex, the thalamus and the brainstem (particularly the PAG). Fibres pass from the PAG down to the reticular formation, making serotonergic connections before the axons continue down the spinal cord to interneurons close to the SG where the predominant neurotransmitters are the enkephalins, which have opiate activity (Marchand, 2008). When stimulated, this system inhibits incoming pain signals via the central actions of serotonin. Additional inhibitory effects come from a separate pathway that is noradrenaline-based. 'ON' and 'OFF' cells also play a role in pain modulation via the descending pathways; 'ON' cells increase pain transmission while 'OFF' cells decrease it (Fields *et al.*, 1995). Opioids and noradrenaline inhibit the 'ON' cells and opioids also stimulate transmission in the 'OFF' cells (Heinricher *et al.*, 1994).

2.9 Pain responses to noxious stimuli

Responses to pain, as a result of nociception in conscious, sentient individuals, can be divided into two categories: physiological and behavioural. Physiological responses are the manifestation of two main organisations within the nervous system: the hypothalamic-pituitary-adrenal (HPA) axis and the sympatho-adrenal system. The HPA axis is hormonally based; once activated, cortisol releasing hormone (CRH) produced in

the hypothalamus travels to the anterior pituitary where hormones such as adrenocorticotropic hormone (ACTH) are produced. These hormones are carried to the adrenal gland where the adrenal cortex produces corticosteroid hormones which are then released into the circulatory system where they have a plethora of effects on processes such as inflammation and metabolism. Corticosteroids exert inhibitory feedback on the hypothalamus to control their own levels. Corticosteroids have a number of effects within the body, including dilation of the pupils, changes in blood pressure and heart rate, increased respiration rate and/or depth of respiration, changes in the temperature of the skin and body, anti-inflammatory effects and metabolic changes.

The sympatho-adrenal system exerts its control on autonomic functions (mainly involuntary functions regulated by the autonomic nervous system) primarily via the catecholamine hormones, adrenaline and noradrenaline. These two hormones are produced by the adrenal medulla (though noradrenaline is also produced by the nerve endings of sympathetic nerve fibres) and share many of the same bodily effects of the corticosteroids.

Physiological responses to pain manifest as a result of activation of the HPA axis and/or the sympatho-adrenal system. These physiological signs which could be used as indicators of the presence of pain are outlined in Table 2.

A number of physiological effects have been described as occurring in human neonates exposed to painful procedures (Anand & Hickey, 1987). These effects included cardiorespiratory changes such as marked increases in heart rate and blood pressure, and hormonal and metabolic changes such as increases in plasma concentrations of adrenaline, noradrenaline and cortisol (Anand & Hickey, 1987). Similarly, rubber-ring castration and tail docking of young lambs causes significant increases in arterial blood pressure, heart rate, plasma ACTH concentrations and plasma cortisol concentrations (Peers *et al.*, 2002).

Table 2. Effects of the hypothalamic-pituitary-adrenal axis and the sympatho-adrenal system in the mammalian body and the accompanying physiological signs that could be used as indicators of pain.

Effects of the HPA and sympatho-adrenal systems	**Potential physiological indicators of pain**
Increased levels of hormones associated with activation of the HPA and sympatho-adrenal systems	Increased blood cortisol concentration Increased concentrations of catecholamine hormones, adrenaline and noradrenaline
Increased depth and rate of heart beat	Increased blood pressure
Blood is shunted away from the periphery to the skeletal muscles, coronary arteries, liver and brain	Increase in core body temperature/ decrease in skin temperature
Dilation of bronchioles	Increase in respiration rate and/or depth of respiration
Mobilisation of glycogen from the liver	Increase in blood glucose concentration
Increased metabolic rate.	Increase in core body temperature

In general, behaviour can be defined as an expression of specific motor patterns, or inhibition of them. Behavioural responses to pain are outward expressions of efforts by the animal to adapt to internal/external flux which can easily be observed. Changes in physiological state can lead to changes in behaviour; however, behaviour is not simply a 'result' of a specific physiological change. Behaviour also has the capacity to feed back on an animal's physiological state. For example, activation of cannabinoid receptors immediately after birth in mice is thought to be essential in stimulating suckling behaviour (Fride, 2008). Without suckling behaviour, which results in the transfer of important maternal proteins and precursors to the newborn (Fride, 2008), successful postnatal growth and development would not be achieved.

Pain behaviours function to protect the animal. Animals express pain in a variety of ways which differ between species, individuals and circumstances. Gregory (2004) listed a number of ways in which pain could be expressed, including:

- Escape reactions,
- Abnormal postures or gaits,
- Vocalisation or aggression during movement/manipulation,
- Restlessness, rolling, kicking etc.,
- Depression, withdrawal and sleeplessness,
- Avoidance behaviour.

The stimulation of escape behaviours is beneficial in eliminating/reducing interaction between the noxious stimulus and the animal. Abnormal postures and withdrawal are likely to contribute to recovery from any tissue damage that may have occurred.

It is obvious that the expressions listed above are not exclusively related to painful experiences; distinguishing pain-related behaviours from non-painful behaviours is the main difficulty in recognising pain in animals (Gregory, 2004). The interface between behaviour and physiology is further complicated by the fact that interpretation of both physiology and behaviour is almost entirely subjective. One of the best examples to illustrate this point is the fluctuation of cortisol levels in response to different stimuli.

Plasma cortisol concentration is known to increase in response to the application of noxious stimuli to an animal. However, plasma cortisol levels in sheep also fluctuate in response to feeding (Slater & Mellor, 1981). So how does the interpreter know if they are witnessing pain or simply part of a normal cyclic pattern? Obviously, the context would clarify the likely resulting experience, but it does illustrate that physiological responses must not be taken at face-value and further investigation is commonly required to ensure that the correct conclusion has been reached.

A good example of behavioural ambiguity is the difference in response of lambs castrated by rubber ring compared with lambs castrated by knife. When lambs are castrated using

the rubber ring method, they display changes in frequency of behaviours such as rolling, abnormal lying and unsteady standing. In comparison, lambs castrated using a knife will sit or stand very quietly and in general appear to be non-responsive. It was initially thought that the rubber ring method of castration was more painful than knife castration due to the increased behavioural activity of the lambs. However, upon closer inspection of physiological indicators of pain, it was found that the reverse was true; castration of lambs by knife causes longer-lasting distress than castration using rubber rings (Lester *et al.*, 1991). The underlying theory is that lambs castrated by knife were so consumed by the pain caused by this method that they were unable to behave in a normal fashion. This misinterpretation could have had serious animal welfare implications had the difference in behaviour not been carefully assessed.

When researchers wish to assess responses to pain, behavioural methods are an attractive choice given their unobtrusive nature. Examples of the use of behavioural methods in the measurement of pain in infant humans, rodents and lambs follow.

Behavioural indices of pain are often used to gauge pain in infant humans. The use of facial expression as an indirect measure of nociceptive activity is perhaps the most commonly used indicator of pain in infants. Indeed, the neonatal facial coding system is considered to be the most accurate and valuable of available pain measures in human infants (Grunau & Craig, 1987 cited in Taddio *et al.*, 1997). This system was used to successfully gauge the pain responses of circumcised infants to subsequent vaccination using three specific facial actions (brow bulge, nasolabial furrow and eyes squeezed shut) to obtain a composite facial behaviour score (Taddio *et al.*, 1997).

Pain assessment in rodents is more difficult than it is in non-verbal humans and other species such as cats and dogs. This difference likely exists primarily due to a lack of familiarity with the rodent behavioural repertoire. Animals such as cats and dogs are more popular as pets than rodent species and, as such, it is easier to bond with them and familiarise oneself with their behavioural patterns. Behavioural assessment in animals requires a solid knowledge of what is considered to be ‘normal’ behaviour. Obviously

this holds for any species of animal that we wish to assess, but the lack of familiarity and decreased preference for rodents increases the difficulty of this task. Behaviours such as removal of self from the pain stimulus, vocalisation (that may be outside the range of human hearing) and aggression can be indicative of pain in rodents (Flecknell & Waterman-Pearson, 2000). Reductions in active and attentive behaviours have been observed in rats that have undergone surgical procedures that are known to be painful (Roughan & Flecknell, 2000). A facial coding method similar to that developed for human neonates has recently been constructed for mice; it would appear that pain in mice elicits facial expressions akin to those displayed by humans in pain (Langford *et al.*, 2010).

Pain behaviours in prey species such as sheep may not be overt. Behaviours elicited are likely to be subtle in their presentation, so researchers must take care in their judgement of pain characteristics such as intensity (Flecknell & Waterman-Pearson, 2000). In adult animals, behaviours such as lip curling and the grinding of teeth can be used as indicators of visceral pain. For lambs, the limited repertoire of behaviours available to them translates to quite distinct behavioural patterns in response to painful stimuli such as castration and tail docking. In their assessment of behavioural responses of male lambs to castration, Thornton and Waterman-Pearson (2002) noted a number of important changes in the behaviour of lambs castrated at different ages; it was assumed that the presence of these patterns was indicative of a level of prolonged acute pain. One week-old lambs displayed decreased gambolling behaviour while four to six week old lambs demonstrated an increased frequency of abnormal postures and reduced their display of normal postures (Thornton & Waterman-Pearson, 2002).

2.10 How do we assess pain?

It can be assumed that the experience of pain differs between species. It is understood that painful experiences also differ between individuals within the same species. With this in mind, measuring pain consistently and accurately is a difficult task. What methods are used to gauge pain in humans and other animals? There are two main categories of

pain assessment: physiological and behavioural. Physiological and behavioural responses to pain were covered in a previous section; assessment of pain is largely based on these responses. Imaging techniques for assessment of pain continue to be developed and refined. Some of these techniques will be explored below.

2.10.1 Physiological assessment of pain

The hormonal basis of the two systems that control physiological responses means that measurement of the appropriate hormones released during their activation is a logical option for assessment of pain. The concentrations of corticosteroids produced as a result of HPA activation and catecholamine hormones (adrenaline and noradrenaline) via activation of the sympatho-adrenal system can be measured using blood samples. Measurement of blood cortisol concentration is the most common. If blood samples are taken over a period of time spanning from before the painful event occurred to a number of hours afterwards, a trace can be prepared which illustrates the peak cortisol concentration and subsequent decline to normal levels. This is a particularly useful tool that can be used to assess the duration of a painful experience.

Physiological responses also include cardiovascular and respiratory changes. Given that the onset of pain causes heart rate and respiration rate to change, logically these factors could all be incorporated into pain assessment methods. However, the alterations in these factors (including hormonal alterations such as those seen with cortisol) are not specific and their isolation with regard to a single stimulus such as pain can be difficult to achieve.

2.10.2 Behavioural assessment of pain

Physiological recordings taken to assess pain in humans and animals can be invasive to collect. Behavioural methods provide a comparatively simple, non-invasive alternative to physiological methods which can be used to obtain information regarding individual responses to painful stimuli. An animal experiencing pain commonly displays a pattern of behaviour that differs from their 'normal' repertoire. By cataloguing behavioural patterns before and after painful events, comparisons can be made to assess the presence and impact of the noxious stimulus. Behavioural methods generally follow this pattern:

1. Observers must familiarise themselves with the behaviours of the species of interest.
2. An ethogram must be developed which contains a list and description of all the behaviours that the observers wish to catalogue.
3. The length of focal observations must be determined.
4. Observers must practise scoring animal behaviour to ensure that they can be accurate and consistent in their observations.
5. Once steps 1-4 have been completed, behavioural observations of experimental animals can be made.

Behaviours related specifically to pain can be clarified using tests to see if their display can be decreased or eliminated by the application of anaesthetics or analgesics. Behaviours which are displayed by animals in pain but decrease or are not displayed by animals that have had anaesthetics or analgesics administered can be considered as pain-related. For example, topical anaesthesia has been shown to significantly reduce the display of pain-related behaviours in lambs exposed to surgical castration plus surgical or hot-iron tail docking (Lomax *et al.*, 2010).

Whether the method used to obtain information regarding the presence, duration and intensity of pain is behavioural, physiological or a mixture of the two, the importance lies in the comparisons that can then be made between experimental groups, animals of

different ages, animals exposed to different noxious stimuli and even animals of different species.

2.10.3 Imaging techniques for the assessment of pain

In their construct of animal welfare, Mellor *et al.* (2009) introduced the role of consciousness; an animal must be conscious to experience any emotion, including pain. However, a noxious stimulus administered to an unconscious animal will produce nociceptive signals that may still reach brain structures that have roles in pain processing and/or perception. Activity in these structures in the unconscious animal is insufficient to produce a conscious pain experience, but nociceptive activity occurs nonetheless (Bromm, 2001). Functional brain imaging techniques such as positron emission tomography (PET) and functional magnetic resonance imaging (fMRI) have begun to be applied in situations where we wish to attribute responses to stimuli in the unconscious individual. Non-invasive measurements of this nature are of obvious value from an ethical standpoint. These techniques are largely based on the measurement of metabolic changes resulting from increased neuronal activity in response to specific stimuli. Increased activity in the brain alters the balance between oxygen supply and demand to the tissues; capillaries dilate and cerebral blood flow changes accordingly (Bromm, 2001). Novel research in this area continues to fine-tune techniques which may be used in the future as highly accurate assessors of human and animal pain.

2.11 Modulation of pain processing

How can processing of nociceptive input and perception of pain be modified in mature animals? Three means of modulation will be discussed in this section: the gate control theory, endogenous control and exogenous control.

2.11.1 The Gate Control Theory

The gate control theory suggests that the perception of pain is modulated by interaction between different neurons. For many types of pain, our immediate reaction to the initial sensation is to rub the damaged area. According to the gate control theory, activity in touch fibres acts to inhibit cells that would transmit the pain signals coming from nociceptors activated by a painful stimulus; it follows that inhibition of such cells should decrease the amount of pain felt. More specifically, the theory proposes that painful experiences are the result of interplay between three systems: communication between the substantia gelatinosa (SG) and the central transmission cells; afferent signalling in the dorsal column; and the activation of neural mechanisms by central transmission cells (Melzack & Wall, 1965). The SG is a functional unit when extends down the length of the spinal cord; it was suggested that it modulates the transmission between peripheral fibres and central cells.

With reference to Figure 1, both large (touch) and small (pain) diameter fibres stimulate the transmission cells; pain is experienced once the output of nociceptive signals transmitted along ascending pathways to the brain from the transmission cells exceeds a threshold level. Simultaneously, input from the large diameter fibres is stimulating the SG which has an inhibitory effect on the conduction of input to the transmission cells. In contrast, input from the small diameter fibres inhibits the SG, acting to keep the 'gate' open and neural input flowing through the transmission cells.

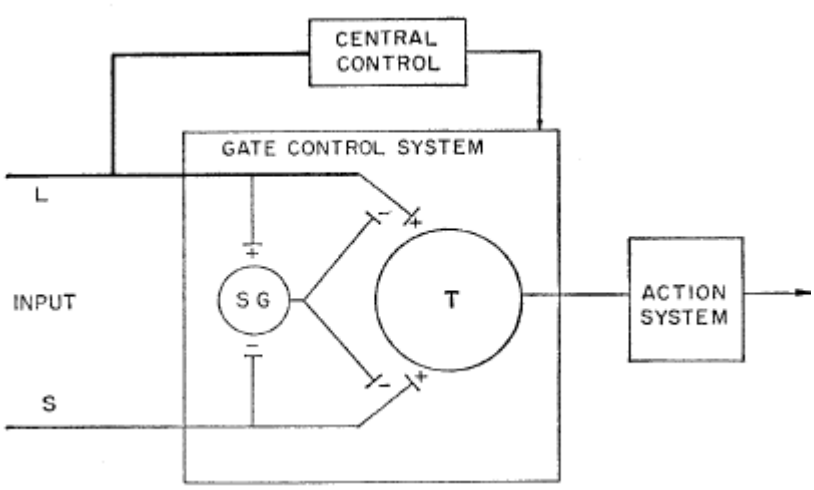

Figure 1. A schematic diagram illustrating the gate control theory of pain modulation (modified from Melzack & Wall, 1965). Large and small diameter fibres project to the substantia gelatinosa (SG) and central transmission cells. Inhibitory effects of the SG on afferent fibre terminals are increased by activity in the large diameter fibres and decreased by activity in the small diameter fibres.

Therefore, the amount of input reaching higher brain centres i.e. pain felt, is a balance between the levels of input coming from the small and large peripheral fibres. While the gate control theory can explain a number of pain phenomena and has made an important contribution to biological and medical sciences, it cannot explain chronic pain problems such as phantom limb pain. It is apparent that the brain does not require the physical presence of the body to still have a sensation of it; Melzack himself said, "the brain itself can generate every quality of experience which is normally triggered by sensory input" (Melzack, 1989 cited in Melzack, 1993).

2.11.2 Endogenous mechanisms

Endogenous mechanisms are another means by which the body can modulate the processing of nociceptive input. Endogenous mechanisms can be divided into excitatory and inhibitory mechanisms. Excitatory mechanisms increase the perception of pain by

stimulating transmission of nociceptive signals to the spinal cord and brain. Central sensitisation can occur at the spinal level as part of excitatory mechanisms. Spinal neurons display an accentuated response to nociceptive (hyperalgesia) and non-nociceptive (allodynia) input (Marchand, 2008). Sensitisation depends on activation of NMDA receptors of spinal neurons, which are activated by a sustained release of glutamate.

In contrast, inhibitory mechanisms act to depress the transmission of nociceptive signals to the spinal cord and brain. A few years after the proposal of the gate control theory, Reynolds showed that stimulation of the PAG has a strong inhibitory effect on pain (Reynolds, 1969). Areas of the rostroventral medulla such as the PAG and the nucleus raphe magnus (NRM) are important serotonergic and noradrenergic descending inhibitory pathways which recruit enkephalinergic interneurons within the spinal cord to achieve the analgesic response (Marchand, 2008).

In the late 1970s, Le Bars and colleagues proposed the diffuse noxious inhibitory control theory (DNIC) (Le Bars *et al.*, 1979). The foundation for this model came from the knowledge that a localised nociceptive stimulus application can produce a diffuse analgesic effect over the rest of the body; this is known as counter-irritation (Marchand, 2008). In the DNIC model, nociceptive signals from an appropriate stimulus will be sent not only to higher centres but also to the PAG and NRM of the brainstem, resulting in inhibitory output at a variety of spinal levels (Le Bars *et al.*, 1979).

The cannabinoid system is a major neurochemical system which has great potential as part of pain management via the application of exogenous cannabinoids. Cannabinoid receptors are localised in neural regions that function in transmission and modulation of pain signals, for example, the dorsal horn, the PAG and the rostral ventromedial medulla (RVM) (Hohmann & Suplita, 2006). Behavioural studies have shown that administration of exogenous cannabinoids can have an antinociceptive effect (Hohmann & Suplita, 2006; Hohmann, 2002).

2.11.3 Exogenous mechanisms

Exogenous management of pain is an important area of medicine. Analgesics are those drugs that relieve pain. There are two divisions of analgesic drug: opioid and non-opioid analgesics. Opioids have a potent effect on pain, acting at a number of points along the pain pathways (periphery, spinal cord and supraspinal sites) to inhibit various nociceptive reflexes (DeLeo, 2006). This is achieved by the inhibition of release of neurotransmitters such as substance P, and the mimicking of endogenous opioids. Non-opioid analgesics include a group known as the non-steroidal anti-inflammatory drugs (NSAIDs). The NSAIDs act by inhibiting cyclooxygenases which lead to a decrease in prostaglandin production. Prostaglandins have a role in activating the inflammatory response which leads to swelling and pain; decreasing the release of prostaglandins post-injury thus minimises swelling and relieves associated pain.

Analgesics act on secondary processed to effectively soften the experience of pain. Anaesthetics function by stopping the pain from being perceived. General anaesthesia involves the administration of drugs which cause the patient to become unconscious. As mentioned previously, an individual must be conscious to perceive and experience pain; the unconscious patient is unable to feel pain. Local anaesthesia (applied topically or by injection) blocks the generation and/or transmission of nerve impulses from injured tissues. The patient remains conscious, but as nociceptive signals fail to make it to supraspinal centres, they cannot be perceived as pain.

2.11.4 Types of stimuli that modulate pain processing

The mechanisms that act in the modulation of pain transmission and processing have been outlined. This section will focus on types of stimuli that activate or inhibit said modulation.

2.11.4.1 Stress-induced hyperalgesia/hypoalgesia

Hyperalgesia refers to an increased sensivity in pain either in the injured tissue (primary hyperalgesia) or in undamaged adjacent tissue (secondary hyperalgesia). Extending this further, stress-induced hyperalgesia (SIH+) is the term given to those situations where stress appears to exacerbate painful experiences (Martenson *et al.*, 2009). The reverse, stress-induced hypoalgesia (SIH-; where intense stress suppresses pain) is also known to occur. SIH- is modulated by brainstem systems, but the mechanism by which SIH+ acts has yet to be confirmed. It has been suggested that the hypothalamic dorsomedial nucleus is a contributor to SIH+ via the RVM and its role in the descending control of nociception (Martenson *et al.*, 2009). Populations of neurons within the RVM can be divided into NEUTRAL, ON and OFF cells. The role of NEUTRAL cells is unclear, but ON cells facilitate nociception and OFF cells suppress it (Martenson *et al.*, 2009). Martenson and colleagues showed that activation of stress-related circuitry in the hypothalamus recruits ON neurons in the RVM to produce hyperalgesia (Martenson *et al.*, 2009).

2.11.4.2 Comfort hypoalgesia

Kangaroo care (KC) is an excellent example of comfort hypoalgesia, where the mother/father holds the human infant to their chest with skin-skin contact, similar to the way a kangaroo holds it's young inside the pouch. Via its positive effects on infant autonomic behaviour and state, this method is used to minimise the risk of side effects that premature infants exposed to pain and stress may develop (Browne, 2004). Johnston and colleagues believe that the skin-skin contact involved in KC has an analgesic effect for preterm neonates exposed to heel-stick blood sampling (Johnston *et al.*, 2003). Other studies have shown maternal presence to have a dampening effect on pain responses in animals (Blass *et al.*, 1995; Walker *et al.*, 2003). Furthermore, a number of animal studies have shown that maternal-infant reunion following a separation causes the release of endorphins (Kalin *et al.*, 1995; Carden *et al.*, 1991). Further research on the underlying physiological mechanisms of KC is required, but it seems that KC reduces

physiologic volatility and facilitates stability that will be of obvious benefit during a painful or traumatic experience.

2.11.4.3 Distraction

Distraction as a means of coping with pain is a component in many pain management systems; indeed it is a way that people will often use intuitively to help lessen pain in themselves and others. This mechanism of modulation is based around the idea that pain requires attention for its full effect to be felt; if attention is directed elsewhere, less attention will be available for pain and less pain will be experienced (Verhoeven *et al.*, 2010). The ability of distraction to consistently detract from the experience of pain is questionable. Studies have shown that certain painful situations, for example, the application of a novel or especially intense painful stimulus, are very difficult to shift attention away from using distraction methods (Crombez *et al.*, 1998; Eccleston, 1994; Legrain *et al.*, 2009). According to models predicting attention capacity, there is a limited quantity of cognitive resources available for use. For distraction to be effective, it must command a greater share of cognitive capacity than the painful stimulus (Kahneman, 1973, cited in Eccleston & Crombez, 1999). Such models have been challenged by motivational counterparts that place more importance on the affective-motivational characteristics of the distraction than the level of cognitive difficulty (Eccleston, 1994).

2.12 Development of pain processing systems

Animals can be placed into one of two groups based on their level of development at birth. Altricial species such as humans and rodents are born in an immature state compared to precocial species such as sheep which are born relatively developmentally mature. Given that developmental state at birth differs between the groups, it is of benefit that the pain processing systems of examplar species be outlined if they are to be accurately compared in any way. What capacity do newborn and very young animals have to feel pain? Firstly, the newborn status of pain componentry in an altricial species

(human) will be described, followed by the status of pain componentry in the lamb, a precocial species.

2.12.1 Altricial species

In previous sections of this review, the components of neural pathways necessary for the processing and perception of pain have been outlined. Here, the development of the human nervous system in relation to pain processing and perception will be covered.

The human nervous system begins to develop early in gestation and continues to do so in postnatal life (Simons & Tibboel, 2006). Given that nociception refers to nervous activity produced by noxious stimuli, a good judge for the level of development of the nervous system is the timing at which the components necessary for nociception are present and functional. In their broadest forms, these components are the peripheral nerves, the spinal cord and the brain. Various studies have surmised that the peripheral nerves and spinal cord are sufficiently developed by birth to support the activity necessary for nociception (Anand & Hickey, 1987; Lee *et al.*, 2005; Simons & Tibboel, 2006). Functional maturity of the cortex is of key importance to nociception. By just 20 gestational weeks, the neocortex has a full quota of 10^9 neurons and within approximately four weeks of this, thalamocortical connections are also established (Anand & Hickey, 1987). The functional maturity of these components and their connections has been indicated via analysis of electroencephalographic patterns, cerebral metabolism and behaviour (Anand & Hickey, 1987). Distinct encephalographic patterns are evident at 30 weeks of gestation, and evoked potentials have been recorded in response to visual, auditory, olfactory and tactile stimuli in preterm infants (Torres & Anderson, 1985). Achievement of maximal metabolic activity in sensory areas of the brain (Chugani & Phelps, 1986) combined with well-defined periods of sleep behaviour (Arduini *et al.*, 1986, cited in Anand & Hickey, 1987) and the EEG findings mentioned above provide sound support for a high level of cortical functionality. Therefore, the anatomical and functional componentry necessary for pain perception are present in the newborn human infant.

2.12.2 Precocial species

As a precocial species, lambs are born at a relatively mature stage of development. They can stand to suckle and move about within a short period of time post-parturition. Given that their physical ability with regard to locomotion is so advanced, it stands to reason that their nervous function may also be at a more advanced stage compared to that of an altricial newborn such as a human.

Mellor and Diesch state that for any animal to experience pain they must be sentient (their nervous system must be developed enough to relay sensory inputs to the higher brain centres) and conscious (Mellor & Diesch, 2006). Patterns of neurological development can be illustrated by looking at the development of electrical activity in the brain via the electroencephalogram (EEG). In the beginning, the EEG pattern is isoelectric. Intermittent spikes in activity begin to appear, followed by more substantial bursts of activity and finally, continuous EEG activity (Diesch *et al.*, 2007). This differentiates into specific sleep-like patterns which later include characteristics indicative of conscious awareness. Despite great advances in our knowledge in this area, the exact time at which both sentience and consciousness are achieved is not yet known for the majority of species. For lambs, it is known that differentiation of EEG activity occurs after approximately 80% of pregnancy has elapsed. However, *in utero* factors maintain the unborn lamb in an unconscious state until immediately after birth (Mellor & Diesch, 2006). While conscious perception is achieved soon after birth, the appearance of all its associated features is likely to be a gradual process that may not be complete for a number of days (Diesch *et al.*, 2007).

Johnson and colleagues found that anaesthetised lambs castrated at 1-2 days of age show a lower EEG response compared to lambs castrated at one week and older (Johnson *et al.*, 2005). Slow regression of hormonal factors with anaesthetic, analgesic and sedative effects has been proposed as the reason for such neural ‘sluggishness’ (Diesch *et al.*, 2007). While lambs of different ages may be qualitatively different in their experience of painful stimulations, the important point to note is not ‘how bad’ the pain is, but whether

it is experienced at all. Newborn lambs have the ability to experience pain and this must be taken into account when carrying out invasive and potentially painful procedures.

2.13 Acute effects of early pain in lambs

As a livestock species, sheep are subjected to a number of procedures in early life as part of standard husbandry for their species. While their practice may be painful or traumatic, these systems exist for justifiable and practical reasons which will be detailed below. For those procedures which cause pain, the acute effect of their application has been well-researched. In the following subsections, castration and docking will be examined with a view to their methods, acute effects of their application in lambs and the effect of age on lamb responses to their application.

2.13.1 Methods of castration and tail docking

Castration and tail docking are standard husbandry procedures on New Zealand sheep farms. In New Zealand, a common method of achieving both castration and tail docking is the use of tight rubber rings applied to the neck of the scrotum or the base of the tail using a scissor-like device known as an elastrator. Blood flow/venous drainage of the testes/tail is prevented (ischemia) (Wood & Molony, 1992) and the resultant dead tissue distal to the ring drops off within a month. As the tail and the testes/scrotum are richly innervated appendages, their removal will result in acute pain lasting a number of hours (Thornton & Waterman-Pearson, 1999). Manipulation of the scrotum, testes and tail involved in the application of the rubber rings elicits an initial nociceptive barrage. After this, the cause of persisting pain appears to come from nociceptive activity in the ischemic tissues, conveyed to the central nervous system via intact nerves from the affected areas (Wood & Molony, 1991). These nociceptors (testicular, in the case of this particular study) can function for more than three hours after ring application (Grubb *et al.*, 1990).

2.13.2 Acute effects of castration and tail docking

The acute effects of early pain in lambs have been well-documented. These can be divided into two groups which will be discussed in that order: physiological effects and behavioural effects.

In a study that looked specifically at acute responses to rubber-ring castration and tail docking, it was found that they caused distinct elevations in arterial blood pressure and heart rate which continue for more than four hours post-treatment (Peers *et al.*, 2002). Similarly, plasma concentrations of cortisol and ACTH peak within the same time at around 60-80 minutes post-treatment (Peers *et al.*, 2002). Another study that investigated the cortisol responses of young lambs to castration and tail docking using different methods found that lambs docked using rubber rings displayed a transient increase in plasma cortisol concentrations (Lester *et al.*, 1991). However in this case, plasma cortisol returned to pre-treatment values or below within four hours of treatment.

Castration and tail docking stimulate behavioural responses in lambs that are suggestive of pain (Dinniss *et al.*, 1999). Behaviours which are rarely displayed otherwise, such as lateral recumbency and increased restlessness are commonly elicited following the application of rubber rings in both tail docking and castration (Mellor & Murray, 1989; Molony *et al.*, 1993; Kent *et al.*, 1995; Lester *et al.*, 1996).

2.13.3 The effect of age on pain responses

Recent behavioural research in lambs has indicated that animals castrated at one day of age are more sensitive to the pain of tail docking at one month than lambs castrated at ten days of age (McCracken *et al.*, 2010). However, gauging the differences between age groups in terms of pain response is difficult given that intensity/activity of behavioural responses also differs with age. To illustrate, Molony and colleagues found that lambs of different ages had different 'normal' behaviour patterns (Molony *et al.*, 1993). A number of explanations for the age behavioural difference to the same stimulus have been

postulated. For example, the maintenance of normal behaviour may have higher importance for younger lambs i.e. changes in behaviour could be attributed to changing survival needs. Ability to respond to pain may develop over time; different stages of neural development will translate to different motor and sensory responses. Similarly, in a study that sought to assess the effect of age on the electroencephalographic response of lambs to castration, the authors concluded that there are qualitative differences between animals of different ages (Johnson *et al.*, 2005).

2.14 Can the development of pain processing systems be modulated?

It is thought that the main target of pain modulation by pain itself is the primary nociceptive circuitry surrounding the area of the insult (Lidow, 2002). Alteration to even a single part of the pain system can easily result in knock-on effects for the rest of the system due to their high level of interconnectivity. While great measures can be taken to shield newborns from pain, avoiding exposure to stress is not so easily handled. Stress can have a range of effects on those individuals which experience it. The possibility that stress in early life, like pain, may also have long-term effects on pain sensitivity is a cause for concern. Unlike pain, which initially affects the nervous system in a localised, specific way, stress often acts more globally by altering systems at their most superior structures (Lidow, 2002). From these points, changes affect the sensitivity of response of primary pain circuits. Pain and stress may also be differentiated in their effect on pain processing systems by the overall patterns of changes in future sensitivity that these stimuli create (Lidow, 2002).

2.15 Evidence of early pain modulating sensitivity in altricial species

Clinical studies have suggested that pain can affect both the short- and long-term experiences of exposed individuals (Taddio *et al.*, 1997; Porter *et al.*, 1999; Anand & Scalzo, 2000). The bulk of the knowledge in this area comes from human and rodent research. In a study investigating the effect of noxious stimulation (neonatal circumcision) on subsequent pain response to vaccination it was found that circumcised

infants had an increased pain response to vaccination (Taddio *et al.*, 1997). At birth, neonatal rat pups have a neurological maturity that is very similar to that of human neonates at 24 weeks of gestation (Anand *et al.*, 1999). Furthermore, their subsequent development follows a similar path as well, making rodents an excellent model species for research regarding early neurological plasticity, where plasticity refers to "changes that occur in the established nervous system (Stucky *et al.*, 2001). Indeed, a neonatal rodent model found that rats that experienced repeated neonatal pain (insertion of a 25 gauge needle through the paw) developed decreased pain thresholds (Anand *et al.*, 1999). Furthermore, as adults these rats displayed a higher preference for alcohol and increased anxiety and defensive withdrawal behaviour.

In their review, Anand and Hickey state that the nervous system is active during prenatal development and that developmental change naturally affects the entire system (Anand & Hickey, 1987). It follows that negative changes such as abnormal or excessive activity (due to pain) in the developing nervous system have the potential to alter normal synaptic development and lead to changes in somatosensory processing (Reynolds & Fitzgerald, 1995; Anand *et al.*, 1999; Ruda *et al.*, 2000). While the intensity and duration of the inflammation caused by the manipulations carried out in these studies were high compared to those caused by standard interventions in human neonates, the existence of even a tendency towards adverse developmental effects needs to be taken into serious consideration. Until conclusive evidence is provided regarding the ability of infants to feel pain and the potentially adverse long-term effects of early pain, ethical considerations require us to assume that infants do experience pain and the possibility of said pain having negative effects in the future must be taken into account.

2.16 Animal welfare implications of early pain in lambs

The National Animal Welfare Advisory Committee (NAWAC) states that castration and tail docking should be performed as early as possible, but not after six months of age without pain relief (NAWAC, 2005). Ideally, castration without anaesthetic or analgesic

should be carried out on lambs up to four weeks of age and tail docking should be undertaken before lambs reach six weeks of age (NAWAC, 2005).

Castration and tail docking are carried out for a number of reasons. In extensive sheep farming systems, male lambs often reach puberty before they are drafted off for slaughter. For that reason, castration of males within the first few weeks of birth is a standard procedure to prevent a) flock disruption due to the sexual activity of young males, b) unwanted pregnancies, and c) indiscriminate breeding (Archer, 2004). Although this is no longer a significant problem, castrations were initially also performed to reduce taint in meat caused by high levels of male hormones. Tail docking is carried out primarily to reduce the risk of disease. The proximity of the tail to the excretory orifices means that it can easily become soiled with faeces and urine. Dirty wool and dags attract flies; flies lay their eggs on the wool and the resultant maggots burrow through the fleece before beginning to feed on the flesh below. The welfare costs of this condition have not been confirmed by quantitative studies but they may be considerable. The removal of part of the tail aims to minimise the degree of soiling that can occur around the posterior end of the animal and therefore decrease attraction to flies.

2.17 Conclusion

Studies using human and rodent models have established that painful experiences in early life can result in persistent changes in pain processing and sensitivity, at least in altricial species.

As part of normal husbandry, castration and/or tail docking is carried out on the majority of New Zealand lambs during early life. The potential for early painful experiences to alter pain sensitivity is thus of particular relevance to lambs and justifies further research. Current animal welfare guidelines in New Zealand recommend that castration without the use of local anaesthesia should be performed as early as possible, with the best results being achieved on those animals less than four weeks of age (NAWAC, 2005). The possibility that early painful experiences could impact on the perception of future painful

events raises some important questions: if early pain does have future effects, do these increase or decrease lamb sensitivity to pain? Does age of early pain influence this effect? Preliminary research has indicated that pain sensitivity in older animals is changed following early noxious stimulation. This research has highlighted acute changes, but the investigation of longer-term change would also be valuable.

Therefore, based on existing research in humans and rodents, longer-term changes in pain sensitivity in lambs were investigated. The study builds on preliminary research carried out within the Institute of Veterinary, Animal and Biomedical Sciences at Massey University (McCracken *et al.*, 2010). There were three main objectives for this study:

1. To determine whether early castration alters sensitivity to subsequent noxious input (tail docking) in lambs.
2. To determine whether age at castration influences changes in pain sensitivity after tail docking in lambs.
3. To determine whether the interval between the two procedures influences changes in pain sensitivity after tail docking.

3.0 Methods and Materials

3.1 Animals

The animals that were used in this study were cared for according to standard farm husbandry practice. Animals remained on Massey University's Moginie Block and Number One Dairy sheep farms throughout the duration of the study. Ewes (both mature and two-tooth) were mated on site approximately 2 weeks apart. The lambs (145 Romney x Poll-Dorset) included in this study were born over a one month period, starting in the middle of August 2008. The original number of lambs entered into the study was 182, a number of these had to be removed from the trial for a variety of reasons including death, illness, mis-mothering and physical loss. Much of said loss can be attributed to the bad weather that experienced throughout the study. Some data was also lost due to videotape errors, further reducing the number of animals from which useable data could be obtained. Most of the lambs were kept in large mob paddocks at both of the sheep farms but a number from Moginie Block were kept in small, separate paddocks (groups of 2-3 ewes and their lambs) for the bulk of the study. For the last two weeks of the study, the small paddocks were opened into 4 main sections to increase grazing area. All animals were grazed on pasture. Individual lambs were identified using coloured paint marks on their flanks. Ear tags were not used due to the possible effects of this noxious stimulation on the pain responses being studied.

3.2 Treatment groups

3.2.1 Experimental procedures

All male lambs were randomly allocated to one of six groups at birth. According to these groups, lambs were either castrated or handled on their first day of age or at 21 days of age (refer to Table 1 below). Treatments were balanced across the two farms by the use of a spreadsheet containing the different groups which had been randomised prior to the start of the trial. For practical reasons, male lambs from multi-offspring births

(comprised of at least two males) were placed in the same treatment group. It is important to note here that by the use of 30-minute pre-docking videotapes, lambs acted as their own controls. Behavioural data obtained from the post-docking videotapes could then be judged in relation to the pre-docking videos.

3.2.2 Castration and handling

The rubber ring method was used to castrate the lambs in this study. In this method, a tight rubber ring is applied to the neck of the scrotum using an elastrator (see Figure 2 below). One person held the lambs while a second person applied the rubber ring to the neck of the scrotum using the elastrator.

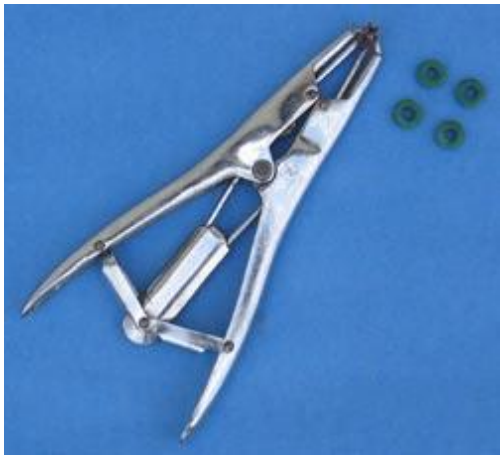

Figure 2. An example of an elastrator, the device used in this study to apply a tight rubber ring to the neck of the scrotum (castration) or the base of the tail (tail docking). Photo taken from http://www.luresext.edu/photos/elastrator.jpg.

The rubber ring was positioned so that the testes lay distal to the ring and the teats remained proximal. Handled lambs underwent the same procedure as castrated lambs, except that the rubber rings were not applied to the scrotum.

Castration of 1-day-old lambs took place in the paddock. Care was taken to ensure that these lambs had stood and nursed (important for the formation of the ewe-lamb bond) before castration was carried out. Lambs castrated at 21 days of age were rounded up

into small yards with their dams, the rubber rings applied, and then released back into the paddock.

Table 3. Experimental schedule for treatment and tail docking of lambs. Age One = the age (in days) of lambs at first treatment (castration or handling); Age Dock = the age in days of lambs at tail docking. Note: the tail docking timetable allowed for a one day leeway on either side of the scheduled day.

Group	N	Day of age	
		Age One	**Age Dock**
1	21	Castrate - 1	21
2	27	Handle – 1	21
3	23	Castrate - 1	42
4	24	Handle - 1	42
5	26	Castrate - 21	42
6	24	Handle - 21	42
Total	145		

3.2.3 Tail docking

Refer to Table 3 for the tail docking schedule for the experimental groups. On the day of tail docking (according to the treatment group), lambs were separated from the main flock with their dams and any siblings. Male lambs to be docked were penned in groups of 2-3, with their dam and siblings in an adjacent pen so that physical (albeit limited) and visual access was possible at all times. Identification marks were re-sprayed on the backs of the lambs and small Velcro bands were placed around the base of the lambs' tails (at the future docking site). These bands consisted of two pieces of overlapping black Velcro which could easily be wrapped around the lambs' tails. The bands were applied so that when observations were made from video-recordings of the pre- and post-docking periods, the observer would be blind to whether the lambs had been docked yet or not.

The period of time lambs spent in the experimental pen can be divided into three phases: acclimation, pre-docking and post-docking. In acclimation, penned lambs were given time to settle, where they were left undisturbed for 30 minutes prior to the pre-docking period. Following the 30 minute pre-docking period which was recorded on camera, tail docking was carried out in the pen. One person entered the pen and held the lamb while the second person removed the Velcro band and used an elastrator to apply a rubber ring to the base of the tail just distal to the caudal tail folds. The Velcro band was replaced to cover the rubber ring and then the lamb was returned to the pen. Following docking, videotaping continued for another 30 minutes and then all lambs received vaccination for scabby mouth via a scratch device applied superficially to the skin of the inner hind-leg. After the session, all lambs, their dams and any siblings were returned to their home paddock.

3.2.4 Behavioural observation

Pens were set up at each farm (one at Moginie Block farm and two at Number One Dairy farm) with appropriate structures for the attachment of overhead video-cameras. Each pen had one camera positioned approximately 2.5 metres above the centre of each pen so that the entire pen could be seen at any given time (see Figure 3 below). Behaviour was recorded for 30 minutes before docking (pre-docking period) and then for a further 30 minutes after docking had taken place (post-docking period). In total, sixty minutes of video footage was obtained for each lamb. Each sixty minute video was split into two separate files: pre- and post-docking. The 296 resultant files were scored blind at a later date using the behavioural observation program, JWatcher (Blumstein *et al.*, 2000). After viewing, information regarding treatment and period was re-linked so that statistical analysis could be carried out.

Behaviours chosen as possible indicators of pain were based on general observation and other behavioural studies of pain responses to castration and tail docking in lambs (Molony *et al.*, 1993; McCracken *et al.*, 2010). Frequency (the number of times a behaviour occurred per unit time) and duration (time spent performing a given behaviour

within total time) of both postural and active behaviours (refer to Table 4). Behavioural observations were completed by the same person (SP) and consisted of continuous scoring for the 30 minute duration of each video file.

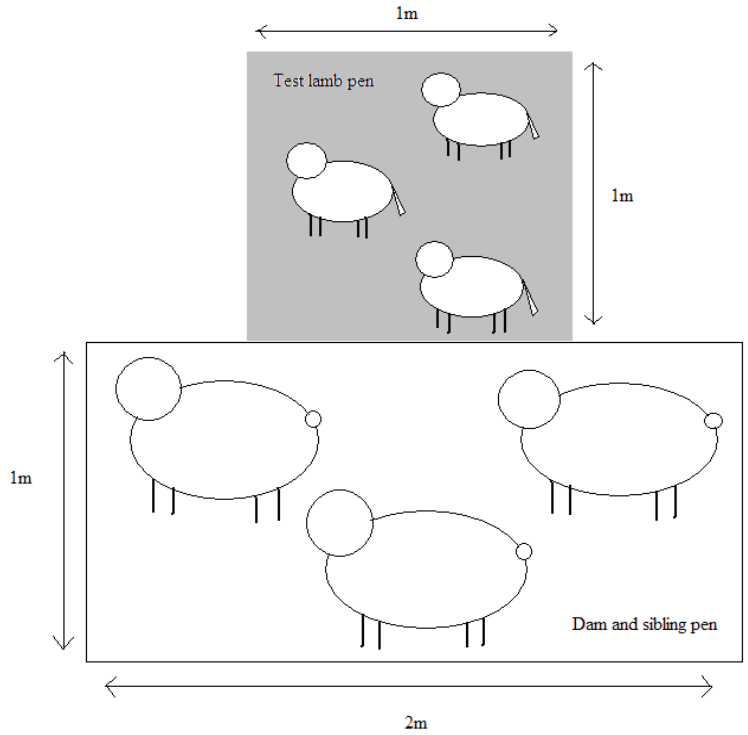

Figure 3. Pen set-up for observation before and after tail docking. The shaded section shows the area viewed by the camera.

Table 4. Ethogram for the observation of pre- and post-docking behaviour in lambs. Modified from Molony *et al.*, 1993 and McCracken *et al.*, 2010.

Behaviour	Event/State	Active/postural	Description
Normal standing (Ns)	State	Postural	Standing postures normally seen in lambs that appear content and aware of their surroundings
Unsteady standing (U)	State	Postural	Abnormal stance (other than statue standing) with ataxia/swaying
Normal lying (Nl)	State	Postural	Ventral recumbency, all legs tucked under or near body (flexed)
Lateral lying (L)	State	Postural	Lying flat on side with both fore and hind limbs extended
Abnormal ventral lying (A)	State	Postural	Ventral recumbency with apparent discomfort
Twisted lying (T)	State	Postural	Ventral recumbency with one or both hind legs extended away from the body
Moving (M)	State	Active	At least three steps in a forward direction
Rolling (roll)	Event	Active	While lying: from one side of the body to the other; from one side of the body to the back and then back to the same side
Kicking (k)	Event	Active	While lying or standing, rapid movement/extension of one or both hind limbs either away from or towards the body
Stamping (st)	Event	Active	Rapid, forceful downward movement of either a fore or hind foot
Jumping (j)	Event	Active	Leaping with two or four legs off the ground at the same time
Shaking (sh)	Event	Active	Forceful voluntary body shake
Tail gazing (tg)	Event	Postural	Turning of the head to look or bite at the tail
Repetitive standing (rp)	Event	Active	Rising from any lying position to a standing position
Ease quarters (eq)	Event	Postural	A pronounced wag of the tail and hindquarters
Backwards walking (bw)	Event	Active	At least three steps in a backwards direction
Restlessness (rest)	Event	Active	The sum of repetitive standing, easing quarters, jumping, rolling, kicking, backward walking, shaking and stamping feet.

3.3 Statistical analysis

Data compiled using JWatcher (Blumstein *et al.*, 2000) were exported into Microsoft Excel for processing. All data (plus 1 to deal with 0 values) were log transformed before analysis to satisfy the assumption of normal distribution and homogeneity of variance (Levene's test). Effects were considered significant if $p < 0.05$.

3.3.1 Separate analyses of event and state data

For each recording period (pre, post), the MIXED procedure of SAS® (Version 9.1, SAS Institute, Cary, NC, USA) was used to complete a two-way ANOVA for each behavioural variable. Data were divided into three subgroups:
Sub group 1: Experimental groups 1 (N=21), 2 (N=27), 3 (N=23) and 4 (N=24).
Sub group 2: Experimental groups 1, 2, 5 (N=26) and 6 (N=24)
Sub group 3: Experimental groups 3-6
For subgroups 1 and 2, the model included the fixed effects of treatment, age at tail docking (Age Dock) and their interactions. For subgroup 3, the model included the fixed effects of treatment, age at castration/handling (Age One) and their interactions. For the overall analysis (all 6 groups included), data were analysed using a factorial model; the model included treatment, Age Dock, Age One, and their interactions.

3.3.2 Repeated measures analyses of event and state data

For each subgroup, both data from both periods (pre and post) were analysed together using the MIXED procedure of SAS® (Version 9.1, SAS Institute, Cary, NC, USA). For subgroups 1 and 2, the model included treatment, Age Dock, period, and interactions and the random effect of lamb within period (Littell *et al.*, 1998). For subgroup 3, the model included treatment, Age Dock, period and interactions and the random effect of lamb within period. For the overall analysis (all 6 groups included), the model included treatment, Age Dock, Age One, period and interactions and the random effect of lamb within period. Using the Akaike's information criterion, a compound symmetry error

structure was determined as the most appropriate residual covariance structure for repeated measures over period within lambs. For all analyses and behaviours, back-transformed least squares means and 95% confidence intervals are presented.

4.0 Results

The data presented in the tables below have been summarised from the separate analyses, as described in the Methods and Materials. Data for the pre-docking period, post-docking period, repeated measures and the overall grouping are displayed separately. All factors (effect of treatment, effect of age at first treatment, effect of age at docking and any interaction effects) were included in each of the four data sets. For ease of reading, least squares means and 95% confidence values for all analyses are presented in Appendices 1-14.

4.1 Effect of Treatment

Pre-docking period:
The overall analysis indicated that treatment had an effect on several lamb behaviours in the 30-minute period before docking (Table 5). Lambs castrated early in life walked backwards less frequently in this period ($F=7.12$, $p<0.01$) and spent less time standing unsteadily ($F=4.03$, $p=0.05$) than lambs that were handled (means and 95% confidence values shown in Appendices 1-2).

From the separate analyses, treatment affected the frequency of backward walking when the age of docking also differed (significant differences for subgroups 1 [$F=4.10$, $p=0.05$] and 2 [$F=7.20$, $p<0.01$]) but not when docking occurred at the same age (no significant difference for subgroup 3 – see Table 5; means and 95% confidence values shown in Appendices 3-8). This suggests a treatment x agedock effect, but this was not confirmed in the overall analysis.

In addition, there was a tendency for treatment to affect the time spent standing normally before docking (overall analysis [$F=3.70$, $p=0.06$] and analysis 1 [$F=3.35$, $p=0.07$], see Table 5), with lambs that were castrated early in life standing in normal postures for less time than lambs that were handled (means and 95% confidence values shown in Appendices 2 and 4).

Post-docking period:

The overall analysis indicated that treatment had an effect on several lamb behaviours in the 30-minute period after docking (Table 6). Lambs castrated early in life stamped their feet more frequently ($F=4.00$, $p=0.05$) during this period than handled lambs (means and 95% confidence values shown in Appendix 1). Treatment also affected the display of the state behaviour, lateral lying. Lambs castrated early in life spent less time in the lateral lying position ($F=4.15$, $p=0.04$) than handled lambs (means and 95% confidence values shown in Appendix 2).

The separate analyses supported both of the above results for foot stamping (analysis 2: $F=4.44$, $p=0.04$) and lateral lying (analyses 2: $F=4.18$, $p=0.04$ and 3: $F=3.98$, $p=0.05$; means and 95% confidence values shown in Appendices 5 and 7). Analysis 1 results also showed that castrated animals spent more time moving continuously around the experimental pen in the post-docking period ($F=7.68$, $p=0.01$) than handled animals (means and 95% confidence values shown in Appendix 4). This effect was only seen in analysis 1 and not in the overall analysis so is likely to be weak.

Repeated measures analysis:

The overall analysis indicated that lambs castrated early in life walked backwards less frequently ($F=4.29$, $p=0.04$) than handled animals (Table 7; Appendix 1). Separate analyses 1 ($F=3.44$, $p=0.07$) and 2 ($F=5.57$, $p=0.02$) indicated a similar effect (Table 7; Appendices 3 and 5). The data from the pre-period analyses showed that castrated animals displayed backward walking less frequently than handled animals; however, there was no significant difference with respect to treatment in the post-period. The effect picked up in the repeated measures analysis can be primarily attributed to the difference between castrated and handled animals seen in the pre-docking period.

In the post-docking period, castrated animals spent less time lying laterally than handled animals. This effect is supported by the repeated measures data from the overall analysis ($F=4.23$, $p=0.04$), analysis 2 ($F=4.29$, $p=0.04$) and analysis 3 ($F=4.11$, $p=0.05$). In

addition, a significant treatment x period effect was found which indicated that in the post-docking period, castrated lambs spent less time lying laterally than handled lambs did (F=4.80, Bonferroni-adjusted p=0.02).

Table 5. Statistical results for the effect of treatment on behaviour of lambs recorded in a 30-minute period before tail docking (pre period) from overall (N=145) and sub-group (1:N=95; 2:N=98; 3:N=97) analyses.

Pre	Overall		Analysis 1		Analysis 2		Analysis 3	
	F	**p**	**F**	**p**	**F**	**p**	**F**	**p**
Backwards walking	7.12	<0.01	4.10	0.05	7.20	<0.01	2.06	0.15
Ease quarters	2.50	0.12	1.54	0.22	1.71	0.19	0.00	1.00
Foot stamping	0.16	0.69	0.15	0.70	0.18	0.67	2.66	0.11
Jumping	3.23	0.07	2.59	0.11	2.73	0.10	1.55	0.22
Kicking	0.34	0.56	0.01	0.94	0.26	0.61	1.58	0.21
Repetitive standing	0.44	0.51	0.38	0.54	0.33	0.57	0.02	0.88
Rolling	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00
Tail gazing	0.11	0.74	0.07	0.80	0.10	0.75	1.16	0.28
Shaking	0.11	0.74	0.00	0.95	0.08	0.78	5.02	0.03
Restlessness	2.12	0.15	0.54	0.46	2.27	0.14	0.10	0.75
Abnormal ventral lying	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00
Lateral lying	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00
Moving	0.68	0.41	0.05	0.82	0.57	0.45	0.01	0.92
Normal lying	0.04	0.84	0.41	0.52	0.04	0.85	0.16	0.69
Normal standing	3.70	0.06	3.35	0.07	2.64	0.11	0.47	0.49
Twisted lying	2.56	0.11	0.00	1.00	1.73	0.19	1.71	0.19
Unsteady standing	4.03	0.05	0.00	1.00	2.73	0.10	2.69	0.10

Table 6. Statistical results for the effect of treatment on behaviour of lambs recorded in a 30-minute period after tail docking (post period) from overall (N=145) and sub-group (1:N=95; 2:N=98; 3:N=97) analyses.

Post	Overall		Analysis 1		Analysis 2		Analysis 3	
	F	**p**	**F**	**p**	**F**	**p**	**F**	**p**
Backwards walking	0.94	0.33	1.10	0.30	1.00	0.32	0.00	0.96
Ease quarters	0.07	0.79	0.11	0.74	0.08	0.78	0.06	0.42
Foot stamping	4.00	0.05	2.76	0.10	4.44	0.04	0.81	0.37
Jumping	0.21	0.65	0.77	0.38	0.21	0.65	0.00	0.95
Kicking	0.51	0.48	0.10	0.75	0.50	0.48	0.01	0.92
Repetitive standing	0.00	0.99	0.12	0.73	0.00	0.99	0.04	0.84
Rolling	0.99	0.32	0.90	0.35	0.93	0.34	1.95	0.17
Tail gazing	0.43	0.51	0.00	0.98	0.45	0.50	0.27	0.60
Shaking	0.98	0.32	2.24	0.14	0.76	0.38	0.00	0.97
Restlessness	0.18	0.67	0.08	0.77	0.22	0.64	0.18	0.67
Abnormal ventral lying	2.16	0.14	1.75	0.19	2.19	0.14	3.36	0.07
Lateral lying	4.15	0.04	0.86	0.36	4.18	0.04	3.98	0.05
Moving	2.68	0.10	7.68	0.01	2.69	0.10	0.81	0.37
Normal lying	0.07	0.79	0.36	0.55	0.06	0.81	0.00	0.98
Normal standing	1.20	0.28	0.70	0.40	1.15	0.29	1.83	0.18
Twisted lying	1.50	0.22	2.33	0.13	1.10	0.30	0.46	0.50
Unsteady standing	0.25	0.62	0.59	0.44	0.25	0.62	0.61	0.44

Table 7. Repeated measures results from overall (N=145) and sub-group (1:N=95; 2:N=98; 3:N=97) analyses of lamb behaviour with regards to the effect of treatment.

RM	Overall		Analysis 1		Analysis 2		Analysis 3	
	F	**p**	**F**	**p**	**F**	**p**	**F**	**p**
Backwards walking	4.29	0.04	3.44	0.07	5.57	0.02	0.79	0.38
Ease quarters	0.32	0.57	0.35	0.56	0.32	0.57	0.06	0.81
Foot stamping	2.00	0.16	0.64	0.43	1.34	0.25	2.29	0.13
Jumping	0.72	0.40	0.01	0.94	1.27	0.26	0.52	0.47
Kicking	0.13	0.72	0.09	0.77	0.61	0.44	0.11	0.74
Repetitive standing	0.08	0.78	0.27	0.60	0.05	0.82	0.02	0.90
Rolling	1.82	0.18	0.90	0.35	0.96	0.33	1.98	0.16
Tail gazing	0.30	0.59	0.01	0.93	0.35	0.56	0.45	0.51
Shaking	0.92	0.34	0.87	0.35	0.48	0.49	0.64	0.43
Restlessness	0.33	0.57	0.22	0.64	2.03	0.16	0.18	0.67
Abnormal ventral lying	3.54	0.06	1.77	0.19	2.22	0.14	3.39	0.07
Lateral lying	4.23	0.04	0.87	0.35	4.29	0.04	4.11	0.05
Moving	0.47	0.49	2.12	0.15	0.01	0.92	0.09	0.77
Normal lying	0.00	0.96	0.09	0.77	0.01	0.93	0.16	0.69
Normal standing	1.48	0.23	0.60	0.44	0.95	0.33	1.71	0.19
Twisted lying	0.05	0.82	2.34	0.13	0.09	0.76	0.27	0.60
Unsteady standing	2.73	0.10	0.59	0.44	3.14	0.08	1.81	0.18

4.2 Effect of Age at First Treatment

Pre-docking period:

In the pre-docking period, lambs that were castrated or handled at 21 days of age displayed a higher frequency of jumping and were more restless than lambs that were castrated or handled at 1 day of age (see Table 8; means ± 95% confidence intervals shown in Appendices 9 and 10). Results for both of these behaviours were supported by both analyses (overall: $F=12.05$, $p=0.00$; and analysis 3: $F=9.73$, $p=0.00$) applied to the data. Lambs castrated or handled at 21 days of age also spent more time standing in unsteady postures (supported by the overall analysis: $F=4.02$, $p=0.05$).

A significant treatment x age at treatment interaction (overall analysis) was found that indicated that lambs castrated at 21 days of age stood unsteadily for longer durations in the pre-docking period than lambs that were castrated at 1 day of age ($F=4.02$, Bonferroni-adjusted $p=0.03$).

Post-docking period:

The overall analysis indicated that lambs castrated or handled on their first day of age moved continuously ($F=3.79$, $p=0.05$) around the experimental pen for longer durations than lambs that were castrated or handled at 21 days of age (see Table 9).

Repeated measures analysis:

The total duration of unsteady standing was found to be significantly greater ($F=4.68$, $p=0.03$) in lambs castrated or handled at 21 days of age (see Table 10). This effect is largely due to the significant difference between the groups in the pre-treatment period.

Table 8. Statistical results for the effect of age at treatment from overall (N=145) and sub-group (3:N=97) analyses of lamb behaviour recorded in a 30-minute period before tail-docking (pre period).

Pre	Overall		Analysis 3	
	F	**p**	**F**	**p**
Backwards walking	0.17	0.68	0.17	0.68
Ease quarters	0.00	1.00	0.00	1.00
Foot stamping	1.19	0.28	1.22	0.27
Jumping	12.05	0.00	9.73	0.00
Kicking	0.27	0.60	0.26	0.61
Repetitive standing	2.91	0.09	3.38	0.07
Rolling	0.00	1.00	0.00	1.00
Tail gazing	0.44	0.51	1.16	0.28
Shaking	0.55	0.46	2.00	0.16
Restlessness	5.76	0.02	5.25	0.02
Abnormal ventral lying	0.00	1.00	0.00	1.00
Lateral lying	0.00	1.00	0.00	1.00
Moving	0.55	0.46	0.52	0.47
Normal lying	0.41	0.52	0.50	0.48
Normal standing	1.54	0.22	1.42	0.24
Twisted lying	2.55	0.11	1.71	0.19
Unsteady standing	4.02	0.05	2.69	1.04

Table 9. Statistical results for the effect of age at first treatment from overall (N=145) and sub-group (3:N=97) analyses of lamb behaviour recorded in a 30-minute period after tail-docking (post period).

Post	Overall		Analysis 3	
	F	**p**	**F**	**p**
Backwards walking	0.07	0.79	0.08	0.78
Ease quarters	0.67	0.41	0.06	0.81
Foot stamping	0.54	0.46	0.52	0.47
Jumping	0.00	1.00	0.00	1.00
Kicking	0.37	0.54	0.35	0.56
Repetitive standing	0.52	0.47	0.64	0.42
Rolling	0.01	0.91	0.02	0.90
Tail gazing	2.24	0.14	2.14	0.15
Shaking	1.25	0.27	2.21	0.14
Restlessness	0.23	0.63	0.19	0.66
Abnormal ventral lying	1.82	0.18	2.26	0.14
Lateral lying	0.40	0.53	0.41	0.52
Moving	3.79	0.05	3.37	0.07
Normal lying	0.00	0.99	0.00	0.98
Normal standing	0.80	0.37	1.11	0.29
Twisted lying	0.52	0.47	0.37	0.55
Unsteady standing	0.52	0.47	0.73	0.40

Table 10. Repeated measures results from overall (N=145) and sub-group (3:N=97) analyses of lamb behaviour with regards to the effect of age at treatment.

RM	Overall		Analysis 3	
	F	**p**	**F**	**p**
Backwards walking	0.19	0.66	0.18	0.67
Ease quarters	0.67	0.41	0.65	0.42
Foot stamping	1.26	0.26	1.20	0.28
Jumping	2.99	0.09	2.54	0.11
Kicking	0.25	0.62	0.23	0.63
Repetitive standing	0.00	0.95	0.01	0.94
Rolling	0.01	0.91	0.02	0.90
Tail gazing	2.43	0.12	2.48	0.12
Shaking	1.19	0.28	2.91	0.09
Restlessness	3.10	0.08	2.77	0.10
Abnormal ventral lying	1.84	0.18	2.28	0.13
Lateral lying	0.38	0.54	0.40	0.53
Moving	1.95	0.17	1.74	0.19
Normal lying	0.31	0.58	0.45	0.50
Normal standing	0.94	0.33	1.30	0.26
Twisted lying	0.47	0.50	0.31	0.58
Unsteady standing	4.68	0.03	3.49	0.06

4.3 Effect of Age at Docking

Pre-docking period:

Backwards walking, repetitive standing, tail gazing, shaking, restlessness, moving and normal lying were displayed more frequently/for longer durations in the pre-docking period by lambs that were docked at 21 days of age rather than those that were docked at 42 days of age (regardless of whether they were castrated or handled, or the time at which castration/handling took place) (see Table 11 for F and p values; means ± 95% confidence intervals can be found in Appendices 11 and 12). All of the effects for these behaviours were supported by at least two analyses (three analyses in the case of the event behaviour, moving). In contrast, jumping and normal standing were displayed more by lambs that were docked at 42 day of age. The effect seen for the state behaviour, normal standing, was supported by two analyses but the effect for the event behaviour, jumping, was only confirmed by one analysis and may therefore be weak.

Post-docking period:

In the post-docking period, lambs that were docked at 21 days of age displayed more repetitive standing, tail gazing, shaking, restlessness, moving, normal standing, kicking, abnormal ventral lying and lateral lying than lambs that were docked at 42 days of age (see Table 12 for F and p values; means ± 95% confidence intervals can be found in Appendices 13 and 14). All of the effects were supported by at least two analyses, with the exception of the event behaviour, repetitive standing. Lambs docked at 42 days of age spent longer durations in the state behaviour, unsteady standing.

Table 11. Statistical results for the effect of age at docking from overall (N=145), sub-group (1:N=95; 2:N=98) analyses of lamb behaviour in the pre-docking period.

Behaviour	Overall		Analysis 1		Analysis 2	
	F	p	F	p	F	p
Backwards walking	3.84	0.05	3.83	0.05	2.57	0.11
Ease quarters	0.00	1.00	1.54	0.22	1.71	0.19
Foot stamping	0.14	0.71	0.12	0.73	2.45	0.12
Jumping	0.82	0.37	1.45	0.23	5.61	0.02
Kicking	0.27	0.60	0.58	0.45	0.01	0.94
Repetitive standing	3.99	0.05	5.12	0.03	0.08	0.78
Rolling	0.00	1.00	0.00	1.00	0.00	1.00
Tail gazing	4.48	0.04	2.91	0.09	7.37	<0.01
Shaking	5.88	0.02	4.44	0.04	2.16	0.15
Restlessness	3.79	0.05	3.92	0.05	0.19	0.66
Abnormal ventral lying	0.00	1.00	0.00	1.00	0.00	1.00
Lateral lying	0.00	1.00	0.00	1.00	0.00	1.00
Moving	5.42	0.02	7.11	<0.01	8.16	<0.01
Normal lying	5.76	0.02	5.54	0.02	2.84	0.10
Normal standing	6.52	0.01	13.09	<0.01	1.30	0.26
Twisted lying	0.00	1.00	0.00	1.00	1.73	0.19
Unsteady standing	0.00	1.00	0.00	1.00	2.73	0.10

Table 12. Statistical results for the effect of age at docking from overall (N=145), sub-group (1:N=95; 2:N=98) analyses of lamb behaviour in the post-docking period.

Behaviour	Overall		Analysis 1		Analysis 2	
	F	p	F	p	F	p
Backwards walking	0.64	0.43	0.58	0.45	0.32	0.57
Ease quarters	0.08	0.78	0.07	0.79	0.32	0.57
Foot stamping	0.01	0.91	0.01	0.92	0.44	0.51
Jumping	0.52	0.47	0.56	0.46	0.54	0.46
Kicking	4.31	0.04	4.77	0.03	7.37	<0.01
Repetitive standing	2.72	0.10	2.39	0.13	5.47	0.02
Rolling	1.96	0.16	1.85	0.18	1.63	0.21
Tail gazing	15.62	<0.01	15.62	<0.01	32.40	<0.01
Shaking	8.99	<0.01	7.85	<0.01	2.95	0.09
Restlessness	9.93	<0.01	9.72	<0.01	17.13	<0.01
Abnormal ventral lying	4.52	0.04	3.74	0.06	12.46	<0.01
Lateral lying	4.65	0.03	4.54	0.04	2.43	0.12
Moving	7.69	<0.01	8.80	<0.01	22.73	<0.01
Normal lying	3.21	0.08	2.54	0.11	2.69	0.10
Normal standing	5.66	0.02	4.55	0.04	10.49	<0.01
Twisted lying	0.08	0.79	0.41	0.52	0.74	0.39
Unsteady standing	6.11	0.01	4.84	0.03	10.16	<0.01

Repeated measures analysis:

The effects seen for repetitive standing, tail gazing, shaking, restlessness, moving, kicking, abnormal ventral lying, and lateral lying were confirmed in the repeated measures ANOVAs which were applied to the sets of analyses (see Table 13 for F and p values). The general trend appears to have been for lambs docked at 21 days of age to display a greater frequency or duration of pain-related behaviours when compared to lambs docked at 42 days of age. The significant results in the RM ANOVA for normal lying are primarily due to the significant increase in this behaviour in animals that were due to be docked on their 21st day of age in the pre-treatment period. Lambs docked at 42 days of age spent longer periods of time in the state behaviour, unsteady standing.

Table 13. Repeated measures results for the effect of age at docking from overall (N=145), sub-group (1:N=95; 2:N=98) analyses of lamb behaviour under a repeated measures test.

Behaviour	Overall		Analysis 1		Analysis 2	
	F	p	F	p	F	p
Backwards walking	3.26	0.07	2.62	0.11	2.18	0.14
Ease quarters	0.00	0.99	0.00	0.99	0.77	0.38
Foot stamping	0.04	0.85	0.02	0.89	2.11	0.15
Jumping	0.00	1.00	0.00	0.99	2.77	0.10
Kicking	4.38	0.04	4.78	0.03	6.66	0.01
Repetitive standing	5.10	0.03	4.76	0.03	4.53	0.04
Rolling	1.93	0.17	1.85	0.18	1.60	0.21
Tail gazing	17.73	<0.01	16.42	<0.01	35.70	<0.01
Shaking	9.67	<0.01	7.86	0.01	2.34	0.13
Restlessness	10.24	<0.01	9.94	<0.01	3.24	0.07
Abnormal ventral lying	4.68	0.03	3.85	0.05	12.67	<0.01
Lateral lying	4.55	0.03	4.63	0.03	2.29	0.13
Moving	8.12	<0.01	10.82	<0.01	16.08	<0.01
Normal lying	8.00	<0.01	7.26	<0.01	4.49	0.04
Normal standing	5.16	0.02	4.13	0.05	10.25	<0.01
Twisted lying	0.06	0.81	0.50	0.48	0.13	0.72
Unsteady standing	1.30	0.26	5.20	0.02	7.76	<0.01

5.0 Discussion

The aim of this study was to investigate whether changes in pain sensitivity occur in lambs in response to standard sheep husbandry procedures. The three main objectives for this study were:

1. To determine whether castration using the rubber ring method alters sensitivity to a subsequent noxious experience, tail docking by the rubber ring method.
2. To determine whether age at castration influences changes in pain sensitivity after tail docking.
3. To determine whether the interval between the two procedures influences changes in pain sensitivity after docking.

The main findings of this study were:

- Castration had an effect on behavioural responses of lambs prior to docking, in response to social isolation and confinement;
- Castration had an effect on behaviour following docking;
- Age at castration did not significantly affect the behavioural response to docking;
- Age at tail docking affected the behaviour of lambs following castration/handling.

5.1 Behavioural observation

Assessment of pain in animals is usually a case of gauging responses to a painful stimulus. In this case, behavioural observation was chosen as the means of assessment. While the concept is simple in that the observations are easy to set up, there are still difficulties associated with such a method. Observation of behaviour in any species requires the observer to possess a good prior knowledge of that species' behaviour; 'normal' must be recognisable for 'abnormal' to be identified. Considerable amounts of time must be taken by the observer to familiarise themselves with the subject species. The observations themselves and their subsequent organisation can also be time-consuming. Computer programs have been developed which can reduce the tedium of this task, but these have their own shortcomings. Behavioural observation generates a massive amount of data which, even once analysed, can remain incredibly opaque.

The behavioural responses of lambs to tail-docking by rubber-ring have been well-documented (Mellor & Murray, 1989; Lester *et al.*, 1996; Graham *et al.*, 1997; Molony & Kent, 1997; Kent *et al.*, 1998; Dinnis *et al.*, 1999). Such responses include an increase in the display of active behaviours and the assumption of abnormal postures that are not seen in lambs that are free of pain and aware of their surroundings. It is generally agreed that these behaviours are indicative of pain and distress. The ethogram for this study (refer to Table 4; adapted from Molony *et al.*, 1993 and McCracken *et al.*, 2010) included a range of behaviours which are known to be indicative of pain as well as others identified in the literature and from pre-observation familiarisation.

5.2 The effect of treatment on response to social isolation and confinement

An analysis of behavioural data from the pre-docking period i.e. the 30-minute period of video-recording before the lambs were docked, uncovered some interesting results. Significant differences between lambs that had been castrated and lambs that had been handled were found before tail docking even took place: castrated lambs walked backwards less frequently, spent less time standing unsteadily and showed a tendency to spend less time standing normally than handled lambs. The pre-docking period of behavioural observation was intended to act as a baseline against which post-docking behaviour could be judged. For differences between castrated and handled animals to be apparent before docking is of interest. Two lines of explanation emerge at this point: i) lambs were responding to a stressor of some sort, perhaps to do with the experimental conditions in the pre-docking observation period, or ii) given that there was no control group for the possible pre-period stressor, there is the possibility that the lambs' behaviour may have been the same even in their undisturbed, natural environment.

For the first few weeks of life, lambs stay within a short radius of their mother. Given their gregarious nature, removal of an individual sheep or a small group of sheep, from their conspecifics is a stressful experience that results in a variety of physiological and behavioural changes (Fordham *et al.*, 1991; van Adrichem & Vogt, 1993; Degabrielle &

Fell, 2001). Confinement in pens has also been shown to have similar, immediate effects (Fordham *et al.*, 1991). In the present study, lambs were separated from their ewes and placed in pens with one or two other lambs. Although mother ewes were kept in an adjacent pen, this separation could still be considered sufficient to induce stress-related effects of social isolation, especially when combined with the effects of confinement in a novel environment. Castration may have affected the lambs in a way that alters responses to future stressors. With the exception of backward walking, castrated lambs could be responding to the stress of social isolation and confinement in an active manner i.e. these lambs spent less time in stationary postures, when compared to control lambs. Increased locomotion in a novel environment has been interpreted as a sign of agitation or fear (Fell *et al.*, 1991); are lambs that have been castrated in early life more sensitive to novel stressors than handled lambs? These findings contrast with those of Moburg and Wood (1981), who found that neonatal stress did not have any effect on response to a subsequent stressor. The effects seen in the present study, while significant and of interest, are not striking. It may be that the impact of social isolation and confinement in this experiment was not enough to elicit stronger responses. Alternatively, castration may simply have changed lamb behavioural in a general sense; the behaviour seen in the pre-period might have occurred regardless of the animals' placement in the experimental environment.

5.3 The effect of treatment on behavioural response to tail docking

With respect to the effect of early castration on behavioural response to subsequent tail docking, a number of significant results were found in the sets of analyses that were applied to the behavioural data. Several behaviours differed in the pre- and post-docking periods, but for the purpose of clarity in this particular section, only those behaviours which differed significantly in the post-docking period and were found to have a significant difference between pre- and post-docking periods overall (as interpreted using the repeated measures analyses) will be discussed.

Lambs which had been castrated at 1 or 21 days of age spent significantly less time lying in a lateral position (lateral lying: a postural state behaviour in which the lamb lies flat on its side with its fore and hind limbs extended) than control lambs which had only been handled. A postural change, such as that demonstrated by increased durations of lateral lying, could be an attempt to protect the tail from further damage (Graham *et al.*, 1997). Options in terms of explanation for the difference between castrated and handled lambs are as follows: 1) Having already experienced acute pain in early life (castration), castrated lambs may have become desensitised to pain and its subsequent application may have resulted in a diminished response; 2) Not having experienced any significant painful events in early life, handled lambs may have shown exaggerated responses to novel stimuli.

Perceived pain is a balance between nociceptive and anti-nociceptive mechanisms which is influenced by the level of threat that the painful stimulus poses and also its novelty (Bingel *et al.*, 2007). 'Habituation' describes the elicitation of progressively smaller responses to repeated stimuli (Glaser & Whittow, 1953). Habituation has been validated for somato-sensory reflexes such as pain (Milne *et al.*, 1991), but such work relates to repetition of the same painful event. In the present study, the painful stimuli differ and no results supported an effect of interval between procedures on behavioural response i.e. the length of time between the two events was such that habituation was unlikely, or its effects were so diluted that they were not visible.

On the other hand, handled lambs may have responded to a novel stimulus (in the form of tail docking) in an exaggerated manner due to their lack of exposure to any painful previous painful procedures. From the reverse perspective, there was obviously something about the experience of castration which altered the lambs in some way that resulted in a depressed behavioural display in response to subsequent tail docking.

5.4 Age at treatment does not have an effect on behavioural response to tail docking

Castration (treatment) or handling (control) of lambs took place on either the first or 21st day of age. While neural development of lambs at 1 day of age is advanced, further development will take place before lambs reach 21 days of age. The capability of noxious activity (in relation to painful events) to upset developmental processes (via the stimulation of activity in the autonomic nervous system) that are still in a state of flux i.e. the developing nervous system, has been indicated in a number of studies (Taddio *et al.*, 1997; Anand *et al.*, 1999; McCracken *et al.*, 2010).

Significant differences were identified for a number of behaviours in the pre- and post-docking periods. These differences were not supported by the results of a repeated measures analysis, so age at castration does not appear to have had a significant effect on behavioural response to tail docking. This contrasts with preliminary work which had shown that lambs castrated at 1 day of age showed an increased behavioural response to docking at one month compared to lambs that were castrated at 10 days of age (McCracken *et al.*, 2010).

5.5 Age at tail docking affects the behavioural response of lambs to the procedure itself

Irrespective of whether they had been castrated or handled early in life, lambs docked at 21 days of age displayed significantly greater frequencies/durations of a number of pain-related behaviours (kicking, tail gazing, shaking, restlessness, abnormal ventral lying, moving and normal standing) than their 42 day old counterparts. This finding is supported by other age-related studies. Johnson *et al.* (2005) found that the EEG responses to castration differ between 2 week-old lambs and 4 week-old lambs. It would appear that in this case, age-related differences are more marked with reference to age at the second procedure than age at the first procedure.

At 21 days of age, lambs may still be relying on their mothers for a degree of protection. In contrast, 42 day old lambs are much less dependent. As a prey species, visibly weakened or ill sheep are at a higher risk of predation than animals that appear to be alert and healthy. Without the guardianship of an older animal, it becomes increasingly important for lambs to adopt the behavioural mannerisms of the rest of the flock. Forty-two day old lambs may be dampening their behavioural responses in an effort to avoid drawing negative attention to them.

In contrast to the other results for the effect of age at tail docking, 42 day old lambs spent longer durations than 21 day old lambs in the abnormal posture, unsteady standing. From extensive observation, unsteady standing often involves hunching of the back and lateral curvature that is commonly accompanied by the performance of the event behaviour, tail gazing. The hunching could be an effort to relieve pain in the tail, and the tail gazing an investigatory reaction to said pain. It has been suggested that because the amount of tissue trapped by the ring is greater in older lambs, afferent activity is increased (Molony & Kent, 1993). Unsteady standing could be an important behaviour for the assessment of pain in response to tail docking specifically.

5.6 Trial conditions: weather, management and equipment

Environmentally, the weather played a major role during the early stages of this trial. Very high levels of rainfall, cold temperatures and high winds did were not ideal conditions for birth and early life of lambs. Many lambs died during this period of bad weather, or became weakened and died at a later date (after being placed in the study). These losses contributed to an overall depletion in numbers of animals available for observation as part of the trial. While efforts were made to avoid disruption to the animals, shifting of lambs and ewes between paddocks occurred with some frequency due to waterlogged paddocks, reduced grazing and the management strategies being practised on the farms. At least twice during the study (prior to tail docking), all animals had to be yarded and penned so that their spray-paint identification numbers (which had

run badly in the rain) could be reapplied. All shifting, yarding and penning must be acknowledged as potential sources of disruption and distress.

Due to the high number of animals required for the study, two sheep farms had to be used. While the farms were run in a similar manner, their layout, features and size differed. Grazing at Dairy Unit One was much better than that at Moginie and as a result, the ewes and lambs at Dairy Unit One were in markedly better condition. Obviously general health and body condition of lambs will have been a contributing factor to their behavioural responses. A randomised selection method was used to allocate lambs into the different treatment groups which should have balanced this variation between farms.

The original plan for the set-up of the pens that would be used to hold animals during behavioural observation was to use large rectangular garden marquees. Unfortunately, these structures did not prove robust in the face of waterlogged ground and high winds. Reinforcements and minor alterations (shortening of the marquee length, alteration of the roof structure, and removal of the canvas tarpaulin roof and sides) had to be made to prolong their life. At Moginie, the structure was torn apart so badly in strong winds that it could not be repaired and an alternative site for behavioural observation had to be found. A small shearing and utility shed on the farm was fitted out with appropriate pens and overhead fixtures so that it could be used. The changes that had to be made to the marquees and the fact that an entirely new location had to be established mid-study meant that the environmental conditions during behavioural observation differed. There was either a full marquee with a roof and sides; a full marquee with no sides; a partial marquee with no sides and no roof; and an indoor pen. Ground underfoot varied as well: grass; hard-packed dirt and stone; and wooden slat flooring.

5.7 Limitations of this study

The main limitations in this study included the number of animals that were available for use in the experiment and the use of behaviour as the sole gauge of pain responses. For financial and practical reasons, the projected number of animals for this study was 180

(note that this number was judged acceptable by power analysis). In total, useable videotape was obtained for 145 lambs from the 182 lambs that were originally entered into the trial; the discrepancy between the total lambs used and the number of lambs from which data could be obtained was due to lamb losses in bad weather, illness, small number of losses due to mismothering and a couple of damaged videos. With approximately 24 animals per treatment group, a larger pool of animals would have been desirable.

The use of behaviour, while a simple and effective method of pain assessment, is itself a limitation. In a perfect situation, it would be advantageous to have a range of measures, both behavioural and physiological. Obviously, behavioural and physiological measurements are not usually able to be collected concurrently, on the same animals. The manipulations that are sometimes involved in obtaining physiological measurements such as blood samples can be invasive and may affect the behavioural responses of animals to experimental treatments. Often, a range of behavioural methods can be used to try and offset disadvantages of using a single method alone. Time constraints in the processing of the data for this study meant that this was not possible, but it would be beneficial to apply appropriate methods to existing data in the future e.g. use visual analogue scales to gauge lamb pain in the pre- and post-docking periods. In addition to further behavioural measurement, the repetition of this study using physiological indices such as plasma cortisol concentration would be valuable.

6.0 Conclusion

Significant differences in behaviour between the castrated and handled animals were evident even before the lambs were docked. Castrated lambs displayed lesser frequencies and durations of a number of behaviours when compared to control lambs. Differences between animals at this stage were not expected. The lack of a control group by which to judge these differences weakens the proposal of any conclusive statements. Further research looking at the effect of castration on behavioural response to general stress is suggested.

Castration did affect the behavioural responses of lambs to subsequent docking. Castrated lambs displayed lesser frequencies and durations of a number of behaviours when compared to control lambs. Whether this difference is due to a diminished response in castrated lambs due to alterations caused by docking, or due to exaggerated responses on the part of control lambs is not clear. Age at castration was not found to have a significant effect on pain behaviour in response to docking, but age at docking did. Younger lambs (21 days of age) displayed higher frequencies and longer durations of pain-related behaviours than their 42-day old counterparts. Repetition of this trial, wither with additional means of behavioural assessment or a solely physiological approach is suggested to further investigate the effects that have been found.

Current guidelines for the practise of painful husbandry procedures in sheep recommend that such practices be carried out as early in life as possible and ideally before lambs reach 6 weeks of age (NAWAC, 2005). Given their tentative nature, the findings of this research do not directly contradict these guidelines. Further clarification of the behavioural difference between castrated and control lambs, and the effect of age at docking on behavioural response may allow for a reassessment of these guidelines at a later date.

7.0 Appendices

Appendix 1. Overall (Groups 1-6; N=145) event behaviour frequency estimates and lower (LL) and upper (UP) 95% confidence values* for pre-treatment, post-treatment and repeated measures analyses – effect of treatment.

Behaviour	Treatment	PRE			POST			REPEATED MEASURES		
		Estimate	LL	UP	Estimate	LL	UP	Estimate	LL	UP
Bw	C	0.46	0.15	0.84	1.60	0.97	2.45	0.93	0.64	1.27
	H	0.21	-0.03	0.51	2.15	1.41	3.11	1.45	1.10	1.87
Eq	C	0.00	-0.03	0.03	0.37	0.15	0.62	0.18	0.10	0.27
	H	0.03	0.00	0.06	0.41	0.20	0.66	0.21	0.14	0.30
St	C	1.02	0.53	1.66	2.64	1.71	3.88	1.81	1.32	2.39
	H	1.18	0.68	1.84	1.41	0.82	2.18	1.32	0.94	1.79
J	C	0.66	0.30	1.12	1.36	0.73	2.23	0.97	0.61	1.41
	H	1.26	0.79	1.85	1.61	0.94	2.51	1.23	0.83	1.70
K	C	0.03	-0.03	0.08	5.48	3.54	8.24	1.56	1.20	1.98
	H	0.05	0.00	0.10	6.74	4.53	9.85	1.66	1.30	2.08
Rp	C	0.26	0.13	0.41	32.88	25.61	42.14	5.27	4.61	6.01
	H	0.20	0.08	0.33	32.94	26.00	41.67	5.14	4.52	5.83
Roll	C	0.00	0.00	0.00	0.24	0.04	0.46	0.09	0.02	0.17
	H	0.00	0.00	0.00	0.39	0.18	0.63	0.16	0.09	0.24
Tg	C	0.06	-0.01	0.13	7.45	5.13	10.66	1.81	1.45	2.22
	H	0.07	0.01	0.14	6.29	4.38	8.89	1.66	1.33	2.04
Sh	C	0.18	0.06	0.32	0.54	0.31	0.80	0.27	0.15	0.40
	H	0.15	0.04	0.28	0.37	0.18	0.60	0.19	0.08	0.30
rest	C	2.71	1.74	4.02	56.12	47.00	66.98	12.99	11.03	15.27
	H	4.06	2.80	5.74	59.14	49.99	69.94	13.88	11.86	16.20

*back-transformed from logged values

Appendix 2. Overall (Groups 1-6; N=145) state behaviour duration estimates and lower (LL) and upper (UP) 95% confidence values* for pre-treatment, post-treatment and repeated measures analyses – effect of treatment.

Behaviour	Treatment	PRE			POST			REPEATED MEASURES		
		Estimate	LL	UP	Estimate	LL	UP	Estimate	LL	UP
a	C	0.00	0.00	0.00	30.08	7.83	108.40	0.18	-0.29	0.97
	H	0.00	0.00	0.00	7.42	1.53	27.06	1.68	0.64	3.38
l	C	0.00	0.00	0.00	184.90	37.58	894.79	10.42	5.02	20.67
	H	0.00	0.00	0.00	1782.62	395.50	8022.36	28.11	14.68	53.04
m	C	61009.42	45095.86	82538.48	189888.96	157359.53	229142.84	106222.43	89217.21	126468.90
	H	72706.51	54452.37	97079.91	152801.27	127671.90	182876.75	97527.51	82400.56	115431.41
nl	C	27.85	5.37	129.55	1.06	0.05	3.04	4.46	1.74	9.88
	H	35.00	7.50	151.51	0.81	-0.05	2.45	4.60	1.88	9.91
ns	C	1425451.69	1318791.63	1540738.11	22.53	5.23	87.91	5217.68	3048.00	8931.33
	H	1584214.67	1470653.36	1706545.00	7.42	1.36	29.00	3278.02	1950.38	5508.93
t	C	0.55	0.05	1.27	488794.75	342140.61	698310.11	838.83	672.77	1045.81
	H	0.00	-0.31	0.45	665636.16	473194.55	936340.93	809.62	654.20	1001.90
u	C	0.82	0.19	1.77	620448.81	499921.32	770034.58	983.76	813.15	1190.13
	H	0.00	-0.33	0.50	574639.55	467387.45	706502.90	786.84	654.57	945.80

Appendix 3. Analysis One (Groups 1-4; N=95) event behaviour frequency estimates and lower (LL) and upper (UP) 95% confidence values* for pre-treatment, post-treatment and repeated measures analyses – effect of treatment.

Behaviour	Treatment	PRE			POST			REPEATED MEASURES		
		Estimate	LL	UP	Estimate	LL	UP	Estimate	LL	UP
bw	C	0.48	0.16	0.88	1.50	0.84	2.38	0.92	0.54	1.39
	H	1.08	0.66	1.62	2.12	1.34	3.14	1.55	1.08	2.13
eq	C	0.00	-0.04	0.04	0.43	0.20	0.71	0.20	0.09	0.31
	H	0.03	0.00	0.07	0.49	0.25	0.78	0.24	0.14	0.35
st	C	1.24	0.65	2.04	2.83	1.80	4.25	1.93	1.28	2.77
	H	1.44	0.83	2.24	1.66	0.98	2.58	1.55	1.01	2.23
j	C	0.29	0.07	0.56	1.73	1.00	2.72	0.88	0.52	1.31
	H	0.59	0.34	0.91	1.26	0.69	2.02	0.90	0.56	1.31
k	C	0.03	-0.02	0.07	6.95	4.62	10.25	1.86	1.38	2.42
	H	0.03	-0.01	0.07	6.36	4.31	9.21	1.75	1.32	2.26
rp	C	0.17	0.06	0.30	36.22	27.56	47.52	5.61	4.71	6.66
	H	0.12	0.02	0.24	33.94	26.23	43.82	5.27	4.46	6.20
roll	C	0.00	0.00	0.00	0.23	0.03	0.46	0.11	0.01	0.21
	H	0.00	0.00	0.00	0.38	0.17	0.63	0.17	0.08	0.28
tg	C	0.09	0.00	0.18	8.34	5.71	11.99	2.19	1.67	2.81
	H	0.07	-0.01	0.16	8.28	5.80	11.67	2.16	1.67	2.74
sh	C	0.13	-0.01	0.29	0.50	0.26	0.78	0.30	0.13	0.49
	H	0.14	0.01	0.29	0.24	0.06	0.47	0.19	0.05	0.35
rest	C	2.10	1.28	3.21	60.49	50.30	72.69	12.80	10.46	15.61
	H	2.63	1.72	3.84	58.26	48.97	69.27	13.66	11.31	16.46

Appendix 4. Analysis One (Groups 1-4; N=95) state behaviour duration estimates and lower (LL) and upper (UP) 95% confidence values* for pre-treatment, post-treatment and repeated measures analyses – effect of treatment.

B	T	PRE			POST			REPEATED MEASURES		
		Estimate	Lower bar	Upper bar	Estimate	Lower bar	Upper bar	Estimate	Lower bar	Upper bar
A	C	0.00	0.00	0.00	55.81	12.69	234.79	6.55	2.72	14.32
	H	0.00	0.00	0.00	14.31	3.08	56.47	2.92	1.03	6.57
L	C	0.00	0.00	0.00	237.29	45.37	1223.51	14.45	5.85	33.85
	H	0.00	0.00	0.00	686.80	149.23	3148.04	25.25	11.32	54.90
M	C	73628.42	56124.19	96591.85	230774.35	192617.80	276489.46	130456.14	107566.86	158216.00
	H	70543.57	54815.58	90784.24	162835.19	137654.35	192622.27	107268.54	89664.23	128329.16
Nl	C	31.57	5.69	157.63	0.65	-0.24	2.59	6.30	1.92	17.27
	H	15.08	2.69	69.04	1.29	0.11	3.71	5.04	1.57	13.16
Ns	C	1500035.50	1417706.94	1587145.01	32.42	6.27	152.60	7088.71	3332.57	15077.12
	H	1611860.05	1529467.43	1698691.17	12.72	2.32	55.64	4709.74	2335.27	9497.49
T	C	0.00	0.00	0.00	573090.12	487420.44	673817.19	757.01	698.14	820.84
	H	0.00	0.00	0.00	680782.76	585657.19	791359.11	825.25	765.44	889.73
U	C	0.00	0.00	0.00	604162.42	470753.50	775378.60	774.57	683.63	877.59
	H	0.00	0.00	0.00	528658.59	419252.91	666614.04	724.31	644.94	813.44

Appendix 5. Analysis Two (Groups 1, 2, 5 & 6; N=98) event behaviour frequency estimates and lower (LL) and upper (UP) 95% confidence values* for pre-treatment, post-treatment and repeated measures analyses – effect of treatment.

Behaviour	Treatment	PRE			POST			REPEATED MEASURES		
		Estimate	Lower bar	Upper bar	Estimate	Lower bar	Upper bar	Estimate	Lower bar	Upper bar
bw	C	0.46	0.15	0.84	1.60	0.98	2.42	0.96	0.63	1.36
	H	1.26	0.81	1.83	2.15	1.44	3.08	1.68	1.24	2.20
eq	C	0.00	-0.04	0.04	0.37	0.16	0.61	0.17	0.08	0.27
	H	0.03	0.00	0.07	0.41	0.21	0.65	0.21	0.12	0.30
st	C	1.02	0.55	1.62	2.64	1.75	3.81	1.72	1.21	2.36
	H	1.18	0.70	1.80	1.41	0.85	2.14	1.29	0.88	1.80
j	C	0.66	0.27	1.17	1.36	0.73	2.23	0.97	0.51	1.56
	H	1.26	0.76	1.91	1.61	0.94	2.51	1.43	0.89	2.12
k	C	0.03	-0.04	0.09	5.48	3.52	8.28	1.57	1.13	2.10
	H	0.05	-0.01	0.11	6.74	4.51	9.89	1.85	1.38	2.40
rp	C	0.26	0.11	0.44	32.88	25.41	42.48	5.52	4.65	6.53
	H	0.20	0.06	0.35	32.94	25.80	41.99	5.37	4.56	6.30
roll	C	0.00	0.00	0.00	0.24	0.04	0.47	0.11	0.02	0.21
	H	0.00	0.00	0.00	0.39	0.18	0.64	0.18	0.09	0.28
tg	C	0.06	-0.01	0.13	7.45	5.18	10.57	2.00	1.54	2.54
	H	0.07	0.01	0.14	6.29	4.42	8.82	1.80	1.39	2.28
sh	C	0.18	0.04	0.35	0.54	0.28	0.84	13.64	11.28	16.45
	H	0.15	0.02	0.30	0.37	0.16	0.63	16.46	13.78	19.63
rest	C	2.71	1.77	3.97	56.12	47.87	65.78	0.35	0.17	0.55
	H	4.06	2.84	5.67	59.14	50.86	68.74	0.26	0.10	0.44

Appendix 6. Analysis Two (Groups 1, 2, 5 & 6; N=98) state behaviour duration estimates and lower (LL) and upper (UP) 95% confidence values* for pre-treatment, post-treatment and repeated measures analyses – effect of treatment.

B	Treatment	PRE			POST			REPEATED MEASURES		
		Estimate	Lower bar	Upper bar	Estimate	Lower bar	Upper bar	Estimate	Lower bar	Upper bar
A	C	0.00	0.00	0.00	30.08	7.90	107.61	4.60	2.01	9.41
	H	0.00	0.00	0.00	7.42	1.55	26.87	1.91	0.60	4.27
L	C	0.00	0.00	0.00	184.90	37.78	890.24	12.21	5.05	27.86
	H	0.00	0.00	0.00	1782.62	397.53	7981.57	40.56	18.65	86.89
M	C	61009.42	43935.47	84718.41	189888.96	157378.03	229115.89	106776.23	85194.85	133824.50
	H	72706.51	53113.65	99526.77	152801.27	127686.91	182855.25	104976.36	84550.49	130336.69
Nl	C	27.85	4.79	142.83	1.06	-0.03	3.37	6.55	2.02	17.89
	H	35.00	6.75	166.29	0.81	-0.12	2.72	6.99	2.32	18.24
Ns	C	1425451.69	1300107.66	1562880.20	22.53	5.05	90.47	5825.66	2976.68	11400.50
	H	1584214.67	1450727.50	1729984.53	7.42	1.30	29.83	3661.86	1924.09	6968.31
T	C	0.55	-0.03	1.47	488794.75	322160.42	741618.81	877.84	635.62	1212.20
	H	0.00	-0.36	0.57	665636.16	446785.44	991687.18	819.49	601.33	1116.66
U	C	0.82	0.09	2.03	620448.81	498453.70	772301.82	1071.23	815.53	1407.02
	H	0.00	-0.39	0.63	574639.55	466106.70	708444.21	760.89	585.94	987.98

Appendix 7. Analysis Three (Groups 3-6; N=97) event behaviour frequency estimates and lower (LL) and upper (UP) 95% confidence values* for pre-treatment, post-treatment and repeated measures analyses – effect of treatment.

B	T	PRE			POST			REPEATED MEASURES		
		Estimate	Lower bar	Upper bar	Estimate	Lower bar	Upper bar	Estimate	Lower bar	Upper bar
bw	C	0.36	0.08	0.72	1.63	1.00	2.45	0.89	0.55	1.31
	H	0.74	0.38	1.18	1.65	1.03	2.48	1.15	0.76	1.62
eq	C	0.00	0.00	0.00	0.39	0.17	0.65	2.21	1.95	2.49
	H	0.00	0.00	0.00	0.43	0.21	0.70	0.20	0.10	0.30
st	C	1.37	0.81	2.10	2.29	1.45	3.42	1.79	1.21	2.53
	H	0.73	0.32	1.25	1.72	1.03	2.64	1.17	0.72	1.73
j	C	0.59	0.21	1.08	1.67	0.94	2.68	1.05	0.57	1.67
	H	1.02	0.55	1.63	1.71	0.98	2.72	1.35	0.81	2.05
k	C	0.00	-0.05	0.06	4.36	2.73	6.70	1.31	0.91	1.78
	H	0.05	0.00	0.11	4.50	2.84	6.88	1.41	1.00	1.90
rp	C	0.14	0.03	0.26	27.94	22.38	34.82	4.74	4.09	5.47
	H	0.13	0.02	0.25	28.83	23.15	35.84	4.80	4.16	5.53
roll	C	0.00	0.00	0.00	0.11	-0.05	0.30	0.06	-0.02	0.14
	H	0.00	0.00	0.00	0.30	0.12	0.52	0.14	0.06	0.23
tg	C	0.03	-0.01	0.07	4.28	2.82	6.31	1.34	0.98	1.76
	H	0.00	-0.04	0.04	3.68	2.39	5.44	1.16	0.83	1.55
sh	C	0.11	0.05	0.18	0.22	0.09	0.37	0.16	0.08	0.25
	H	0.01	-0.04	0.07	0.22	0.09	0.38	0.11	0.03	0.20
rest	C	2.63	1.66	3.97	48.48	40.05	58.64	12.36	10.03	15.18
	H	2.38	1.48	3.61	45.75	37.87	55.23	11.61	9.43	14.23

Appendix 8. Analysis Three (Groups 3-6; N=97) state behaviour duration estimates and lower (LL) and upper (UP) 95% confidence values* for pre-treatment, post-treatment and repeated measures analyses – effect of treatment.

B	T	PRE			POST			REPEATED MEASURES		
		Estimate	Lower bar	Upper bar	Estimate	Lower bar	Upper bar	Estimate	Lower bar	Upper bar
A	C	0.00	0.00	0.00	11.91	3.28	37.90	2.59	1.08	5.22
	H	0.00	0.00	0.00	1.98	-0.02	8.08	0.73	-0.01	2.00
L	C	0.00	0.00	0.00	55.88	11.42	259.46	6.41	2.46	14.85
	H	0.00	0.00	0.00	512.37	109.67	2380.50	21.66	9.51	47.83
M	C	51173.67	37818.19	69245.51	150571.50	123970.53	182880.34	87315.96	70133.63	108707.80
	H	52353.86	38591.92	71023.20	132653.06	109042.26	161376.24	83332.01	66802.58	103951.36
Nl	C	6.25	0.90	26.72	0.25	-0.21	0.98	1.99	0.50	4.98
	H	9.67	1.76	40.24	0.26	-0.21	1.00	2.67	0.82	6.38
Ns	C	1581998.32	1461675.17	1712226.31	6.76	1.59	22.28	3509.10	2027.78	6072.02
	H	1645573.30	1519401.92	1782221.97	1.65	-0.13	7.02	2086.66	1199.50	3629.44
T	C	0.55	-0.02	1.45	496877.10	328194.26	752258.03	879.16	639.21	1209.03
	H	0.00	-0.37	0.59	609197.84	400966.64	925568.03	779.55	565.09	1075.26
U	C	0.82	0.10	2.00	687418.49	575111.18	821657.06	1125.53	865.49	1463.61
	H	0.00	-0.40	0.66	760323.05	635145.65	910170.93	870.92	667.95	1135.48

Appendix 9. Analysis Three (Groups 3-6; N=97) event behaviour frequency estimates and lower (LL) and upper (UP) 95% confidence values* for pre-treatment, post-treatment and repeated measures analyses – effect of age at first treatment.

Behaviour	Treatment	PRE			POST			REPEATED MEASURES		
		Estimate	Lower bar	Upper bar	Estimate	Lower bar	Upper bar	Estimate	Lower bar	Upper bar
bw	1	0.49	0.17	0.89	1.57	0.95	2.40	0.96	0.60	1.40
	21	0.59	0.27	1.00	1.71	1.08	2.54	1.08	0.71	1.52
eq	1	0.00	0.00	0.00	0.48	0.25	0.77	0.22	0.12	0.33
	21	0.00	0.00	0.00	0.35	0.14	0.59	0.16	0.07	0.26
st	1	1.25	0.71	1.96	2.23	1.39	3.36	1.70	1.12	2.43
	21	0.82	0.40	1.36	1.77	1.08	2.69	1.24	0.79	1.82
j	1	0.32	0.01	0.74	1.69	0.94	2.73	0.88	0.44	1.47
	21	1.42	0.86	2.14	1.69	0.97	2.67	1.55	0.97	2.30
k	1	0.01	-0.04	0.07	4.87	3.05	7.49	1.44	1.01	1.95
	21	0.04	-0.02	0.09	4.03	2.54	6.15	1.28	0.90	1.74
rp	1	0.06	-0.04	0.18	30.24	24.13	37.84	4.76	4.09	5.50
	21	0.21	0.10	0.34	26.63	21.46	32.98	4.79	4.16	5.50
roll	1	0.00	0.00	0.00	0.19	0.02	0.40	0.09	0.01	0.18
	21	0.00	0.00	0.00	0.21	0.04	0.41	0.10	0.02	0.19
tg	1	0.03	-0.01	0.07	4.89	3.23	7.20	1.47	1.08	1.92
	21	0.00	-0.04	0.04	3.19	2.06	4.74	1.05	0.75	1.40
sh	1	0.03	-0.03	0.09	0.15	0.02	0.30	0.09	0.01	0.17
	21	0.09	0.03	0.15	0.30	0.16	0.46	0.19	0.11	0.28
rest	1	1.71	0.97	2.73	48.53	39.95	58.91	10.57	8.52	13.07
	21	3.53	2.35	5.14	45.70	37.98	54.96	13.55	11.09	16.52

Appendix 10. Analysis Three (Groups 3-6; N=97) state behaviour duration estimates and lower (LL) and upper (UP) 95% confidence values* for pre-treatment, post-treatment and repeated measures analyses – effect of age at first treatment.

Behaviour	Treatment	PRE			POST			REPEATED MEASURES		
		Estimate	Lower bar	Upper bar	Estimate	Lower bar	Upper bar	Estimate	Lower bar	Upper bar
A	1	0.00	0.00	0.00	10.31	2.67	33.83	2.36	0.92	4.88
	21	0.00	0.00	0.00	2.40	0.14	9.14	0.85	0.07	2.17
L	1	0.00	0.00	0.00	119.13	24.47	565.54	9.89	4.01	22.66
	21	0.00	0.00	0.00	242.06	52.98	1093.43	14.41	6.26	31.72
M	1	56009.81	41149.53	76236.44	160844.57	131931.27	196094.29	94730.32	75761.60	118448.26
	21	47833.38	35142.40	65107.34	124180.56	102462.63	150501.77	76809.76	61840.13	95403.02
Nl	1	5.25	0.59	23.51	0.26	-0.21	1.01	1.80	0.38	4.68
	21	11.39	2.29	45.64	0.25	-0.21	0.97	2.92	0.97	6.78
Ns	1	1669607.96	1540264.87	1809812.57	5.90	1.25	20.14	3395.16	1940.49	5939.73
	21	1559380.77	1442049.19	1686258.97	1.98	0.01	7.83	2156.70	1253.47	3710.28
T	1	0.00	-0.37	0.60	602473.13	394756.92	919486.75	776.43	560.96	1074.54
	21	0.55	-0.02	1.43	502423.17	333422.26	757084.90	882.60	643.98	1209.48
U	1	0.00	-0.40	0.67	684263.62	570489.16	820728.45	828.65	633.65	1083.56
	21	0.82	0.11	1.98	763828.59	640304.06	911182.88	1183.05	912.24	1534.18

Appendix 11. Analysis One (Groups 1-4; N=95) event behaviour frequency estimates and lower (LL) and upper (UP) 95% confidence values* for pre-treatment, post-treatment and repeated measures analyses – effect of age at docking.

Behaviour	Age dock	PRE			POST			REPEATED MEASURES		
		Estimate	Lower bar	Upper bar	Estimate	Lower bar	Upper bar	Estimate	Lower bar	Upper bar
bw	21	1.07	0.64	1.62	2.02	1.26	3.05	2.02	1.26	3.05
	42	0.49	0.17	0.88	1.57	0.91	2.46	1.57	0.91	2.46
eq	21	0.03	0.00	0.07	0.44	0.21	0.70	0.44	0.21	0.70
	42	0.00	-0.04	0.04	0.48	0.25	0.77	0.48	0.25	0.77
st	21	1.42	0.81	2.25	2.16	1.33	3.27	2.16	1.38	3.20
	42	1.25	0.67	2.03	2.23	1.38	3.39	2.23	1.38	3.39
j	21	0.55	0.29	0.86	1.29	0.70	2.09	1.29	0.70	2.09
	42	0.32	0.10	0.59	1.69	0.99	2.65	1.69	0.99	2.65
k	21	0.04	0.00	0.08	8.98	6.14	12.94	8.98	6.14	12.94
	42	0.01	-0.03	0.06	4.87	3.18	7.24	4.87	3.18	7.24
rp	21	0.25	0.13	0.37	40.62	31.25	52.73	40.62	31.25	52.73
	42	0.06	-0.04	0.17	30.24	23.11	39.48	30.24	23.11	39.48
roll	21	0.00	0.00	0.00	0.41	0.19	0.68	0.41	0.19	0.68
	42	0.00	0.00	0.00	0.19	0.00	0.42	0.19	0.00	0.42
tg	21	0.14	0.05	0.23	13.71	9.70	19.22	13.71	9.70	19.22
	42	0.03	-0.05	0.12	4.89	3.26	7.14	4.89	3.26	7.14
sh	21	0.25	0.10	0.42	0.62	0.37	0.92	0.62	0.37	0.92
	42	0.03	-0.09	0.17	0.15	-0.03	0.36	0.15	-0.03	0.36
rest	21	3.14	2.09	4.56	72.57	60.79	86.59	72.57	60.79	86.59
	42	1.71	1.01	2.66	48.53	40.49	58.14	48.53	40.49	58.14

Appendix 12. Analysis One (Groups 1-4; N=95) state behaviour duration estimates and lower (LL) and upper (UP) 95% confidence values* for pre-treatment, post-treatment and repeated measures analyses – effect of age at docking.

Behaviour	Treatment	PRE			POST			REPEATED MEASURES		
		Estimate	Lower bar	Upper bar	Estimate	Lower bar	Upper bar	Estimate	Lower bar	Upper bar
A	21	0.00	0.00	0.00	2.02	1.26	3.05	7.81	3.47	16.39
	42	0.00	0.00	0.00	1.57	0.91	2.46	2.36	0.70	5.66
L	21	0.00	0.00	0.00	0.44	0.21	0.70	36.01	15.94	79.86
	42	0.00	0.00	0.00	0.48	0.25	0.77	9.96	3.99	23.06
M	21	92733.88	71383.78	120469.48	2.16	1.33	3.27	147486.69	122532.87	177522.31
	42	56009.81	43080.80	72818.86	2.23	1.38	3.39	94882.02	78731.24	114345.89
Nl	21	82.81	17.22	384.54	1.29	0.70	2.09	14.68	5.49	36.85
	42	5.25	0.35	27.88	1.69	0.99	2.65	1.81	0.16	5.83
Ns	21	1448152.71	1371437.57	1529159.12	8.98	6.14	12.94	9844.80	4767.54	20328.01
	42	1669607.96	1580913.44	1763278.53	4.87	3.18	7.24	3391.08	1633.83	7037.17
T	21	0.00	0.00	0.00	40.62	31.25	52.73	806.38	746.03	871.61
	42	0.00	0.00	0.00	30.24	23.11	39.48	774.80	716.44	837.91
U	21	0.00	0.00	0.00	0.41	0.19	0.68	678.46	601.74	764.95
	42	0.00	0.00	0.00	0.19	0.00	0.42	826.99	732.91	933.13

Appendix 13. Analysis Two (Groups 1, 2, 5 & 6; N=98) event behaviour frequency estimates and lower (LL) and upper (UP) 95% confidence values* for pre-treatment, post-treatment and repeated measures analyses – effect of age at docking.

Behaviour	Treatment	PRE			POST			REPEATED MEASURES		
		Estimate	Lower bar	Upper bar	Estimate	Lower bar	Upper bar	Estimate	Lower bar	Upper bar
bw	21	1.07	0.64	1.61	2.02	1.31	2.96	1.52	1.10	2.04
	42	0.59	0.27	0.99	1.71	1.09	2.52	1.08	0.74	1.49
eq	21	0.03	0.00	0.07	0.44	0.22	0.69	0.22	0.13	0.32
	42	0.00	-0.03	0.04	0.35	0.15	0.57	0.16	0.07	0.25
st	21	1.42	0.87	2.14	2.16	1.39	3.16	1.78	1.26	2.43
	42	0.82	0.41	1.33	1.77	1.12	2.62	1.24	0.83	1.75
j	21	0.55	0.19	1.02	1.29	0.68	2.12	0.87	0.44	1.43
	42	1.42	0.87	2.12	1.69	1.00	2.63	1.55	0.98	2.28
k	21	0.04	-0.02	0.10	8.98	5.99	13.25	2.21	1.66	2.86
	42	0.04	-0.03	0.10	4.03	2.57	6.09	1.28	0.91	1.73
rp	21	0.25	0.10	0.42	40.62	31.51	52.30	6.18	5.23	7.27
	42	0.21	0.07	0.37	26.63	20.76	34.08	4.79	4.05	5.64
roll	21	0.00	0.00	0.00	0.41	0.19	0.68	0.19	0.09	0.29
	42	0.00	0.00	0.00	0.21	0.03	0.43	0.10	0.01	0.20
tg	21	0.14	0.06	0.21	13.71	9.78	19.07	3.10	2.48	3.83
	42	0.00	-0.06	0.07	3.19	2.10	4.66	1.05	0.75	1.40
sh	21	0.25	0.10	0.42	0.62	0.35	0.94	0.42	0.24	0.63
	42	0.09	-0.04	0.24	0.30	0.10	0.55	0.19	0.04	0.36
rest	21	3.14	2.10	4.53	72.57	62.02	84.88	16.57	13.77	19.91
	42	3.53	2.43	5.00	45.70	39.22	53.23	13.55	11.30	16.22

Appendix 14. Analysis Two (Groups 1, 2, 5 & 6; N=98) state behaviour duration estimates and lower (LL) and upper (UP) 95% confidence values* for pre-treatment, post-treatment and repeated measures analyses – effect of age at docking.

Behaviour	Treatment	PRE			POST			REPEATED MEASURES		
		Estimate	Lower bar	Upper bar	Estimate	Lower bar	Upper bar	Estimate	Lower bar	Upper bar
A	21	0.00	0.00	0.00	75.91	21.24	264.97	7.81	3.77	15.30
	42	0.00	0.00	0.00	2.40	0.02	10.39	0.85	0.01	2.37
L	21	0.00	0.00	0.00	1363.17	287.31	6453.80	34.61	15.42	76.23
	42	0.00	0.00	0.00	242.06	52.58	1101.62	14.39	6.22	31.77
M	21	92733.88	66965.25	128418.30	233630.41	193930.60	281457.19	145844.04	116594.74	182430.86
	42	47833.38	34847.45	65658.40	124180.56	103601.59	148847.22	76855.86	61768.19	95628.82
Nl	21	82.81	16.04	411.35	1.99	0.42	5.29	14.44	5.22	37.28
	42	11.39	1.63	57.37	0.25	-0.39	1.58	2.91	0.61	8.49
Ns	21	1448152.71	1321822.53	1586556.61	65.47	16.29	254.50	9878.33	5079.54	19209.80
	42	1559380.77	1426867.01	1704201.13	1.98	-0.20	10.04	2159.08	1127.40	4134.02
T	21	0.00	-0.37	0.59	647580.54	428323.34	979074.46	814.17	591.24	1121.01
	42	0.55	-0.02	1.43	502423.17	336046.57	751172.55	860.00	629.22	1175.30
U	21	0.00	-0.40	0.66	466772.78	375656.09	579990.08	690.18	526.79	904.15
	42	0.82	0.11	1.98	763828.59	618350.29	943533.30	1180.93	907.21	1537.13

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