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Behavioural assessment of pain in dairy cattle with mastitis

By

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TABLE OF CONTENTS

Page

I. REVIEW PAPER

II. EXPERIMENTAL PAPER

LIST OF TABLES

Page

LIST OF FIGURES

Page

I. REVIEW PAPER

II. EXPERIMENTAL PAPER

Assessing pain in dairy cattle

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Abstract

Assessing pain and discomfort experience in cattle is one of the main concerns of farm animal welfare science. Both behavioural and physiological measures have been used as indicators of pain; however, due to impracticability and invasiveness that physiological measures involve, behavioural measures are currently the most used parameter to assess pain in cattle. The scientific assessment of pain has been focused on farm procedures such as dehorning, branding and castration. Nonetheless, research on pain related to diseases is also getting stronger due to the impact on the farm's economy and cows' welfare. Mastitis has been classified as one of the most important, frequent and painful diseases in dairy cattle; however pain alleviation is not considered common part of mastitis therapy, unless cows have evident systemic illness. Pain assessment due to mastitis has been done primarily using models of experimental-induced mastitis. Physiological measures such as temperature and heart rate have been used to evaluate the efficacy of different analgesic drugs after inducing mastitis. Behavioural measures based mainly on pain sensitivity and activity behaviours have been used as indicators of pain for mastitis. Although the valuable information provided for the available studies, further research in this area is required. Combining different measures used for pain assessment associated with mastitis but also successful methodologies used to evaluate pain and discomfort in other diseases and farm procedures, it is possible to improve pain assessment in mastitic cows and subsequently improve management and welfare.

Key words: Pain, Cattle, Mastitis, Pain assessment.

1. Introduction

Assessment and alleviation of pain in animals have been always linked to the attitude that society has towards them. In the past, it was believed that animals were not capable of suffering. Descartes was one of the strongest precursors of the idea that animals could not reason, think, feel pain and suffer (Hellebrekers, 2000). However, this belief started to change when Jeremy Bentham (18th century) promoted the idea that animals can experience suffering as humans do (Troyer, 2003; Fraser, 2008). More recently, from 1950's onwards, attitudes toward animals have had a profound positive change due to the emergence of an ethical concern about the welfare and quality of life of farm animals (Appleby and Hughes, 1997; Fraser, 2008). As a consequence the science of animal welfare was created with its main fundament to prevent pain and suffering, and promote positive experiences in animals (Yeats and Main, 2008).

Pain assessment in cattle is a difficult task for welfare researchers, veterinarians and farmers, not only because of the lack of self-report that characterise non-human animals, but also because cattle evolved as prey species, tending to hide any sign of weakness as an evolutionary strategy for survival. Cattle show signs of pain only when the level is extremely high (Phillips, 2002), for example, mastitic cows express signs of pain only when they are suffering severe clinical mastitis with systemic illness (Fitzpatrick et al., 1999). In cases of less severe mastitis, clinical signs are less evident, making more difficult pain detection and the implementation of analgesic treatment (Fitzpatrick et al., 1999).

Mastitis is one of the three major causes of economic losses, and the second cause of culling in the dairy industry (Blowey and Edmondson, 2010; CCIL, 2010 respectively). The Food and Agriculture Organization of the United Nations (FAO, 2011) and the Farm

Animal Welfare Council (FAWC, 1997) declared it as one of the most painful diseases in dairy cows, with detrimental effects on physical and mental wellbeing. As a result, mastitis has attracted the attention of scientists to improve strategies of prevention, control and treatment to subsequently improve the management of mastitis on farms and welfare of cows.

The aim of this review is to describe the current understanding of pain experience and assessment in dairy cattle with mastitis, highlighting the general concept of pain, including the pathophysiological process behind this affective state and the ways in which pain can be assessed. Furthermore, this review will focus on the ways in which pain has been specifically assessed in cattle and the current situation of pain assessment in cows with mastitis.

2. What is pain?

2.1. Definition of Pain in Animals

Pain in humans has been defined by the International Association for the Study of Pain (IASP) as *"An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage"* (Merskey and Bogduk, 1994). This definition remarks the subjectivity of a painful experience and the importance of verbal self-reports as part of the behavioural pattern that a human being expresses during a painful experience (Bateson, 1991). Nonetheless, verbal self-report has a limited use in animals; therefore, all tempts of trying to define pain have been focused on behavioural and physiological changes, which are considered the basis of pain assessment in non-human beings. Currently, there is no standard and unique definition of pain in animals; however in 1997, Molony (cited by Rutherford, 2002) defined it as "... *an aversive sensory and emotional experience representing an awareness by the animal of damage or threat to the integrity of its tissues. It changes the animal's physiology and behaviour to reduce or avoid the damage, to reduce the likelihood of recurrence and to promote recovery..."* integrating in a good way the different components of pain suitable for animals, and hence, this review will be based on this definition.

2.2. Classification and Functionality of Pain

Pain can be classified by anatomic source, duration and functionality. Pathological pain states can be caused mainly by tissue or nerve damage (somatic or visceral pain, and neuropatic pain, respectively).

The classification based on duration helps to identify when pain is beneficial or is functional for the animal (Zulkifli and Siegel, 1995; Dawkins, 1998). During acute pain, the animal enters in to a protective and recuperative stage, known as ‘adaptive affective state’ (Millan, 1999; Fraser, 2008). In this stage, pain provides an incentive that promotes that the animal performs actions to stop or alleviate the damage in short-term; prioritising specific biological functions that will help to avoid the noxious stimuli (Bateson, 1992; Broom, 2001; Gregory, 2004). In addition, this stage motivates the ill individual to rest and save energy in order to have a faster recovery (Rutherford, 2002; Fraser, 2008). In the long-term, the animal will learn to avoid specific situations that it had associated with a previous unpleasant experience (Bateson, 1992; Broom, 2001). Conversely, chronic pain, which generally lasts more than the recovery time, has neither physiological purpose nor adaptive value and is known as ‘non-functional pain’ (Millan, 1999; Rutherford, 2002).

2.3. Pathophysiology of Pain

Pain can be divided into two important components: a sensory discriminative component (physiological side of pain) and an emotional or affective component (Rutherford, 2002; Smith, 2009). The former is also known as nociception, and refers to neurophysiological process of detection, transduction and transmission of noxious stimuli to the central nervous system (CNS). The latter relates to the perception and conscious awareness of an aversive sensation that it is triggered when the brain interprets the noxious information received and produces the sensation of pain (Hellebrekers, 2000; Kopf and Patel, 2010).

The nociceptive process (Figure 1) starts with the detection of noxious stimuli by peripheral sensory neurons (nociceptors). When nociceptors are stimulated, they transduce the noxious stimulus, which is mainly mechanical, thermal or chemical, into electrical energy and the action potential is transmitted towards the CNS through their afferent fibres (Hellebrekers, 2000). The classification of nociceptors is based on the type of nerve fibre. There are myelinated fibrers (Aδ-type) that transport the impulse at high speed (5-30 m/s) and unmyelinated fibres (C-type) that transport the impulse at low speed (0.5-2 m/s). The Aδ-fibres correspond to nociceptors specialised in detecting chemical and mechanical stimuli. They produce the sensation of sharp-fast pain such as pain felt during tissue compression; while the C-fibres are related to polymodal nociceptors and produce the sensation of delayed dull pain (Hellebrekers, 2000; Smith, 2009).

The activation of the nociceptors and therefore the intensity of pain sensation are modulated by the degree of tissue damage and a number of chemical substances released during this process such as prostaglandins and histamine. Additionally, this complex chemical signalling protects the injured area by influencing the animal to behave in a certain way to keep that area away from other stimuli, complying with the protective function of pain (Kopf and Patel, 2010).

After nociceptors are stimulated, the afferent fibres transport the impulse to the spinal cord, where pain information is subjected to modulation by local interneurons and descending and inhibitory neurons. This modulation may produce an immediate response (reflex responses) or transmit the information directly to the brain, which will transmit the correspondent response through the descending pathways (autonomic activity) (Rutherford, 2002). In the case of visceral pain, there is no reflex action because pain related to visceral organs is only transmitted by C-fibre nociceptive nerves (Viñuela-Fernández et al., 2007; Kopf and Patel, 2010).

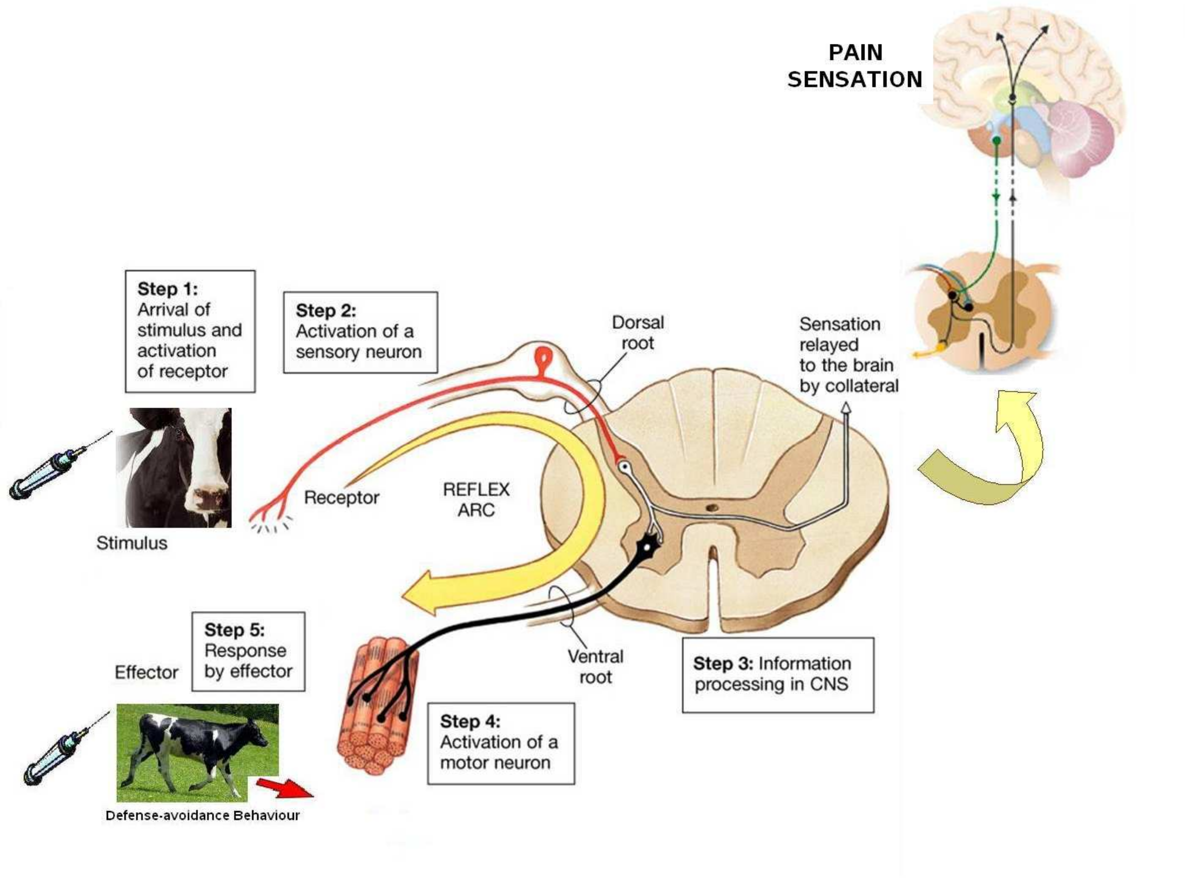

Figure 1. Nociceptive process and the production of pain sensation when noxious information is interpreted by the brain (modified from Morainevalley, 2011).

3. How is Pain Measured?

Reliable, sensitive and valid pain assessment methods are a fundamental part of animal welfare science and veterinary medicine (Rutherford, 2002). Reliability means that measures taken in animals with similar health and environmental conditions must provide similar results when the measure is repeated (reproducible). This can be evaluated testing for intra-observer (same observer re-score the animals in different occasions) and inter-observer (different observers score the animals independently) reliability (Weary et al., 2006). However, in some cases reliability is difficult to reach due to the variability that exists between individuals. On the other hand, sensitivity refers to measures that co-vary with the degree of pain that the animal is experiencing; ideally the methods used should discriminate between different levels of pain. Finally, validity refers to the real measure of signs associated with pain and not with a different source. Additionally, due to the lack of self-report in animals, it is important to compare and correlate the measure that is being used with other independent measures for a better validation of the methodology.

The evaluation of pain in animals is mainly based on behavioural and physiological changes. Both need to take into account the normal behavioural repertoire and physiological rates of the animal, but also the individual variability (Martini et al., 1999; Hellebrekers, 2000). Additionally, it is important to consider whether the pain that is being assessed has significance for the animal based on the magnitude of the response that it expresses and the effort that it does to avoid the painful stimulus (Rutherford, 2002).

Regardless of the methodology used (behavioural or physiological measures), they need to be validated to establish if the observed responses are pain-specific rather than simple reflexes. To validate a measure, it needs to be subjected to studies that evaluate the animal's responses exposed to four different treatments (Table 1) that combine the absence or presence of a painful condition, and whether or not analgesic treatment is used (Weary et al., 2006; Rushen et al., 2008). The most useful measure should find a difference between the painful treatment (pain condition without analgesic treatment) and the other treatments, but no difference among these other treatments (Weary et al., 2006).

Table 1. Standard treatments necessary to validate a pain assessment measure. Concept described by Weary et al. (2006).

Four Treatments*	Analgesic treatment (A)	Non analgesic treatment (a)
Painful condition (P)	PA	Pa
Non painful condition (p)	pA	pa

When analgesic treatment is not implemented, the comparison between an animal in a painful condition *versus* an animal without painful condition (Pa *vs.* pa) helps to understand if there are behavioural or physiological changes related to the pain experience. In addition, the use of analgesic treatment helps to distinguish between pain responses and other effects associated with the assumed painful condition. The use of analgesics can be done in two ways: first, using analgesics to evaluate if the animal returns to normal behaviour and physiological measures after analgesic administration (Bateson, 1991; Rutherford, 2002; Sneddon, 2003). Second, using consumer demand or preferences test, where the animal had the opportunity to choose the analgesic to alleviate the pain (Yeats and Main, 2008).

3.1. Physiological Measures

Physiological measures evaluated during pain assessment are related to stress responses produced by the sympathetic-adrenomedullary (SA) and hypothalamic-pituitary-adrenocortical (HPA) axis that allow the animal to have available resources necessary to solve the problem that is coping with (Wiepkema and Koolhaas, 1993; Rutherford, 2002; Gregory, 2004; Weary et al., 2006). The activation of the SA axis affects the cardiovascular and gastrointestinal system, adrenal gland and exocrine glands (Moberg and Mench, 2000), generating a variety of short-term clinical changes such as an increase in heart rate, changes in body temperature, defecation and urination, and increase in plasma adrenaline levels (‘fight-flight’ responses). The activation of the HPA axis affects the entire metabolism through indirect interaction with other systems such as reproductive and immune. Some physiological measures related to this axis are increased cortisol levels (in plasma, saliva, faeces and urine) (Rutherford, 2002; Fraser, 2008), increase in plasma-ACTH and glucose levels, and decrease in insulin levels (Benson et al., 2000).

3.2. Behavioural Measures

Behaviour is commonly used to evaluate pain in human neonates and infants as a way to replace verbal self-report. Additionally, it is the most used parameter and less invasive way to assess pain in animals (Rutherford, 2002). Behavioural measures can be evaluated using subjective or objective methodologies (Weary et al., 2006).

3.2.1. Subjective Behavioural Measures

Subjective methodologies are related to unquantified personal judgement and descriptive scoring systems, where in some cases, the veracity of the data cannot be verified, although this does not mean less accuracy. These methodologies are important to veterinarians because they are easily applied in their daily work (Weary et al., 2006). Within the different types of subjective behavioural measures of pain, the Visual Analogue Scale (VAS) is one of the most common techniques, where an observer estimates the level of pain that an animal is experiencing using a scale that goes from 'no pain' to the 'worst possible pain' (usually from 0 to 10 or 0 to 100) (Rutherford, 2002; Viñuela-Fernández et al., 2011). Other examples of subjective behavioural measures are simple descriptive scales (SDS) such as the Obel score system and the numerical rating score system (NRS) used for assessing lameness in horses and cows (Viñuela-Fernández et al., 2011; Flower and Weary, 2006). These descriptive scales rely on the presentation of specific behaviours such as foot lifting while resting or head bobs to describe pain intensity assigning an index value, but still are dependent on the quality of observer's training.

3.2.2. Objective Behavioural Measures

Objective methodologies to assess pain are related to detailed quantification of behaviours clearly and rigidly defined. This strict definition of behaviours makes them easier to verify and increases intra and inter observer reliability (Rutherford, 2002). Quantification of specific behaviours based for instance on frequency and duration have helped to differentiate healthy animals from those that are suffering a painful condition, highlighting differences that may not be detected by subjective measures. For example, Chapinal et al. (2010), using objective behavioural measures, found that lame cows lied in average 15.8 minutes more per bout compared with sound cows.

Complementarily, whichever behavioural measure is used, subjective or objective, there are three main classes of measures useful for the assessment of pain: measure of pain-specific behaviours presentation, measure of the decline in frequency or magnitude of maintenance behaviours, and choice-preference tests (Weary et al., 2006; Rushen et al., 2008). The evaluation of pain-specific behaviours is the most common class. It assesses pain based on the presentation of defensive-avoidance behaviours that animals perform to protect the injured area. Nociceptive thresholds measurement is a way to evaluate these behaviours, measuring the withdrawal responses in animals exposed to painful thermal or mechanical stimuli. The second class focuses on continual observation of changes in the animal's basic behavioural repertoire identifying changes in activity, food and water intake. Choice-preference tests for pain assessment can be done using trials that allow the animals to choose to alleviate pain sensation by discriminating between treatments with and without analgesics (Weary et al., 2006).

4. Pain in Cattle

In the UK, but with worldwide influence, the FAWC (1979) proposed the 'Five Freedoms' as guiding principles for animal welfare (OIE, 2002; Fraser, 2008). Within these freedoms, the third freedom *'Freedom of pain, injury and disease'* highlight the importance of preventing and alleviating pain and suffering in farm animals (FAWC, 2009). Complementarily, the official definition of animal welfare given by the World Organization for Animal Health (OIE, 2010) highlights the importance of preventing suffering defining good animal welfare as: '*An animal is in a good state of welfare if... it is healthy, comfortable, well nourished, safe, able to express innate behaviour, and if it is not suffering from unpleasant states such as pain, fear, and distress. Good animal welfare requires disease prevention and veterinary treatment, appropriate shelter, management, nutrition, humane handling and humane slaughter/killing...*'.

Consequently, cattle welfare research has focused on diagnosis, alleviation and prevention of pain, discomfort and suffering. The evaluation of these negative states is mainly done in relation with the most common diseases and farm practices in dairy and beef farms. Indeed, the majority of the animal welfare legislation around the world is focused on painful procedures done in farms (von Keyserlingk et al., 2009), which generates the necessity of creating scientific bases that support these codes and legislations.

4.1. Limiting Factors Affecting Alleviation of Pain in Cattle

There are three main limiting factors that affect the appropriate alleviation of pain in cattle: natural behaviour of cattle, human's pain perception towards cattle and the economic cost of pain relief.

Cattle' stoicism and their tendency to hide pain behaviours as an instinct of survival create challenges in identifying pain experience, and thus, its alleviation. Cattle evolved as prey species, tending to react less to painful stimuli as an evolutionary strategy, prioritizing the need to escape (Phillips, 2002). However, there is no evidence to affirm that cattle do not experience pain (Viñuela-Fernández et al., 2007).

Pain is traditionally overlooked by farmers and veterinarians (Viñuela-Fernández et al., 2007). Two surveys of bovine veterinarian attitudes toward analgesia in Canada and the United States reported a variation between individuals on pain perception and analgesic drugs' administration, influenced by gender, age and educational environment (Hewson et al., 2007; Fajt et al., 2011). Both studies respectively showed that 2.9% and 3.7% of the respondents did not provide any analgesic to cows undergoing caesarean section. Fajt and collaborators (2011) also found that women estimated higher (on average 0.4 points more) than men in a pain score scale associated with standard practices and medical conditions. Additionally, they found that individuals who grew-up on farms estimated lower (on average 0.5 points less) in a pain score scale than those not raised on a farm.

Finally, the other limiting factor is that producers are not willing to use analgesics due to the cost. Hewson et al. (2007) reported a strong agreement between veterinarians about the necessity of having more long-acting, cost-effective analgesics with smaller withdrawal periods.

4.2. Pain Assessment in Cattle

The development of validated measures of pain is one of the most important concerns of cattle welfare science (von Keyserlingk et al., 2009). The scientific assessment of pain in cattle is based on measures of productivity, behaviour and physiology (Rushen et al., 2008).

Physiological measures are particularly useful in cattle when behavioural changes are subtle. However, they are less useful for on-farm assessment due to their impracticability (e.g. technology and equipment required) and invasiveness associated with these processes (e.g. restrain for blood sampling) that can increase distress in the animals, thus, compromising the reliability of the results (Weary, et al., 2006). Grandin (1993) reported increased behavioural agitation during restraint and handing for blood sampling and weight measurement of cattle. Wohlt et al. (1994) reported significant increases in plasma cortisol related to handling prior to dehorning. The disadvantages of physiological measures have resulted in pain-related behavioural indicators and changes in maintenance behaviours to be the most common parameters to evaluate pain in cattle. They have been used to assess if farm procedures or diseases cause pain and to test the efficacy of analgesic therapies (Weary et al., 2006).

Generally, farmers use changes in cows' appearance (depressed, innapetent, weight loss) along side some changes in quantitative measures (e.g feed intake, milk yield) to identify cows that are in pain (Kemp et al., 2008).

Below, I will describe how pain-specific behaviours and changes in maintenance behaviours have been used to evaluate the effects of pain associated with different procedures carried out on farms and some health problems (lameness and mastitis) that affect cattle.

4.2.1. Pain-Specific Behaviours and On-Farm Procedures

Pain-specific behaviours are usually observed when the cow or the injured area is manipulated (Weary et al., 2006). Quantitative measurements of these behaviours have been used for the evaluation of pain experience in cattle during the performance of typical farm procedures.

This has been the case for dehorning in calves between 2 to 6 weeks of age and mature cattle (Stafford and Mellor, 2005a). Caustic paste and hot-iron dehorning are the most common methods used (Rushen et al., 2008), the latter being more common (Faulkner and Weary, 2000). Pain-specific behaviours such as ear flicking, head shaking, head rubbing, tail flicking and food stamping have been used for the evaluation of pain related to dehorning. Faulkner and Weary (2000) found that hot-iron dehorned calves without receiving analgesic treatment performed 8 times more head shaking, 20 times more ear flicking and twice as many head rubbing behaviours than ketoprofen (Non-steroidal anti-inflammatory NSAID) treated calves during the first 24-h after dehorning. Similarly, Heinrich and collaborators (2010) reported that calves not receiving pre-analgesic treatment with meloxicam (NSAID) had higher frequency of ear flicks and head shakes per hour (4.1 and 5.5 times more respectively) than analgesic treated calves 48 hours after hot-iron dehorning. Additionally, they evaluated nociceptive thresholds with a pressure algometer, finding that meloxicam treated calves withstood more pressure following dehorning compared to calves without analgesic (2.13 Kgf and 1.68 Kgf respectively).

Pain related to tail docking has been evaluated using pain-specific behaviours in calves and adult cows. This procedure is performed in dairy cows in some countries (e.g. Canada and the United States) under the anecdotal argument of improving udder and milk hygiene , cleanliness of milking parlours and milker comfort (Eicher et al., 2000; Tom et al., 2002b; Rushen et al., 2008). Rubber ring and hot-iron are the methods involved, the former being the most common (Tom et al., 2002a). Behaviours directly associated to tail docking include tail grooming and tail position. Rubber ring tail docked calves had in average higher frequency of tail grooming on day 0, 1 and 5 after docking compared to hot-iron tail docked calves (3.7, 5.5 and 4.5 times more respectively) and control group (2.8, 5.0 and 4.8 times more respectively) (Tom et al. 2002b). Similarly, rubber ring tail docked cows held their tails in a raised position significantly less than the non-tail docked cows in the first 24-h after docking (2.4 times less) (Tom et al., 2002a). Additionally, one week post-docking, the number of docked cows that remained with the tail in pressed position (against their body) was two times higher than the number of non-tail docked cows. In conclusion, tail docking caused mild discomfort (Tom et al., 2002a); and hot-iron was lesser aversive than rubber ring (Tom et al., 2002b).

Castration in cattle has been the best researched in terms of effects of pain on cattle welfare (Rushen et al., 2008). Pain-specific behaviours have been used as a manner of comparing the different existing methods (Stafford and Mellor, 2005b). The most common methods used in cattle are based on the removal of the testicles by surgery, by crushing (Burdizzo) or by constriction (rubber rings or latex bands); the latter being the most painful procedure (Rushen et al., 2008). Molony and collaborators (1995) reported that restless behaviours (footstamping/kicking, head turning, tail wagging and stretching) were significantly higher in rubber ring castrated calves during the first 3 hours after castration compared to burdizzo, surgery and burdizzo combined with rubber ring and non-castrated calves. For instance, rubber ring castrated calves performed 24 times more footstamping and kicking than non-castrated calves, and 4 times more than calves castrated with the other methods. For castration, research on pain has generated important evidence causing significant changes in welfare legislation in some countries (e.g. in Austria it is mandatory to use effective anaesthesia in combination with analgesia for castration) (Thüer et al., 2007).

Pain assessment of branding methods (hot-iron and freeze branding) has been focused on quantifying behaviours such as vocalizations, kicking, falling down in the chute, tail-flicking and escape-avoidance behaviours (Lay et al., 1992; Schwartzkopf-Genswein et al., 1997). These behaviours have been directly associated with the degree of pain sensation experienced by the animal during branding, with greater frequency for hot-iron branding. Schwartzkopf-Genswein et al. (1997) reported that 30% of hot-iron branded calves performed more than 20 tail flicks during branding compared to just 7% of freeze branded calves. The percentage of calves that kicked, fell in the chute and vocalised during hot-iron branding were higher (20, 15 and 24% respectively) compared to freeze branding calves (1, 5 and 2 % respectively). Similarly, hot-iron branded calves performed more escape-avoidance reactions (measured as the number of lines crossed during branding) than freeze branded calves (11 *vs.* 5 lines) (Lay et al., 1992). Additionally, measures of exertion-force and duration (obtained from headgate load cells, headgate strain gauges and squeeze chute load cells) have been used as a way to evaluate discomfort during branding. Schwartzkopf-Genswein et al. (1997) found that the average of the exertion-force measures was higher in hot-iron branded calves compared to freeze branded and non-branded calves (7, -2 and -5 millivolts respectively).

In contrast with the procedures mentioned above that happens once or less in a cow's life, there is a procedure that occurs daily throughout the productive life of a dairy cow: milking. Depending on external factors (such as milking type system and handlers' behaviours) and internal factors (such as health and lactation stage) milking can be a stressful procedure that generates discomfort in cows. The discomfort can be expressed by the performance of restless behaviours such as defecation-urination, stepping, lifting and kicking. Cows with few or considerable teat lesions were three times more likely to kick at least once during milking compared to cows without (Rousing et al., 2004). Similarly, cows with a painful condition (lameness) were more reactive during milking than sound cows (Hassall et al. 1993).

4.2.2. Pain-Specific Behaviours and Lameness in Cattle

Lameness is widely regarded as one of the major causes of economic losses in farms but also as a concern for animal welfare (Rushen et al., 2008). It is generally accepted that lame cows experience pain and/or discomfort when walking or standing (Rushen et al, 2008). Consequently, detection and alleviation of lameness, especially those at early stages, are an important part of cattle welfare research. Several studies in lameness have focussed on the development of reliable automatic detection systems to avoid issues of reliability and time-consuming gait scoring systems (e.g. Flower and weary, 2006). Special attention has been addressed to changes in weight distribution to identify discomfort and pain associated with lameness. Neveux and collaborators (2006) reported that cows applied 10% less weight on the front hoof that was on an uncomfortable surface compared to the other three hooves that were on a comfortable surface. However, when the uncomfortable surface was under a hind hoof, the difference on the applied weight was only between hind hooves (cows did not shift weight to front hooves). Additionally, Neveux et al. (2006) used the variability of weight applied to the legs as a specific measure of discomfort and pain. The variability of weight (using standard deviation of weight as a measure) applied between contralateral legs increased by 50% and 100% when an uncomfortable surface was under the back hooves and under the front hooves respectively. Similarly, Rushen et al. (2007) reported that lame cows applied 10% less weight on the injured leg compared to the contralateral leg and to healthy cows. Additionally, lame cows had an increase in more than 50% in the standard deviation of weight on the injured and the contralateral leg compared to healthy cows (from 15 to 45 Kg). Weight distribution has been also used as a measure to evaluate pain mitigation. Lidocaine (local anaesthetic) treated lame cows increased the percentage of weight applied to the injured leg (4% more), and decreased the standard deviation of weight on the injured and the contralateral leg by 25 Kg (Rushen et al., 2007). Ketoprofen (NSAID) had a positive effect in lame cows reducing the standard deviation of weight compared with the day before and after analgesic treatment (before analgesic: 35 Kg, analgesic day: 25 Kg; post-analgesic day: 30 kg) (Chapinal, et al. 2010).

The evaluation of nociceptive thresholds has been used widely for the detection of post-operative pain in horses (Rédua et al., 2002). This is another useful way to determine if a cow is experiencing pain by inducing the expression of pain-specific behaviours. A negative correlation exists between the severity of lameness and mechanical nociceptive thresholds in lame cows. As lameness increases, nociceptive thresholds significantly decrease (Whay et al. 1997). Lame cows with sole ulcer and white line disease had lower nociceptive thresholds (hypersensitive) 28 days after detection compared to sound cows, demonstrating that these two types of lameness cause long-lasting pain experience in cows (Whay et al., 1998).

4.2.3. Maintenance Behaviours and Pain in Cattle

In addition to pain-specific behaviours, researchers have also focused on changes in the animal's normal behavioural budget. Behavioural activity, particularly lying behaviour, is an indicator used to assess changes in cattle welfare particularly for comfort, pain and disease evaluation such lameness and mastitis (Trénel et al., 2009; Mattachini et al., 2011). The reason for this is that lying is considered a high-priority activity, where cows rest and sleep as part of restorative processes; with a higher motivation to perform this behaviour more than feeding and social behaviour (Metz, 1985; Krohn and Munksgaard, 1993).

Generally, dairy cows lie down between 8-15 hours per day; this period is divided into 8-10 bouts approximately with a duration that can vary between few minutes to more than 3 hours (Krohn and Munksgaard, 1993). Normally, cows switch sides (left-right) between lying bouts (Forsberg et al., 2008). Tucker et al. (2009) reported that cows do not have any laterality preference, spending 51% of their total lying time per day on the left side with some individual preferences. However, it has been found that cows in later stages of pregnancy tend to lie down more on the left; this is because foetus' location is mainly on the right side as the rumen is occupying the left (Forsberg et al, 2008). Additionally, ruminally cannulated cows tend to lie down more on the right side (Forsberg et al., 2008; Tucker et al., 2009).

Chapinal et al. (2010) demonstrated that daily activity (including lying, standing and walking) is a good measure for detecting lameness and measuring pain mitigation. Lame cows spent one hour longer lying down and had longer lying bouts (16 minutes more per bout) than sound cows. Furthermore, lame cows had a lower walking speed than sound cows (0.14 m/s less). However, analgesic therapy did not have significant effects on activity. Despite these results, authors clarified that variation in lying behaviour between cows is very high and it is difficult to find significant differences between lame and non lame cattle. Similar results were found by Blackie et al. (2011) where lame cows also spent 2 hour longer lying than sound cows.

5. Mastitis and Pain in Dairy Cattle

Mastitis is one of the most important and frequent diseases in dairy cattle, causing direct and indirect economic losses. Direct costs are related to discarded milk and treatment costs. On the other hand, indirect costs are related to penalties because of high somatic cell count (SCC) and culling-replacement rates (Hillerton, 1998; Bradley, 2002; Blowey and Edmondson, 2010). The average cost of a clinical mastitis case has been estimated between £100 and £200 (Blowey and Edmondson, 2010). Additionally, the average incidence of mastitis can vary between 20-50 cases for 100 cows per year (e.g. UK: 40-50 cases; Canada: 23 cases. Blowey and Edmondson, 2010; Olde Riekerink et al., 2008 respectively).

As well as the economic implications, welfare issues should not be underestimated. Mastitis has been classified as one of the most painful diseases and a major welfare problem in cows (FAWC, 1997; Broom and Fraser, 2007; FAO, 2011). Nevertheless, analgesic treatment is not routinely used as part of mastitis therapy on farm due to the three limiting factors that pain alleviation has on farms (see section 3.1). Therefore, the development of objective methods for assessing pain in mastitic cows is required (Fitzpatrick et al., 1999).

5.1. Aetiology

Mastitis is the result of an inflammatory response in the udder that is initiated due to the proliferation of micro-organisms that traversed the teat canal. Mastitis pathogens are classified as contagious or environmental based on the reservoir and mode of transmission (Smith, 2009). The major contagious pathogens are *Streptococcus agalactie* and *Staphylococcus aureus*. The main reservoirs of these pathogens are infected mammary glands. Environmental pathogens are mainly coliforms (*Escherichia coli* and *Klebsiella spp.*) and environmental S*treptococci*. Coliforms are more commonly associated with sawdust-bedding; and environmental S*treptococci* with sand-bedding (Zdanowicz et al., 2004).

5.2. Inflammation and Pain During Mastitis

The inflammatory response during mastitis is initiated by the multiplication of pathogens in milk and mammary tissue. While the pathogens are multiplying, polymorphonuclear neutrophils are attracted to the site of the infection. These defense cells produce oxidants and proteases that not only destroy the pathogens, but also some epithelial cells, causing tissue damage and decreasing milk production (Lauzon et al., 2006). The secretion of dead cells into the milk results in high SCC (more than 200.000- 250.000/mL that is usually the maximum recommended) (Smith, 2009; Viguier et al., 2009). The severity of this inflammation response determines whether mastitis is classified into subclinical or clinical (Viguier et al., 2009). Subclinical mastitis is the predominant type in dairy cows; however, it is difficult to diagnosis due to the absence of visible indicators. Contrarily, clinical mastitis produces notorious abnormalities in milk and udder appearance. In cases of mild clinic mastitis, only changes in milk are evident (changes in color, viscosity and consistence). In moderate cases, changes of udder are showed. In severe cases, changes in milk and udder are accompanied by systemic illness (fever, lethargy, depression) (Smith, 2009; Viguier et al., 2009).

5.3. Treatment

Different factors influence mastitis treatment on farms, but the more influential are the severity of clinical signs, milk and meat withdrawal periods of the drugs and treatment costs (Smith, 2009). Mastitis treatment is mainly based on antibiotics. However, supportive treatment can be used to help the cow to recover from the infection, alleviating any sign of suffering. Analgesic therapy is also recommended for severe cases of clinical mastitis (Fitzpatrick et al., 1999). However, the limited availability of long-lasting cost-effective analgesics for cattle and the long withdrawal periods outweigh the benefits that an analgesic therapy can bring to a mastitic cow (Hewson et al., 2007; Smith, 2009).

5.4. Pain Assessment in Cows with Clinical Mastitis

It is generally accepted by veterinarians that cows with severe cases of mastitis are experiencing pain based on the evident clinical signs and the general ill condition expressed by the cow; therefore analgesics are usually administrated (Hewson et al., 2007; Fajt et al., 2011). However, in the case of less severe mastitis, clinical signs are less evident; making the pain detection more difficult for farmers and added to the cost of analgesics the possibilities to use pain alleviation treatments within the common mastitis therapy is greatly reduced (Fitzpatrick et al., 1999).

Traditional research on mastitis has mainly focused on epidemiological studies of aetiology, antibiotic therapy, methods of diagnosis, control and prevention (e.g. Calderon and Rodríguez, 2008; Olde Riekerink et al., 2008). To date, very little research has focused on developing objective methodologies of measuring pain associated with mastitis, and the possible benefits that analgesic therapy can have on cows' welfare.

Studies on analgesic therapy have remarked the importance of pain mitigation in cows with mastitis (e.g. Banting et al., 2008; Wagner and Apley, 2004). Production, physiological and behavioural measures have been used to evaluate the efficacy of anti-inflammatory drugs using models of clinical mastitis induced by *Escherichia coli* lipopolysaccharide (LPS). The most used productive measure has been milk production. Wagner and Apley (2004) found that there were no significant differences in the rate of recovery of milk production after the induction of mastitis between non-analgesic and analgesic treated mastitic cows. However, flunixin meglumine (NSAID) treated mastitic cows had a lower decrease in milk production after mastitis induction compared to isoflupredone (steroidal anti-inflamatory) treated mastitic cows and non-analgesic treated mastitic cows (38 Kg *vs.* 30 and 32 Kg respectively). Within physiological measures, temperature, heart and respiratory rate, rumen motility and udder size have been widely used. Wagner and Apley (2004) also found that flunixin meglumine preserved rumen motility, reduced rectal temperature and heart rate in mastitic cows during the first 14 hours after mastitis induction compared to non-analgesic treated cows (average for the 14 hours: rumen motility, 4.25 *vs.* 3.7 contractions/2 minutes; temperature, 39.3 *vs*. 40.1 °C; heart rate, 86 *vs.* 98 beats/minute).

Similar results were found by Banting and collaborators (2008), where ketoprofen (NSAID) treated mastitic cows had a decrease in temperature, respiratory rate and udder size six hours after mastitis induction compared to non-analgesic treated mastitic cows (temperature, 38.5 *vs*. 40.5 °C; respiratory rate, 21 *vs*. 26 breaths/minute; udder size, normal size *vs.* still more than 50% increase). Additionally, this study also found that ketoprofen had positive effects on rumen motility that returned to normal rates 24 hours after mastitis induction compared with control cows (rumen motility, 4 *vs*. 2 contractions/2 minutes).

On the other hand, research on pain based on behavioural measures is not abundant. Nonetheless, the existing studies have used subjective and objective measurements of pain-specific and maintenance behaviours to evaluate pain sensitivity, and effects of pain and analgesic treatments in cows with either experimental-induced or natural occurring mastitis.

Fitzpatrick and collaborators (1999) evaluated pain sensitivity in cows with natural mastitis and the effects of flunixin meglumine (NSAID) using an objective measure: quantification of nociceptive thresholds. They specifically found that mastitic cows that received a single dose of the analgesic had higher nociceptive thresholds than non-treated cows, showing a reduced sensitivity to pain. However, the single dose of analgesic was only effective for mild mastitis cases, suggesting that repeated doses might be necessary for moderate-severe cases. Similarly, Bating et al. (2008) recorded pain responses to udder palpation to evaluate the efficacy of Ketoprofen (NSAID) in cows with experimental-induced mastitis. Pain responses were assessed using a subjective behavioural measure: VAS (10 cm). After mastitis induction, both control and analgesic treated cows had an increase in the pain score (analysed using the areas under the response curves: 15.6 and 16.8 mm respectively). However, after 24 hours, treated cows scored significantly lower than control cows (2.8 vs. 8.6 mm), showing that Ketoprofen had a positive effect on reducing pain sensitivity.

More recently, Kemp et al. (2008) evaluated mechanical nociceptive thresholds (using a gas-driven ramped device) and hind leg stance (measuring the distance between the cow's hocks) as pain-specific behavioural measures to evaluate changes in pain sensitivity and standing posture in cows with mild and moderate mastitis. Cows with mild and moderate clinical mastitis had significantly wider hock-to-hock distance by 272 mm and 271 mm respectively compared to control cows (225 mm). This indicates that cows with mastitis changed their posture as a result of the udder inflammation, and this measure was considered a reliable sign of severity of mastitis. They evaluated nociceptive thresholds applying pressure against the lateral condyle of the metatarsal bone of the hind legs, finding that in mastitic cows the leg on the mastitic side had lower nociceptive thresholds than the contralateral leg (26.2 KPa and 39.4 KPa respectively). However, the pressure withstood by control cows was similar to the pressure withstood by the leg on the mastitic side in mastitic cows (28.3 KPa). The authors concluded that higher levels of pressure on the contralateral leg on mastitic cows compared to control cows was because mastitic cows withstood more pressure due to the reluctance to shift weight to the leg on the mastitic side.

Recent researches have focused on pain sensitivity (Fitzpatrick et al., 2011) and changes in maintenance behaviours (Siivonen et al., 2011) as objective measures of pain experienced during experimentally-induced mastitis (*Escherichia coli* LPS). Fitzpatrick et al. (2011) evaluated nociceptive thresholds (using an algometer device) applying pressure to the udder of mastitic cows. They found that meloxicam (NSAID) treated mastitic cows withstood on average 2 lbs more of pressure on the mastitic quarter compared to the healthy quarter. Contrarily, non-analgesic treated mastitic cows withstood on average 2.5 lbs less of pressure on the mastitic quarter compared to the healthy quarter. The authors of this study concluded that pain pressure sensitivity is a potential objective measurement of pain due to clinical mastitis in dairy cows.

Most recently, Siinoven and collaborators (2011) reported that on the induction day, mastitic cows took 253 more steps, spent 2 hours less lying down and 65 minutes less lying on the mastitic quarter side compared to the day before induction. The authors concluded that mastitis had a strong effect on the lying rhythm and that although pain was not directly evaluated, changes in behaviour might indicate that a sort of discomfort in the udder caused prolonged standing in mastitic cows.

6. Conclusions and Recommendations

Different farm practices and diseases cause pain in cows at different stages throughout their lives. Although these pain experiences are initially beneficial for the animals triggering physiological and behavioural changes with the purpose of stopping tissue damage and promoting recovery, unnecessary pain needs to be prevented and controlled properly no manner the cause.

Mastitis is one of the most important and frequent disease, causing economic losses, and threatening cows' welfare due to the lack of pain mitigation. The lack of pain control is in part due to different perceptions of pain in cattle, deficiency in the ability of detecting pain, and because the high cost that implies analgesics' use. As a result, during the last decade, welfare researchers have been trying to develop more objective and quantified methods for assessing pain due to mastitis. However, the information available is not abundant. Physiological measures have provided valuable information about the positive effects that analgesics have on mastitic cows; nevertheless, they have a weakness due to their invasiveness and impracticability for on-farm assessments. Current methods of pain evaluation based on behavioural measurements has the limitation to be restricted to the assessment of a particular aspect of pain, primary changes in pain sensitivity or activity, leaving on the side the importance of comparing measures for a better validation of the methodologies proposed. The majority of studies have focused on comparing measures between analgesic and non-analgesic treated mastitic cows. However, it may be necessary to compare pain measures between mastitic and non mastitic cows; then proceed with comparisons using analgesic treatment.

Clearly, further research in this area is required to achieve more reliable, sensitive, and practical pain methodology to improve pain assessment in dairy cows with mastitis and subsequently their management and welfare. An integration of successful methodologies used in mastitis and lameness pain research (e.g. activity, nociceptive thresholds, weight distribution and hind leg stance) with behavioural measures (e.g. behaviours during milking) routinely used in the evaluation of farm practices can provide interesting and useful results.

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Development of behavioural methodologies to evaluate pain in dairy cows with mastitis

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Abstract

Mastitis is a frequent and painful disease in dairy cows. However, pain detection and alleviation in mastitic cows has been overlooked. The objective of this study was to develop methodologies to measure pain in dairy cows with clinical mastitis based on objective and quantitative behavioural measures. Forty-two lactating cows were used: 14 mastitic cows and 28 control cows. Mastitic and control cows were subjected to evaluation of pain responses on D1 (mastitis detection day), D2, D3 and 7 days after the last antibiotic treatment (D10+). Pain responses were evaluated by measuring lying behaviour, reactivity during milking (stepping, lifting and kicking), weight distribution and hock-to-hock distance. Overall, mastitic cows lied down 69 minutes less on D2 compared to control cows (p=0.01). The percentage of time lying on the mastitic quarter side did not differ significantly between control and mastitic cows. Nonetheless, laterality of lying had a high variability between individuals for both treatments. In average, control cows spent 46.95 ± 9.46% lying on the right side; however some individuals had a marked side preference. Similarly, mastitic cows showed a marked preference despite the tendency to lie down less time on the mastitic side (43.21 ± 23.95%). Restlessness during milking did not differ between treatments. Restless behaviours differed significantly within mastitic cows between days. Frequency of kicks per minute was higher on D1 comparing with D2 (*p*=0.019), frequency of lifts was higher on D1 and D2 comparing with D10+ (*p*=0.009 and *p*=0.001 respectively), and frequency of steps was higher on D2 comparing with D10+ (*p*=0.032). The variability of weight that mastitic cows applied to the leg on the mastitic quarter side was much higher on D1 compared to D10+. For control cows the variability of weight was higher on D1 compared to D2 and D3. The hock-to-hock distance did not differ between control and mastitic cows. Mild clinical mastitis might not cause sufficient discomfort or pain to markedly observe changes in behaviours. However, cows showed differences in lying time and reactivity during milking, and slight differences in laterality of lying. The lack of knowledge about cows' lying side preference and the possible effects of no familiarisation period with the scale made interpretation of these differences as pain-specific responses difficult. To further develop of methodologies for assessing pain in mastitic cows, it is worth applying the methodologies used in this study to cows with moderate-severe mastitis, followed by their validation using analgesic treatment.

Key words: Cattle, pain, mastitis, behaviours, pain assessment.

1. Introduction

Mastitis is a costly disease affecting dairy cattle. Economic losses result from discarded milk, treatment, and culling-replacement (Hillerton, 1998; Bradley, 2002; Blowey and Edmondson, 2010; CCIL, 2010). Mastitis is likely a painful disease and a major welfare problem in cows (FAWC, 1997; Broom and Fraser, 2007; FAO, 2011). Within the 'Five Freedoms', '*Freedom of pain, injury and disease*' highlights the importance of preventing and alleviating suffering in farm animals (FAWC, 2009), including pain related to mastitis.

Pain assessment and alleviation in cattle are limited by three factors. Firstly, cattle evolved as prey species tending to react less to painful stimuli as an instinct of survival (Phillips, 2002). Secondly, pain is traditionally underestimated by farmers and veterinarians (Viñuela-Fernández et al., 2007). The last limiting factor is related to high costs and withdrawal periods associated with analgesics. Consequently, analgesics are not an essential part of mastitis therapy (Fitzpatrick et al, 1999; Hewson et al, 2007).

The development of objective and quantitative methods for assessing pain in cows with mastitis has become an aim of welfare scientists (Fitzpatrick et al, 1999). Research has focused on evaluating the effects of analgesic therapy on physiological and behavioural measures with experimentally-induced and natural mastitis (Fitzpatrick et al, 1999; Wagner and Apley, 2004; Banting et al, 2008; Fitzpatrick et al, 2011). However, few studies have evaluated the effects of pain due to mastitis on behaviour as a first step before implementing an analgesic treatment (Kemp et al, 2008; Siinoven et al, 2011).

A combination of behavioural measures for pain and discomfort assessment may be necessary to detect pain from mastitis. Thus, the objective of this study was to develop methodologies to assess pain in cows with natural clinical mastitis. The assessment of pain was based on a combination of objective and quantitative measures of lying behaviour, restless behaviours during milking, weight distribution among legs and hind leg stance.

The hypothesis of this study was that mastitic cows would spend less time lying and, when lying, they would spend less time lying on the mastitic quarter side compared to control cows based on the findings of Siinoven et al. (2011). They found that experimentally-induced mastitic cows spent less time lying and less time lying on the mastitic side after the induction of mastitis compared to the day before induction. Based on the results of Rushen et al. (2007), where lame cows had a higher variation of weight applied to the injured leg than sound cows, we predicted that mastitic cows would have a higher variation of weight applied to the leg on the mastitic quarter side compared to control cows. Reactivity has been used as a measure of stress in cattle during milking. Hassall et al. (1996) and Rousing at al. (2004) found that lame cows and cows with lesions on the teat respectively, were more restless on their feet while being milked. Therefore, we predicted that mastitic cows would be more reactive during milking than control cows. Based on the results of Kemp et al. (2008), where cows with mild and moderate mastitis had a wider hock-to-hock distance than healthy cows, we predicted that mastitic cows would have wider hock-to-hock distance than control healthy cows. A pre-experiment was conducted to evaluate the effect of IceTags (accelerometers) on lying behaviour and laterality of lying of lactating cows, to be able to ensure that in the main study IceTags were not going to affect laterality of lying of mastitic cows.

2. Materials and Methods

2.1. Animals and Housing

Primiparous and multiparous lactating Holstein cows (*Bos taurus*) with body condition score (BCS) > 2 and sound walking gait assessed by using the numerical rating score system (Flower and Weary, 2006) were selected. Cows were housed in sand-bedded freestalls (2.4 m long x 1.18 m wide x 0.40 m deep) with access to at least one stall per cow at the University of British Columbia's Dairy Education and Research Centre (Agassiz, Canada). Cows were fed a TMR twice daily (45.5% concentrate and 54.5% forage on a dry matter basis). Water was supplied *ad libitum*. The cows were milked twice daily (05:00 and 15:00 h). All the procedures related to animals in this experiment were approved by the UBC Committee on Animal Care (the University of British Columbia).

2.2. General Procedures and Measures

2.2.1. Accelerometers

Hobo Pendant G Acceleration Data Loggers (Onset Computer Corporation, Pocasset, MA – dimensions mm: 33 high x 60 wide x 25 deep approximately) were programmed to record the position of the cow as standing or lying down using a logging interval of 1 reading per minute and *g* forces as a unit (Chapinal et al. 2009a). The y-axis was used to evaluate lying behaviour; and the z-axis was used to determine laterality of lying. The hobo loggers were attached with Vet Wrap (Co-Flex, Andover Coated Products Inc., Salisbury, MA) at the level of the middle part of the metatarsus. The data collected by the Hobos were downloaded using Onset HOBOware® Lite Software Version 2.2.1 (Onset Computer

Corporation, Pocasset, MA) and exported to Microsoft Excel® (Microsoft Corporation). Then Macro Hobo 3D Microsoft Excel® was used to modify and edit data.

IceTag automatic recording devices (IceRobotics© Ltd, Edinburgh, UK - dimensions mm: 65 high x 60 wide x 30 deep approx.) were programmed to record cows' activity (standing and step count) with a sampling rate of 16 readings per second. The IceTag devices were attached to the hind legs above the fetlock (Chapinal, et al., 2010a). The data collected by the IceTags were wirelessly downloaded to a computer using Ice Reader Desktop Download System and the Ice Tag Analyser™ 2010 Software (IceRobotics© Ltd). However, data from IceTags were not used for this project; it will be used as part of the larger project.

2.2.2. Behaviours at the Milking Parlour

The behaviours of the cows during milking were individually recorded using a video camera Panasonic SDR-H85PC (Panasonic Shikoku Electronics Indonesia). Videos took place during the time of milking that included three periods mentioned by Cavallina et al. (2006): pre-milking (from when the cow entered her milking place until the milking unit was attached), milking (from directly after attachment until the complete removal of the milking unit), and post-milking (from the removal of the milking unit after the post-dip was applied to the last teat).

CowLog® Software (Hänninen and Pastell, 2009) was used to score behaviours related to reactivity of cows during milking including the three periods mentioned above. Table 1 and Figure 1 describe the ethogram of the behaviours scored. The scorings were done by a single observer who was completely blind to the treatments. To evaluate intra-observer reliability, the observer scored 8 videos twice chosen at random after three time points: 1.

finishing training period, 2. at a mid point and 3. at the end, when all video were completed. Spearman correlation between the two scores was high (*rs* = 0.92-0.99).

Table 1. Behaviours related to reactivity of the cows recorded at the milking time.

Behaviours	Definition
Step	The hoof is lifted off the ground without going higher than the upper part of the dew-claw
Lift	The hoof is lifted off the ground higher than the upper part of the dew-claw but lower than the middle point between the dew-claw and the point of the hock
Low kick	The hoof is lifted off the ground higher than the middle point between the dew-claw and the point of the hock but lower than the point of hock
High kick	The hoof is lifted off the ground higher than the point of hock

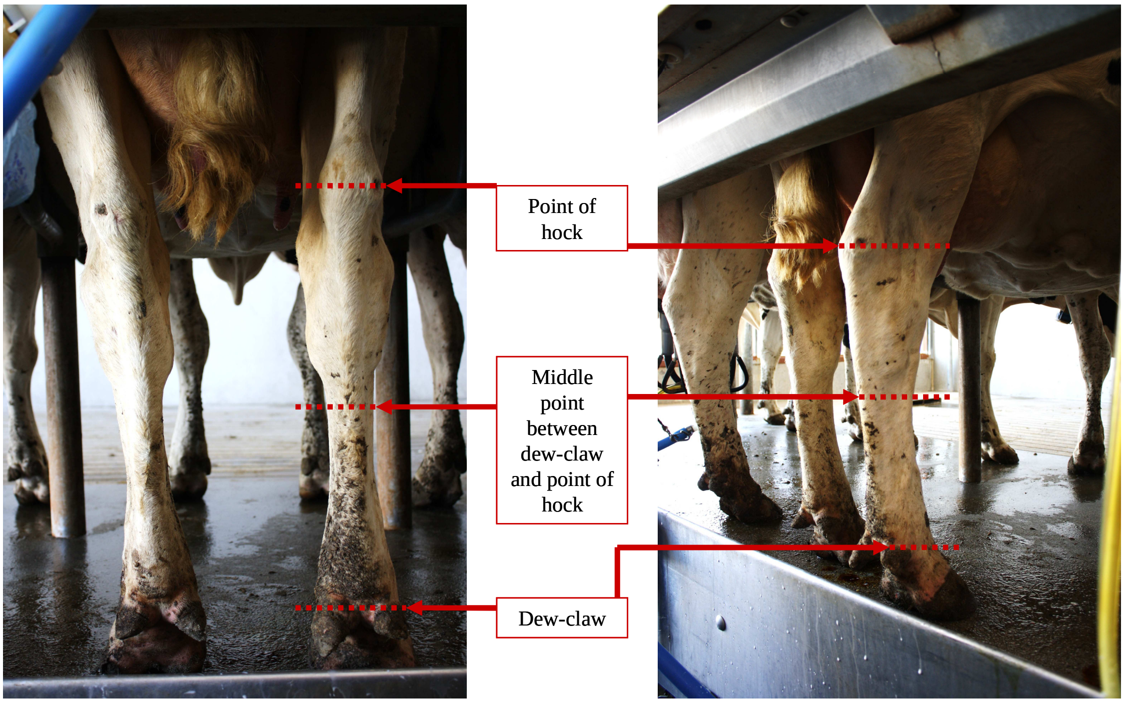

Figure 1. Anatomical parts of the hind legs used to define the behaviour related to reactivity of cows during milking.

2.2.3. Weight Distribution

The distribution of weight between the cow's four legs was recorded while the cow stood on a force plate scale for 5 minutes following the procedure previously described by Neveux et al. (2006) and Chapinal et al. (2009b, 2010a, 2010b) (Figure 2). It was not possible to habituate or familiarise the cows to the scale during this study unlike previous studies (Chapinal et al, 2009b; 2010a; 2010b; Pastell et al, 2010) because it was not known when a cow would be diagnosed mastitic. However, to avoid the development of negative associations related to the scale, all the animals were rewarded with food once they were standing calm and head restrained in the scale and before release. For detailed information see Appendix 1.

Figure 2. Cow standing on the force plate scale.

2.2.4. Hock-to-Hock Distance

The distance between hocks was measured based on the methodology used by Kemp et al. (2008). The distance was taken twice using a retractable measuring tape having as a reference points the Calcanean tuberosities (point of hock) of both hind legs (Figure 3). This procedure was done while the cows were standing on the force plate scale.

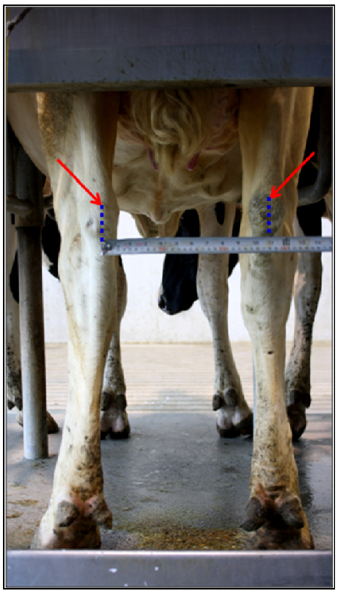

Figure 3. Measurement of hock-to-hock distance. Red narrows: Calcanean tuberosities.

2.3. Specific Experimental Procedures

2.3.1. Experiment 1: IceTag Trial

The aim of this experiment was to evaluate whether there were effects of position (lateral or medial), number (one or two) and location (left or right hind leg) of IceTags on cows' lying behaviour. Sixteen cows were randomly split into two groups (Group Medial n=8 and Group Lateral n=8). The Ice Tag devices were always attached on the medial side of the hind leg for Group Medial, and always attached on the lateral side for Group Lateral.

Both groups were balanced for parity, days in milk (DIM), BCS and body weight (BW) (mean ± SD, Group Medial: parity = 1.63 ± 0.74; DIM = 132.87 ± 54.30; BCS = 3.12 ± 0.42; BW = 642.5 ±168.61 Kg; Group Lateral: parity = 1.5 ± 0.75; DIM = 118 ± 61.03; BCS = 2.78 ± 0.49; BW = 621.3 ± 48.53 Kg).

Hobo loggers were attached to all the animals on day (D) 0 at 10.00 h. Hobos were programmed to start recording data at 00:00 h on D1 of the trial. Hobo side location (left or right hind leg) was balanced by attaching the hobo to half of the cows (n=8) on the left hind leg, and on the right hind leg for the other half of cows (n=8). This balance was equally applied to both groups.

The cows were assigned to four treatments in a latin square design. All cows had one hobo attached and the four treatments were: no IceTags attached as a control (C); one IceTag on the left hind leg (L), one IceTag on the right hind leg (R); one IceTag on both hind legs (B). Each treatment lasted for 6 days, and a total of 24 days of accelerometer data was collected. All the cows participated in all the four treatments.

2.3.2. Experiment 2: Evaluation of Pain Responses in Cows with Clinical Mastitis

Pain responses were evaluated measuring lying behaviour and laterality of lying, behaviours during milking, body weight distribution and hock-to-hock distance. A methodology for measuring mechanical nociceptive thresholds started to be developed in this study but is still in progress and validation (see Appendix 2).

2.3.2.1. Selection of Cows

Forty-two cows were recruited for this experiment. Fourteen cows with clinical mastitis in only one quarter were selected (mean ± SD; parity = 3.92 ± 1.77; DIM = 185.14 ±

104.06; BCS = 3.35 ± 0.42; gait score = 1.64 ± 0.49). Mastitis was detected by the milker using visual inspection for abnormalities in the foremilk (flakes, clots or blood) and the udder (redness, heat and swelling). A milk sample from the mastitic quarter was taken for bacteriological culture on the day of detection and prior to milking (see Appendix 3 and Table 2). The samples were sent to the Animal Health Centre AAVLD – Accredited Laboratory, Ministry of Agriculture, Abbotsford, BC, Canada for analysis. Following the farm procedures, mastitic cows received an intramammary infusion of antibiotic (Cefa-Lak®, Fort Dodge, Pfizer) as a mastitis treatment twice daily at each milking for three or four consecutive days starting on the mastitis detection day.

Table 2. Pathogens isolated from milk samples of mastitic cows.

Group	Cow ID	Microorganism isolated
1	9029	No bacteria isolated
2	7118	*Enterococcus faecium*
3	5014	*Enterococcus faecium*
4	9957	No bacteria isolated
5	5053	No bacteria isolated
6	7109	*Enterococcus faecium*
7	6047	*Enterococcus faecium*
8	3072	*Enterococcus faecium*
9	4064	*Klebsiella oxytoca*
10	4040	*Klebsiella oxytoca*
11	3056	*Escherichia coli*
12	3126	No bacteria isolated
13	6022	*Escherichia coli*
14	5016	*Klebsiella oxytoca*

Twenty-eight control cows were recruited for this experiment (mean ± SD; parity = 3.75 ± 1.50; DIM = 173.29 ± 87.66; BCS = 3.20 ± 0.50; gait score = 1.75 ± 0.44). Two control cows were assigned to each mastitic cow to avoid problems of losing any control cow due to illness, high SCC, lameness, or difficulty in training to use force plate scale. Control cows were selected based on parity and DIM to match the mastitic cow. Each mastitic cow and the two control cows were assigned a group number from 1-14. A sample of milk from each control cow was collected and analysed for somatic cell count (SCC) to confirm the absence of mastitis (samples were sent to the Pacific Milk Analysis Lab, Chilliwack, BC, Canada). For selection, control cows needed to have SCC less than 250,000 cells/ml (Smith, 2009; Viguer et al, 2009) at the beginning of the trial (mean ± SD; SCC = 63,643 ± 76,177); but also for the mean of the previous 3 months (mean ± SD; SCC = 27,000 ± 30,000). Additionally, at the end of the trial, a milk sample from both mastitic and control cows were analysed for SCC. Both mastitic and control cows needed to have SCC less than 250,000 cells/mL to be considered healthy (mean ± SD; 179,800 ± 196,400 and 58,460 ± 80,320 respectively).

2.3.2.2. Timeline

Each mastitic cow and her two controls were subjected to evaluation of pain responses on D1, D2, D3 and D10+ (7 days after the last antibiotic treatment) of the trial. D1, D2 and D3 were known as mastitis treatment days, where the cows received antibiotic treatment. D10+ was considered a baseline where the mastitic cows were mastitis free. The mean (± SD) duration of the trial for each group was 10.35 days (± 0.49); and the mean (± SD) number of days between D3 and D10+ was 7.93 days (± 0.62).

There were two timelines depending on whether the cows were detected on the morning or afternoon milking. Timelines are detailed in Appendices 4A and 4B. All the cows were subjected to a series of procedures following the timeline depending on the detection time.

2.3.2.3. Procedures

IceTags and Hobos were attached on D1 to mastitic cows and control cows to continuously record lying behaviour and activity from D1 to D10+. Based on the results of the Experiment 1 (see section 2.3.1 and 3.1.), it was decided to attach one IceTag to the lateral side of both hind legs of each cow. One Hobo was attached to measure lying behaviour and the laterality of lying. IceTag and Hobos were removed on the day after D10+ at the same time of the day that they were attached on D1 to be able to complete cycles of 24 hours.

Weight distribution, behaviours during the milking, and hock to hock distances (see section 2.2) were recorded for D1, D2, D3 and D10+ for the mastitic cows and the controls. Video recording of behaviours during milking were always at the same milking depending on whether mastitic cows were detected at either am or pm milking.

2.4. Data and Statistical Analysis

All descriptive analysis was performed in Excel®; and statistical analyses were performed with SAS V9.2.

2.4.1. Experiment 1

For the three behaviours evaluated (total lying, lying on the left side and lying on the right side), a single average per cow on a 24 hours basis was calculated for total time performing the behaviour (min/day), frequency of bouts (bouts/day) and average duration of bouts (min/bout). This was done for each of the four treatments in both medial and lateral groups. During the experiment one cow developed a swelling on the left hock, and her data was discarded.

Data was not normally distributed and variances were not homogeneous ($p < 0.05$ Shapiro-Wilk Test and Bartlett's Test respectively), therefore a Kruskal-Wallis Test was selected for comparing groups and to investigate whether there was an effect of the position of the IceTag (medial or lateral) and number of Ice Tags attached (one or two) on the lying behaviour of the cows. Furthermore, a Kruskal-Wallis Test was also used to evaluate the treatment effect intra-group and the group effect intra-treatment. Differences between groups were not found (see Appendix 5); therefore comparisons between treatments were done compiling data from both groups for each of the variables analysed using Kruskal-Wallis Test.

2.4.2. Experiment 2

Data from four of the 28 control cows was discarded because of illness (lameness and displaced abomasum), high SCC at the end of the trial (>250,000 cell/mL). Consequently, of the total 14 groups used in the study (one mastitic cow plus two control cows per group), 10 groups had complete data for the two control cows; therefore an average between them was calculated to get a single set of data for each procedures done.

Of the 14 mastitic cows recruited, four cows had mastitis on a back quarter and ten on a front quarter. From the four cows with mastitis on a back quarter, three had mastitis on the right and one on the left. From the ten cows with mastitis in a front quarter, three had mastitis on the right and seven on the left. Data from one cow on D10+ was discarded because she did not recover from mastitis (SCC = 817,000 cells/mL). Unreliable data for lying laterality of one mastitic cow was discarded due to problems related to a twisted hobo. Additionally, one group (n=3, 1 mastitic and 2 control cows) did not have data of weight distribution, algometer test and hock-to-hock distance due to unforeseen circumstances.

2.4.2.1. Activity

Data from the hobos was used to measure lying behaviour and lying laterality. Data from IceTags will be used as part of the larger project and are not reported here. The variables calculated on a 24 hours basis were total lying time (TLT), number of lying bouts (NLB), mean duration of lying bouts (MDLB) and percentage of total time lying on the mastitic side (PTLM) that in control cows correspond to total time lying on the homologous side. Differences between days (D1, D2, D3, D10+) for mastitic and control cows, and differences between treatments (control *vs.* mastitic) within days were calculated. Data was not normally distributed and variances were not homogeneous ($p < 0.05$, Shapiro-Wilk Test and Bartlett's Test respectively). Wilcoxon Signed Ranks Test was used to compare the differences. Cows detected at morning milking had a total of 21 hours recorded for lying behaviour whereas cows detected during afternoon milking had only 9 hours recorded for lying behaviour on D1. D1 was not used due to these large differences in recorded lying data.

2.4.2.2. Behaviours at the Milking Parlour

A mean of the number of behaviour's frequency per minute was calculated for: step, lift and kick (low kick and high kick were combined for the statistical analysis in a single category), to allow comparisons between milking of different lengths. The difference in frequency per minute between days for both treatments (mastitic *vs.* control); and the difference between treatments by day were calculated. Data was not normally distributed and variances were not homogeneous ($p < 0.05$, Shapiro-Wilk Test and Bartlett's Test respectively). Wilcoxon Signed Ranks Test was used to compare the differences.

2.4.2.3. Weight Distribution

The differences between treatments (mastitic *vs.* control) on the standard deviation (SD) over time (a measure of weight shifting) and the percentage (%) of weight (Kg) applied to the leg on the mastitic side were compared by days. Additionally, the differences were compared between days for each treatment. Data for the SD of weight was normally distributed and the variances were homogeneous ($p > 0.05$); contrary to the percentage of weight data ($p < 0.05$) (Shapiro-Wilk Test and Bartlett's Test). Student paired *t* Test was used to compare the differences for the SD of weight. Wilcoxon Signed Ranked Test was used to compare the differences for the percentage of weight.

2.4.2.4. Hock-to-Hock Distance

A single mean per cow was calculated by day from the two daily measures taken. The difference in hock-to-hock distance between treatments (mastitic *vs.* control) was calculated by day. Additionally, the differences between days were also calculated for each treatment. Data was not normally distributed and variances were not homogeneous ($p<0.05$, Shapiro-Wilk Test and Bartlett's Test respectively). Wilcoxon Signed Ranks Test was used to compare the differences.

3. Results

3.1. Experiment 1

3.1.1. Total Lying Time and Lying Bouts

There were no significant differences ($p > 0.05$) in total lying time, frequency of lying bouts and average duration of lying bouts between any of the treatments we tested. Whether the IceTag was attached on the left or on the right leg, or to either both legs or none of them (Table 3).

Table 3. Median (25th percentile -75th percentile) time spent lying down, frequency of lying bouts and mean duration of lying bouts of cows with no IceTag (C), cows with an IceTag on either the left (L) or right (R) hind leg or with an IceTag on both hind legs (B).

Total lying time (minutes/24 h)			Frequency of lying bouts (bouts/24 h)		
Treatment	n	Median (P25 - P75)	Treatment	n	Median (P25 - P75)
C	15	778.8 (746.8 - 819.5)	C	15	9.6 (8.8 - 11.5)
L	15	792.6 (765.1 - 813.0)	L	15	10.6 (9.0 - 11.0)
R	14	765.1 (744.8 - 810.3)	R	14	10.1 (9.6 - 11.1)
B	15	783.6 (711.0 - 813.0)	B	15	10.0 (8.6 - 11.5)
χ^2†		1.32	χ^2†		0.04
p-value		0.72	*p*-value		0.99

Mean duration of lying bouts (minutes/bout)		
Treatment	n	Median (P25 - P75)
C	15	74.7 (70.3 - 90.5)
L	15	74.9 (69.4 - 83.7)
R	14	74.3 (65.3 - 85.6)
B	15	76.4 (69.0 - 89.1)
χ^2†		0.44
p-value		0.93

† χ^2 = Test statistic for Kruskal-Wallis Test
P25 = 25th percentile
P75 = 75th percentile

3.1.2. Laterality of Lying

There were no significant differences ($p > 0.05$) on total lying time, frequency and mean duration of lying bouts on the left side between treatments (Table 4). Similarly, there were no significant differences ($p > 0.05$) on total lying time, frequency and mean duration of lying bouts on the right side between treatments (Table 5).

Table 4. Median (25th percentile -75th percentile) time spent lying down on the left, frequency of lying bouts on the left and mean duration of lying bouts on the left of cows with no IceTag (C), cows with an IceTag on either the left (L) or right (R) hind leg or with an IceTag on both hind legs (B).

Total lying time on the left side (minutes/24 h)			Frequency of lying bouts on the left side (bouts/24 h)		
Treatment	n	Median (P25 - P75)	Treatment	n	Median (P25 - P75)
C	15	395.8 (365.3 - 430.3)	C	15	5.8 (4.3 - 8.0)
L	15	411.8 (379.6 - 444.8)	L	15	5.6 (5.3 - 9.6)
R	14	387.7 (371.1 - 399.6)	R	14	5.9 (4.6 - 6.8)
B	15	396.5 (376.8 - 426.0)	B	15	5.8 (5.3 - 8.3)
χ^2†		2.01	χ^2†		1.59
p-value		0.56	*p*-value		0.66

Mean duration of lying bouts on the left side (minutes/bout)		
Treatment	n	Median (P25 - P75)
C	15	67.6 (58.1 - 77.7)
L	15	59.6 (44.2 - 84.2)
R	14	66.8 (60.9 - 91.9)
B	15	73.4 (49.4 - 77.4)
χ^2†		0.49
p-value		0.92

† χ^2 = Test statistic for Kruskal-Wallis Test
P25 = 25th percentile
P75 = 75th percentile

Table 5. Median (25th percentile -75th percentile) time spent lying down on the right, frequency of lying bouts on the right and mean duration of lying bouts on the right of cows with no IceTag (C), cows with an IceTag on either the left (L) or right (R) hind leg or with an IceTag on both hind legs (B).

Total lying time on the right side (minutes/24 h)			Number of lying bouts on the right side (bouts/24 h)		
Treatment	n	Median (P25 - P75)	Treatment	n	Median (P25 - P75)
C	15	379.5 (366.8 - 417.8)	C	15	5.3 (4.8 - 6.5)
L	15	379.8 (311.0 - 421.3)	L	15	6.6 (5.3 - 7.8)
R	14	373.3 (331.8 - 415.5)	R	14	5.9 (5.5 - 6.8)
B	15	351.1 (315.6 - 420.6)	B	15	6.0 (5.0 - 7.8)
χ^2†		1.63	χ^2†		2.65
p-value		0.65	*p*-value		0.44

Mean duration of lying bouts on the right side (minutes/bout)			
Treatment	n	Median (P25 - P75)	
C	15	73.1 (62.1 - 82.8)	
L	15	61.1 (46.6 - 78.1)	
R	14	61.5 (57.8 - 74.6)	
B	15	60.1 (51.1 - 77.9)	† χ^2 = Test statistic for Kruskal-Wallis Test
χ^2†		4.07	P25 = 25th percentile
p-value		0.25	P75 = 75th percentile

3.2. Experiment 2

3.2.1. Lying Behaviour

Mastitic cows spent significantly less time lying down on D2 than control cows (Table 6). Looking at the medians, mastitic cows also spent less time lying on D10+ compared to control cows, following the same tendency as D2 but this was not significant. There were no significant differences ($p > 0.05$) between mastitic and control cows for frequency and mean duration of lying bouts at each time (Table 7 and 8). Similarly, there were no significant differences between days for total lying time, frequency and mean duration of lying bouts within each treatment ($p > 0.05$).

Table 6. Differences on the total lying time between control and mastitic cows by days, and between days for each treatment: control and mastitic cows.

Variable		Total lying time (minutes/24-h)					
Treatment		Control cows		Mastitic Cows		Diff between C-M	
		n	Median (P25 - P75)	n	Median (P25 - P75)	*S*-value†	*p*-value
Day	D2	14	776.5 (701.9 - 811.9)	14	707.5 (562.8 - 809.0)	38.0	0.01*
	D3	14	724.0 (674.9 - 796.9)	14	740.5 (559.8 - 790.3)	19.5	0.24
	D10+	14	784.5 (702.8 - 827.4)	13	654.0 (598.0 - 790.0)	26.5	0.06
		S-value†	*p*-value	*S*-value†	*p*-value		
Diff between days	D3 - D2	-17.5	0.29	-1.5	0.95		
	D10+ - D2	7	0.68	4.5	0.78		
	D10+ - D3	23.5	0.15	-2.5	0.89		

C=Control cows P25= 25th percentile † *S*-value = Test statistic for Wilcoxon sign ranked test
M=Mastitic cows P75= 75th percentile * Significant difference ($p<0.05$)

Table 7. Differences on the frequency of lying bouts between control and mastitic cows by days, and between days within control and mastitic cows.

Variable		Frequency of lying bouts (bouts/24-h)					
Treatment		Control cows		Mastitic Cows		Diff between C-M	
		n	Median (P25-P75)	n	Median (P25-P75)	*S*-value†	*p*-value
Day	D2	14	10.0 (7.9-11.4)	14	11.0 (8.0-12.0)	-8.5	0.61
	D3	14	9.3 (8.5-10.8)	14	9.0 (8.0-10.8)	7.0	0.64
	D10+	14	9.8 (9.0-10.9)	13	10.0 (6.0-13.0)	-5.5	0.65
		S-value†	*p*-value	*S*-value†	*p*-value		
Diff between days	D3 - D2	-4.0	0.71	-24.5	0.09		
	D10+ - D2	1.0	0.98	2.0	0.89		
	D10+ - D3	3.0	0.86	11.5	0.25		

C=Control cows P25= 25th percentile † *S*-value = Test statistic for Wilcoxon sign ranked test
M=Mastitic cows P75= 75th percentile

Table 8. Differences on the mean duration of lying bouts between control and mastitic cows by days, and between days within control and mastitic cows.

Variable		Mean duration of lying bouts (minutes/bouts)					
Treatment		Control Cows		Mastitic Cows		Diff between C-M	
		n	Median (P25-P75)	n	Median (P25-P75)	*S*-value†	*p*-value
Day	D2	14	78.54 (72.70-91.80)	14	70.45 (59.04-85.76)	12.5	0.46
	D3	14	81.58 (72.16-89.10)	14	81.82 (67.74-98.78)	0.5	1.00
	D10+	14	80.37 (73.29-90.40)	13	84.40 (54.69-100)	5.5	0.73
		S-value†	*p*-value	*S*-value†	*p*-value		
Diff between days	D3 - D2	-8.5	0.62	18.5	0.26		
	D10+ - D2	11.5	0.5	1.5	0.94		
	D10+ - D3	20.5	0.21	-6.5	0.68		

C=Control cows
M=Mastitic cows
P25= 25th percentile
P75= 75th percentile
† *S*-value = Test statistic for Wilcoxon sign ranked test

The percentage of time lying on the mastitic quarter side did not differ significantly between control and mastitic cows on D2, D3 and D10+; and there were no significant differences between days for each treatment (Table 9). Although for control cows there were no significant differences on the laterality of lying between days; Figure 4 shows the mean percentage of time lying on the right side for the length of the trial (10.35 ± 0.49 days). There was a high variability between individuals. On average, for a 24h period, control cows spent 46.95 ± 9.46 % (mean ± SD) of the time lying on the right side (min, 28.34%; max, 69.27%). For mastitic cows, Figure 5 shows the mean percentage of time lying of D2 and D3 on the mastitic side, there was also a high variation between individuals. On average, mastitic cows spent 43.21 ± 23.95 % (mean ± SD) of the time lying on the mastitis side. The variation in lying on the mastitic side ranged from 0.00 % to 83.06 % (min-max). Individual graphs of laterality of lying for mastitic cows are shown in Appendix 6.

Table 9. Differences on the percentage of time lying on the mastitic side between control and mastitic cows by days, and between days within control and mastitic cows.

Variable		Percentage of time lying on the mastitic quarter side					
Treatment		Control Cows		Mastitic Cows		Diff between C-M	
		n	Median (P25-P75)	n	Median (P25-P75)	*S*-value†	*p*-value
Day	D2	13	47.7 (44.4-53.3)	13	52.9 (28.0-58.2)	-1.5	0.94
	D3	13	53.2 (49.6-61.7)	13	41.9 (29.9-53.0)	21.5	0.14
	D10+	13	48.6 (38.3-61.5)	12	44.4 (29.4-71.4)	7.0	0.62
		S-value†	*p*-value	*S*-value†	*p*-value		
Diff between days	D3 - D2	20.5	0.21	-1.7	0.20		
	D10+ - D2	8.5	0.62	5.0	0.73		
	D10+ - D3	-10.5	0.54	18.0	0.17		

C=Control cows P25= 25th percentile † *S*-value = Test statistic for Wilcoxon sign ranked test
M=Mastitic cows P75= 75th percentile

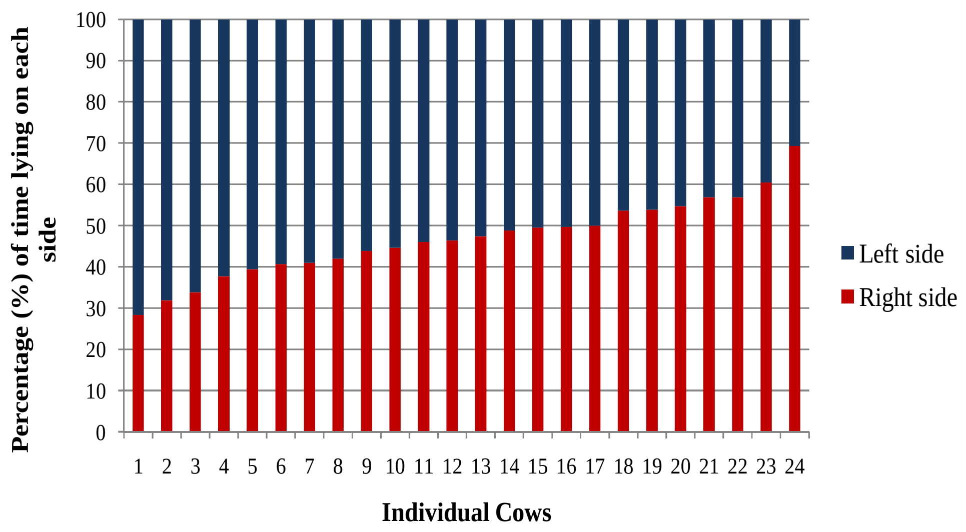

Figure 4. Distribution of the mean percentage of time that control cows (n=24) spent lying on each side (right vs. left) for a total of 10 days.

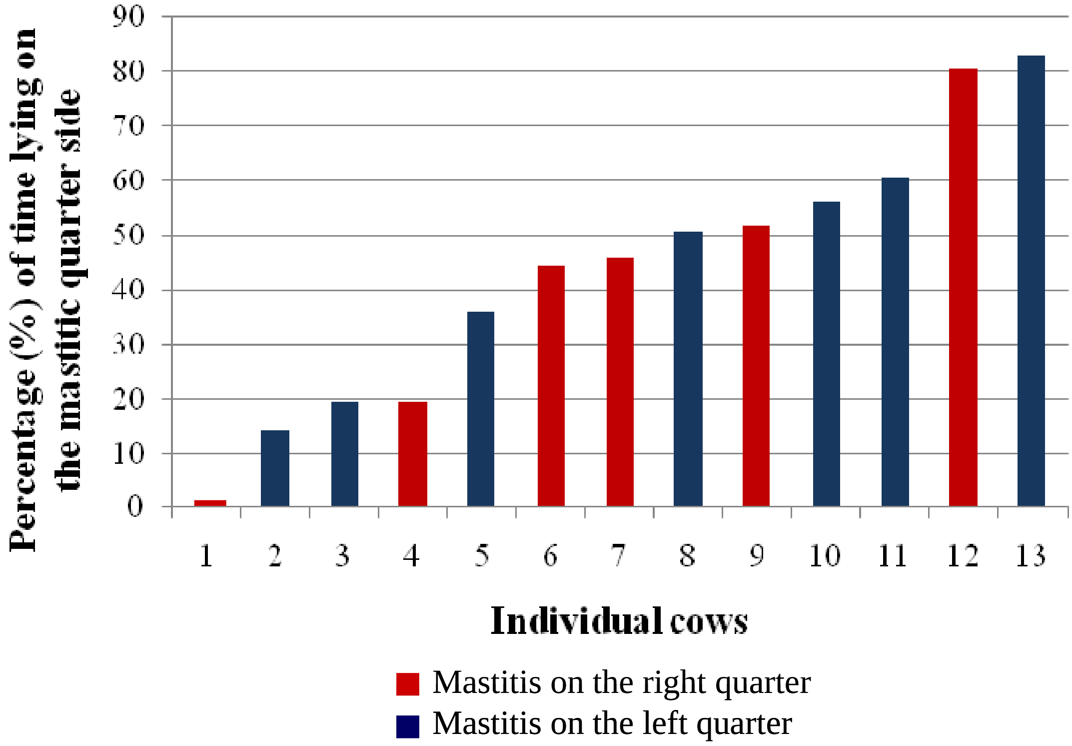

Figure 5. Mean percentage of time that mastitic cows (n=13) spent lying on the mastitic quarter side during D2 and D3.

The time spent lying on the mastitic quarter side and the results of the bacteriological culture for mastitic cows are shown in Figure 6. From the four mastitic cows that spent less than 30% of their time lying on the mastitic side, three of them had no bacteria isolated in their milk samples; and one had *Klebsiella oxytoca*. The two mastitic cows that spent more than 60% of their time lying on the mastitic side had *Enterococcus faecium*.

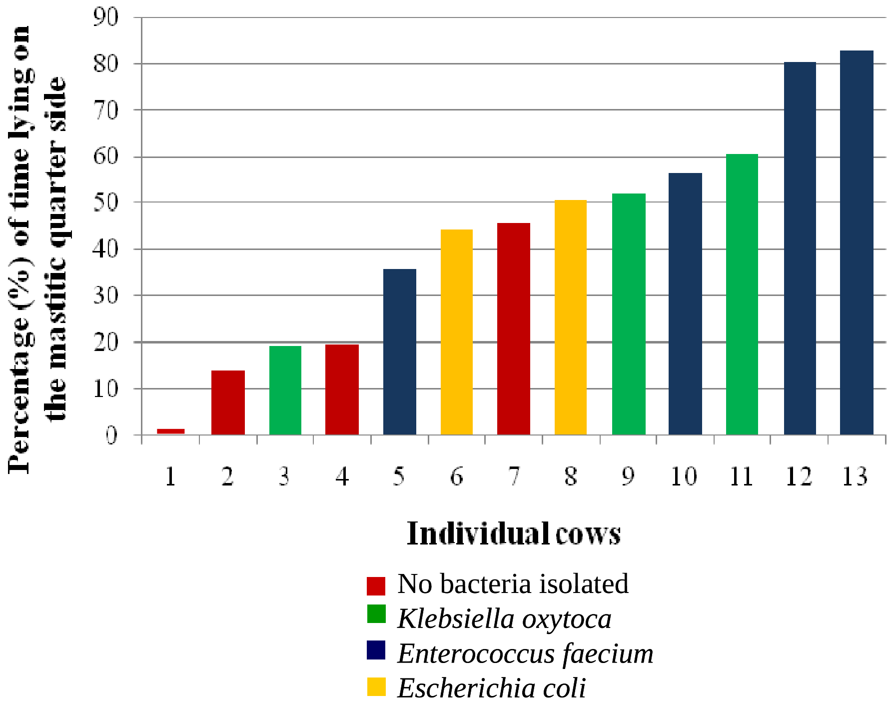

Figure 6. Bacteriology culture and the percentage of time lying on the mastitic side during a 24-h period for D2 and D3 for mastitic cows (n=13).

3.2.2. Behaviours at milking parlour

There were no significant differences ($p > 0.05$) between control and mastitic cows on the frequency of steps, lifts and kicks per minute within days (Table 10). Nonetheless, significant differences were found within mastitic cows across time (Table 11). Mastitic cows had a higher frequency of kicks per minute on D1 compared to D2, a higher frequency of lifts on D1 and D2 compared to D10+. Additionally, mastitic cows had a higher frequency of steps on D2 compared to D10+. Mastitic cows also tended to have a higher frequency of steps on D1 compared to D2, and a higher frequency of lifts on D3 compared to D10+ but they were not significant. There were not significant differences between days for control cows ($p > 0.05$).

Table 10. Differences on the frequency of steps, lifts, kicks per minute during the milking between control and mastitic cows by day.

Frequency of steps per minute						
Day	Control Cows		Mastitic Cows		Diff between C-M	
	n	Median (P25 - P75)	n	Median (P25 - P75)	*S*-value†	*p*-value
D1	14	3.05 (2.48 - 4.39)	14	2.23 (1.46 - 3.61)	-8.5	0.62
D2	14	2.7 (2.24 - 3.57)	14	2.20 (1.96 - 4.42)	18.5	0.26
D3	14	2.54 (2.18 - 2.91)	14	1.80 (1.73 - 2.00)	-17.5	0.29
D10+	14	2.20 (1.88 - 3.81)	13	2.07 (1.15 - 2.44)	-12.5	0.41

Frequency of lifts per minute						
Day	Control Cows		Mastitic Cows		Diff between C-M	
	n	Median (P25 - P75)	n	Median (P25 - P75)	*S*-value†	*p*-value
D1	14	0.51 (0.21 - 1.30)	14	0.70 (0.24 - 1.08)	2.5	0.90
D2	14	0.45 (0.15 - 0.80)	14	0.33 (0.16 - 1.02)	5.5	0.7
D3	14	0.81 (0.22 - 1.15)	14	0.42 (0.05 - 0.75)	-11.5	0.50
D10+	14	0.45 (0.13 - 1.10)	13	0.15 (0.00 - 0.39)	-10.5	0.49

Frequency of kicks per minute						
Day	Control Cows		Mastitic Cows		Diff between C-M	
	n	Median (P25 - P75)	n	Median (P25 - P75)	*S*-value†	*p*-value
D1	14	0.06 (0.00 - 0.26)	14	0.10 (0.00 - 0.13)	-7.5	0.49
D2	14	0.03 (0.00 - 0.18)	14	0.00 (0.00 - 0.07)	-5.5	0.57
D3	14	0.04 (0.00 - 0.21)	14	0.00 (0.00 - 0.07)	-9.5	0.37
D10+	14	0.03 (0.00 - 0.21)	13	0.00 (0.00 - 0.14)	-10.0	0.19

P25= 25th percentile
P75= 75th percentile

† *S*-value = Test statistic for Wilcoxon sign ranked test

Table 11. Differences on the frequency per minute of steps, lifts and kicks between days for mastitic cows.

Treatment	Day difference	Difference in steps' frequency per minute			Difference in lifts' frequency per minute			Difference in kicks' frequency per minute		
		N	*S*-value†	*p*-value	N	*S*-value†	*p*-value	N	*S*-value†	*p*-value
Control Cows	D2 – D1	14	-24.5	0.135	14	-23.5	0.109	14	-13.5	0.12
	D3 – D2	14	-11.5	0.501	14	-1.5	0.940	14	3.5	0.76
	D3 – D1	14	4.5	0.807	14	24.5	0.094	14	9.5	0.37
	D10+ – D1	14	-17.5	0.295	14	-8.5	0.587	14	8.5	0.43
	D10+ – D2	14	4.5	0.807	14	16.5	0.273	14	9.5	0.30
	D10+ – D3	14	-13.5	0.426	14	-2.5	0.862	14	1.5	0.91

Treatment	Day difference	Difference in steps' frequency per minute			Difference in lifts' frequency per minute			Difference in kicks' frequency per minute		
		N	*S*-value†	*p*-value	N	*S*-value†	*p*-value	N	*S*-value†	*p*-value
Mastitic Cows	D2 – D1	14	28.5	0.07	14	-5.5	0.76	14	-22.5	0.01*
	D3 – D2	14	7.5	0.66	14	-5.0	0.73	14	-12.5	0.23
	D3 – D1	14	-19.5	0.24	14	2.5	0.90	14	7.5	0.15
	D10+ – D1	13	-20.5	0.16	13	-32.0	0.01*	13	-3.0	0.74
	D10+ – D2	13	-30.5	0.03*	13	-37.0	0.001*	13	1.0	0.93
	D10+ – D3	13	-15.5	0.30	13	-21.0	0.06	13	-1.0	0.92

P25= 25th percentile
P75= 75th percentile
† *S*-value = Test statistic for Wilcoxon sign ranked test
* Significant difference (p<0.05)

3.2.3. Weight distribution

There were no significant differences between control and mastitic cows on the SD of weight and the percentage of weight applied to the leg on the mastitic quarter side within days (Table 12 and 13). Specifically for the SD of weight, a significant difference for mastitic cows was found between D1 and D10+. Mastitic cows had higher SD of weight on D1 compared to D10+ (mean ± SD: D1, 44.46 ± 14.24 Kg; D10+, 36.44 ± 12.04 Kg) (Table 14). For control cows, the SD of weight on D1 (mean ± SD: 40.31 ± 7.48 Kg) differed significantly from D2 (mean ± SD: 35.82 ± 8.01 Kg) and D3 (mean ± SD: 35.25 ± 9.23 Kg) (Table 14). Between the other days there were no significant differences for both treatments ($p > 0.05$).

For the percentage of weight applied to both hind legs, there were no significant differences ($p > 0.05$) between legs within days for both control and mastitic cows (Figure 7).

Table 12. Differences on the SD of weight (Kg) of the leg on the mastitic quarter side between mastitic and control cows for each day.

Day	SD of weight (Kg) of the leg on the mastitic quarter side					
	Mastitic Cows		Control Cows		Diff between M-C	
	n	Mean ± SD	n	Mean ± SD	*t*-value†	*p*-value
D1	13	44.46 ± 14.24	13	40.31 ± 7.48	1.10	0.29
D2	13	36.14 ± 14.96	13	35.82 ± 8.01	1.23	0.24
D3	13	36.55 ± 17.56	13	35.25 ± 9.23	1.25	0.23
D10+	12	36.44 ± 13.27	13	35.76 ± 12.04	0.23	0.81

C=Control cows
M=Mastitic cows

† *t*-value = Test statistic for Student paired *t* Test

Table 13. Differences on the percentage of weight applied to the leg on the mastitic quarter side between mastitic and control cows for each day.

Day	Percentage of weight applied to the leg on the mastitic quarter side					
	Mastitic Cows		Control Cows		Diff between M-C	
	n	Median (P25 - P75)	n	Median (P25 - P75)	*S*-value†	*p*-value
D1	13	49.72 (45.31 - 52.79)	13	49.66 (47.69 - 52.48)	1.50	0.95
D2	13	54.17 (49.09 - 55.64)	13	49.43 (46.40 - 5258)	11.50	0.45
D3	13	49.94 (45.71 - 52.75)	13	51.27 (47.06 - 54.28)	4.50	0.79
D10+	12	52.62 (50.87 - 57.47)	13	50.03 (48.45 - 53.63)	15.00	0.27

C=Control cows P25= 25th percentile
M=Mastitic cows P75= 75th percentile
† *S*-value = Test statistic for Wilcoxon sign ranked test

Table 14. Differences on the SD of weight (Kg) of the leg on the mastitic quarter side between days for both mastitic and control cows.

Treatment	Mastitic Cows				Control Cows			
Differences between days	n	Mean ± SD	*t*-value†	*p*-value	n	Mean ± SD	*t*-value†	*p*-value
D2 – D1	13	-4.79 ± 12.95	-1.34	0.20	13	-6.08 ± 8.11	-2.70	0.01*
D3 – D2	13	-2.21 ± 9.25	-0.86	0.40	13	-2.80 ± 9.14	-1.10	0.29
D3 – D1	13	-7.01 ± 13.15	-1.92	0.07	13	-8.88 ± 9.13	-3.50	0.004*
D10+ – D1	12	-9.11 ± 12.98	-2.43	0.03*	13	-4.55±9.28	-1.76	0.10
D10+ – D2	12	-2.91 ± 10.63	-0.95	0.36	13	1.52 ± 13.56	0.40	0.69
D10+ – D3	12	-2.15 ± 10.97	-0.68	0.51	13	4.33 ± 11.11	1.40	0.18

* Significant difference ($p<0.05$) † *t*-value = Test statistic for Student paired t Test

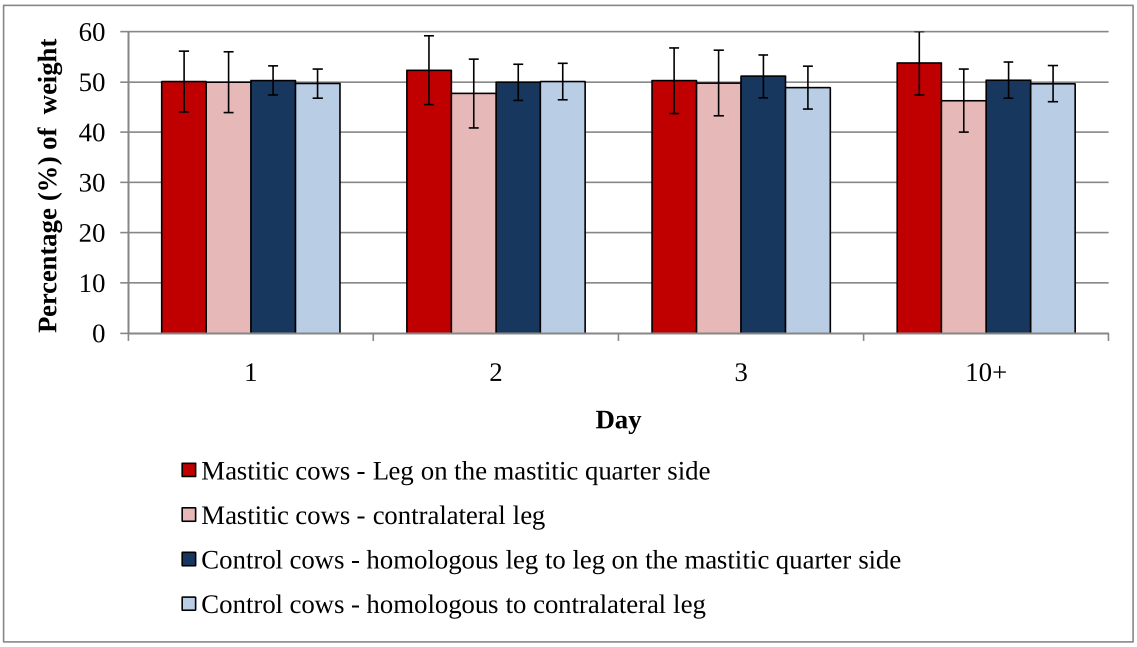

Figure 7. Mean ± SD of the percentage of the percentage of weight applied on both hind legs across time for control and mastitic cows (no significant differences at each day).

3.2.4. Hock-to hock distance

There were no significant differences ($p > 0.05$) in the hock-to-hock distance between control and mastitic cows across time (Table 15). Similarly, there were no significant differences between days for each treatment in the hock-to-hock distance ($p > 0.05$).

Table 15. Differences on the hock-to-hock distance between control and mastitic cows by days, and between days within control and mastitic cows.

Variable		Difference of hock-to-hock distance					
Treatment		Control Cows		Mastitic Cows		Diff between C-M	
		n	Median (P25 - P75)	n	Median (P25 - P75)	*S*-value†	*p*-value
Day	D1	13	29.7 (28.0 - 31.1)	13	27.0 (23.0 - 32.5)	8.50	0.57
	D2	13	29.7 (27.6 - 31.8)	13	27.0 (22.4 - 32.5)	17.00	0.25
	D3	13	30.0 (27.5 - 31.3)	13	26.0 (24.0 - 31.0)	14.00	0.34
	D10+	13	28.5 (27.0 - 38.8)	12	26.0 (23.9 - 31.5)	8.00	0.55
		S-value†	*p*-value	*S*-value†	*p*-value		
Diff between days	D2 – D1	7.5	0.62	-8.5	0.45		
	D3 – D2	3.0	0.81	-4.0	0.80		
	D3 – D1	-2.0	0.91	4.0	0.80		
	D10+ – D1	-15.0	0.31	3.0	0.84		
	D10+ – D2	-20.0	0.12	12.0	0.37		
	D10+ – D3	-21.5	0.15	11.0	0.35		

M=Mastitic cows

C=Control cows

† *S*-value = Test statistic for Wilcoxon sign ranked test

4. Discussion

4.1. Effect of IceTags on cows' lying behaviour

Monitoring behaviours of dairy cows is an important part of welfare and productivity assessment on farms (Trénel et al, 2009). The majority of the research addressed to validate data loggers for behavioural monitoring has been focused on accuracy based on comparisons with data from analysis of video recordings or direct observation (Trénel et al, 2009; Ledgerwood et al, 2010). Nowadays, new data loggers are been developed by the industry. However, there is no previous research on the possible effects that data loggers might have on cows' lying behaviour. Using the newest model of IceTag data loggers, this study showed that the medial or lateral attachment of IceTags on one or both hind legs did not affect the cows' lying behaviour. Cows did not change their lying side preference after the attachment of the devices. These results contradict the anecdotal evidence reported by Grubmüeler et al. (personal communication cited by Tucker et al, 2009) who found that cows spent less time lying on the side corresponding to the leg where the data logger was attached (40% on side with logger, 60% on side without, compared with 50-50% in cows without logger). From this study, we can conclude that after attaching IceTags and starting behavioural recording 14 hours after attachment, the devices provide reliable data without affecting lying behaviour; therefore a 14 hours habituation period is sufficient to allow the animal to get used to hold the device. An additional study might be carried out if effects of IceTags during these first 14 hours want to be known. This experiment is a pioneer study in the area that encourages continuing evaluating whether data loggers affect cows' behaviour such as walking and stepping.

4.2. Methodologies to evaluate pain in dairy cows with mastitis

This study described a behavioural based methodology to evaluate pain in dairy cows with naturally occurring clinical mastitis that provided valuable information but also showed limitations.

The evaluation of lying behaviour and variation of lying laterality are important measures of cows' welfare and comfort (Forsberg et al, 2008; O'Driscoll et al, 2008). Mastitic cows spent less time lying on the day after mastitis detection compared to control cows. The same tendency, but not significant, was observed for the following day and 7 days after antibiotic treatment. The decreased lying time compared with healthy cows may be because of pain or discomfort experience by mastitic cows felt when lying down due to the compression of the udder against the stall/cubicle base, opting then for standing up. Similarly, Siinoven and collaborators (2011) found that cows with experimental-induced mastitis tended ($p < 0.07$) to spend less time lying on the induction day than the control day (one day before induction). They suggested that mastitic cows may have reduced motivation for lying than healthy cows due to the discomfort or pain experienced in the udder and therefore reluctance to lie on the udder. The present study found that within mastitic cows there were no significant differences between each day on lying time, including the baseline day (D10+). The explanation for this might be related to the avoidance-learning process that pain experience involves, where pain acts as a reinforcer in learning to avoid future risk of damage (Broom, 2001). Learning to avoid negative experiences such as fear and pain have been tested in calves and cows subjected to aversive handling procedures, finding that calves and cows are able to discriminate between negative and positive situations after having experienced them (de Passillé et al, 1996;

Pajor et al, 2000 respectively). In the present study, mastitic cows might have learned that pain or discomfort in the udder was alleviated when they avoided lying down during the first days of mastitis infection; therefore, they preferred to continue lying down less to avoid feeling pain.

Previous reports in literature have suggested that pronounced changes in laterality of lying may indicate that cows are uncomfortable, becoming a suitable indicator of cows' welfare (Ledgerwood et al, 2010). In our study, it was found that although there were no significant differences on the percentage of time lying on the mastitic side on D2, D3 and D10+ between and within control and mastitic cows, laterality of lying had a high variability between individuals. This study reports approximately 53:47 % of time lying on left and right for control cows which is in agreement with the literature (Forsberg et al, 2008; Tucker et al, 2009; Ledgerwood et al, 2010). However, on consideration of individual cows and not the average, both control and mastitic cows showed a marked lying side preference. For control cows, both right and left side preference's range was between 30 to 70% approximately. Mastitic cows were more extreme in their lying side preferences than any control cows, where 4 mastitic cows were under the minimum and 2 were over the maximum value for control cows. Although the average shows no side preference, each individual is unique and different. To be able to use laterality of lying as an indicator of pain due to mastitis, the individual's lying side preferences must be first known by the farmer or the scientists to be able to conclude whether a certain cow is feeling pain or discomfort based on changes in her preference.

Restlessness behaviours have been used to evaluate stress during milking that may affects cows' welfare (Rushen et al, 2001). In the present study, a higher frequency of kicking, lifting and stepping in milking during the first three days after mastitis detection might be explained by the pain and discomfort experienced by the cow due to mastitis. Usually, the increase of cows' movements is associated with agitation during a stressful situation (Grandin, 1993, 1997). The findings of the present study are consistent with findings of other studies that have evaluated restless behaviours in cows. Although they did not evaluate effects of pain associated with mastitis, they did show that cows increased their reactivity when experiencing discomfort or fear. For instance, Chapinal et al. (2011) found that cows had an increase in the frequency of steps over one hour of forced standing. Furthermore, Peters and collaborators (2010) found that cows subjected to aversive handling before going to the milking were more reactive in the milking parlour. Additionally, Gygax at al. (2008) found that cows with high SCC had a higher rate of stepping during milking. This finding might explain the higher frequency of restless behaviour in our mastitic cows during the first three days after mastitis detection where it is expected that cows will have higher SCC. However, during the first three days after mastitis detection, mastitic cows received an intra-mammary infusion of antibiotic twice a day. It is unknown what side effects this antibiotic and the manipulation of the udder during the infusion might have on the cows. It is possible that it may cause mild irritation in the udder and consequent discomfort that might be expressed by the cows during the direct manipulation of the udder while they are milking.

Mastitic cows had a higher variability in the weight over time that they applied to the leg on the mastitic side (a measure of weight shifting) when mastitis was detected compared when healthy on D10+. This result might confirm the formulated hypothesis (mastitic cows will shift more weight) based on the findings of Rushen et al. (2007) where lame cows had a much higher variation of weight on both the injured and the contralateral leg than healthy cows. Similarly, Neveux and collaborators (2006) showed that the variability in weight distribution increased when cows were standing on uncomfortable surfaces. It can be suggested that mastitic cows had a higher variability on weight on D1 due to the inflammation and pain felt in the affected quarter, which started to decrease slightly due to the effect of the local antibiotics that cows received on D1. However, in this study, control cows also showed a variation on weight between days, being higher for D1 comparing with the two following days. These differences within healthy cows and within mastitic cows specifically for D1 that decreased over time might also suggest that the variation on weight may not be due to pain or discomfort (in mastitic cows) but for a novel environment effect. Cows in this study did not have a familiarization period with the scale conversely to other studies in weight distribution, where cows had always familiarization periods with the scale (novel object) by standing on it 4 times/day for at least 4 days before the recordings started (Chapinal et al, 2009b; Chapinal et al, 2010a; Chapinal et al, 2010b; Pastell et al, 2010). The reason for no implementation of familiarization period in this experiment was because the experiment depended on when a cow was diagnosed with mastitis so the identification of the cow and her detection day where unknown until changes in milk appearance were detected. The implications of these findings for future on-farm applications of the force plate scale for evaluation of pain due to mastitis but also for lameness detection are that either the entire herd needs to be familiarized with the scale or simply the scale needs to be located in a familiar environment for the cows, where they do not notice any novelty that can cause stress and anxiety such as the milking parlour or ideally in an automatic milking system.

The hock-to-hock distance is a measure of hind leg stance, which has been reported to be wider in mastitic cows due to the inflammation during the beginning of the disease (Kemp et al., 2008). However, in our study the hock-to-hock distance did not differ between control and mastitic cows; and the distance did not present the expected reduction at the end of the trial, on D10+, when the cows were healthy. The implementation of this measure for assessing mastitis severity and indirectly pain as has been suggested by Kemp et al. (2008) might be useful for moderate-severe mastitis when changes in udder appearance are evident, but not for mild mastitis where only changes in milk appearance are evident which has been the case for the animals recruited in our experiment.

The results of bacteriological culture of 13 mastitic cows' milk samples showed the isolation of different environmental pathogens, being consistent with the results of other studies, where environmental pathogens were the most common isolated microorganisms (Sargeant et al., 1998; Olde Riekerink et al., 2008). The isolation of *Enterococcus faecium* was expected based on the results of Zdanowicz and collaborators (2004), where Streptococci were more commonly isolated from teats of sand housed cows compared to sawdust housed cows. Although the isolation of *Escherichia coli* and *Klebsiella oxytoca* (coliforms) has been highly related to sawdust bedding (Zdanowicz et al., 2004), they were isolated in two and three cows respectively. Four cases of no bacteria grown were obtained; being consistent with other studies where culture-negative milk samples represented a large part of the culture results of clinical mastitic cows (Sargeant et al., 1998; Olde Riekerink et al., 2008). Interestingly, a great proportion of culture-negative clinical mastitis cases have been attributed to *Escherichia coli* (Schukken et al., 1988; Zorah et al., 1993; Olde Riekerink et al., 2008). This suggests that these four cases could be caused by *Escherichia coli*. Although coliforms are catalogued as the major cause of disease and severe clinical mastitis in cows (Smith, 2009), in our study, mastitic cows with a positive result to coliforms in milk culture did not have physical signs of udder inflammation or systemic illness. However, three of the mastitic cows with no bacteria isolation and one with

Klebsiella oxytoca were the cows with the lowest extreme values for percentage of time lying on the mastitic side; the two cows with the highest extreme values correspond to cows with *Enterococcus*.

The results of this study were limited by the type of mastitis that the recruited cows had (mild mastitis). Mild mastitis may not have been painful enough to cause changes in behaviour. However, pain sensation cannot be denied, and based on the findings it can be affirmed that the cows were at least in a level of discomfort that affected their lying behaviour and reactivity during milking. As a consequence, the methodologies proposed in this study need to be re-evaluated using cows with moderate and severe mastitis, where behavioural differences due to pain compared to healthy cows might be found. If the scale for weight distribution and laterality of lying are included in these methodologies, cows need to be familiarised with the scale, and side preferences must be known before starting the evaluation.

5. Conclusion

Using the methodologies proposed in this study, cows with mild clinical mastitis showed basic differences in lying time and reactivity during milking compared to healthy cows. Furthermore, they showed some slight differences in laterality of lying and weight distribution; however, these responses cannot be interpreted as pain-specific responses due to the limitations that these behavioural measures presented in this study related to the lack of knowledge about previous lying side preferences of the cows and familiarisation period with the scale. To continue the development of methodologies for assessing pain associated with mastitis, it is worth applying the behavioural measures used in this study in cows with moderate-severe mastitis. However, to implement weight distribution and laterality of lying as measures for pain assessment, the scale must be placed in a familiar environment for the herd and previous records about lying side preferences of the cows in healthy conditions need to be carried out. Following this, validation of the methodologies using analgesic treatment to confirm that the responses are specific-pain behaviours is needed.

Acknowledgments

The development and the culmination of this dissertation have been one of the most important challenges that I have had to face throughout my life. However, this has been a challenge that I have not faced alone; different people in one way or another helped me to reach my goals with their knowledge, support, guidance, patience and love.

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General Discussion of Results and Criticisms of Methodology

The initial project proposed for my dissertation was ‘Effects of analgesics on pain due to mastitis in cows’ that had the objective of evaluating the impact that pain and its alleviation using analgesic treatment have on cows with mastitis based on changes in behaviour, body weight distribution and nociceptive thresholds. This project was divided into two stages. The objective of the first stage was to compare pain responses between control and mastitic cows. In the second stage, mastitic cows without analgesic treatment and mastitic cows with analgesic treatment were going to be compared. However, due to lack of time it was possible only to reach part of the first stage, and I said ‘part’ because the project was developed in one farm (University of British Columbia’s Dairy Education and Research Centre) where the type of mastitis that they had was mild clinical mastitis, and cows just had changes in milk appearance but signs of evident udder inflammation and systemic signs were not common. Therefore, after this initial step of looking at changes in behaviour due to pain in the less severe clinical mastitis, the methodologies proposed in this study would need to be evaluated in other farms where moderate and severe cases of clinical mastitis are more common, before continuing with the second stage of this project.

One of the major limitations that the project had was the time regarding mastitis detection and the starting of behavioural recordings. Because the project used natural mastitis, control over the occurrence of each mastitic case was not feasible; therefore, to record behaviours from the beginning of the disease and ideally before, to be able to have a better baseline data, was not possible. An easy solution for this would be to experimentally induce mastitis to the cows. However, this would not fulfil the ‘Three R’s’, specifically the Third R “Refinement”, that are part of the guidelines for research with animals, because if there is a possibility to do the same research avoiding inflicting disease and unnecessary pain to the animals, natural mastitis occurrence is the correct choice. Besides, outcomes from research done with real conditions are more applicable in farm for future improvements of cows' welfare.

Another limitation of the project was that mastitic cows received a treatment that control cows did not, the antibiotic treatment as part of the mastitis therapy established on the farm. The issue with this is that it is unknown the possible effects that this intra-mammary injection could have on mastitic cows' behaviours, therefore we could not ensure that the changes in behaviour (mainly in lying behaviour and restless behaviours during milking) found in the study are related to pain or to the possible irritation that the drug produced. As a consequence, the next step is to first ensure that there is no effect of the antibiotic on cows' behaviours. Currently, this small project is started to be planned.

The evaluation of mechanical nociceptive thresholds using an algometer was part of the methodologies to be developed in this study because it has shown success measuring pain sensation related to dehorning in calves. However, this technique specifically for assessment pain in mastitis is still unclear, although is being worked by other research teams. Consequently, the development of the methodology took a lot of time (and still needs improvements and re-evaluation) because simple questions such as which location on the udder to apply the pressure were unknown. To improve the use of the algometer as a possible tool to measure pain in mastitic cows, first, it needs to be tested in healthy cows to ensure consistency in the applied pressure and in the response of the animals but also to test possible handler effects.

Appendices

Appendix 1. Force Plate Scale

The body weight distribution of cows was measured using a scale (Pacific Industrial Scale Co. Ltd., Richmond, British Columbia, Canada) that was composed by 4 independent recording units (12 cm high x 59 cm wide x 99 cm long). The units were covered with textured rubber mats (1.5 cm thick). The scale was fitted in a 1.9 m x 1.3 m enclosure. The recorded weights, corresponding to vertical load applied by the cow, were automatically transmitted via electrical signal to a computer at a rate of 14 readings/sec. Cow Weight Software version 2.2 (Pacific Industrial Scale Co. Ltd) was used to provide a real-time display of the weight applied to each of the 4 units. The data were automatically exported to Microsoft Excel® (Microsoft Corporation). The scale was calibrated periodically during the study and tested for weight homogeneity and accuracy between the four plates before being used.

Appendix 2. Mechanical Nociceptive Thresholds: Algometer Test

The evaluation of mechanical nociceptive thresholds in the cows was evaluated by applying pressure on the udder using an algometer with a pain threshold capacity of 50 lbs (FDX Force Ten Algometer™, Wagner Instruments, Greenwich, CT, USA). The algometer was set up in Kgf as a unit of force, and it was programmed to record using the Peak Mode – Run Compression Test.

This procedure was carried out while the cows were standing on a force plate scale. Pressure was applied on the quarters to be evaluated depending on the experiment on mastitic and control cows. Each quarter was divided in three areas: top, middle and bottom (Figure a). Pressure was applied twice per area during 5 seconds or less if the cow displayed/showed avoidance reaction to the stimulus. The avoidance reaction was defined as a vigorous tail swishing, shifting of weight, stepping, lifting or kicking. As soon as the cow performed any of the avoidance reactions or the five seconds ended, the pressure was immediately stopped and the value of pressure applied and the absence or presence of response recorded.

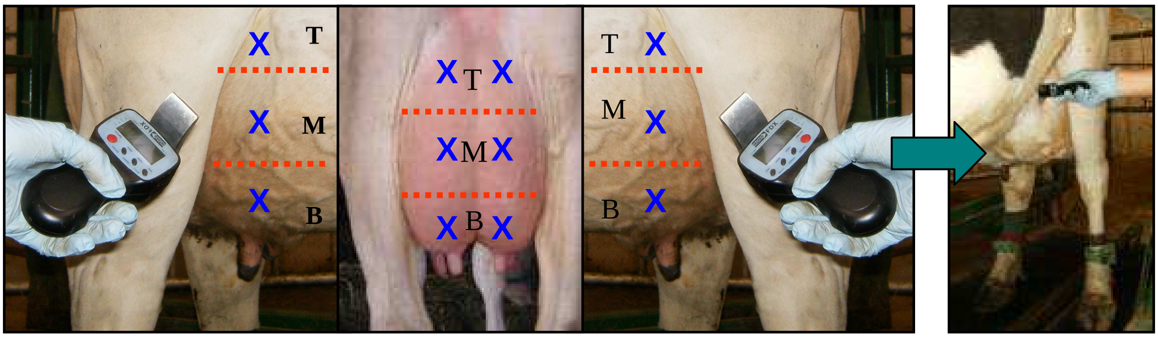


Figure a. Areas per quarter for pressure application

T=top; M=middle; B=bottom.

Appendix 3. Milk Sampling for Bacteriological Culture

Milk sampling was carried out following the procedure established by the University of British Columbia's Dairy Education and Research Centre. The procedure was done before cows were milked and is described below:

1. Two or three strips of milk were pulled out to flush contaminating bacteria out of the teat end.
2. The teat was pre-dipped with 0.5% Iodine Solution during 30 seconds.
3. The teat and teat end was wiped dip off with a clean paper towel.
4. The end of teat was cleaned using alcohol swabs until the swabs come back clean.
5. Without touching the prepared teat end, the sterile sample tube was opened and two or three strips of milk (the first must be discarded) were collected into the tube on an angle of 45°.
6. The sample was frozen (-20°C) until they were sent to the commercial laboratory to be cultured and analyzed.

Appendix 4A. Timeline for Procedures Based on Mastitis Time Detection: Morning Milking

This time line is an example using data from one of the mastitic cows that was in the trial. The cow was detected on the 17th of April during morning (AM) milking. Her last antibiotic treatment day was on the 20th of April on the morning milking; therefore D10+ started on the 27th at the morning milking (see Diagram 1).

Appendix 4B. Timeline for Procedures Based on Mastitis Time Detection: Afternoon Milking

This time line is an example using data from one of the mastitic cows that was in the trial. The cow was detected on the 15th of April during afternoon (PM) milking. Her last antibiotic treatment day was on the 18th of April in the am milking; therefore D10+ started on the 24th of April at afternoon milking (see Diagram 2).

Diagram 1. Morning (AM) detection

Day	Date	Time	Procedure
D1	17.04.2011	08:00 h	**AM milking** average detection time Beginning of day 1 Milk sampling for bacteriology culture from mastitic cows
		10:00-14:00 h	Weight distribution Algometer test Hock-to-hock distance Attachment of Ice Tags and Hobos
		15:00-19:00 h	**PM milking** Milking sampling for SCC from control cows Behavioural video recording of mastitic and control cows
		07:59 h	End of day 1
D2	18.04.2011	08:00 h	Beginning of day 2
		10:00-14:00 h	Weight distribution Algometer test Hock-to-hock distance
		15:00-19:00 h	**PM milking** Behavioural video recording of mastitic and control cows
		07:59 h	End of day 2
D3	19.04.2011	08:00 h	Beginning day 3
		10:00-14:00 h	Weight distribution Algometer test Hock-to-hock distance
		15:00-19:00 h	**PM milking** Behavioural video recording of mastitic and control cows
	20.04.2011	17:59 h	End of day 3
D10+	27.04.2011	08:00 h	Beginning day 10+
		10:00-14:00 h	Weight distribution Algometer test Hock-to-hock distance
		15:00-19:00 h	**PM milking** Milk samples for SCC from mastitic and control cows Behavioural video recording of mastitic and control cows
	28.04.2011	07:59 h	End day 10+
D10+ + 1		>08:00 h	Remove Ice Tag and Hobos

Day 10 = 7 days after last antibiotic treatment

Day 10+ + 1= day after day 10+

Diagram 2. Afternoon (PM) detection

Day	Date	Time	Procedure
D1	15.04.2011	18:00 h	**PM milking** average detection time Beginning of day 1 Milk sampling for bacteriology culture from mastitic cows
	16.04.2011	5:00-09:00 h	**AM milking** Milking sampling for SCC from control cows Behavioural video recording of mastitic and control cows Attachment of Ice Tags and Hobos
		10:00-14:00 h	Weight distribution Algometer test Hock-to-hock distance
		17:59 h	End of day 1
D2		18:00 h	Beginning of day 2
	17.04.2011	5:00-09:00 h	**AM milking** Behavioural video recording of mastitic and control cows
		10:00-14:00 h	Weight distribution Algometer test Hock-to-hock distance
		17:59 h	End of day 2
D3		18:00 h	Beginning day 3
	18.04.2011	5:00-09:00 h	**AM milking** Behavioural video recording of mastitic and control cows
		10:00-14:00 h	Weight distribution Algometer test Hock-to-hock distance
		17:59 h	End of day 3
D10+	24.04.2011	18:00 h	Beginning day 10+
	25.04.2011	5:00-09:00 h	**AM milking** Milk samples for SCC from mastitic and control cows Behavioural video recording of mastitic and control cows
		10:00-14:00 h	Weight distribution Algometer test Hock-to-hock distance
		17:59 h	End day 10+
D10+ + 1		>18:00 h	Remove Ice Tag and Hobos

D10+ = 7 days after last antibiotic treatment

D10+ + 1= day after day 10+

Appendix 5. Complementary results of Experiment 1: IceTag Trial

There were no significant differences ($p > 0.05$) in total lying time, number of lying bouts and average duration of lying bouts between groups (medial and lateral IceTag attachment). No significant differences were also found between any of the treatments we tested. Whether the IceTag was attached on the left or on the right leg, or to either both legs or none of them. There were no significant differences when comparing each of the treatments between medial and lateral groups (Table A).

There were no significant differences ($p > 0.05$) between group, treatment within group and group within treatment on total lying time, lying bouts and average duration of lying bouts on the left side (Table B). Similarly, there were no significant differences ($p > 0.05$) between group, treatment within group and group within treatment on total lying time, lying bouts and average duration of lying bouts on the right side (Table C).

Table A. Effect of group and IceTags treatments on total lying behaviour.

Total Lying Time (min/24 h)		Group Medial (Median P25-P75)	Group Lateral (Median P25-P75)	χ^{2*}	*p*-value
	Total	784.7 (735.4-805.05)	763.5 (755.1-814.9)	0.01	0.91
Treatments	C	781 (746.8-819.5)	778.3 (743.6-818.2)	0.12	0.73
Treatments	L	786 (776.3-813)	796.6 (760.5-812.1)	0.03	0.86
Treatments	R	761.8 (710.2-810.3)	765.2 (752-819.2)	0.15	0.70
Treatments	B	795.2 (698.6-813)	758.2 (720.3-810.2)	0.01	0.91
	χ^{2*}	1.26	0.74		
	p-value	0.74	0.86		

Lying bouts (bouts/24 h)		Group Medial (Median P25-P75)	Group Lateral (Median P25-P75)	χ^{2*}	*p*-value
	Total	9.6 (8.6-11)	10.5 (9.6-11.5)	1.62	0.20
Treatments	C	9.3 (8.8-10.8)	10.8 (9.4-12.3)	2.11	0.15
Treatments	L	10.8 (8.5-11.5)	10.5 (9.5-10.9)	0.00	1.00
Treatments	R	10.3 (9.1-11)	10.08 (9.6-11.7)	0.42	0.52
Treatments	B	9 (8.6-11.5)	10.7 (9.8-1)	1.95	0.16
	χ^{2*}	0.40	0.41		
	p-value	0.94	0.94		

Average duration of lying bouts (min/bout)		Group Medial (Median P25-P75)	Group Lateral (Median P25-P75)	χ^{2*}	*p*-value
	Total	82.4 (72.05-90.5)	72.6 (62.6-83.4)	1.08	0.30
Treatments	C	88.3 (74.7-90.5)	72.2 (64.4-83.9)	1.93	0.16
Treatments	L	79.99 (69.4-91.1)	74.05 (65.4-83.4)	0.66	0.42
Treatments	R	74.3 (72.05-86.4)	72.02 (62.4-83.6)	0.82	0.37
Treatments	B	81.8 (69-92.7)	70.5 (61.6-81-5)	1.08	0.30
	χ^{2*}	0.79	0.38		
	p-value	0.85	0.94		

Treatments: C=No IceTag; L= IceTag on the left hind leg; R=IceTag on the right hind leg; B=IceTag on both hind legs.
P25= 25th percentile.
P75= 75th percentile.
* χ^2: Test statistic for Kruskal-Wallis Test.

Table B. Effect of group and IceTags treatments on lying on left side.

Total Lying Time (min/24 h)		Group Medial (Median P25-P75)	Group Lateral (Median P25-P75)	χ^{2*}	*p*-value
	Total	399.8 (381.9-436.4)	406.6 (368.08-426.3)	0.01	0.91
Treatments	C	383.3 (365.3-432.3)	403.4 (352.4-437.8)	0.21	0.64
Treatments	L	424.3 (389.8-432.1)	410.7 (356.1-445.9)	0.12	0.73
Treatments	R	387.7 (384.6-397.5)	385.3 (357.7-449.5)	0.02	0.90
Treatments	B	394.8 (376.8-461.8)	400.5 (376.7-417.2)	0.12	0.73
	χ^{2*}	2.78	0.29		
	p-value	0.43	0.96		

Lying bouts (bouts/24 h)		Group Medial (Median P25-P75)	Group Lateral (Median P25-P75)	χ^{2*}	*p*-value
	Total	6.4 (5.5-7.6)	6.2 (5.1-8.2)	0.01	0.91
Treatments	C	5.8 (5.1-9.6)	6 (4.8-9)	0.34	0.53
Treatments	L	7.1 (5.1-9.6)	5.5 (5.3-9.8)	0.01	0.91
Treatments	R	5.9 (5.3-6.8)	5.7 (4.5-7.8)	0.07	0.80
Treatments	B	5.8 (4.3-7.1)	6 (5.4-7.4)	0.03	0.86
	χ^{2*}	1.62	0.69		
	p-value	0.65	0.88		

Duration of lying bouts (min/bout)		Group Medial (Median P25-P75)	Group Lateral (Median P25-P75)	χ^{2*}	*p*-value
	Total	65.6 (59.8-77.7)	64.8 (53.01-83.6)	0.12	0.73
Treatments	C	67.6 (63.6-81.3)	67.9 (49.5-76.2)	0.48	0.49
Treatments	L	59.6 (44.2-88.6)	70.4 (45.2-84.2)	0.12	0.73
Treatments	R	66.2 (61.7-70.3)	71.4 (56.1-95.6)	0.02	0.90
Treatments	B	73.5 (49.2-80.5)	72.1 (55.2-76.09)	0.05	0.82
	χ^{2*}	0.73	0.46		
	p-value	0.87	0.93		

Treatments: C=No IceTag; L= IceTag on the left hind leg; R=IceTag on the right hind leg; B=IceTag on both hind legs.
P25= 25th percentile.
P75= 75th percentile.
* χ^2: Test statistic for Kruskal-Wallis Test.

Table C. Effect of group and IceTags treatments on lying on right side.

Total Lying Time (min/24 h)					
		Group Medial (Median P25-P75)	Group Lateral (Median P25-P75)	χ^{2*}	*p*-value
	Total	384.9 (336.2-424.5)	373.8 (343.4-408.4)	0.05	0.82
Treatments	C	415.6 (363.5-440)	368.8 (315.9-411.1)	0.12	0.73
	L	361.6 (289.1-422)	378.1 (368.1-399.1)	0.05	0.82
	R	375.2 (323.1-415.5)	380.3 (332.2-417.7)	0.02	0.90
	B	351.1 (315.6-428)	373.3 (345.7-410)	0.21	0.64
	χ^{2*}	1.51	0.39		
	p-value	0.68	0.94		

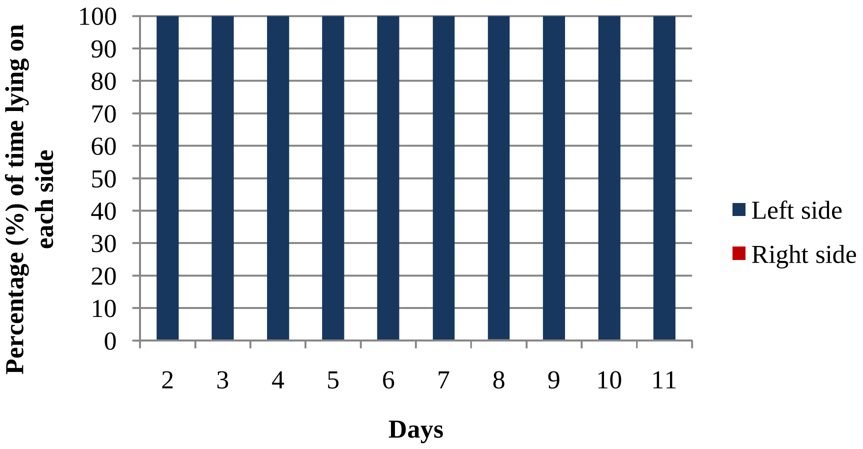
Lying bouts (bouts/24 h)					
		Group Medial (Median P25-P75)	Group Lateral (Median P25-P75)	χ^{2*}	*p*-value
	Total	6.2 (5.4-6.6)	6.08 (5.2-7.5)	0.12	0.73
Treatments	C	5 (4.6-5.8)	5.91 (5.3-7.3)	2.28	0.13
	L	6.6 (6.6-6.8)	5.66 (5.3-8)	0.09	0.77
	R	5.8 (5.5-6.1)	6.2 (5.2-7)	0.27	0.60
	B	6 (5-6.5)	5.5 (4.9-8)	0.17	0.68
	χ^{2*}	6.84	0.23		
	p-value	0.08	0.97		

Duration of lying bouts (min/bout)					
		Group Medial (Median P25-P75)	Group Lateral (Median P25-P75)	χ^{2*}	*p*-value
	Total	67.7 (56.6-74.1)	66.5 (51.8-75.9)	0.00	1.00
Treatments	C	80.6 (65.3-88.09)	65.5 (59.01-75.3)	3.01	0.08
	L	58.8 (46.6-67.6)	38.8 (45.9-78.2)	0.21	0.64
	R	64.7 (57.8-74.6)	61.5 (56.4-74.6)	0.00	1.00
	B	60.08 (51.05-80.3)	64.8 (50.5-75.9)	0.01	0.91
	χ^{2*}	5.93	0.14		
	p-value	0.12	0.99		

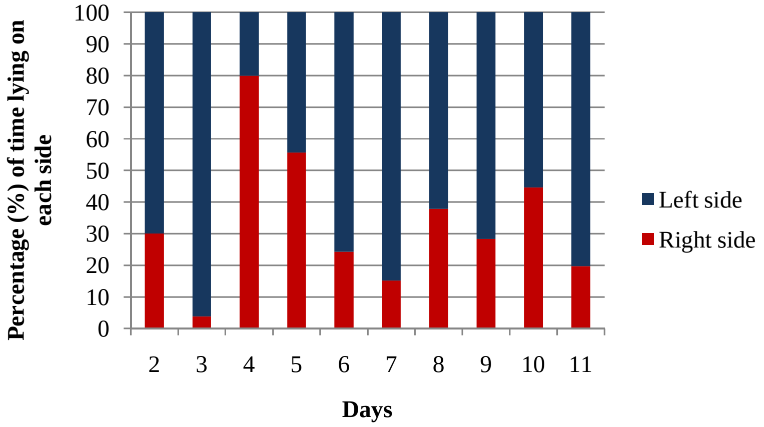
Treatments: C=No IceTag; L= IceTag on the left hind leg; R=IceTag on the right hind leg; B=IceTag on both hind legs.
P25= 25th percentile.
P75= 75th percentile.
* χ^2: Test statistic for Kruskal-Wallis Test.

Appendix 6. Laterality of lying for each individual mastitic cow

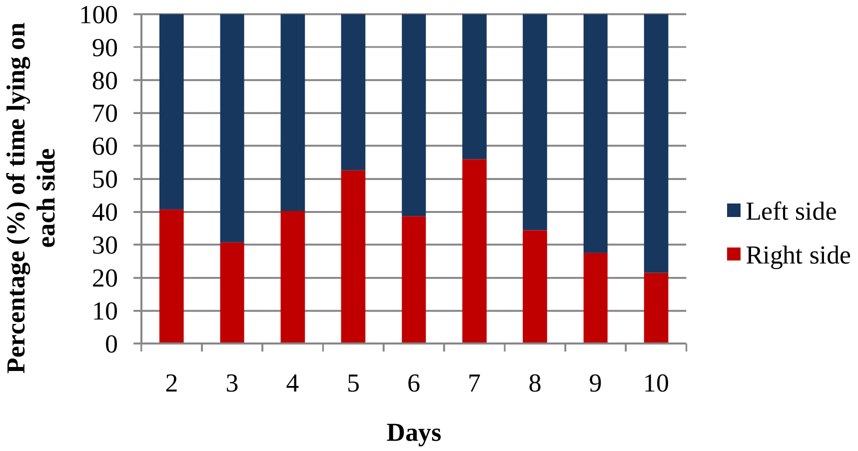
Mean percentage of time lying on each side (left *vs.* right) in a 24 hour period starting on the day after mastitis detection (D2) and finishing on D10+ (in this case day 11) of cow 9029 (mastitis on the right).



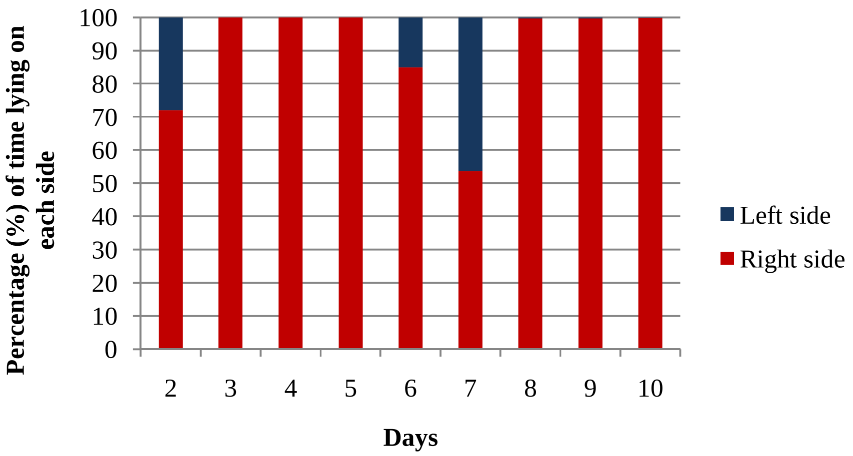
Mean percentage of time lying on each side (left *vs.* right) in a 24 hour period starting on the day after mastitis detection (D2) and finishing on D10+ (in this case day 11) of cow 7118 (mastitis on the left).



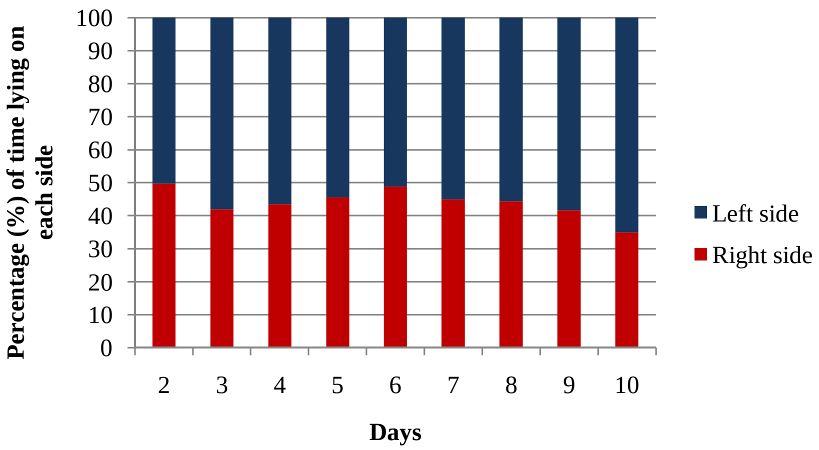
Mean percentage of time lying on each side (left *vs.* right) in a 24 hour period starting on the day after mastitis detection (D2) and finishing on D10+ (in this case day 10) of cow 5014 (mastitis on the right, this cow was still with mastitis on D10+).



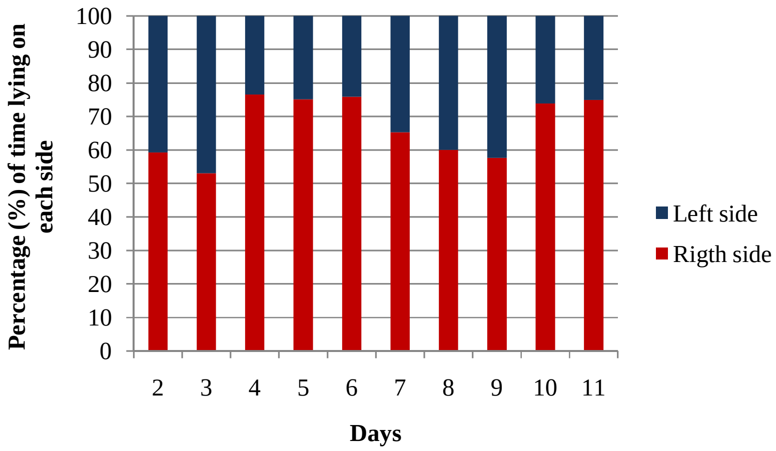
Mean percentage of time lying on each side (left *vs.* right) in a 24 hour period starting on the day after mastitis detection (D2) and finishing on D10+ (in this case day 10) of cow 9957 (mastitis on the left).



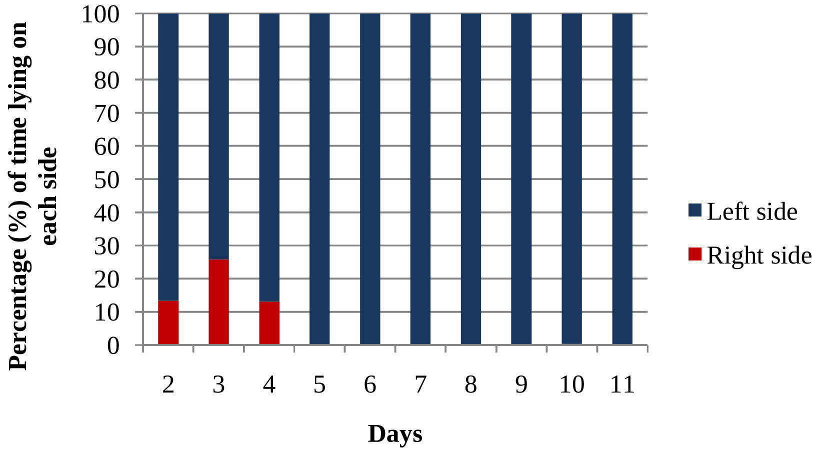
Mean percentage of time lying on each side (left *vs.* right) in a 24 hour period starting on the day after mastitis detection (D2) and finishing on D10+ (in this case day 10) of cow 5053 (mastitis on the right).



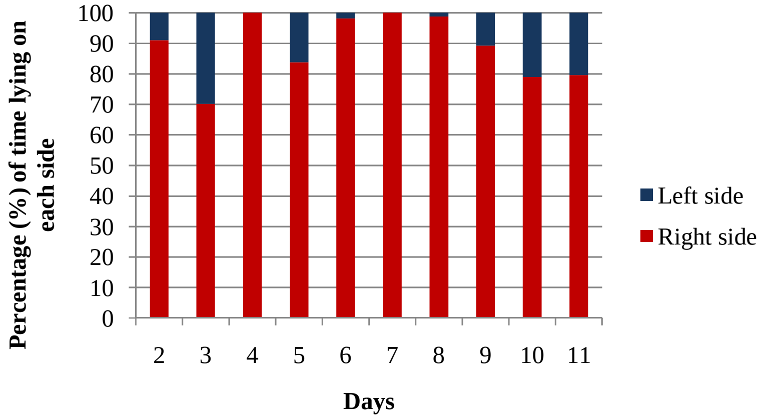
Mean percentage of time lying on each side (left *vs.* right) in a 24 hour period starting on the day after mastitis detection (D2) and finishing on D10+ (in this case day 11) of cow 7109 (mastitis on the right).



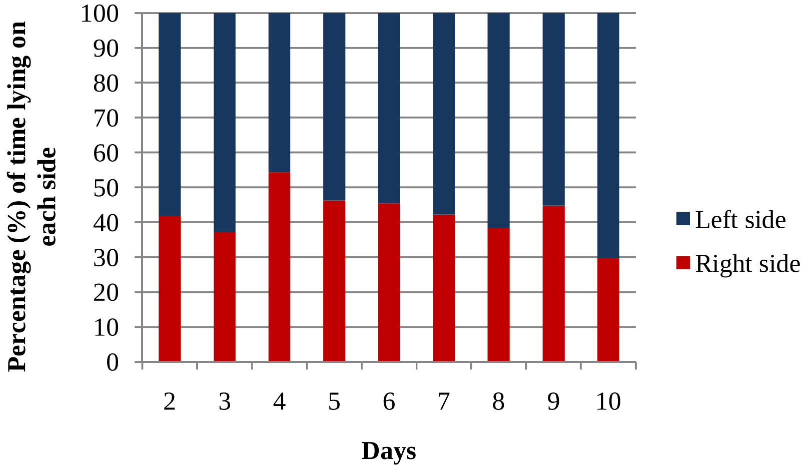
Mean percentage of time lying on each side (left *vs.* right) in a 24 hour period starting on the day after mastitis detection (D2) and finishing on D10+ (in this case day 11) of cow 3072 (mastitis on the left).



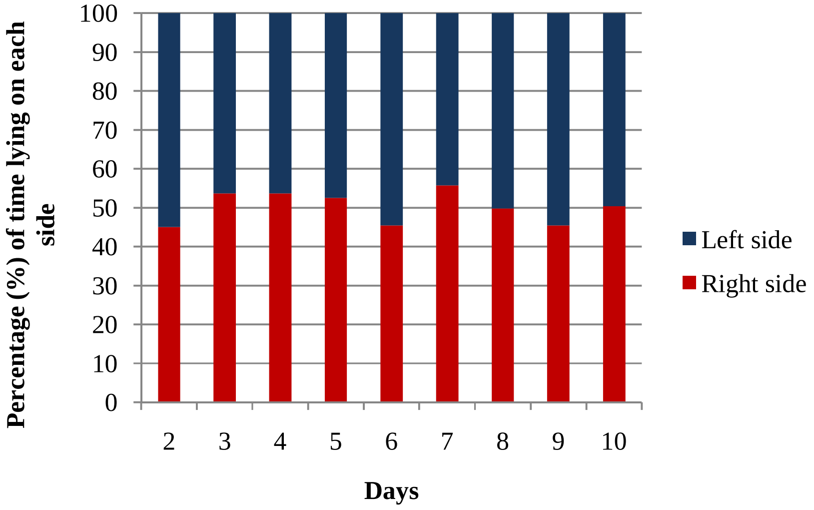
Mean percentage of time lying on each side (left *vs.* right) in a 24 hour period starting on the day after mastitis detection (D2) and finishing on D10+ (in this case day 11) of cow 4064 (mastitis on the left).



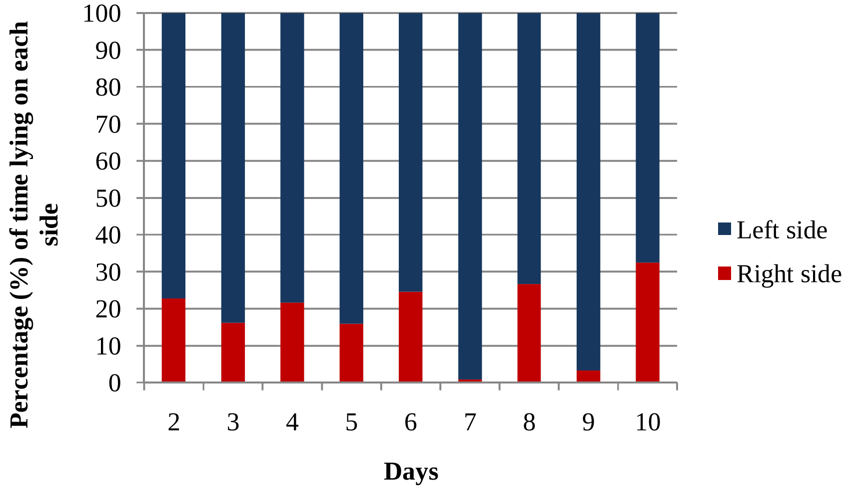
Mean percentage of time lying on each side (left *vs.* right) in a 24 hour period starting on the day after mastitis detection (D2) and finishing on D10+ (in this case day 10) of cow 4040 (mastitis on the left).



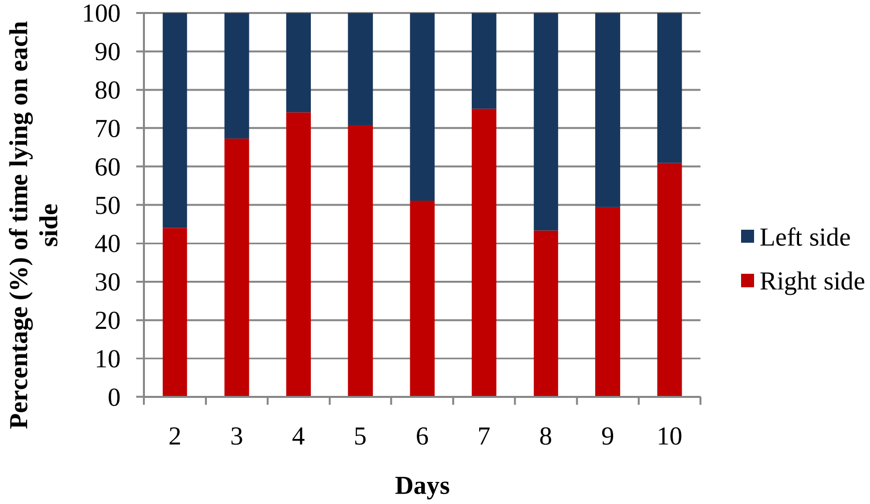
Mean percentage of time lying on each side (left *vs.* right) in a 24 hour period starting on the day after mastitis detection (D2) and finishing on D10+ (in this case day 10) of cow 3056 (mastitis on the left).



Mean percentage of time lying on each side (left *vs.* right) in a 24 hour period starting on the day after mastitis detection (D2) and finishing on D10+ (in this case day 10) of cow 3126 (mastitis on the right).



Mean percentage of time lying on each side (left *vs.* right) in a 24 hour period starting on the day after mastitis detection (D2) and finishing on D10+ (in this case day 10) of cow 6022 (mastitis on the left).



Mean percentage of time lying on each side (left *vs.* right) in a 24 hour period starting on the day after mastitis detection (D2) and finishing on D10+ (in this case day 10) of cow 5016 (mastitis on the right).