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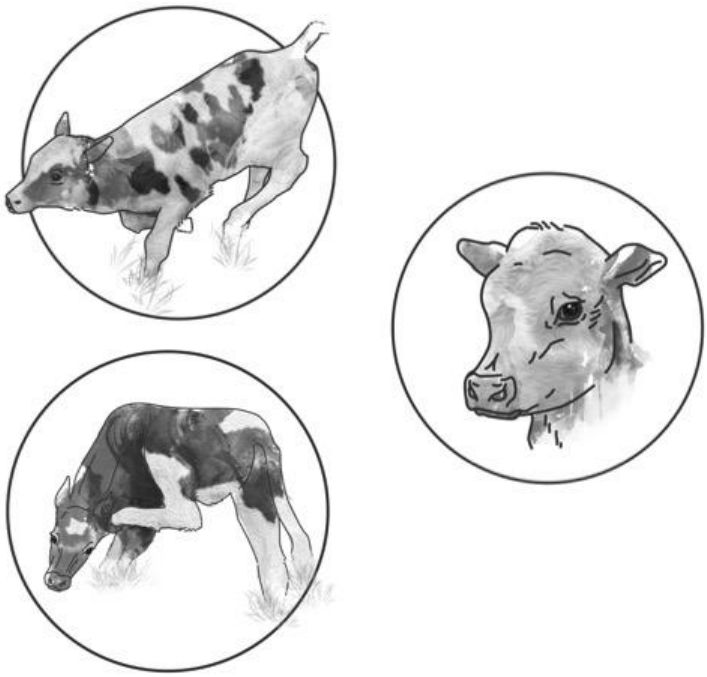

The effect of meloxicam treatment after disbudding on pain-related behaviours and weight gain in Danish Holstein calves.

- A comparative study between one day and four days of treatment.

Adam Rääf (rwc467) & Signe Rejnhardt Olsen (jrb642)

Supervisors: Karina Bech Gleerup, DVM, Ph.D. and Nina Dam Otten, DVM, Ph.D, Dipl. ECAWBM-AWSEL.

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Name of department:	Department of Clinical Veterinary Medicine
Author(s):	Adam Rääf, rwc467 Sadelmakarebyn 4b 21840 Bunkeflostrand Sweden Signe Rejnhardt Olsen, jrb642 Markmandsgade 5, 1 th 2300 Copenhagen S Denmark
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Supervisor:	Karina Bech Gleerup, DVM, Ph.D. Nina Dam Otten, DVM, Ph.D, Dipl. ECAWBM-AWSEL.
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Adam Rääf

Signe Rejnhardt Olsen

Abstract

Disbudding of calves has proven to be a painful procedure, but is still a common practice in Danish milk-producing herds. The lack of evidence on the effect of repeated treatments with NSAIDs in the period after disbudding constitutes the basis for this current study. Reducing post-surgical pain would increase the welfare of the calves, and potentially increase their weight gain.

Aim of the study: This study is carried out as a double blinded case-control study on Danish Holstein calves, focusing on the animal-based indicators of welfare, weight and behaviour. The study evaluates the weight gain and behaviours of calves treated with meloxicam for four days following disbudding, compared to calves treated only on the day of disbudding.

Methods: 56 calves were included in the study. One day prior to disbudding, all calves were weighed and observed for 90 minutes in total, to establish a baseline value of behaviour and weight. Calves were paired by weight and age. All calves were disbudded by heat cauterization on day 0, using sedation, local anaesthetic and meloxicam. The following 3 days, calves allocated to the treatment group received treatment with meloxicam, while the control calves did not receive any treatment. All calves were continuously observed for 30 minutes prior to-, and 60 minutes after milk feeding, for 4 days after disbudding, and weighed on day 4, and day 14.

Results: The study found and increased weight gain during day -1-4 for the treatment group. Furthermore, the study found an overall increase in pain specific behaviours on day 1, suggesting an insufficient coverage of the pain from disbudding by the NSAID treatment. However, the treatment group expressed significantly lower numbers of pain specific behaviours and *pain face* on day 3, in addition to an increased amount of time spent playing, compared to the control group. The calves in the treatment group spent significantly more time on oral abnormalities and less time on resting, on day three and four, compared to the control group.

Conclusions: There is a significant effect of a four-day treatment with meloxicam following disbudding, which decreases pain related behaviours and increases weight gain, play and oral abnormalities, during the first four days after the procedure. This shows a significant benefit of the treatment, in reducing post-surgical pain and increasing weight gain. The effect of the treatment may be maintained by the administration of meloxicam every other day, or even increased by a longer treatment. However, further studies need to be carried out to prove this proposal.

Keywords: Disbudding, calves, welfare, meloxicam, NSAID, pain, behaviour, weight gain

Resumé

Afhorning af kalve er en smertefuld procedure, men er stadig normal procedure i danske malkekvægsbesætninger. Manglen på studier omkring behandling med NSAIDs i en længere periode efter afhorning, har dannet grundlag for dette studie. Hvis smerten efter afhorning kan reducers, kan kalvenes velfærd, og muligvis tilvækst forbedres.

Formål: Studie er designet som et dobbeltblændet case-control studie på Dansk Holstein kalve, som fokuserer på de dyre-baserede indikatorer vægt, og adfærd som udtryk for velfærd. Studiet evaluerer effekten af fire dages behandling med meloxicam, på tilvæksten og adfærd efter afhorning, sammenlignet med en enkelt behandling omkring afhorningstidspunktet.

Metode: 56 kalve var inkluderet i studiet. Alle kalvene blev vejet og observeret dagen før afhorning, for etablering af en standardadfærd og -vægt til sammenligning med observationer fra de følgende dage. Kalvene blev parret, efter vægt og alder. Alle kalve blev afhornet med brændejern under sedation, og med brug af lokalanaestesi og meloxicam. Kalve i behandlingsgruppen blev behandlet med meloxicam de efterfølgende tre dage. Kalvene blev observeret 30 min. før- og 60 min. efter fording, i fire dage efter afhorning, og vejet igen på dag 4 og 14.

Resultater: Studiet fandt øget tilvækst i behandlingsgruppen mellem dag -1 og 4. Overordnet fandtes significant øgning i mængden af smertespecifik adfærd på dag 1, hvilket viser at meloxicam ikke dækker smarten efter afhorning tilstrækkeligt. Behandlingsgruppen udtrykte significant lavere mængde af smerteadfærd og *smerte-ansigt* på dag 3, og øget legeadfærd, sammenlignet med control gruppen. Kalve i behandlingsgruppen brugte også mindre tid på at hvile og mere til på orale abnormaliteter på dag 3 og 4, sammenlignet med kontrolgruppen.

Konklusion: Fire dages meloxicambehandling efter afhorning, nedsætter smerte-relateret adfærd og æger tilvækst, legeadfærd og oral abnormaliteter, gennem de første fire dage efter proceduren. Den fordelagtige effekt af meloxicambehandlingen kan muligvis opretholdes ved behandling hver anden dag, eller øges ved en længerevarende behandling. Disse muligheder bør dog udersøges i fremtidige studier for at kunne verificeres.

Foreword and acknowledgements

This study is a result of our master's thesis in veterinary medicine at the University of Copenhagen, Faculty of Health Sciences, Department of Clinical Veterinary Medicine. The purpose of the study is to serve as a pilot study, to encourage further future research into the husbandry and management of painful procedures in calves, and disbudding in particular.

This project could not have been achieved without the great contributions and patience from our supervisors, assistant professor Karina Bech Gleerup, DVM, Ph.D. and assistant professor Nina Dam Otten, DVM, Ph.D, Dipl. ECAWBM-AWSEL.
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List of abbreviations

COX-1, 2, 3:	Cyclooxygenase-1, 2, 3
GH:	Growth hormone
H1, 2 & 3:	Herd 1, 2 & 3
IGF-1:	Insulin-like growth factor 1
IL-1β:	Interleukine-1β
IL-6:	Interleukine-6
I.M.:	Intramuscular
NSAIDs:	Non-Steroidal Anti-Inflammatory Drugs
P.O.:	Per oral
S.C.:	Subcutaneously
TMR:	Total Mixed Ratio
TNF-α:	Tumor Necrosis Factor alpha

Adam Rääf, rwc467

Signe Rejnhardt Olsen, jrb642

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1 Introduction

Disbudding of calves is proven to be a painful procedure, but is still common practice in Danish milk-producing herds. During the past two decades, many studies have been published regarding post-operative analgesic treatment and the effect on pain related behaviour, stress and weight gain. Most studies have focused on the use of sedation and local anaesthesia, while some studies also evaluates the effect of non-steroidal anti-inflammatory drugs (NSAIDs). Several studies on calves have shown that one dose of NSAID before or during the disbudding procedure, decrease the amount of pain related behaviours and serum cortisol levels in the following period. There is to our knowledge lacking studies comparing behaviour and weight gain in calves treated with NSAIDs at the time of disbudding, with calves treated at disbudding, and the following 3 days. The aim of this study was to evaluate the effect of administration of meloxicam for four days after disbudding on weight gain, and the number and duration of pain related behaviours. Proper analgesic treatment after disbudding is a welfare issue, as Danish legislation states that animals has to be kept from hunger, thirst, pain, suffering, permanent injury, major disadvantages, fear, and be housed with regards to their physical and natural behavioural needs (*Bekentgørelse af dyreværnsloven*, 2016)

1.1 Outline and hypothesis'

This project focuses on weight gain and the pain-related behavioural following disbudding. For this purpose, to separate hypothesis' was formulated.

1.

H_0: Calves treated with meloxicam for four days following disbudding, do not gain significantly more weight within both 4 and 14 days after the procedure, compared to calves treated only on the day of disbudding.

H_1: Calves treated with meloxicam for four days following disbudding, gains significantly more weight within 4 and 14 days after the procedure, compared to calves treated only on the day of disbudding.

2.

H_0: Calves treated with meloxicam for four days following disbudding, do not express a lower number of pain-related behaviours within 4 days after the procedure, compared to calves treated only on the day of disbudding.

H_1: Calves treated with meloxicam for four days following disbudding, express a lower number of pain-related behaviours within 4 days after the procedure, compared to calves treated only on the day of disbudding.

2 Background

2.1 Disbudding and alternative methods

Disbudding and dehorning of cattle is common practise throughout the world. It is done to reduce damage and bruising on other cattle, as well as making handling safer and easier for workers in modern production stables (Goonewardene *et al.*, 1999; Oliver, 2009). Fights and bruising amongst the cattle has a negative impact on both animal welfare economics. A study from Australia in 1974 concluded that the amount of trimmed meat in the beef cattle industry due to bruising was 0.77 kg for hornless cattle and 1.59 kg for horned cattle. (Meischke, Ramsay and Shaw, 1974) The 2010/2011 Canadian beef quality audit states, that the economic loss due to bruising was $2.10/carcass, or $6.7 million in total (*National Beef Quality Audit, 2010/11 Plant Carcass Audit*, 2011)

Gottardo *et al.*, 2011, found that 80.5 % of all farms answering a questionnaire in the northeast part of Italy disbudded all young calves. They state that this is consistent with the mean value reported by SANCO in 2009 for the 27 countries in the EU (Oliver, 2009; Gottardo *et al.*, 2011). Disbudding involves removal of horn-producing cells, and should be done in calves under 2 months of age. At a later point the horn bud will be more attached to the frontal bone demanding amputation which is more painful for the animals (Stafford and Mellor, 2005).
The study from Italy showed that the mean age for disbudding calves were 30-39 days, hot-iron disbudding was the most common practice (90.6 %), and that farm personnel was the main person in charge for the procedure (Gottardo *et al.*, 2011). The same study showed that only 14.5 % of the farms used some sort of pre-operative treatment, and that the post-operative treatment consisted of analgesia in 5 % of the farms, and antibiotics in 32.0 % of the farms. The remaining farms (63 %) did not provide post-operative treatment. Lack of treatment was mainly either due to the high cost of the drugs or the owners lack of willingness to pay for the drugs or the veterinarians service (Hewson *et al.*, 2007).
According to Danish legislation, only heat cauterization may be used in Denmark. In other parts of the world there are several other methods to disbud calves, including cryosurgery, caustic paste and scoop dehorning.
The genes that codes for horns are autosomal recessive genes, in contrary to the polled dominant gene, which is why it is easy in theory to breed polled cattle(Long and Gregory, 1978).

However, the breeding of Holstein bulls for dairy production has been slow in the developing. In 2013, there were 68 registered genomic polled Holstein bulls in the American National Association of Animal Breeders, within these, 6 were active in breeding (Spurlock, Stock and Coetzee, 2014).
The concerns about using polled bulls' semen for AI of dairy cows, has included productivity of milk. In 2013, the 20% best polled bulls, still had lower average Net Merit Predicted Transmissive Ability value, NM PTA, at 590, compared with the horned bulls of 761 in 2013 (Spurlock, Stock and Coetzee, 2014). The NM PTA is a value, expressing the genetic potential for the traits enrolled in the calculation. The traits include, milk-production (milk-yield, fat-yield, protein-yield), daughter pregnancy rate, udder composition, feet and leg composition etc. The average bull worldwide will have a NM of 100. Hence, the polled bulls in the U.S. still lacks some scores on traits, which might mean that their daughters have a lower total life yield, and hereby production profit, than a daughter of a horned bull.

2.2 Legislation on disbudding

In Denmark, legislation on disbudding of animals is incorporated in the animal welfare act in executive order number 828 by 07/11/1997 on disbudding of animals (Bekendtgørelse om afhorning af dyr, 1997).
According to this executive order, disbudding of animals may only be performed by a veterinarian and while using sedatives. The producer may perform disbudding of calves younger than 3 months if a veterinarian immediately before the procedure administers sedative drugs.
When disbudding calves younger than 3 months, the following rules should be considered:

1. The procedure may only be conducted with an electrically or gas-heated iron. Those irons should be heated to at least 600°C and should maintain this temperature throughout the whole disbudding procedure. Electrically/heated irons should meet the safety requirements stated in the strong current legislation.
2. The hair around the horn buds should be clipped before the disbudding, and if the horn bud is longer then 1 cm it is to be cut off with a knife immediately before the disbudding.
3. The animal should be restrained so that the head cannot be moved during the procedure.
4. The disbudding should be carried out on one horn bud at the time, without pauses and should be completed within 20 seconds per bud. One may not burn several times per horn bud.

5. Cleaning of the iron should be conducted between burning of each bud with a steel brush.
6. If an electrically-heated iron is used, the handler should be aware of the legislation on use and control of electrical tools and the demands of protection against indirect touch, e.g. by use of a residual-current device.

Disbudding by the use of caustic agents, elastics and similar products are illegal.

2.3 Anatomical considerations

The horn consists of specialized skin composed of hard keratin, similar to hoofs of ungulates or claws of carnivores. The corneal process develops as an exophysis to the frontal bone, which is then covered by dermis and epidermis. It is in the epidermal stratum corneum that the hard keratin is located (Bacha and Bacha, 2000).

At approximately one month post-partum, the epidermis proliferates and form horn buds. During the next two months, the corneal processes becomes hollow and the frontal sinus extends into the horn cavity (Sinowatz, 2010). It is recommended that disbudding is carried out before this point. The main innervation of the horn buds is by the cornual branch of the zygomaticotemporal nerve, which originates from the trigeminal nerve (the fifth cranial nerve). Other nerves influencing the area are the supraorbital nerve and infratrochlear nerve. The corneal nerve runs through the temporal fossa, caudally from the eye together with the corneal artery and vein, and is protected by the temporal line. Halfway between the eye and the basis of the horn, it is only covered by fascia and muscles, why this is a good place for local analgesia when disbudding (Weaver, St Jean and Steiner, 2005; Reese *et al.*, 2009).

2.4 Pain and inflammation caused by disbudding

The definition of pain according to the International Association for the Study of Pain is described as: ‘An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage’ (Muir, 2015).

This damage can be caused by mechanical or thermal stimuli of high intensity, as in the case of disbudding. The thermal stimulus caused by disbudding causes a fourth-degree burn, as it is defined as tissue trauma that extends through the dermis and causes trauma to underlying tissue (MacPhail, 2013; Gaughan, Hanson and Divers, 2014).

Acute responses to pain in an animal are: withdrawal reflexes and activation of the hypothalamopituitaryadrenal axis (HPA-axis), which leads to increases in multiple stress

hormones, including adrenocorticotropic hormone (ACTH) and growth hormone (GH). By activation of the sympathetic nervous system there is an increase in heart rate, blood pressure, and the secretion of cortisol and catecholamines. Changes in behavioural patterns are also seen, depending on severity and origin of the pain (Muir, 2015; Wiese and Yaksh, 2015).

To describe the physiologic pain responses generated by a stimulus the term nociception is used. The pain sensation starts with an activation of nociceptors. Nociceptors are found in many organs, amongst others in the skin, articular capsules, ligaments, and connective tissue in visceral organs (Almeida, Roizenblatt and Tufik, 2004). Nociceptors are free nerve endings specialized in the detection of a harmful or potentially harmful stimulus. The nociceptors are activated when the stimulus becomes greater than the threshold. If activated, the stimulus is transduced into action potentials. The action potentials travels along the axons of the nerves, and are transmitted to the spinal cord, where a modulation takes place before they are projected to the brain for perception.

Nerves are classified according to their myelinisation and fibre diameter, which affects the velocity that action potentials can be conducted in (Grimm *et al.*, 2007). The strongest nerve fibres are the myelinated somatic fibres, classified as A-fibres, and includes Aα, Aβ, Aδ and Aγ. The major functions of the mentioned are innervation of skeletal muscles, proprioception, obtaining of muscle tonus, sensation of pressure, sensation of temperature and fast nociceptive response, and the development of hyperalgesia or allodynia following chronic nociceptive stimuli. Type B fibres are pre-ganglionary autonomic fibres, and type C-fibres are non-myelinated fibres responsible for post-ganglionic sympathic innervation and the transmission of visceral pain, slow pain, nociception and the sensation of temperature (Grimm *et al.*, 2007; Steeds, 2016).

In the acute pain phase in burn wounds, high-threshold Aδ-fiber nociceptors are activated, and are responsible for the first sensation of pain. Later on, the C-fibre nociceptors are activated and are responsible for the second onset of pain and signals of tissue damage and inflammation (Basbaum *et al.*, 2009; Santana, 2014; Muir, 2015).

In the case of disbudding, the nerves involved are somatic afferent fibres, and since the pain originates in the head region, it is transduced and directly transmitted to the brain via the trigeminal nerve (Purves *et al.*, 2004; Muir, 2015).

Trauma such as disbudding causes tissue damage. The trauma initiates an acute local inflammation, by the immediate release of preformed vasoactive, chemotactic and pro-

inflammatory substances from both epithelial cells, fibroblasts, C-reactive nerve fibres and endothelial cells (Ackermann, 2012). The inflammatory substances initiating the inflammation are amongst others histamine, bradykinin, prostaglandins, nitric oxide, substance P and components of the complement system. These factors mediate the recruitment and activation of immune cells, vasodilation and the activation of endothelium, increasing the local blood supply and permeability of vessels. The recruitment and activation of immune cells results in a further increase in the release of pro-inflammatory mediators, such as cytokines. The main cytokines included are TNF-α, IL-1β, IL-6 and IL-8. Physical trauma and chemical stimuli by components of the compliment cascade facilitates the release of arachidonic acid from membrane phospholipids of almost any cell type in the tissue (Marchand, 2008; Ackermann, 2012; Santana, 2014).

The arachidonic acid can be metabolized by COX-enzymes, lipoxygenase or cytochrome p450. The metabolites are prostaglandins, tromboxanes, leukotrienes or epoxyeicosatrienoic acids, respectively. There are three different COX enzymes, COX-1, COX-2 and COX-3, whereas COX-1 is present in most tissues, and is functioning in the homeostasis and the protection of gastric mucosa. COX-2 is derived from leukocytes and endothelial cells, and is expressed during inflammation. COX-3 is present in the cerebral cortex of humans and dogs (Ackermann, 2012). The inflammatory response to tissue damage lowers the nociceptors threshold, contributing to an increase in pain sensitivity (Basbaum *et al.*, 2009; Rittner, Machelska and Stein, 2010; Santana, 2014).

In some cases, such as hyperalgesia and allodynia, the balance of the nociceptors' thresholds is altered and hyperalgesia or allodynia occurs. Hyperalgesia is described as generally increased pain sensitivity, which makes a painful stimulus become more painful than normal. Allodynia is described as the situation when a non-painful stimulus, becomes painful (Basbaum *et al.*, 2009; Gaynor and Muir, 2015).

2.5 Burn wounds and healing

Burn wounds occur when thermal energy is applied at a rate that exceeds the body's ability to absorb and dispel it. The degree of injury depends on the temperature of the heat source, the time of contact and tissue conductance.

The burn wound is classified according to extension of tissue trauma (MacPhail, 2013; Gaughan, Hanson and Divers, 2014):

- First-degree or superficial burns: Includes only epidermis. The traumatized area is thickened, painful and erythematous. The epidermis is usually shedded. Healing is rapid and it usually takes 3-6 days.
- Second-degree or partial-thickness burns: Causes major destruction of cutis. Tissue inflammation and edema located in subcutis appears. The hair is not easily epilated. The damage is progressive during the first 24 hours due to prostaglandins, proteolytic enzymes and vasoactive substances. Healing takes weeks to months and often cause extensive scarring. Can progress to third-degree burn, especially if bacterial infections manifests.
- Third-degree or full-thickness burns: A dark brown, leathery and insensitive eschar is formed. All skin structures are destroyed. Since all nerves in the area is destroyed, this burning is less painful than first- or second-degree burns. Subcutaneous edema and necrosis due to superficial vascular thrombosis and deep vascular permeability. Healing by contraction, reepithelialisation or reconstruction.
- Fourth-degree burns: Tissue trauma that extends through the dermis and causes underlying trauma to muscles, fat fascia, ligaments and bone. Otherwise the same characteristics as third-degree burns. Healing by secondary intention or reconstruction.

In humans, contact with a heat source of 70°C for only one second creates a full-thickness burn, or a third degree burn (MacPhail, 2013).

Burn wounds cause cell destruction, which leads to tissue inflammation. Schwartzkopf-Genswein and Stookey investigated the tissue inflammation following hot-iron branding in cattle using infrared thermography, and found significant evidence that tissue inflammation is still present 168 h after hot-iron branding (Schwartzkopf-Genswein and Stookey, 1997).

Other studies have found that an initial increase in blood flow is seen 30 minutes after thermal trauma, followed by a decrease in blood flow for at least 6,5 hours. After one hour, a significant local inflammatory response with dermal elevations of IL-6 and IL-1β occurs (Tarnow *et al.*, 1996; Junger, Moore and Sorkin, 2002; Ipaktchi *et al.*, 2006).

Junger, Moore and Sorkin, 2002, also concluded that full-thickness burns may cause a secondary hyperalgesia in the surrounding unburned tissue.

2.6 Appropriate analgetic treatments

As stated earlier, the use of sedation is a requirement by law when disbudding. The use of local anaesthetics and non-steroidal anti-inflammatory drugs (NSAIDs) are on the other hand a recommendation.

Stewart *et al.*, 2009 found that local anaesthetics abolishes pain from disbudding for 2-3 hours post-surgery, and that treatment with NSAIDs prolongs the time of pain relief (Stewart *et al.*, 2009). The same results regarding local anaesthesia has been shown in several other studies, amongst others Graf & Senn, 1999 and Stafford and Mellor, 2005 (Graf and Senn, 1999; Stafford and Mellor, 2005). Those studies investigated eye temperature, cardiac responses, plasma concentration of vasopressin, cortisol and ACTH. They also looked at behavioural parameters; tail swishing, head moving, tripping, rearing, head moving and feeding behaviour. The overall conclusion from these studies is that disbudding is a painful procedure, and that local analgesia is a good pain relieving treatment for the first 2-3 hours after disbudding. Stafford and Mellor, 2005 and Stewart et al., 2009 also concluded, that to avoid the rise of disbudding-related behaviours and the peak in cortisol levels as the local analgesics wears of, this nerve block should be combined with a NSAID to get a post-surgery pain relieving effect for up to 24 hours.

2.6.2 Meloxicam, mechanism of action and pharmacokinetics

NSAIDs are anti-inflammatory, analgesic and antipyretic drugs that inhibit the enzyme cyclooxygenase (COX), which plays a role in the producing of prostaglandin and thromboxane and has an immunosuppressive effect (Barkin, 1998).

Meloxicam is a NSAID registered for postoperative pain due to disbudding in calves, why it is the drug of choice when disbudding calves in Denmark. It has both a peripheral and a central analgesic effect. Meloxicam classified as a moderately selective COX-2 inhibitor (Barkin, 1998; Plumb, 2008). This is important to us because COX-2 selective NSAIDs has been shown to be anti-hyperalgesic (Lees, 2011). The only registered adverse effect when given subcutaneously (S.C.) to cattle is a small swelling at the injection site. In calves that were given a S.C. dose of 0,5 mg/kg BW, a C_{max} of 2,1 µg/ml was seen after 7,7 hours, with a t½ of 26 hours (*Produktresumé, Metacam*, 2007).

2.7 Normal growth and the effects of pain-induced stress on growth

In a study on social behavior of young Danish dairy Holstein calves, the weight gain for 0-3 weeks of age, was measured to 0,541 ± 0,02 kg/day. Calves at the age of 3-6 weeks had an average daily weight gain at 0,823 ± 0,294 kg (Duve and Jensen, 2012).

Group housed calves has shown to have a significant higher daily gain of 990 (±40) g/day, compared to individually housed calves at 850 (±40) g/day, when receiving an enhanced milk diet of 9 L/day from day 3-28 and 5L from day 29-42 besides the concentrate (Jensen, Duve and Weary, 2015). Body weight gain for the calves fed standard milk amount and concentrate in that study was 810 – 840 (±40) g/day, regardless if they were housed individually or in groups of two.

The average daily weight gain of calves, can be altered under the influence of pain or stress, following three main reasons; less time spend eating, high circulating levels of plasma cortisol altering other endocrine factors, and altered sleep patterns (Friess *et al.*, 1995; Moberg *et al.*, 2000). These processes are proposed as physiological stress and activation of the HPA-axis, and altered somatotropin release. These changes results in an increased GH release, as a consequence of the cortisol provoked reduction of IGF-1 secretion from the liver (Friess *et al.*, 1995; Moberg *et al.*, 2000). Cortisol acts to preserve easily available energy from the blood where it inhibits the uptake of glucose to the peripheral tissue by reducing the IGF-1 secretion and effect of insulin. This leads to the inhibition of anabolic processes, such as energy preservation and growth (Moberg *et al.*, 2000). Studies on humans reveals that the release of growth hormone is initiated during the initial period of the sleep, and cortisol is rising at the end of a period of sleep (Friess *et al.*, 1995). This suggests that GH secretions can be decreased when calves are deprived from rest, and if not deprived from resting, the high circulating levels of cortisol can result in disturbances of the sleep. Several studies have measured the effect of NSAIDs on the daily weight gain of calves after disbudding, with different results. Heinrich *et al.*, 2010, did not find any significant difference in feed intake between calves receiving a 0,5 mg/kg I.M. injection of meloxicam prior to dehorning. However, there was a trend for the treated calves to consume more feed on day 1, than day 0, which was not applicable for the control group (Heinrich *et al.*, 2010).

Faulkner and Weary, 2000, tested the effect of ketoprofen after disbudding, and found the treated calves gaining 1,2 ± 0,4 kg within the first 24 hours after the procedure, compared to the control group gaining 0,2 ± 0,4 kg (Faulkner and Weary, 2000).

However, the long-term effects of NSAID-treatment on weight gain, has shown different results. One study found a weight gain on 1,2 kg/day up to 13 days after disbudding and castration, by providing ad libitum access to salicylate (2,5-5 mg/ml) in the drinking water, in contrast to the control group with a daily weight gain of < 0,2 kg/day (Baldridge *et al.*, 2011). Another study on meloxicam administration prior to dehorning without local anaesthetics, found an average daily gain of 1,05 ± 0,13 kg/day in contrast to 0,4 ± 0,25 kg/day, within the first 10 days after dehorning, when given placebo treatment (Coetzee *et al.*, 2012). Other studies found no differences in weight gain between treated and non-treated calves 16 days after the procedure has been done (Grøndahl-Nielsen *et al.*, 1999; Bates *et al.*, 2016). The study by Bates et al., 2016, showed a significant increase in growth for up to 16 days with the use of NSAIDs when calves were disbudded without any other pain relieve. In contrary to NSAIDs, the use of local anaesthetics and sedation alone made an increase in growth for up to 30 days (Bates *et al.*, 2016).

2.8 Normal behaviour for calves aged 2-7 weeks, and common behavioural abnormalities related to the housing conditions

The behaviours of calves aged 14-49 days include sleeping, feeding, rumination, social interactions and explorative behaviour including playing and grooming, and possibly adjacent abnormal behavioural patterns.

2.8.1 Sleeping behaviour

Young animals generally have a higher demand for rest, than older animals resulting in a decrease in lying time with age (Duve and Jensen, 2012). Duve and Jensen found that calves at the age of 49 days spent on average 10,2 hours lying down, while calves at the age of 2 days spent on average 13,8 hours between 06:00 and 22:00.

It has been shown, that calves up to 96 days of age spends 38-53 % of their time inactive, in the period between 7:00 and 17:35 every day, and calves housed 2 by 2 had a lying time of 70 % during 24 hours (Veissier *et al.*, 1997; Veissier, Ramirez De La Fe and Pradel, 1998; Chua *et al.*, 2002a). Von Keyserlingk *et al.*, 2006 suggests that calves have a distinct diurnal resting pattern, with more resting at night, than day. They found that calves at the age of 5 - 32 days, housed

individually, were lying down on average 44±0,4 min/hour for all 24 hours a day. Between 00:00 and 06:00 the calves rested almost 60 minutes/hour. The lying time decreases during the day with the least rest between 18:00 and 00:00. In this period the average lying time was under 40 min/hour. Around feeding at 08:00 and 18:00, there were almost no rest (von Keyserlingk *et al.*, 2006).

When moving calves together in group pens, they generally spend less time lying down the first week, and there was a tendency to lie down closer to a former neighbour from the previous housing facility, whereas they lay down closer to a random pen mate, 5 days after introduction to the group pen (Duve and Jensen, 2012). There was no difference on the lying time at 49 days of age (5 days after introduction to group pen), between calves that had previous been held in single pens with limited access to a neighbour and calves previously held in group pens (Duve and Jensen, 2012).

Lying behaviour has also been shown to rely on the environmental factors in the pen, as the calves seek dry soft bedding. In contrary to a total lying time of around 17 hours/day, calves would lie down on wet bedding for a maximum of 5,3 hours ± 1,1 hour, and a minimum of 0 hours, depending on the dry matter in the bedding (Camiloti *et al.*, 2012). The temperature and humidity also plays a role in the comfort of the calves, as younger animals can have difficulties with their thermoregulation, and heat-stress can be a factor of importance for their welfare and growth (Hill *et al.*, 2011). The best housing for optimal weight gain of calves at 5 to 56 days of age has been found to be nursery pens with straw bedding and cooling in warm weather at daytime (Hill *et al.*, 2011).

2.8.2 Feeding and oral abnormalities

Artificially weaned calves and calves housed without the dam can develop oral abnormalities. Oral abnormalities including cross-sucking, tongue rolling, and licking on inventory. Cross-sucking is seen when a calf is sucking on a pen-mate to satisfy their needs for sucking. This can later on develop into intersucking between cows, and thereby milk waste for the farmer (Mahmoud, Mahmoud and Ahmed, 2016).

The cross-sucking of calves are found to be triggered by an insufficient way of feeding the calves in the milk feeding period. Non-nutritive sucking, can be elicited when drinking milk, as the motivation to suck is stimulated by the ingestion of milk, and cross-sucking is a redirection of the natural sucking behaviour of the calves (Jensen *et al.* 2003). The amount of milk ingested,

also influences cross-sucking behaviour, as calves fed small amounts of milk has an increased duration of oral abnormalities, than calves fed large amounts of milk (de Passillé *et al.*, 1992; Rushen and Depassille, 1995).

A study on sucking behaviour of twelve heifer calves at 7, 14 and 28 days of age, suggests that the average time spent sucking per feeding was 6,52 ± 0,60 min., at 7 days of age, and increased slightly to 8,77 ± 0,85 min. at 28 days of age (Lidfors, Jung and de Passillé, 2010).

The total nursing time, that calves fed by teat buckets spend sucking, has been measured to 47 minutes per day, whereas bucket fed calves only spend 1 minute and 49 seconds, feeding. Furthermore teat fed calves consumed almost double the amount of milk, consumed by the bucket fed calves at the age of 1-4 weeks (Appleby, Weary and Chua, 2001). When comparing restrictional teat-bucket fed calves, with ad libitum teat-bucket fed calves, both groups spent approximately six minutes sucking on either milk feeding teat or dry teat per visit (Miller-Cushon *et al.*, 2016).

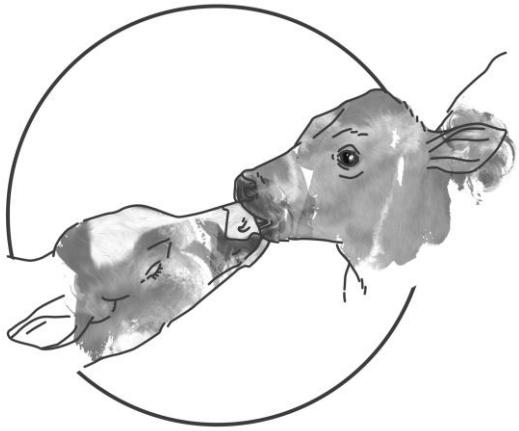

Figure 2.1: Calves cross-sucking. Illustrations by Pawel Nowakowski

However, the restrictional fed calves had more frequent visits to either of the teats, resulting in a longer total sucking time per day (Miller-Cushon *et al.*, 2016). This suggests that restrictional feeding leads to an exacerbated motivation to suck (Miller-Cushon *et al.*, 2016).

It is discussed whether the actual time spent sucking on milk feeding teats has an impact on the development of cross-sucking. However, the amount of milk consumed seems to play a greater role in preventing cross-sucking, than the duration of the milk intake since calves in a positive energy balance having the lowest rate of cross-sucking (Jung and Lidfors, 2001; Roth *et al.*, 2009). The concentration of the milk also influences the motivation to suck, as non-nutritive sucking is proven to increase with the concentration of formula in the milk. However, there is no difference between real cow's milk and sufficiently concentrated milk replacer formula, in the ability to elicit non-nutritive sucking (De Passillé, Rushen and Janzen, 1997). The most non-nutritive sucking is performed within 15 minutes after a meal (Lidfors, 1993).

Post-prandial sucking can be seen as a result of insufficient energy intake, concentration and amount of the milk and insufficient stimulation of sucking. However, it can also be seen as an

expression for hunger in the milk feeding period, as the frequency of cross-sucking between calves generally decreases after weaning (Lidfors, 1993). Cross-sucking can persist between the same calves after weaning, which suggests that there is a social component in the sucking. A study by De Passillé, Borderas and Rushen, 2011, found that the frequency of cross-sucking decreased when moving heifers to another barn, while the few persistent cross-suckers increased the duration of their sucking. Furthermore, they were sucking the same pen mate every time (De Passillé, Borderas and Rushen, 2011). They found no correlation between weaning method and-age, with the incidence of cross-sucking development.

Calves licking on inventory can be seen both as an expression for hunger, or as a result of social deprivation, as calves housed in group pens has a lower frequency of licking on inventory, compared to individually housed calves (Veissier, Ramirez De La Fe and Pradel, 1998).
Pen housed calves and calves kept on fields with high loads of pasture, spend significantly less time obtaining feed, but spend more time on oral behaviours, compared to the calves on the low pasture field (Ishiwata *et al.*, 2008). The oral behaviours included self-grooming, allogrooming, licking inventory and playing with their tongue. The calves in pens or high pasture fields, spend around 8% of their time on oral behaviours and 32% on eating, compared to the low pasture calves, spending 59% of the time eating (Ishiwata *et al.*, 2008).
Calves at 6 weeks of age, in the middle of their weaning period from 5-7 weeks, spend between 46,8 – 56,7 min/day feeding solid food. The difference in mean feeding time was depending on whether the calves was presented for at mixed ratio with concentrate and chopped hay < 2,5 cm or if they had concentrate and hay served separately (E K Miller-Cushon *et al.*, 2013). Calves up to eight weeks of age fed with the mixed ratio, generally spend more time feeding, and had a lower intake of food per minute, compared to the separate fed calves (E K Miller-Cushon *et al.*, 2013). Another time budget suggests that calves up to 96 days of age, fed with milk replacer and solid mixed ratio starter food, on average spends 10% of the time between 7 and 17:35 chewing, including rumination and feeding. This seen with the majority of intake of solid feed after morning milk feeding and the majority of rumination occurring during the inactive periods of the day (Veissier, Ramirez De La Fe and Pradel, 1998).

2.8.3 Social behaviour and play

If the calf was housed with the dam, the nursing and contact time right after birth would be initiated, when the dam seeks the calf. This switches over the first 8 days, and the calf will be initiating the contact between cow and calf, and the cow will increase her socialization with other cows (Jensen, 2011). Over time, the calf would spend more and more time with other calves in the herd. Subtracting the time suckling the dam, the calf will, by the age of 2-3 weeks, spend more time with other calves, than with the dam (Munksgaard and Søndergaard, 2006). The social behaviour among calves are described by Reinhardt, Mutiso and Reinhardt, 1978 on *Bos Indicus* calves, with mounting and pushing with the heads of the calves against each other, as the most frequent social gestures, and is defined as play behaviour (Reinhardt, Mutiso and Reinhardt, 1978).

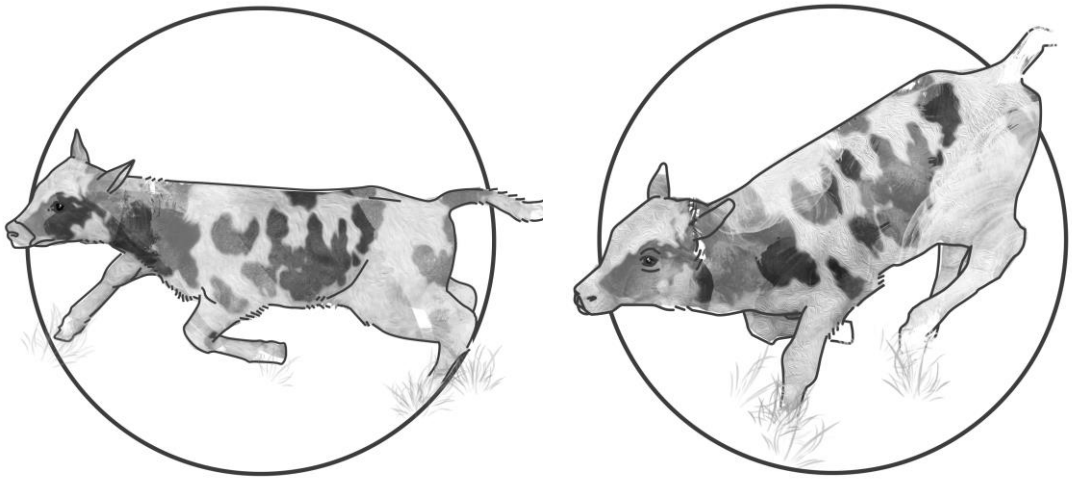

Figure 2.2a & 2.2b: Calves expressing play behaviour. Illustrations by Pawel Nowakowski.

Calves also express play behaviour alone, evaluated as locomotor play and straw play behaviour. Both behaviours can be expressed parallel to another calf, or alone. Locomotor play can consist of several movements, where the calf is galloping, changing directions, jumping, kicking with its hind legs, bucking and twisting its neck or head (Jensen, Duve and Weary, 2015). Straw play consists of periods where the calf is butting the straw in the stall or rubbing its head against it (Jensen, Duve and Weary, 2015).

The duration of social play behaviour and locomotor- or straw play is found to be a good indicator for the well-being of the calves (Jensen, Duve and Weary, 2015). Hence, decreased play behaviour, can indicate reduced welfare. Play behaviour is proven to be minimized around weaning (Krachun, Rushen and de Passillé, 2010; Jensen, Duve and Weary, 2015). Jensen, Duve

and Weary, 2015, found that group housed calves has a median daily playtime of 130 seconds per 24 hours.

Chua *et al*., 2002, estimated that calves housed in pairs in the age of 1 - 8 weeks spent approximately 30 minutes per 24 hours on social contact, and Duve and Jensen, 2012, found that calves housed in groups spent approximately 78 minutes in body contact with pen mates in the time period between 06:00 and 22:00, without any difference in age and previous housing conditions.

Table 2.1. The estimated time budget for calves, from the articles referred to in the text.

Behaviour	**Average seconds/24 h**	**Seconds at daytime (6:00-22:00)**
Lying time	60.480[2]	36.720[7]
	63.360[1]	
Standing time	4.121[2]	
Self-grooming	2.652[2]	
Moving	1.236[2]	
Playing	130[6]	
Social contact	1.754[2]	4.680[7]
Sucking on teat	3.257[2]	2.820[3]
Feeding solid food or chewing		2.808 – 3.402[4]
		5.760[5]
Cross-sucking	144[2]	

[1]Von Keyserlingk *et al.,* 2006.
[2]The time budget is done for a calf of 1-8 weeks, over a 24-hour period, housed in a group of minimum two individuals, with restrictive feeding and unlimited access to a non-nutritional teat. The percentage value are not significant, and therefore only a ruff estimate (Chua *et al.*, 2002a).
[3]Measured on calves fed ad libitum teat-feeding, on calves at 1-4 weeks of age (Appleby, Weary and Chua, 2001).
[4]Calves fed mixed ratio of chopped hay and concentrated feed, in the middle of the weaning period, at the age of 6 weeks (E K Miller-Cushon *et al.*, 2013).
[5]Only in the period between 7:00 and 17:35, with calves of up to 96 days of age (Veissier, Ramirez De La Fe and Pradel, 1998).
[6]estimated median by (Jensen, Duve and Weary, 2015)

According to Duve and Jensen, 2012, heifers have more active social behaviour, than bulls. Active social behaviour includes *social sniff* (sniffing to penmates with muzzles touching or within one- muzzle width of another calf), *allogrooming* (with the tongue of one calf in contact with the fur of another calf), *mock fight* (two calves pressing foreheads against each other) and *social butt* (rubbing the head against another calf). Heifers spent approximately 9 minutes, compared to bulls, that spent around 6 minutes with active social behaviour, per period of social activity (Duve and Jensen, 2012).

2.9 Motivation and priorities within normal behaviours

The behaviours described in the previous section, drinking milk, sucking, resting and play, are all behaviours that assumingly are motivated by both internal and external stimuli. When calves were deprived to rest, they spend 95% of the time resting, when they were allowed to (Munksgaard *et al.*, 1999). Cows rest up to 50% more in the resting periods after being deprived from lying down for 3 hours (Metz, 1985). This suggests that resting is highly motivated by internal stimuli, with concerns to Lorenz's psychohydralic model of motivation. This model suggests that the need to perform an internally stimulated behaviour builds up over time, if the animal is prevented from expressing that behaviour (Mason and Bateson, 2009). Furthermore, the frequency of change in behaviour has been shown to increase, when the cows were deprived from lying down (Munksgaard *et al.*, 1999). This might be a sign frustration, as the animal is willing to try other behaviours to satisfy the need for resting (Munksgaard *et al.*, 1999). Cows deprived from both social contact and lying down did also shift between different behaviours more frequent, than cows not deprived of social contact and resting. The proportion of eating increased for cows deprived of lying down, and self-grooming increased for the cows deprived of social contact (Munksgaard and Simonsen, 1996). Milk-drinking behaviour of calves may also be highly internally motivated, as the frequency and duration of visiting the milk-feeding teat or non-nutritional teat was increased when fed restrictively (Lidfors, 1993; Miller-Cushon *et al.*, 2016)(Miller-Cushon *et al.*, 2016). There is also a component of delayed negative feedback, combined with positive feedback in drinking and sucking behaviour, as increasing concentration of the milk, increased the non-nutritional sucking, and especially the lactose has shown to motivate the calves to suck (De Passillé, Christopherson and Rushen, 1993; De Passillé, Rushen and Janzen, 1997) The motivation to perform oral abnormalities are also higher in both restrictively fed and socially deprived calves (Veissier, Ramirez De La Fe and Pradel, 1998).

The motivation for playing was found to be highly internally motivated as well when measured on both rats and calves, as the frequency of play behaviours increased after a deprivation period (Jensen and Kyhn, 2000; Holloway and Suter, 2004). The motivation to perform locomotor play was also increased with the amount of days of locomotor activity deprivation (Jensen, 1999, 2001). Jensen *et al.*, 1999 showed no difference in time spend on locomotor play for calves at the age of 7-9 weeks in a well-known environment whether they had 1,5 m^2 or 4 m^2. However, calves at 10 weeks of age showed more locomotor play when let out in an arena after a period of

less space, suggesting that the age might alter the motivation for locomotor play (Jensen *et al*, 1999). Locomotor activity seems to be of higher priority than food, as locomotor deprived animals choose a large space over a smaller space with food (Jensen et al., 1999). However, there might be an issue measuring the motivation for the two activities, when the animals are not deprived from feed prior to the preference test. In a deprivation study done on lactating cows, with only limited access to perform social behaviours, lie down or eat, the proportion of time lying down increased, while the proportion of time spent on social behaviours and eating remained the same (Munksgaard *et al.*, 2005).

2.10 Pain specific behaviours

The term pain covers both an emotional response and a physiological response to a noxious stimulation. The animals physiological, behavioural and productive response to pain can be used to assess the degree of distress, and hereby the degree of pain the animal feels (Rutherford, 2002).

Physiological responses to pain are elicited by the activation of the sympathetic nervous system, and in relation to that the HPA-axis (Rushen *et al.*, 2008). Previous studies on dehorning of calves has used heart rate and serum cortisol as physiological indicators of pain (Boandl, Wohlt and Carsia, 1989; Wohlt *et al.*, 1994; Grøndahl-Nielsen *et al.*, 1999; Stafford and Mellor, 2005; Stewart *et al.*, 2009). The behavioural alterations might be a better indicator of stress, as alterations in the balance of positive and negative behaviour might have a direct consequence in the welfare of the animals (Moberg *et al.*, 2000). To use specific behaviours as indication of stress, there has to be an understanding of both the neuroendocrine processes and motivations to express the specific behaviour (Moberg *et al.*, 2000). Pain assessment by behavioural monitoring can be done using three different strategies; monitoring of pain specific behaviours, decline in frequency or duration of certain behaviours or choice of preference (Rushen *et al.*, 2008).

Acute pain can elicit following behaviours; increased attention to the damaged region, vocalization, reluctance to move or abnormal gait and aggression or quietness (Gaynor and Muir, 2015). Acute pain from the dehorning of calves without sedation or analgesics, has previously been monitored as escape behaviours such as back- or forward movements, rearing, tail wagging, tripping on forelegs and falling down (Taschke and Fölsch, 1994; Graf and Senn, 1999; Grøndahl-Nielsen *et al.*, 1999). These studies suggest that dehorning without analgesic treatment is unacceptable, with concerns to the experience of acute pain during the procedure.

The pain specific behaviours that can occur following disbudding includes headshaking, ear flicking, tail swishing, scratching or rubbing the horn buds with the hind feet or against the inventory and restlessness seen as frequently lying down and getting up (Taschke and Fölsch, 1994; Morisse, Cotte and Huonnic, 1995; Graf and Senn, 1999; Grøndahl-Nielsen *et al.*, 1999; Faulkner and Weary, 2000; Heinrich *et al.*, 2010; McMeekan *et al.*, 2011; Mintline *et al.*, 2013).

A study has found increased frequency in head shaking and ear flicking for 12 hours, respectively 24 hours, after hot iron disbudding in calves sedated with 0,2 mg/kg xylazine I.M., and administered 4,5 ml of 2% lidocaine HCl bilaterally in the occipital groove and local anaesthetic (Faulkner and Weary, 2000). Heinrich et al., 2010, found behavioural changes up to 44 hours after disbudding. Other studies have found that the increased expression of pain specific behaviours in calves after disbudding, occurs within 4 hours after the procedure (Taschke and Fölsch, 1994; Morisse, Cotte and Huonnic, 1995; Graf and Senn, 1999; McMeekan *et al.*, 2011). However, the study by Morrise *et al.*, 1995, only monitored for pain specific behaviours by continuous video monitoring for 4 hours before and after disbudding.

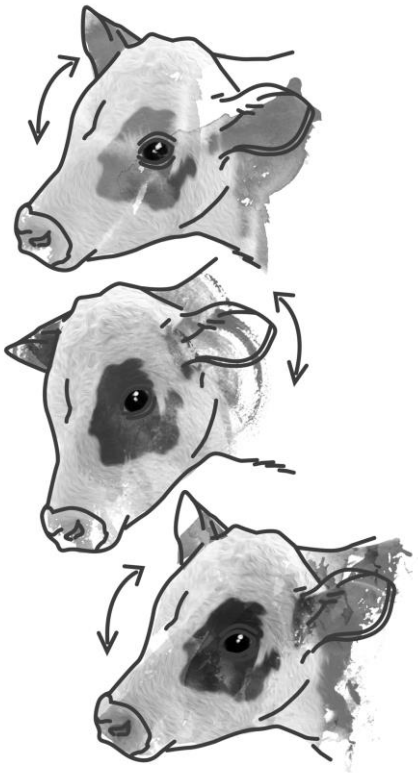

Figure 2.3: Head shaking calf. Illustrations by Pawel Nowakowski

The effect of administration of NSAIDs (Ketoprofen, Carprofen, Meloxicam and Flunixin) during and after disbudding has been evaluated several times (Heinrich *et al.*, 2009, 2010; Coetzee *et al.*, 2012; Huber *et al.*, 2013; Espinoza *et al.*, 2016; Stock *et al.*, 2016). Ketoprofen has been found to have significant effect on reducing both head shaking and ear flicking after disbudding, with the administration of 3 mg/kg 10% liquid ketoprofen P.O. in the milk, before and 2 and 7 hours after the procedure (Faulkner and Weary, 2000).

Meloxicam treatment has been found to reduce head shaking, but with no significant effect on head rubbing and tail swishing (Heinrich *et al*, 2010).

However, the sensitivity of the disbudding wounds has been shown to be elevated for up to 75 hours after disbudding (Heinrich *et al.*, 2010; Mintline *et al.*, 2013). Measures of plasma cortisol level after disbudding with subcutaneously injection of lidocaine bilaterally in the occipital groove, has shown a fast increase in plasma cortisol that peaks 1 hour after the procedure, but rises even higher at 24 hours after the procedure, in calves disbudded with the use of local anaesthetics (Morisse, Cotte and Huonnic, 1995).

These results are suggesting that the inflammatory mediators of the wound or neural alterations associated with disbudding, has an impact on the nociceptive threshold for a longer period, than suggested by the previous behavioural studies. Facial expression has also been shown to be significantly different for horses and cows with pain compared to animals not suffering from pain. (Gleerup *et al.*, 2015).

Figure 2.4: Head scratching calf. Illustrations by Pawel Nowakowski.

The characteristics of a bovine pain face, have been described by Gleerup *et. al.*, 2015, as potential tension in four areas, the facial muscles, the muzzle, the eyes and the ears. The muscles enrolled in the expression is noted in the following parenthesis. Tension in facial muscles, can be seen as tension from the muzzle and alongside the head (zygomatic muscle, buccinator muscle) (Popesko, 2007; Liebich, Maierl and König, 2009; Gleerup *et al.*, 2015). Tension around the nostrils that can result in dilation of the nostrils and/or grooves around-, and tension in the lips (levator muscle of the upper lip, nasolabial levator muscle, canine muscle, apical dilator muscle of nostrils) (Popesko, 2007; Liebich, Maierl and König, 2009; Gleerup *et al.*, 2015). The eyes can be seen with grooves above the eyes and/or the calf can appear absent with an *empty look* (levator of the medial angle of the eye, orbicular muscle of eye) (Popesko, 2007; Liebich, Maierl and König, 2009; Gleerup *et al.*, 2015). The ears can possibly be seen lowered with and altered angle, that makes the pinnae of the ear horizontal, or can be turned straight backwards without an obvious reason (parotid-auricular muscle) (Popesko, 2007; Liebich, Maierl and König, 2009; Gleerup *et al.*, 2015). The current study has based the observations of pain face and pain induced altered angulation of the ears on the study by Gleerup et al., 2015.

2.11 Alterations in frequency or magnitude of normal behaviours

In relation to disbudding, alteration in frequency or duration of the following behaviours are seen: time resting or sleeping, feeding, playing and grooming (Morisse, Cotte and Huonnic, 1995; Graf and Senn, 1999; Grøndahl-Nielsen *et al.*, 1999; Faulkner and Weary, 2000; Stafford and Mellor, 2005; Taschke and Fölsch, 1994; Heinrich *et al.*, 2010; Mintline *et al.*, 2013)
Play behaviours has been found to be decreased up to three hours after disbudding, and social behaviours in general up to 24 hours after disbudding (Taschke and Fölsch, 1994; Morisse, Cotte and Huonnic, 1995; Mintline *et al.*, 2013). For up to 12 hours after disbudding, a decrease in feeding time and an increase resting time has been seen (Morisse, Cotte and Huonnic, 1995; Taschke & Fölsch, 1994). However, there was no significant alterations of feeding, locomotion or resting behaviours in the study done by Faulkner-Weary, 2000.
Ratio of standing and lying time was found to be similar in 24 hours prior to and 24 hours after disbudding (Morisse, Cotte and Huonnic, 1995). However, this study by Morrise et al., 1995, shows no indication of how the distribution is throughout the 24 hours around disbudding, as observations were done in one minute every 15 minutes, throughout 24 hours before- and after disbudding. The ratio was then calculated as a percentage of the time.
In a study done over 7 days, comparing calves treated with one dose of 0,5 mg/kg oral meloxicam on the day of disbudding (day 0), the calves were shown to spend more time lying down on day 1, 2, 3 and 4 compared with the non-treated control group. The two groups had similar lying patterns after day 5 (Theurer *et al.*, 2012). By daily monitoring in 4-5 hours on day 0, 1 and 2, the similarity in time spent on lying, grazing or ruminating, tail shaking and ear flicking, was approximately the same between calves treated with ketoprofen and local anaesthetic prior to disbudding, and calves that were sham disbudded (McMeekan *et al.*, 2011).
A greater proportion of the calves disbudded without receiving any analgesic treatment were lying down two hours after the procedure. Furthermore, a greater proportion of the total number of calves receiving either local anaesthetic, ketoprofen or none of these, were lying down 4 hours after disbudding, compared to the calves receiving both local anaesthetic and ketoprofen during disbudding (McMeekan *et al.*, 2011).
Pain is also affecting the emotional state of the calves, as calves might experience a negative emotional stat for up to 22 hours (Neave *et al.*, 2013). This negative emotional state might alter their motivation for expressing normal behaviours, as this study showed a decrease in motivation for feeding (Neave *et al.*, 2013).

3 Materials & Methods

3.1 Herds

Three herds of 23 (H1), 24 (H2) and 12 (H3) Danish Holstein heifer calves were included in the study. H2 and H3 were housed at the same location. The herds were selected on the basis of sufficient number of calves. The farms were located in the western Denmark in cooperation with Skovbjergs Veterinary Team.

3.2 Animals

The study was done on Holstein heifer calves, also known as Holstein-Friesian, which is the main breed used for dairy production in Denmark. The origin of Holstein cattle is in the Northern part of the Netherlands, Friesland and the region of southern Denmark, known as the Slesvig-Holstein region. The breed is included the species called *Bos Taurus*.
Inclusion criteria were milk-fed, healthy Danish Holstein heifer calves aged 10 – 60 days, due for disbudding at the start of the study. They should have been housed in the same pen for at least five days prior to the start of the study. Health status was evaluated through a thorough clinical examination (see table 3.3) on the day prior to disbudding (day -1). The exclusion criteria for day -1 can be seen in the Table 5.1.
To estimate a difference between means when calculating on the weight, the sample size was calculated to a minimum of 25 animals per group, with a standard deviation (SD) of 0,15, confidence level of 0.95 and a power of 0,8.
Number of calves included in the study was 64, a number later reduced to 56. Three calves were excluded due to disease and 1 was a non-dairy breed. The respective pairs were excluded, resulting in a sample size of 56 (see appendix 1).

3.3 Housing and feeding

In all herds, calves were housed in group pens (6x4 meters) with straw bedding. The number of calves per pen was 6 at H1 and 10 at H2 and H3, which gives a space of 4 m^2/calf at H1, and 2.4 m^2/calf at H2 and H3.

H1 had two water cups per pen and fed ad libitum hay and concentrate. H2 and H3 had one water cup per pen and fed calf muesli ad libitum and Total Mixed Ratio (TMR) cow ration twice daily after milk feeding. None of the herds offered dry teats

3.3.1 Milk-feeding

Both herds were restrictively fed milk twice daily. H1 were fed at 05:00 in the morning and 17:30 in the evening. H2 and H3 were fed around 10:00 in the morning and 17:30 in the evening. H1 were fed milk in a common trough, whereas H2 and H3 were fed twice per feeding in individual 5 litre bowls. All calves were fed a combination of milk from cows with too high a cell count, mixed with milk formula, resulting in a dry matter percentage of 16% at H1 and 14% at H2 and H3. Furthermore, the milk for H2 and H3 had pectin and Fibermax® (Vitfoss, Gråsten, DK) added to it.

Table 3.1 Criteria for the exclusion of calves on day -1.

Organ system	Definition of exclusion criteria
Pathology in airways	Uni- or bilateral mucopurulent discharge from nostrils. Abnormal lung sounds on auscultation of the lungs.
Pathology in the gastrointestinal tract	Diarrhea (characterised by profuse watery manure of normal or abnormal colour) and dehydration (characterised by skintugor > 3 seconds on the side of the neck, sticky or dry oral mucosa or an abnormal eyeball position (enophtalmus)).
Other causes of exclusion	Suspicion of otitis (head tilt, altered angle of one ear) Arthritis, characterised by increased heat, swelling and soreness of one or more joints by palpation and altered gait on the affected limb. Fever, characterised by a rectal body temperature > 40 °C Abnormal general appearance, characterised by minimal or no responsiveness to surroundings. Conformational abnormalities, characterised by angular limb deformities or flexor limb deformities that inhibit a normal gait and thereby locomotor activity. Medical treatment from one day prior to, or during the study. Non-dairy breeds

3.4 Experimental design

The study was done in two sections, one part focusing weight gain, and one part focusing on pain related behaviour, facial expression and ear position. The experimental design was done as case-control study over time. The timeline of the study is presented in table 3.2.

Table 3.2: Timeline of the study.

Procedure	Day -1	Day 0	Day 1	Day 2	Day 3	Day 4	Day 14
Disbudding		+					
Treatment meloxicam		+	(+)	(+)	(+)		
Clinical examination	+		+	+	+	+	
Video recording	+	+	+	+	+	+	
Direct observation	+	+	+	+	+	+	
Weighing	+					+	+

(+): Calves in the treatment group were treated with meloxicam S.C.

The calves were randomly allocated to treatment and paired between treatment groups according to age and weight on day -1. The allocation of the calves to either treatment or control group was done by the author's supervisors, so that the observers were blinded to the treatments during the whole trial period.

All calves received the same treatment on day 0, 10 minutes prior to disbudding, which consisted of a fixed dose 2 ml Metacam® (20 mg/ml meloxicam, Boehringer Ingelheim Vetmedica, Inc.) S.C. The following 3 days (day 1-3), the treatment group received one dose of Metacam® (20 mg/ml meloxicam, Boehringer Ingelheim Vetmedica, Inc.), equal to 0,5 mg/kg meloxicam per day. Treatments were conducted every day around noon, whilst the control group received no treatment. The treatments at day 1-3 were conducted by veterinarians from Skovbjergs Veterinary Team.

3.5 Disbudding procedure

The disbudding took place at day 0 between 08:30 and 12:00. At all herds, the person in charge was a veterinarian from Skovbjergs Veterinary team, assisted by the observers.

The calves were sedated with a fixed dose of 1,5 ml Rompun® Vet. (20 mg/ml xylazinhydrochlorid, intramuscularly Bayer, Animal health division).

When the calves were lying down, local anaesthetic was injected with a 1,5-inch needle directed between the lateral canthus of the eye and the horn bud, into the occipital groove, where the whole dose was deponated to anaesthetize the corneal nerve. On H1 and H3, the local anaesthetic used was 5 ml of Pronestetic (40 mg/ml procainhydrochloride and 0,036 mg/ml epinephrintartrat, FATRO S.p.A., Bologna, Italy). At H2 the local anaesthetic used was 8 ml of Procamidor Vet. (20 mg/ml procainhydrochloride, Salfarm Danmark A/S). The differences between those two drugs are the concentration and the added epinephrinetartrat in Pronestetic. The epinephrinetartrat gives a prolonged duration for Pronestetic is 45-90 minutes

(*Produktresumé, Pronestetic*, 2016) due to the vasoconstrictive effects. The shorter duration of Procamidor is 30-60 minutes, with a T½ at 1-1,5 hours (*Produktinformation, Procamidor*, 2016). The difference in local anaesthetic used, was due to the cooperating veterinarian's available products. In conjunction with the local anaesthetic, all the calves were treated with a fixed dose of 2 ml/calf of Metacam® inj. (20 mg/ml meloxicam, Boehringer Ingelheim Vetmedica, Inc.) administered subcutaneously. Ten to fifteen minutes after the local anaesthetics were distributed, the disbudding procedure started. The horns were cut off at the horn base with a hoof knife, if the horn bud was large enough.

The irons were gas-heated until they were glowing red, which is approximately 600°C, and the horn buds were cauterized for approximately 20 seconds.

3.6 Clinical examination

Each pen was randomly assigned to each observer, and the calves were clinically examined on day -1, 1, 2, 3 and 4 (see table 3.2.). Any notable clinical findings in accordance with exclusion criteria are noted in appendix 1. Clinical examination was conducted between 15:00 and 18:00 on day -1, and between 09:00 and 12:00 before the treatments on day 1-4. The definitions of the parameters evaluated in clinical examinations can be seen in table 3.3. Each calf was examined by the same observer every day.

3.7 Weighing

The calf where weighed on day -1, 4 and 14 on a Tokyo transportable horse scale (Horse weigh, Powys, UK) (see table 3.2.). The scale was calibrated with a water container weighing 11.2 +/- 0,1 kg before and twice during all weighing sessions. The calves were either individually herded or caught with a halter, and guided onto the scale. Some calves were weighed twice to control the scale.

Table 3.3: Clinical recordings and definitions

Clinical parameter	Definition
General attitude	Normal bright, alert and responsive, or depressed, in comparison with the rest of the pen mates.
Temperature (°C)	Rectal temperature, measured with a thermometer.
Heart rate	Obtained by auscultation of the heart.
Respiration rate	Obtained by counting the respiration from a distance when possible, or when auscultating.
Central circulation	Auscultation of the heart: two clearly divided heart sounds without

	murmurs, or arrhythmias.
Peripheral circulation	Capillary refill time (CRT) on vaginal mucosa, evaluation of temperature of ears, back and legs.
Respiration	Inspection of breathing pattern (thoracic/abdominal) and auscultation of the lung field bilaterally, with normal being thoracoabdominal.
Hydration	Normal or abnormal position of the eye in the orbit, skin-tugor on the neck, palpation of the oral mucosa and CRT, with dehydration characterized as sunken eyeballs, sticky or dry oral mucosa and CRT and skin tugor > 3 seconds.
Eyes	Position of eyelids and cilia angle. Epiphora or orbital exudation., If present characterized as serous, cloudy of mucopurulent.
Ears	Angulation of ears and head, in relation to otitis. If present, characterized as, uni- or bilateral drop of ears, with or without head tilt.
Nose	Nasal discharge. If present characterized as serous, cloudy of mucopurulent.
Faeces	Consistency and coloration, with normal being dry-cow manure and light brown to dark yellow in colour.
Skin and hair	Signs of lesions, hair loss or ectoparasites.
Swing auscultation	Auscultating for abnormal sounds of diarrhea on the right side of the calf, characterized as splashing sounds.
Filling of the rumen and abdominal shape	Inspection of the filling of the abdominal space behind the last rib on the left side of the calf, and the general impression of the abdominal shape, with normal characterized as either well filled rumen or tight round shape of the abdomen, depending on the age and function of the rumen.
Body condition	Normal, thin or emaciated.
Hyperalgesia testing	By gentle palpation on dorsal part of the neck, to evaluate the activation of the panniculus reflex as a measure of sensitivity.

3.8 Video recording and direct observation

The calves where video monitored for 90 minutes around milk-feeding on day -1 – 4 (see table 3.2.), with 30 minutes before milk feeding in the evening, and 60 minutes after milk-feeding. The cameras used were GoPro Hero4 cameras with housing that could be mounted on the side of the pens at H1, and on a wall opposing the pens at H2 and H3. The cameras held SD-card ranging from a size of 32-64 GB. When change of battery was necessary, this was timed with the feeding when possible, to disturb the calves as little as possible. The total time of 90 minutes was chosen as one battery could record for approximately 100 minutes. The time around feeding was chosen to observe a period when the calves were both passive and active, and to be able to measure the time from feeding to first attempt to lie down.

At the same time of video recording, the calves were observed undisturbed, from a distance with binoculars to score whether the calves were expressing the described characteristics of *pain face* or *pain ears*. The characteristics of *pain face* and *pain ears* as illustrated in figure 3.1a and 3.1b. Both observers took part in the direct observing. At H1, the direct observation was conducted from an open area in front of the pens, at minimum distance of 8 meters. The observers were positioned at the observation points from approximately 15 minutes before the cameras were started, so the calves were used to their presence. At H2 & H3, the direct observation was conducted from an elevated position behind a wall of approximately 3 meters, 3 meters from the pens. Both observers had received an introduction to the observation of *pain face* and *pain ears* prior to the study.

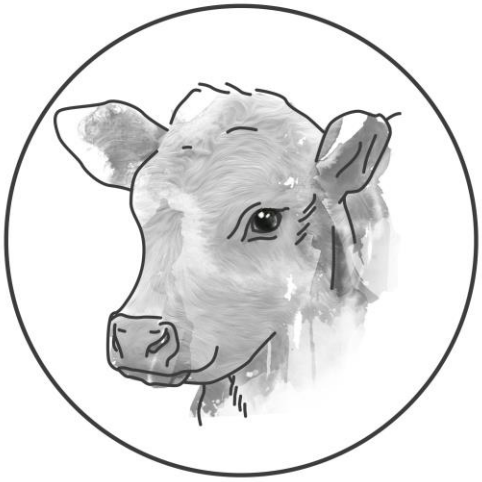

Figure 3.1a: Calf with normal face.
Illustrations by Pawel Nowakowski.

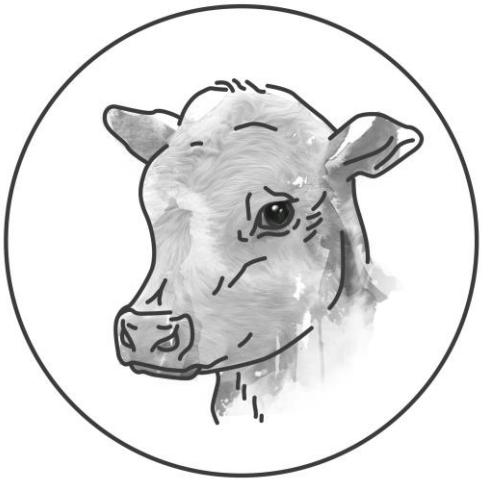

Figure 3.1b: Calf with pain face and pain ears.
Illustrations by Pawel Nowakowski.

3.9 Video analysis

To get an equal number of calves in both treatment and control group, and maximize the sample size, the video length was reduced from 30 minutes before feeding and 60 minutes after feeding to 14 minutes before feeding and 25 minutes after feeding, which resulted in a sample size of 50 calves. Video scoring was conducted using the program ChronoViz for iOS.
The video analysis of each calf, were conducted by the same observer throughout the study. Video sequences ranged between 0,5 - 20 min. When scoring the calves, an annotation was made every time the calves expressed any of the behaviours described in table 3.4. The scoring was

done for 90 minutes per calf and day, with 30 minutes prior to-, and 60 minutes after feeding, with the annotations subtracted according to the fixed time frame afterwards.

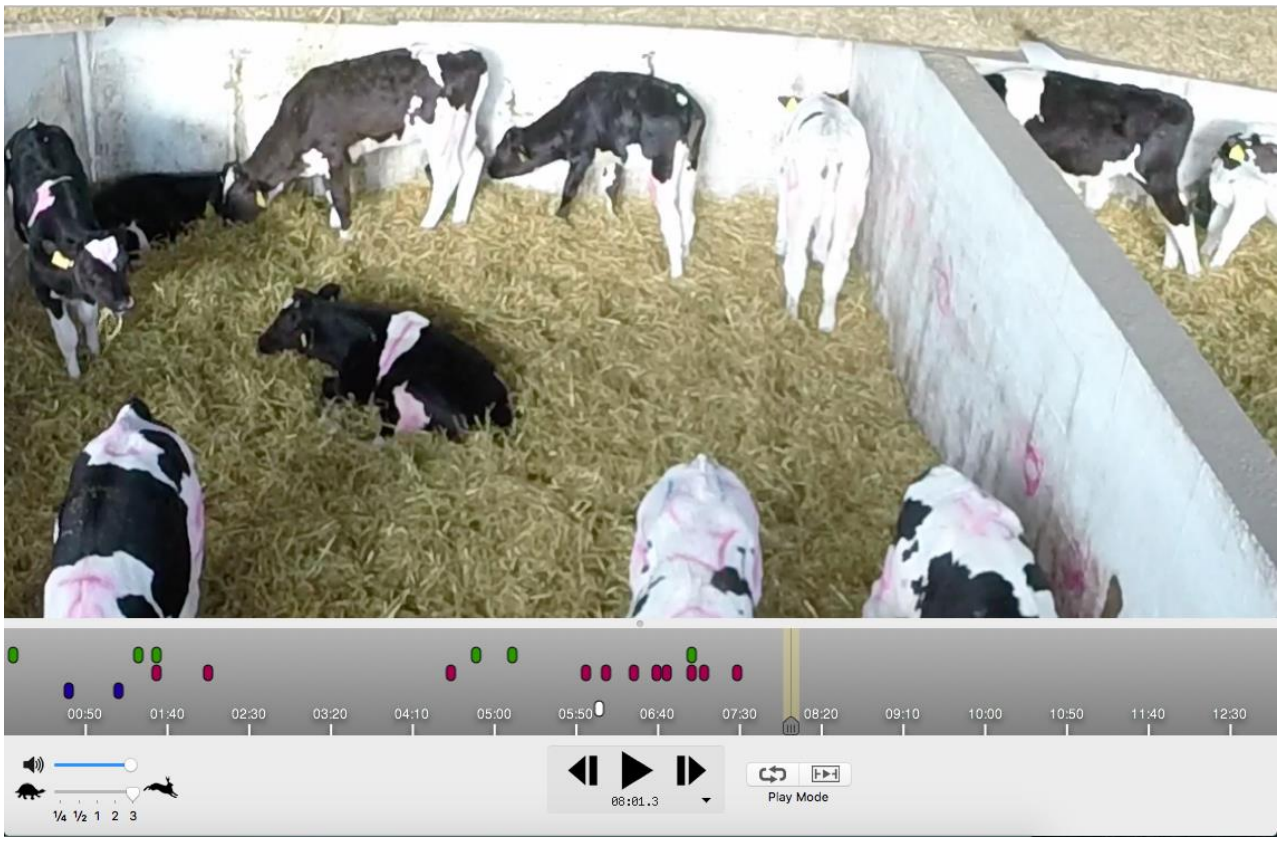

Figure 3.2: Screenshot from the scoring of calves using ChronoViz.

The videos were viewed and scored in an increased speed of 1 – 4x real-time. The annotations were done discriminating between points and durations, according to the definitions in table 3.4. The data was then exported through csv-files to Microsoft Excel, where descriptive statistics where analysed before the final statistical analysis where conducted using Graphpad Prism for iOS. Both observers were blinded to treatments throughout the whole video analysis period.

Table 3.4: Definitions of behaviours evaluated from the video monitoring.

Recording type[1]	**Behaviour**[2]	**Definition**
I	Head rubbing/scratching	Scratching around the wounds with hind feet or scratching wound area against inventory (fig. 2.4).
I	Head shaking	Shaking or throwing the head in any direction, without any obvious reason. Violent or non-violent (fig. 2.3).
I	Ear flicking	Flicking the ears, backwards and forwards, without an obvious reason.
I	Tail swishing	Swishing the tail from left to right in a quick motion, without an obvious reason.

C	Trying to lie down or lying down for the first time.	Carpus is bended, or the whole body is resting on the bedding (with bended front- and hind legs either in sternal recumbency or lateral recumbency). Head in any position.
C	Play	Locomotor play Galloping: moving continuously forwards in high speed (fig. 2.2a & 2.2b). Jumping: front legs and hind legs are lifted and stretched forwards alternately, while moving forwards. Bucking: Hind legs are lifted from the ground and stretched out. Kicking: One or two hoofs on hind legs are lifted from the ground above knee level, and stretched backwards, kicking in a posterior direction. Straw play: Carpus is bended bilaterally, while neck or head are forced through bedding material. Social play head pressing: pressing head against any part of another calf. bullfighting: calves standing with foreheads against each, or opposes each other with the head lowered prior to touching forehead against forehead. mounting: jumping on to another calf from either sides, with both front legs lifted from the ground, or rubbing ventral head on the sacral area of another calf prior to jumping.
C	Oral abnormalities	Non-nutritional sucking on any part of the body of another calf, or licking on walls or inventory of the pen.
C	Time resting/sleeping	Lying down in either sternal recumbency or lateral recumbency with the head in any position.

[1] C = continuous recording, I = instantaneous recording

[2] Jensen *et al.*, 1998, Jensen *et al.*, 2011, Duve and Jensen, 2012, Graf and Senn, 1999,

3.10 Statistical analysis

For the statistical analysis, the program Graphpad Prism for iOS was used.

To evaluate the effect of 4 days of meloxicam treatment on weight gain and pain-related behaviours during 14 days after disbudding, the hypothesis' were tested using a 95 % confidence interval. All data were analysed for normal distribution, and several non-parametric analytical tests were used to confirm or reject the null hypothesis', see table 3.5.

The majority of the data not normally distributed, and the sample size were relatively small, why

non-parametric tests were used. For the behavioural data, a non-parametric Man-Whitney test were used between the groups, and a Wilcoxon Signed Rank paired t-test within the groups. The weight calculations were done using the Wilcoxon paired t-test, even though the data sets followed a normal distribution. Table 3.5 lists the different analyses conducted, with the respective sample size and method of analysis used.

Table 3.5: Number of calves and method of analysis

Analysis	**Number of calves**	**Method of analysis**
Pain specific behaviour (ear flick, head shake, head rubbing/Scratching) Tail swish Play behaviours Time lying resting Oral abnormalities	*n* =50	D'agostino & Pearson normality test. Within groups: Wilcoxon matched pairs signed rank test (paired non-parametric two-tailed exact p-value (*p*=0,05) with 95% CI). Between groups: Mann-Whitney test (unpaired, non-parametric, two-tailed exact p-value(*p*=0,05) with 95% CI).
Time lying down 1st time after feeding	*n* = 34	D'agostino & Pearson normality test. Within groups: Wilcoxon matched pairs signed rank test (paired non-parametric two-tailed exact p-value (*p*=0,05) with 95% CI). Between groups: Mann-Whitney test (unpaired, non-parametric, two-tailed exact p-value(*p*=0,05) with 95% CI).
Pain face	*n* =20-28	Within and between groups: Two-sided p-value by Fisher's exact test (chi-squared). OR with 95% CI with Baptista-Pike method.
Pain ears	*n*=14-30	Within and between groups: Two-sided p-value by Fisher's exact test (chi-squared). OR with 95% CI with Baptista-Pike method.
Hyperalgesia	*n* =56	Within and between groups: Two-sided p-value by Fisher's exact test (chi-squared). OR with 95% CI with Baptista-Pike method.
Weight	*n* =56	D'Agostino & Pearson normality test. Wilcoxon matched pairs signed rank test (paired non-parametric two-tailed exact p-value (*p*=0,05) with 95% CI)

4 Results

The study on weight was done on 56 Holstein calves with a mean age of 34,43 days ±SD of 13,96 days, and a mean weight of 69,03 kg ±SD of 13,41 kg. The analysis of behaviour was done on 50 of the mentioned 56 calves, as 6 were excluded due to technical issues in the video recording on day -1. The 50 calves included in the majority of the behavioural analysis had a mean age of 32,46 days ±SD of 13,4 days, and a mean weight of 67,45 kg ±SD of 13,05 kg. The results of all analysis' conducted, were calculated using Prism. The results are further presented in Appendix 3.

4.1 Weight

There was no significant difference found, when comparing bodyweight of calves, between treatment and control on day -1, 4 or 14. The was a significant difference found in the total weight gain pr. calf, when comparing the treatment group with the control group on day -1 – 4 (see figure 4.1).

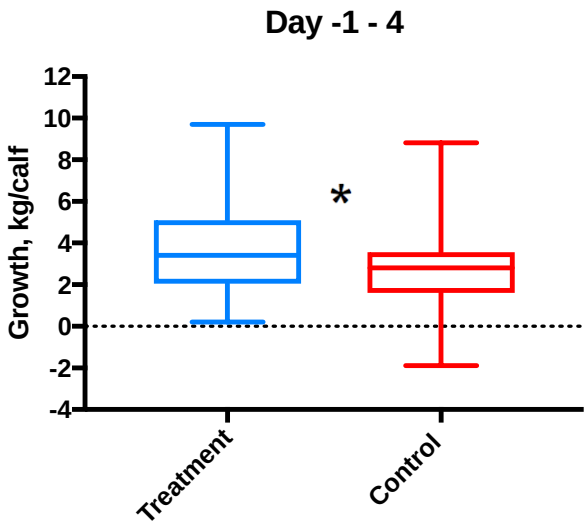

Figure 4.1: Total weight gain/calf, between day -1-4.
* Significant value, $p<0,05$

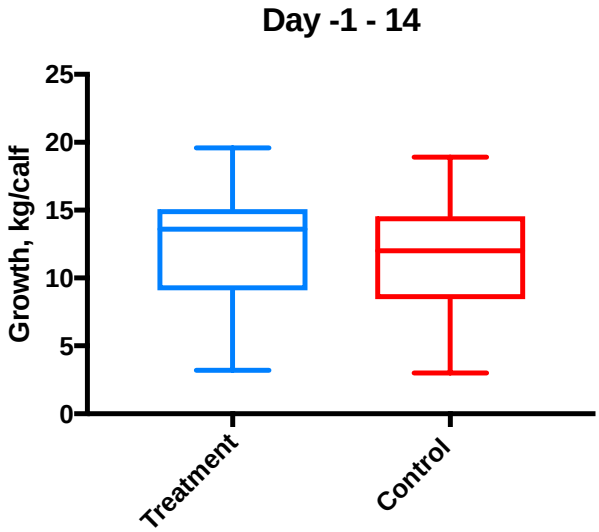

Figure 4.2: Total weight gain/calf, between -1-14.

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Signe Rejnhardt Olsen, jrb642

Table 4.1: Mean total weight gain pr. calf in between groups. Control *n* = 28, Treatment *n* = 28. Unit measured = kg.
* Significant value, *p*<0,05

	Control			Treatment			*p*-value
Period (day)	Mean	95% CI	±SEM	Mean	95% CI	±SEM	
-1-4	2,664	[1,824;3,504]	0,4094	3,918	[2,972;4,864]	0,4609	0,0345*
4-14	8,939	[7,628;10,25]	0,6391	8,439	[7,238;9,641]	0,5856	0,2556
-1-14	11,55	[9,982;13,12]	0,7658	12,36	[10,75;13,97]	0,7843	0,5608

Table 4.2: Mean bodyweight pr. calf in between groups. Control *n* = 28, Treatment *n* = 28. Unit measured = kg.

	Control			Treatment			*p*-value
Day	Mean	95% CI	±SEM	Mean	95% CI	±SEM	
-1	68,98	[63,75;74,2]	2,546	68,9	[63,74;74,07]	2,519	0,3351
4	71,78	[66,49;77,08]	2,581	72,82	[67,63;78,01]	2,53	0,4896
14	80,71	[75,89;85,54]	2,35	81,26	[75,96;86,56]	2,583	0,9294

4.2 Behaviour

Time resting or sleeping:

There was significant difference between the groups on day 4 (*p*=0,0473) (see table 4.3). Within the groups there was significant difference between all days in both the treatment group (*p* < 0,0001 all days) and control group, on day -1-1 (*p*=0,0007), day -1-2 (*p*=0,0147), day -1-3 (*p*=0,0013), and day 1-4 (*p*=0,0081) (see appendix 3).

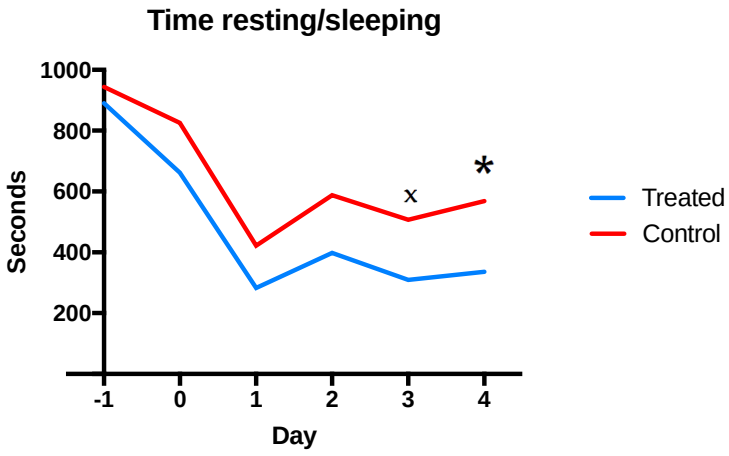

Figure 4.3: Seconds of time resting or sleeping, mean value.
* Significant value, *p*<0,05; ^{x}p<0,06

Table 4.3: Time resting or sleeping, Control *n = 25*, treatment *n = 25*.
* Significant value, *p*<0,05; ^{x}p<0,06

	Control			Treatment			*p*-value
Day	Mean	95% CI	±SD	Mean	95% CI	±SD	
-1	943,5	[726,9;1160]	524,8	890	[707,1;1073]	443,1	0,7437
0	825,4	[566,5;1084]	627,2	661,8	[459,9;863,6]	489	0,4074
1	422	[290,6;553,3]	318,2	282,9	[158,8;407]	300,6	0,1137
2	588,3	[398,5;778,1]	459,9	397,5	[290,5;504,5]	259,2	0,1970
3	506,8	[339,5;674,1]	405,3	309,5	[179,3;439,7]	315,5	0,0546^{x}
4	568,4	[399,8;737,1]	408,5	336,5	[190,4;482,7]	354	0,0473*

Adam Rääf, rwc467

Signe Rejnhardt Olsen, jrb642

Time from feeding to first attempt at lying down: Between the groups there were significant difference on day 3 (p=0,0403)(see table 4.4). Within the treatment group there was significant difference between day -1 and 1 (p=0,0150), day -1 and 3 (p=0,0305) and day -1 and 4 (p = 0,0032). Within the control group there were significant difference between day -1 and 1 (p=0,0024) (see appendix 3).

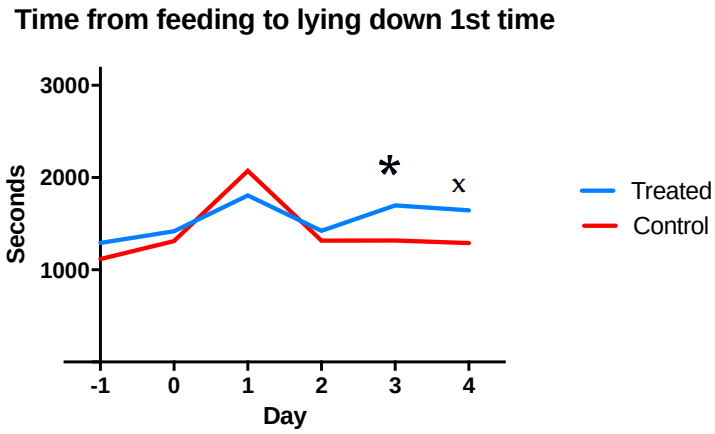

Figure 4.4: Seconds from feeding to first attempt of lying down, mean value.
* Significant value, p<0,05; ^{x}p<0,06

Table 4.4: Seconds from feeding to first attempt of lying down. Control n = 25, Treatment n = 25
* Significant value, p<0,05; ^{x}p<0,06

	Control			Treatment			p-value
Day	Mean	95% CI	±SD	Mean	95% CI	±SD	
-1	1117	[952,9;1280]	318,3	1291	[1053;1529]	463,5	0,2630
0	1310	[921,8;1699]	755,4	1418	[1010;1826]	793,2	0,5922
1	2074	[1530;2617]	1057	1808	[1540;2075]	520,2	0,4745
2	1315	[1050;1580]	515,3	1423	[1239;1607]	358,4	0,5460
3	1319	[1044;1594]	535,3	1698	[1458;1938]	466,5	0,0403*
4	1289	[1034;1545]	496,9	1644	[1393;1895]	488,6	0,0586 ^{x}

Play behaviour:
Significant difference between the groups was found on day -1 and 3 (p=0,0229, p=0,0174)(see table 4.5). Within the treatment group, significant difference was found between day -1 and 1 (p=0,0153). Within the control group, significant difference was found between day -1 to 2 and day -1 to 4 (p=0,0215, p=0,0172)(see appendix 3).

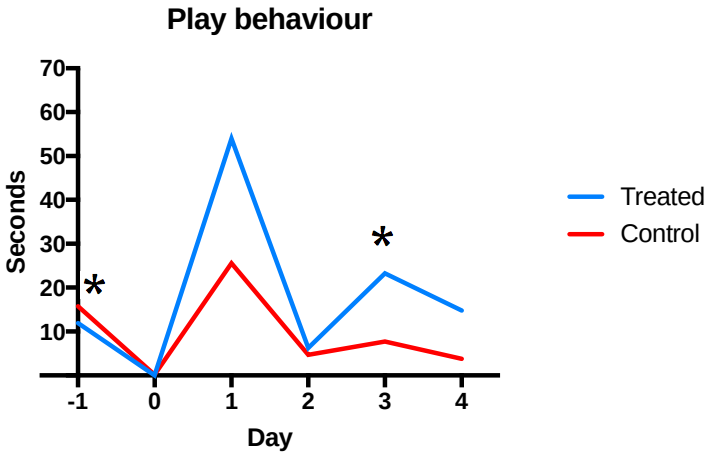

Figure 4.5: Seconds of play behaviour, mean value.
* Significant value, p<0,05

Adam Rääf, rwc467
Signe Rejnhardt Olsen, jrb642

Table 4.5: Play behaviours. Control n = 25, Treatment n = 25
* Significant value, p<0,05

	Control			Treatment			p-value
Day	Mean	95% CI	±SD	Mean	95% CI	±SD	
-1	15,8	[-6,52;30,4]	21,8	11,9	[1;4]	44,7	0,0229*
0	0,12	[0;0]	0,6	0	[-0,128;0,368]	0	>0,9999
1	25,6	[0,624;50,5]	60,4	54	[18,6;89,4]	85,7	0,2053
2	4,68	[-0,135;9,49]	11,7	6,28	[-1,84;14,4]	19,7	0,9334
3	7,72	[-1,12;16,6]	21,4	23,2	[10;36,4]	32	0,0174*
4	3,76	[-1,4;8,92]	12,5	14,8	[-5,69;35,3]	49,6	0,7616

Oral abnormalities:

Significant difference was seen between the groups on day 3 and 4 (p=0,0209, p=0,0020)(see table 4.6). No significant difference was seen within either of the groups.

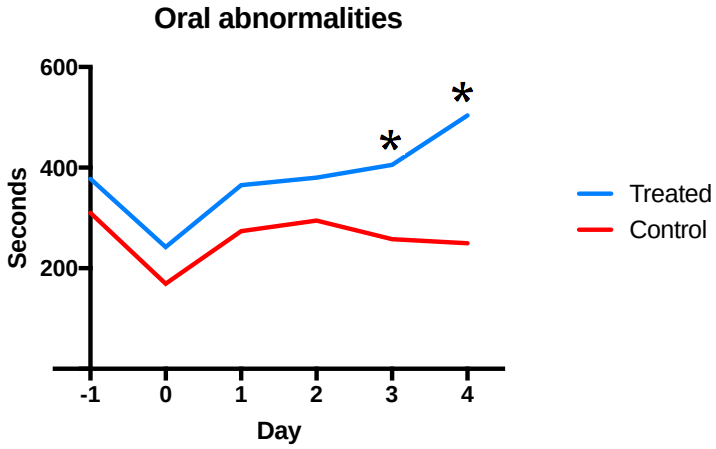

Figure 4.6: Seconds of oral abnormalities, ie: cross-sucking or licking on inventory, mean value.
* Significant value, p<0,05

Table 4.6: Seconds of oral abnormalities, ie: cross-sucking or licking on inventory. Control n = 25, Treatment n = 25
* Significant value, p<0,05

	Control			Treatment			p-value
Day	Mean	95% CI	±SD	Mean	95% CI	±SD	
-1	310,4	[229,1;391,7]	197	377,6	[287,4;467,8]	218,5	0,2548
0	169,4	[80,66;258,1]	214,9	242,3	[148,3;336,4]	227,9	0,2140
1	273,9	[195,3;352,5]	190,4	365,3	[283,5;447,1]	198,2	0,1111
2	294,7	[203,5;385,9]	220,9	380,4	[296,6;464,2]	203	0,1522
3	257,5	[194,8;320,2]	151,8	405,9	[314,5;497,2]	221,3	0,0209*
4	249,9	[167,8;331,9]	198,8	503,6	[370,5;636,7]	322,5	0,0020*

Pain specific behaviours:

Significant differences between the treatment- and control group were seen on day 3 (p=0,0279)(see table 4.7). Within the groups there was significant difference in the treatment group between day -1 and day 1 (p=0,0001), and in the control group between day -1 and day 1 (p=0,0024)(see appendix 3).

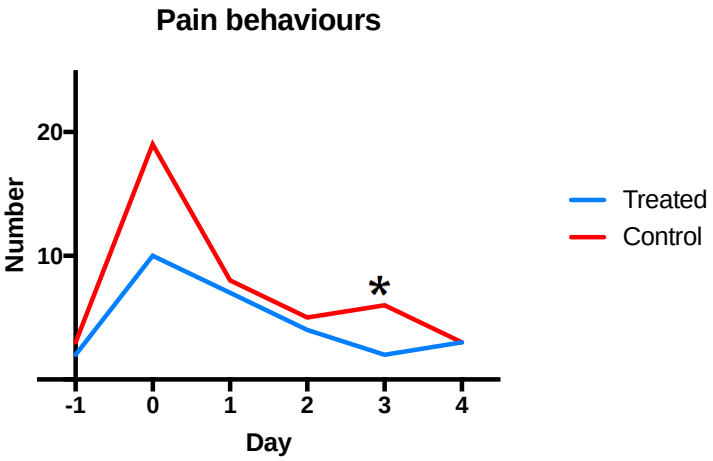

Figure 4.7: Total number of pain specific behaviours; ear flicks, head shakes and head scratch or head rub, median. * Significant value, p<0,05

Table 4.7: Total number of pain specific behaviours; ear flicks, head shakes and head scratch or rub. Control group n = 25, treatment group n = 25. * Significant value, p<0,05

	Control		Percentiles		Treatment		Percentiles		p-value
Day	Median	95% CI	10%	90%	Median	95% CI	10%	90%	
-1	3	[1;5]	0,6	11,4	2	[1;4]	0	5,4	0,2507
0	19	[11;29]	0,6	121	10	[4;41]	1	158,6	0,6753
1	8	[6;12]	1,6	28,6	8	[4;11]	1	18,4	0,4602
2	5	[3;8]	0	13,4	4	[1;6]	0	12,2	0,2089
3	6	[2;9]	1	12,8	2	[1;4]	0	9,8	0,0279*
4	3	[2;9]	0,6	15,6	3	[1;5]	0	13,6	0,3851

Tail swishing:

There was no significant difference between treatment and control groups on any day (see table 4.8). There was significant difference within the treated group when comparing day -1 with day 1 (p <0,0001), day 2 (p=0,0426) and day 4 (p=0,0267). There was significant difference within the control group, when comparing day -1 with day 1(p=0,0144) and 4 (p=0,0131)(see appendix 3).

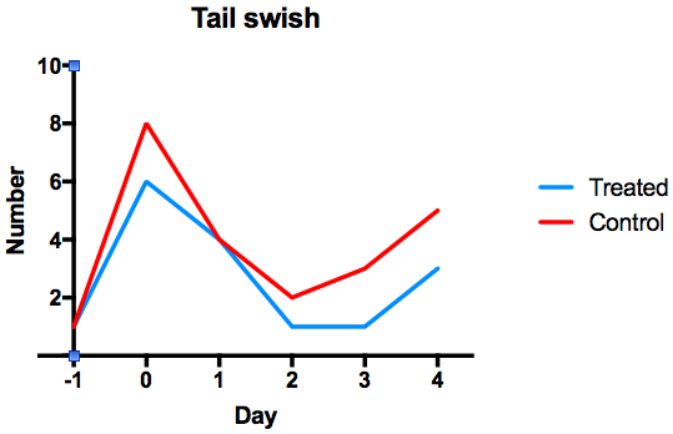

Figure 4.8: Tail swishing, median value.

Adam Rääf, rwc467

Signe Rejnhardt Olsen, jrb642

Table 4.8: Tail swishing, control group *n = 25*, treatment group *n = 25*.

Day	Control Median	95% CI	Percentiles 10%	90%	Treatment Median	95% CI	Percentiles 10%	90%	*p*-value
-1	1	[0;5]	0	10,4	1	[0;2]	0	9	0,9314
0	8	[3;17]	0	84,4	6	[1;42]	0	130,4	0,9269
1	4	[2;5]	0	22,6	4	[2;16]	1	23	0,5264
2	2	[0;4]	0	7,4	1	[0;6]	0	31,8	0,8225
3	3	[0;5]	0	16,6	1	[0;4]	0	25	0,6661
4	5	[3;8]	0	18,6	3	[1;6]	0	13,2	0,1880

4.3 Pain face, pain ears and hyperalgesia

Pain face: There were not seen any significant difference in number of calves displaying pain face between the two groups (see table 4.9). In the treatment group, there was significant difference between day -1 vs. 1 (p=0,0012), 2 (p=0,0022) and 4 (p=0,0106). In the control group, there was significant difference on all 4 days compared to day -1 (p=0,0010, $p < 0,0001$, p =0,0002 and p=0,0019) (see appendix 3).

The observations of both *pain face* and *pain ears* were done in the transition period from feeding to resting, when the calves were undisturbed. This specific moment occurred almost simultaneously in all pens, as the calves tended to lie down and rest whenever the pen-mates did. The short window of time, where the observation of facial expressions was possible, resulted in a varying sample size each day.

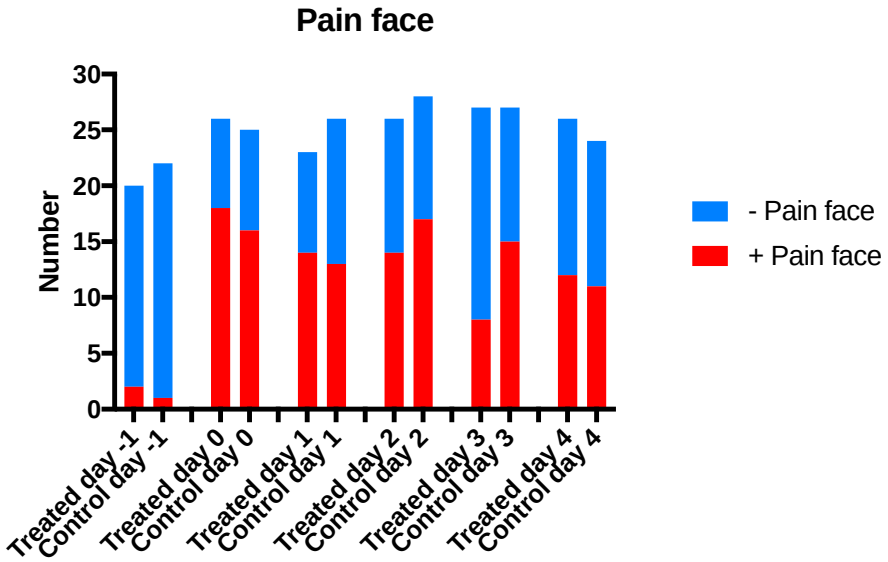

Figure 4.9: Number of calves with pain face.

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Signe Rejnhardt Olsen, jrb642

Table 4.9: Pain face registrations.

Day	Control *n*	Treatment *n*	OR	95% CI	*p*-value
-1	22	20	2,333	[0,25;35,21]	0,5976
0	25	26	1,266	[0,42;3,799]	0,7712
1	26	23	1,556	[0,489;4,43]	0,5675
2	28	26	0,755	[0,252;2,188]	0,7836
3	27	27	0,337	[0,118;1]	0,0978
4	24	26	1,013	[0,323;3,202]	>0,999

Pain ears: There were not seen any significant difference in number of calves displaying pain face between the two groups (see table 4.10). In the treatment group, there was significant difference only between day -1 vs. 1 (p = 0,0428). In the control group, there was seen significant difference between day -1 and all other days (p = 0,0027, p = 0,0075, p = 0,0410 and p = 0,0081)(see appendix 3).

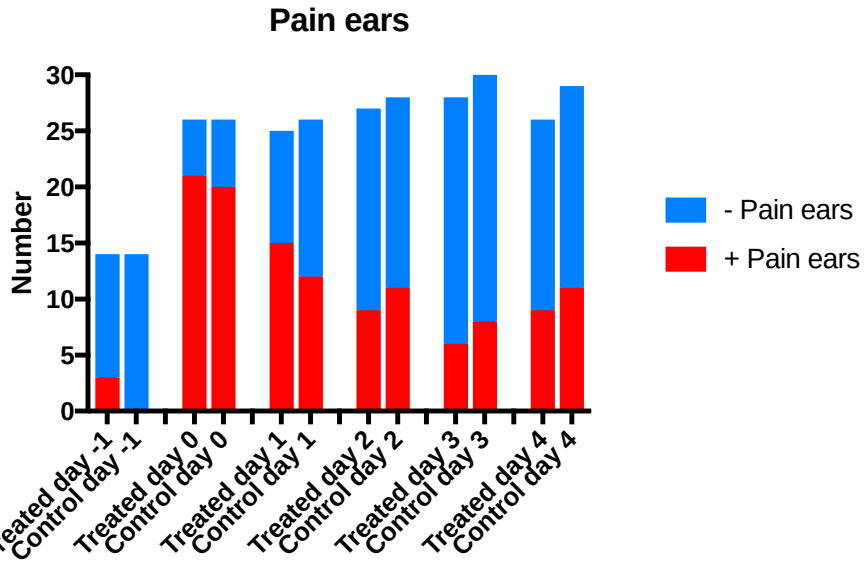

Figure 4.10: Number of calves with pain ears.

Table 4.10: Pain ear registrations.

Day	Control *n*	Treatment *n*	OR	95% CI	*p*-value
-1	14	14	∞	[0,933;∞]	0,2222
0	26	26	1,26	[0,365;4,555]	>0,9999
1	26	25	1,75	[0,582;5,185]	0,4043
2	28	27	0,773	[0,24;2,334]	0,7808
3	30	28	0,75	[0,221;2,346]	0,7624
4	29	26	0,866	[0,27;2,624]	>0,9999

Adam Rääf, rwc467
Signe Rejnhardt Olsen, jrb642

Hyperalgesia: When analysing hyperalgesia testing, no significant differences either between or within the 2 different groups where found.

Disease prevalence: When investigating the disease prevalence as noted in appendix 1, there were 6 calves in each group that were noted for disease during the study period.

There were no complications following the procedure seen during the study period.

5 Discussion

Animal welfare can be assessed, as earlier mentioned, by physiological, productive and behavioural measures. This study focuses on the animal-based indicators, behaviour and weight gain, as indicators of welfare in the days following dehorning.

By comparing the calves in the study, the difference in housing facilities between H1 and the remaining two herds (H2 and H3), needs to be taken into account, as the results are discussed. The main differences were feeding strategy, as H2 and H3 were fed twice in 5 litre bowls, in relation to the common trough in H1. Furthermore, H2 and H3 were fed milk with several additives, partly to slow down the passage time of the manure and ad volume to the milk. The dry matter in the milk differed with two percent between H1 and H2/H3, which might add another influencing factor to the initiation of cross-sucking, as the concentration of the milk plays a role in the motivation to suck (De Passillé, Rushen and Janzen, 1997). The motivation to suck is also influenced on the amount of milk served. However, the amounts fed were not strictly measured.

The solid feed, also differed between the two locations of calves, as H1 were fed concentrate and hay, and H2 and H3 were fed calf muesli, with small amounts of TMR cows feed added twice a day.

5.2 Weight

From the literature, Danish Holstein calves of 0-8 weeks have an estimated weight gain ranging from 541-990 grams/day, under normal non-stressful conditions (Duve and Jensen, 2012; Jensen, Duve and Weary, 2015). The interval is the results of a diet consisting of either 5 litres of milk/day and concentrate, or 9 litres of milk/day and concentrate. The calves in this current study were fed approximately 5 litres twice a day. The weight estimates are in the or disturbed by the disbudding, as it reduces the effect of GH and inhibits the release of IGF-1 and other growth-related factors (Moberg *et al.*, 2000). However, studies on the effect of treatment with one dose of NSAIDs at the time of disbudding, estimates an average daily weight gain of 1,2 kg the first 24 hours (with ketoprofen treatment), and increased feed intake for the treated calves treated with meloxicam, compared to control calves on day 1 (Faulkner and Weary, 2000; Heinrich *et al.*, 2010).

Furthermore, Baldridge *et al.,* 2011, found a significant greater daily weight gain for up to 13 days, if the calves were treated continuously with salicylate compared to no treatment. (Baldridge *et al.*, 2011). The expectations for this current study was an increased weight gain for the treated calves in the period of day -1 – 4, and hereafter a more equal weight gain for the two groups for the rest of the study period. If the analgesic treatment was sufficient, the treatment group would maintain an average weight gain of approximately 990 g/day, throughout treatment period.

The results of this current study revealed an average daily weight gain in the control group of 533 g/day from day -1-4, (2,66 +/- 0,409 kg for the whole period), and 784 g/day for the in the treatment group (3, 91 +/- 0,461 kg for the whole period). This is a significantly higher weight gain for the treated calves with 1,25 kg/calf for this period, corresponding to an increased weight gain of 251 g/day. This indicates that a prolonged treatment with meloxicam has an effect on the inflammatory response and that the decrease in weight gain compared to the expected weight gain of 990 g/day can be reduced.

Considering the period of day 4-14, the average daily gain in this current study was more equal between the two groups, as the treated group had a gain of 844 g/day, and the control group had a gain of 894 g/day. Hence, there was an overall gain closer to the expected value of approximately 990 g/day.

The results of this study show a tendency for a higher total weight gain per calf in the treatment group in the period of day -1-14, but not a significant increase compared to the control group. There is no evidence of a significantly greater weight gain after day 4, however the effect of the treatment on weight gain might be related to the duration of the treatment, as Baldridge *et al.*, 2011, showed a significant increase in average daily weight gain during the treatment period of 13 days. As the inflammatory process can last for at least 3 to 7 days, a treatment period longer than ours might have given another result.

Meloxicam inhibits the natural immune response by both inhibiting the release of pro-inflammatory cytokines locally and by suppressing the inflammatory activation of white blood cells in the blood via COX enzyme regulation (Ackermann, 2012). As inflammatory processes are still developing after three days, as shown by Schwartzkopf-Genswein and Stookey, 1997, there might be an increased local inflammation and activation of neutrophils, when the effect of meloxicam wears off. This might cause a rising physiological and behavioural distress on day 4, which potentially decreases the weight gain in the period day 4-14 in the treatment group.

To make the best possible estimate, the calves were paired on day -1. The analysis of the weight within the pairs on day -1, showed no statistical significant differences, which suggests that the pairings were successful, and the results of the weight analysis, are valid.

5.3 Behaviour

This study evaluates the amount of pain specific behaviours, and estimates the behavioural alterations within a fixed time frame, for the purpose of evaluating the distress of calves for 4 days after disbudding.

To summarize the expected alterations in normal behaviour of calves in distress, would be a higher frequency of shifting behaviours, if calves are deprived from resting behaviour, and an increase in the duration of resting periods (Munksgaard *et al.*, 2005). Furthermore, lying down seems to be of highest priority over playing, social contact and eating (Jensen, 1999, 2001, Munksgaard *et al.*, 1999, 2005). The priorities within normal behaviours, could be lying, locomotor play, social activities and eating, in the written order. All of them internally motivated to some extent. The highest frequency and duration of non-nutritive sucking could be observed when the calves are fed inadequate amounts of milk, with adequate energy concentration (de Passillé *et al.*, 1992; Rushen and Depassille, 1995; De Passillé, Rushen and Janzen, 1997; Jung and Lidfors, 2001; Jensen and Budde, 2003; Nielsen, Jensen and Lidfors, 2008; Roth *et al.*, 2009). Duration and frequency of play, can be seen when the calves have their physiological need covered, and can be used as a measure of welfare (Jensen, Duve and Weary, 2015).

Pain induced by disbudding might also alter motivation for normal behaviours on the basis of negative emotions, as Neave *et al.*, 2013, showed a decrease in feeding motivation after disbudding.

With regards to the literature, the estimated resting time for the calves in this study was expected to be approximately 64% during the 16 hours from 0600 to 2200 (see table 2.1). However, the amount of rest can vary throughout the day, in different environments and with the age of the calf (Chua *et al.*, 2002b; Camiloti *et al.*, 2012; Duve and Jensen, 2012).

The calves of this current study had a baseline mean resting period at day -1 of 890-943,5 seconds, which is 40% of the time observed. The period observed were centred around feeding, which naturally stops any resting in the pen, for several minutes. However, it corresponds very

well with the estimate from Von Keyserlink et al., 2006, finding that calves at the age of 5-32 days, has different resting patterns during the day, with the least rest between 6 pm and midnight. The general time spent inactive/resting in this study, decreases significantly within both treatment and control group, throughout the period from day -1 – 4. This correlates with the studies proving that the disbudding procedure increases plasma cortisol (Morisse, Cotte and Huonnic, 1995; Graf and Senn, 1999; Grøndahl-Nielsen *et al.*, 1999), and the calves sleep less, but spent more time inactive, because of high plasma cortisol levels (Friess *et al.*, 1995). Contrary to this result, Taschke & Fölsch, 1993 and Morrise *et al.,* 1995, found increased resting periods for up to 12 hours after disbudding. Theurer *et al.*, 2012, found the calves treated with one dose of meloxicam at disbudding, having an increase in resting time the first four days after disbudding. By the fifth day, the resting times were equal to the control group that did not receive meloxicam. None of the two groups received corneal nerve blocks with a local anaesthetic (Theurer *et al.*, 2012).

The tendency in this current study points towards an increase in rest within the control group from day 1 – 4, compared to the treatment calves. With a significant difference between the two groups on day 4. Hence, the tendency for the control group to spent more time inactive, could possibly be explained by a higher level of distress in the control calves. McMeekan *et al.,* 2011, also found that a greater proportion of calves not receiving analgesics were lying down two hours after disbudding, compared to calves given analgesics. Furthermore, calves treated with ketoprofen had a lying period equal to calves sham disbudded.

In the current study, the calves had a decrease in time spent resting on day 1, in both the treatment and the control group. On day 1 there is also seen an increase in both play behaviour and time from feeding to first time trying to lie down. This might indicate that some of the discomfort from day 0 has worn off, and the motivation to express social behaviour is high, as the calves have been deprived from playing on day 0. Another explanation could be an expression of discomfort, if the calves express escape behaviours, when galloping around. The ethological registrations from the video analysis on play, includes both social play and locomotor play, which is also defined as the calf galloping around. However, the specific pain behaviours do not peak on day 1 in any of the groups, which we would expect if the calves were even more in distress, compared to day 0.

The results of the analysis on first attempt to lie down after feeding, shows an increase in time from day -1 to day 0 and 1. The general tendencies are that it takes longer time for the calves to lie down first time after feeding during the whole study period. This result correlates with the decreased resting time.

Play behaviour can be measured as a sign of wellbeing of the calves, however, the time spent on play behaviour under normal, non-stressful conditions have been estimated to 130 seconds per 24 hours (see table 2.1). The results of this current study show a baseline mean value at day -1 of 11,9 seconds in the control group and 15,8 seconds in the treatment group, during the 39 minutes observed. The unexpected significant difference in baseline values between the control and the treatment group adds an uncertainty to the results of the play analysis. However, the differences in time spend playing between the treatment and control group are only significant on day –1 and 3. In relation to that, the general alterations in playtime after disbudding has not been shown to last for more than 3 hours (Mintline *et al.*, 2013). The study by Mintline *et al*. 2013, only monitored that calves for 10 minutes at 3 and 27 hours after disbudding. This current study reveals a decrease in time playing and a rise in pain specific behaviours in the control group 72 hours after the procedure, which might suggest a positive effect of the meloxicam treatment, as the same tendency is not seen in the treatment group. Considering the difference in space available for locomotor play, studies have shown that there is no difference in the time spend on playing behaviours by calves of 7-9 weeks, when having of 1.5 m^2 available compared to 4 m^2 (Jensen *et al.*, 1999). Hence, the difference between 4 m^2 in H1, and 2.4 m^2 in H2/H3, should not make any difference.

According to the literature, the reasons for developing oral abnormalities could be a mix of restrictive feeding, sufficient concentration of milk in the formula, and too little time spent drinking milk, which can lead to frustration.
The time spend eating solid food, can also elicit oral abnormalities, as licking on inventory, and licking on pen-mates has been shown to increase when calves were fed separate rations of concentrate and hay, instead of a mixed ratio (Ishiwata *et al.*, 2008; E.K. Miller-Cushon *et al.*, 2013). In this current study, the feeding differed between H1 and H2/H3. H1 were fed hay and concentrate separately, and H2/H3 were fed a mixed ratio and no separate hay. However, the

difference in cross-sucking between the herds has not been measured, why possible differences might cause an increased deviation of the overall results of cross-sucking.
There is also a social aspect of cross sucking, as there is a tendency for the calves to suck on the same pen-mate when cross-sucking (De Passillé, Borderas and Rushen, 2011). Social deprivation has also been shown to elicit oral abnormalities, which in theory should result in an increased duration of oral abnormalities on day 1, correlating with the increased play behaviour in the treatment group. There is no significant increase, but the tendency is there.
The time spend on oral abnormalities on day -1 (baseline) in this study was 310-378 seconds within 2340 seconds observed. There were no significant differences seen between the baseline level compared to day 1, 2, 3 or 4. However, there is a decrease in mean on day 0, which possibly could be a result of the increased time spend on pain behaviours. The motivation for rest has been proven higher than the motivation for both social and eating activities (Munksgaard *et al.*, 1999). This may explain why there is no significant decrease in resting behaviour on day 0, but a decrease in time spent on oral abnormalities.
There is a significant difference in time spent on oral abnormalities between the groups on day 3 and 4, with twice as high a mean of oral abnormalities in the treatment group on day 4. This difference could potentially be seen due to a decrease in time spent on expressing pain-specific behaviours.
The number of pain-specific behaviours expressed, are increased in this current study on day 0, which we would expect after the disbudding. Pain-specific behaviours has been shown to be increased for at least 4 – 24 hours in calves dehorned with the use of local anaesthetics only, and can possibly be increased for up to 44 hours (Heinrich *et al.*, 2010). The study by Heinrich *et al.*, 2010, found a reduction in pain specific behaviours (head shakes and ear flicks) in calves treated with meloxicam prior to disbudding. The results of this current study, finds no significant difference in pain specific behaviours on day -1 compared to day 2, which could be an expected result, since both control and treatment group had meloxicam administered on day 0. Hence, there is a possibility that the analgesic effect of meloxicam is still present for up to 48 hours. There is a 30% increase in pain-specific behaviours on day 3 in the control group, compared to the treatment group, which is a significant difference between the two groups. This result suggests, that the continuous treatment with meloxicam, makes a difference after 48 hours. The difference on day 3, could be result of a continuous accumulative anti-inflammatory effect of the daily administration of meloxicam, with a $T_{1/2}$ of 26 hours. However, there is a rise in pain

specific behaviours seen on day 4 in the treatment group, which does not support the theory of an accumulative effect or longer duration of the analgesic effect than 24 hours. The rise could possibly be due to the activation of the immune response, which has been inhibited by the administration of meloxicam on from day 0 to 3 which could potentially lead to an increased inflammation on day 4.

When looking at the number of tail swishes expressed in both groups, there is a significant increase from day -1 to 1 and day -1 to 4 within both groups. This could indicate that the tail swishes are correlated to the pain behaviours, as both factors had a significant increase between day -1 and 1. However, there is no significant difference between groups on any of the days, as seen in the number of pain specific behaviours. On day 2 and 3, the median in the treatment group is back to baseline level, suggesting a positive effect of the treatment. The control group, has a higher median on day 2 and 3, compared to baseline level.

The general tendencies within both pain specific behaviours and tail swish were, that the treated group had a lower median value of both pain specific behaviours and tail swish, than the control group, during the whole study-period.

5.3 Pain face, pain ears and hyperalgesia

Pain face in calves has not been described before, why this study was carried out using descriptions of facial expressions of pain in cows, and a positive *pain face* scoring was interpreted as a sign of pain. The reason for the direct use of the described mimic of the cow's pain face projected on the calves, is that calves may have fully developed facial muscles, as there are descriptions of fully developed nerve innervation already at the time of birth (Taschke and Fölsch, 1994).

We would expect all calves to have a facial expression of pain during both day one and day two, as there is a significant increase in pain specific behaviours in both groups on these days. However, the sensitivity of the wounds and the surrounding tissues remains increased for up to 75 hours (Heinrich *et al.*, 2010), with a possibly decrease of the nociceptive threshold.

In this this study, a significantly increased number of calves expressed a *pain face* on day 1, 2 and 4, compared with day -1. Furthermore, the number of calves expressing a *pain face* was significantly increased in the control group, on day 3.

If the *pain face* correlates with the expression of pain-specific behaviours, there should also be a significant increase in number of calves expressing pain face on day 3 in the control group, which is confirmed in this study.
The expression of pain, by alterations of the ears, *pain ears*, shows no significant difference between the two groups, and has an odds ratio around 1 (0,72-1,26), which suggests that there is almost equal number of calves expressing *pain ears* in the treatment and the control group.
The registrations on hyperalgesia, show no significant differences between the two groups. However, there is an odds ratio of 0,3 on day 2, which might suggest that there is a tendency to have a higher risk of hyperalgesia for the control calves on day 2.

5.5 Legislation

The legislation prescribes that disbudding has to be performed with the use of sedation but not NSAID. However, this study did not include a control group, not receiving an NSAID. Several studies have already proved the positive effects of a single dose during disbudding, and our group found it unethical to include such group. With regards to the literature, this study and the EU-project Welfare Quality®, the reasons not to prescribe even a single dose of NSAIDs during disbudding raises ethical considerations and legislation in the subject should be evaluated.

5.6 Study evaluation – bias and confounding factors

The animals in our study population were on only two different locations and were not randomly selected. Several different herds, and a larger sample size, would probably give a more exact estimate of the effect of four days of treatment with meloxicam.
Concerning the technical data obtainment, the optimal solution for securing standardized video recordings, would be a separate power supply for each of the cameras. This current study has used cameras running on batteries, which lead to interrupted recording periods, hence disturbing the calves when change of batteries were necessary.
During the data obtainment, the video recordings were scheduled according to feeding times, however, the feeding times varied from day to day of about +/- 30 minutes
The varying feeding times, resulted in alterations in video length in between the days, which again resulted in a shorter fixed time frame (14 minutes prior to-, and 25 minutes after feeding) of the behavioural analysis.
The video recording was also interrupted, when the calf managers were treating sick calves in the

period after feeding.

The statistical analysis was performed with non-parametric tests, due to our small sample size. This method has a higher specificity than parametric tests. A higher specificity results in a higher frequency of false negative results, why a result with a significant value using non-parametric tests is a true rejection of the null hypothesis.

When evaluating outliers of the weight calculations, two calves at H1 had weight loss in the period of day -1–4, with a difference in weight of -1,2 kg and -1,8 kg. Both calves were located in the control group. Their corresponding calf in the treatment group had a weight gain of 9,7 kg and 2,6 kg, respectively. The negative weight balance of the control calves might be due to an altered protocol during the data-obtainment on day -1 at H1, as the calves were weighed approximately 2 hours after the milk feeding. The reason why the mentioned outliers are included in the study, is due to the biological analogy, that the metabolism of the calves, probably gives an output of urine and faeces within two hours after feeding, which balances the possible differences in weight, and to avoid decreasing the numbers of calves in the project further. This could though potentially cause a false high weight on day -1, which would lead to a negative difference in growth during the period of day -1–4. This difference would also be applicable on the calves in the treatment group, why the results of the difference in weight gain between the two groups would potentially be the same.
However, the negative results of the two calves with weight loss, might lower the mean value of the control group more than the corresponding treated calves increases the mean of the treatment group, causing a lower overall gain in the herd.

When evaluating the behavioural data, day 0 reveals 13 outliers in relation to pain specific behaviours. 12 of these outliers are housed at the same location, and 9 calves (4 from the treatment, and 5 from the control group) are from the same herd, H2 (see appendix 2). The weather on day 0, for H2 was rainy and windy, and the pens were faced west, directly towards the wind. Furthermore, the local anaesthetic used on H2, consisted of 8 ml of Procamidor Vet. (20 mg/ml procainhydrochloride, Salfarm Danmark A/S) in relation to 5 ml Pronestetic (40 mg/ml procainhydrochloride and 0,036 mg/ml epinephrintartrat, FATRO S.p.A., Bologna, Italy) for H1 and H3. They might increase the median on day 0 but since there are an almost equal

number of calves in both the treatment and control group, both medians should be increased, and the results still comparable.

There was a single outlier in the treatment group from H3 on day 4, with a number of tail swishes of 330. This calf had profuse diarrhoea, which might explain the restlessness and great amount of tail swishes seen on the video monitoring. Furthermore, this calf did not lie down or drink on day 4, as it trotted around in the pen after feeding. The decrease rest and the tail swishes might be an indicator of pain, however the pain specific behaviours were not increased. Since we used the median to evaluate the tail swish results, this calf did not have any influence on the result.

6 Conclusions

Treatment with meloxicam at the day of disbudding does not entirely cover the post-surgical pain and distress generated by disbudding by heat cauterization. Treatment with meloxicam for four days after disbudding, in comparison to a single dose during disbudding, results in significantly increased growth within the first four days after disbudding and several significant positive effects on day three (last day of meloxicam treatment). The positive effects of the treatment on day three after disbudding includes less expression of pain specific behaviours and *pain face* and an increased time spent on play behaviours.

There was a tendency for the treated calves to spend less time resting during the trial period, compared to the control group, which is not completely justified as an either positive or negative tendency. However, there was a significant decrease in resting throughout the whole period in both groups compared to day -1, which suggests an altered resting pattern after disbudding that lasts for at least four days.

There were no significant differences seen in the number of tail swishes between the two groups. The general tendency of this study shows a decrease of all pain-related behaviours in the treated group compared to the control group.

These results show that treatment with meloxicam for four days, in contrast to one, lowers the post-surgical pain after disbudding, contributing to an increased welfare of the calves in this period. However, the weight gain within 14 days after disbudding is not significantly altered by the extended period of treatment.

7 Perspectives

Based on our conclusion, further studies should be made to investigate the long-term effects and benefits of a prolonged meloxicam treatment, involving more animals and a longer study period. As this current study shows no significant difference in pain specific behaviours between the two groups on day 2, the single treatment during disbudding on day 0 might have a beneficial effect for up to 48 hours. Further studies should also consider, if the beneficial effects of an extended period of four days of meloxicam treatment can be achieved with the administration of meloxicam every other day.

Moreover, the possible correlation between the behaviours evaluated in this study should be analysed using regression models, involving more factors than this study had the capacity to analyse. These regression models could potentially evaluate the possible correlation between pain related behaviours and oral abnormalities, pain specific behaviours and *pain face* and time resting or sleeping and duration of play.

The positive effects of the prolonged treatment, shown in this study, can potentially be extended with a further extended treatment-period, as seen in studies on the effect of salicylate treatment for 13 days (Baldridge *et al.,* 2011).

In relation to the Danish legislation, saying that the animals should be kept from pain, suffering and major disadvantages, there is a motivation for further research on pain relieve during and after disbudding.

The EU-project, Welfare Quality® has developed a protocol for the assessment of both positive and negative welfare indicators (Forkman and Keeling, 2009), which includes 12 welfare criteria. Criteria number 8 describes the absence of pain induced by management procedures, such as dehorning. Their assessment of good animal welfare around disbudding, is either no disbudding, or disbudding at three weeks of age, using anaesthetics, and the administration of analgesics for 3-7 days after the procedure (Forkman and Keeling, 2009).

The recommendations within this protocol are supported by this study, and might be a good recommendation for an increasing the welfare of calves in the future.

However, a breeding programme for the development of polled bulls with an NM-index at the same level of horned cattle, is also a great alternative for avoiding disbudding distress in dairy calves.

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