Final report

Improving animal welfare in the red meat industry – pain relief

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Prepared by: Peter White, Dominique Van der Saag, Sabrina Lomax, Merran Govendir
University of Sydney

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Abstract

Pain resulting from routine livestock husbandry practices is well recognised and poses a threat to animal welfare and production. This project was undertaken to examine novel and traditional methods of measuring pain to allow for improving pain treatment using existing and new therapeutics, with a focus on the development of those for long term analgesia. Multiple studies were undertaken to develop facial grimace scores (FGS), test potential physiological biomarkers of pain, examine long term behavioural responses, and further explore routine measures of pain. Other studies were conducted to develop a modified release injectable analgesic based on meloxicam, and a modified method of delivery of meloxicam via feed. While FGS and biomarkers demonstrated some correlation with pain, they proved inconsistent in the farm setting. Ear-tag based accelerometers appear to be a useful tool for monitoring behaviour changes in response to acute and long-lasting pain. A modified release injectable formulation of meloxicam was shown to extend the putative duration of action of the drug. The addition of meloxicam to feed similarly increased the time period that potential analgesia could occur. Industry would benefit from the further development of long-acting analgesic products to improve animal welfare and ensure consumer confidence in animal products.

Executive summary

Background

The pain experienced by livestock undergoing routine animal husbandry procedures is a primary animal welfare concern in Australia and a well-recognised threat to the animal production industries. Providing red meat producers with tools to reduce the impacts of aversive husbandry procedures is one step toward improving animal welfare and consumer confidence. While much research has already been conducted into reducing the adverse effects of husbandry procedures, there remains room for improvement in both the measurement of pain and the treatment of pain. The purpose of this project was twofold. It aimed to improve the methods used for the accurate measurement of pain and pain relief treatments, and to develop alternative therapies for improved pain relief. The results of this research will be used to continue work in the development of appropriate treatments for pain relief to benefit animal welfare.

Objectives

This project consisted of 4 objectives.

1. **Assessment**: Develop a robust "pain model" by using routine and novel markers of pain that objectively assess pain during husbandry interventions.

2. **Evaluation**: Compare the effect on pain response of the different methods of husbandry procedures and other deleterious impacts to determine systematic ranking of welfare impacts on beef cattle and sheep farms.

3. **Amelioration**: Compare the efficacy of existing and new analgesics, including the development of a prolonged pain management methodology.

4. **Extension**: A set of 'best practice' welfare standards suitable for use on-farm will be developed by application of the 'pain model' markers to painful husbandry procedures, and broader applications (including therapy of other deleterious welfare impacts). Understanding gained from the pain assessment and amelioration studies will then enable examination of pain management regimes for other on-farm health issues and ailments.

The timing of this project largely coincided with the Covid -19 Pandemic in 2020 and 2021. The multiple announcements regarding Public Health Orders had major impacts on the ability to travel and to conduct research both in NSW and interstate. Despite this fact, the project objectives were satisfactorily met.

Methodology

A series of on-farm experiments were conducted to develop and test the use of novel and routine markers of pain in cattle and sheep including lamb and calf facial grimace scales, physiological biomarkers, and technology derived behaviour states. These new and existing methodologies were used to compare the methods used to castrate and tail dock lambs, and to evaluate the duration of pain and the efficacy of new and existing analgesic strategies.

NB: Due to the Covid-19 Pandemic and Public Health Orders, some large-scale studies planned to be conducted in Queensland could not be undertaken and were instead performed on a smaller scale in New South Wales.

Results/key findings

Pain Assessment

Studies were conducted to develop and trial facial grimace scores in calves and lambs. These methods for monitoring and measuring pain response were found to be highly variable and as such were not further developed in the project. A large-scale study undertaken in conjunction with project P.PSH.0819 'Objective measures of welfare' using ear tag technology to monitor pain responses was found to effectively demonstrate both short-term and long-term effects of castration and dehorning on weaner cattle. Individual biomarkers identified in previous literature reviews were also examined however provided inconsistent conclusions in regard to pain responses.

Pain Amelioration

This project demonstrated the inflammatory and wound healing response resulting from routine husbandry procedures can last several weeks, particularly for methods utilising rubber rings, resulting in additional welfare impacts in some instances, such as secondary wound infection. The project also found that current analgesic options do not appear to have animal welfare or production benefits.

This project has demonstrated the feasibility of extending pain relief using a modified release injectable formulation of meloxicam and through meloxicam-medicated feed. These options offer potential solutions to address the long-term pain associated with painful husbandry procedures, though they require further research to elucidate commercial viability and safety.

Benefits to industry

The key outcomes from this program further highlight the need for industry to address long-term pain associated with husbandry procedures such as dehorning to adequately address animal welfare. This may be achieved using existing therapeutics in the form of a slow release injectable or feed additive.

Future research and recommendations

Further research is required to develop strategies to provide long lasting pain relief and wound management following aversive husbandry procedures. The development of a modified release injectable drug would allow producers the option to provide a one-time long-acting analgesic. Likewise, the development of feed additives to address pain could also provide producers with an alternative to providing long-acting analgesia, within suitable production contexts.

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1. Background

1.1 Introduction

The issue of improving welfare during aversive routine husbandry procedures has emerged as a leading welfare concern for all livestock industries in Australia. Growing consumer awareness of painful procedures and practices poses a significant threat to the red meat industry. Providing adequate and long-lasting pain relief will address many of these concerns. At the commencement of this project (2017) approximately 4% of beef producers were regularly using pain relief for routine husbandry procedures compared to 30% in 2021 (Australian Beef Sustainability Annual Update 2021). This project addresses the MLA Animal Welfare Strategic Plan through the development of novel methods of assessing and managing pain associated with husbandry interventions commonplace in extensive sheep meat and beef production systems with the aim of developing and promoting the uptake of best practice pain management approaches that can be readily adopted by producers.

1.2 Previous Research

This research builds on the success of previous research investigating pain relief in sheep and beef cattle (P.PSH.0654) which highlighted the positive but limited benefits of commercially available farmer applied pain relief products. It also highlighted the need for investigating more robust and efficacious methods to assess animal pain and the use of pain relief therapeutics.

A limiting factor in the use of pain relief on farm may be attributed to a lack of registered products suitable for use by livestock producers without the need for costly veterinary intervention. The development of the topically applied anaesthetic (Trisolfen®, Dechra Veterinary Products Australia Pty. Ltd, NSW Australia) for use by producers in the mulesing of lambs was the first of several developments aimed at addressing pain in farmed animals (Lomax, Dickson, Sheil, & Windsor, 2010). Consequently, this product has been registered for use in lambs and pigs for tail docking and castration and in cattle for castration, disbudding and dehorning.

Likewise, the development of the non-steroidal anti-inflammatory (NSAID) drug meloxicam in a gel formulation (Ilium Buccalgesic OTM®, Troy Laboratories Pty Ltd, NSW, Australia) designed for buccal administration was developed to provide an adjunct to local anaesthetic for castration and dehorning in calves and for use in tail docking, castration and mulesing in lambs (Saag et al., 2018; Small, Belson, Holm, & Colditz, 2014; Van der Saag et al., 2018).

Despite these developments in the availability of farmer applied pain relief products addressing some aspects of the acute post procedure pain caused by husbandry procedures, there are still questions that remain unanswered. While much has been done to assess the efficacy of currently available products using methods currently available, there is room for improvement in the measurement of animal pain. In addition to this, the products developed for farmer application to date do little to address the pain of the procedure itself and most likely have a limited duration of effect.

Pain measurement in animals is an important factor in addressing animal welfare and in assessing the efficacy of applied pain relief. The use of existing behavioural and physiological methods of pain assessment are limited in that they are all nonspecific indicators and are affected by multiple factors.

A multimodal approach has been shown to be more reliable but further development of a pain assessment tool would benefit the investigation of pain and pain relief.

1.3 Project Aims

The aim of this project was to investigate a multimodal approach to pain relief utilising longer acting/long term anti-inflammatory drugs utilising novel drug delivery methods in addition to topical anaesthesia to address the gaps highlighted in previous research. In addition, recent work investigating pain measurement techniques was continued in this project to rank painful procedures more accurately and to determine the efficacy of pain relief protocols. The outcome will provide producers with an affordable, efficacious and practical protocol for delivering pain relief on farm.

While other projects have thoroughly investigated the use of pain relief in the acute phase of responses to routine procedures using common pain measurement methods, importantly this project aimed to highlight effective pain measurement tools with the goal of using such tools to compare different methods of performing routine husbandry procedures and to measure the efficacy of pain relief medications, over both acute and longer-term periods. In addition, the project aimed to investigate novel pain relief formulations and delivery methods, with a goal of increasing the time over which pain relief is provided to animals.

2. Objectives

1. **Assessment**: Develop a robust "pain model" by using routine and novel markers of pain that objectively assess pain during husbandry interventions.

   In this objective we aimed to continue to develop current methodologies for pain assessment and build on previous work investigating a "pain model" using novel pain markers (Hazel et al., 2016). Facial Grimace Scores were developed and trialled and found to be highly variable. Individual biomarkers were examined with inconsistent results. Novel ear tag technology was employed in partnership with project P.PSH.0819 'Objective measures of welfare', that effectively showed short- and long-term responses to pain.

   Initially it was planned to continue work on development of a conscious electroencephalography (EEG) protocol originally commenced in another project. While EEG has some applications under Halothane anaesthesia, use of a conscious model was not found to be practical enough for use in this project (Harris, White, Hall, Saag, & Lomax, 2021; Harris, White, Mohler, & Lomax, 2020).

2. **Evaluation**: Compare the effect on pain response of the different methods of husbandry procedures and other deleterious impacts to determine systematic ranking of welfare impacts on beef cattle and sheep farms.

   Findings from Objective 1 were used to provide a framework "Pain Model" to objectively assess pain associated with husbandry and disease in sheep and beef cattle. Multiple methods were used to assess painful procedures and the impact of pain relief on procedures.

   a. An original aim was to use the above 'pain model' to directly compare different procedures (e.g. ring versus surgical castration) and establish the best practice

approach for each method. Our investigations revealed that surgical castration, hot-iron branding, amputation dehorning and spaying via the Willis Dropped Ovary Technique (WDOT) are the primary methods used by most producers for justifiable reasons. Therefore, the need to compare different methods for these procedures was not justified in this project.

b. There is minimal research on the long-term pain, morbidity and mortality associated with routine husbandry procedures. It has been observed that longer-term impacts lasting several weeks may occur following some procedures. Further investigation and documentation of long-term pain was performed in conjunction with project P.PSH.0819 'Objective measures of welfare'.

3. **Amelioration**: Compare the efficacy of existing and new analgesics and methods of administration to address short- and long-term pain, including the development of a prolonged pain management methodology.

   This objective aligns with findings from Objective 1 and 2. The impacts of current and novel analgesics and methods of administration to address the short and long-term pain associated with husbandry procedures in addition to other ailments in sheep and cattle was investigated.

   a. Using findings from Objective 1 objective assessment of the effects of various anaesthetic/analgesic combinations for the different techniques was made.
   b. Following on from Objective 2 this objective examined the use of long-acting analgesic drugs.

      Several long-acting formulations were trialled utilising meloxicam as the target drug. While a successful long-acting formulation was developed, staff losses and the impact of the Covid -19 pandemic prevented further development of this product. The delivery of meloxicam as an additive to feed was also successfully trialled.

4. **Extension**: A set of 'best practice' welfare standards suitable for use on-farm will be developed by application of the 'pain model' markers to painful husbandry procedures, and broader applications (including therapy of other deleterious welfare impacts).

   A survey of beef and sheep producers assessing knowledge, attitudes and skills regarding animal pain determined that most producers believe that pain relief should be provided to treat pain associated with animal husbandry procedures. Determining best practice to provide long-term pain relief is a priority for future research.

3. Methodology

3.1 Investigation into novel markers of pain in lambs

3.1.1 Animals

Thirty-five lambs (6 to 8 weeks of age; 17 males and 18 females) requiring routine 'marking' (that is, castration and / or tail docking) were sourced from the University of Sydney owned farm, 'Mayfarm' in Camden, NSW. The experiment was approved by the University of Sydney Animal Ethics Committee (approval number 2018/1440).

3.1.2 Experimental design

Prior to the experimental period, lambs were kept with their mothers in a nearby paddock. On the day of experimentation, lambs and their mothers were quietly moved into the sheep handling yards. Lambs were then drafted from their mothers immediately prior to commencement of experimental activities. For treatment, blood sampling and video recording of facial expressions, lambs were restrained in a lamb marking cradle. Following treatment and video recording of facial expressions, lambs were held in small yards (5m x 5m) adjacent to the handling facilities for video recording of their behaviour. Lambs were returned to the paddock with their mothers immediately following completion of experimental activities.

3.1.3 Treatments

Lambs were randomly allocated to one of four treatment groups: (1) sham tail docking (STD) (*n* = 9 females); (2) sham tail docking and sham castration (STDC) (*n* = 8 males); (3) hot-iron tail docking (TD) (*n* = 9 females); and hot-iron tail docking and castration (TDC) (*n* = 9 males). Lambs in the STD and STDC treatment groups will be surgically castrated and hot-iron tail docked immediately following the completion of experimental activities.

3.1.3.1 Sham castration and tail docking

The testicular area and / or tail area were gently physically manipulated for 15 seconds without any physical injury occurring.

3.1.3.2 Surgical castration

Surgical castration was performed using a clean, sharp surgical knife and involved the following steps:

1. Excising the distal skin of the scrotum to expose the testes.
2. Extracting each individual testicle from the scrotum by traction, exposing the spermatic cord.
3. Transecting the spermatic cord approximately 10 cm proximal to the head of the epididymis.
4. Cleaning the surgical knife with disinfectant between lambs to ensure its sterility.

3.1.3.3 Hot-iron tail docking

Hot-iron tail docking was performed using a gas-heated tail docking knife and involved the following steps:

1. Heating up the gas-heated tail docking knife for 10 to 20 minutes before treatment.
2. Locating the area between the second and third coccygeal joints of the tail via palpation.
3. Positioning the blade of the gas-heated tail docking knife at the area between the second and third coccygeal joints of the tail and squeezing the lever to cut through the tail. During the cut, the tail was held out flat at 90° to the lamb's body to prevent burning the anus or vulva.
4. Cleaning the cutting edge of the knife with a wire brush between lambs to ensure the temperature of the knife was not affected by foreign matter.

3.1.4 Data collection

3.1.4.1 Facial expressions

The face of each lamb was video recorded from the side angle using hand-held video cameras for 1 minute prior and 5 minutes following treatment.

3.1.4.2 Plasma biomarkers

Blood samples (9 mL) were collected into EDTA vacutainers at 1 minute prior to and 1 hour following treatment via jugular venepuncture. Blood samples were immediately placed on ice and were centrifuged at 1600 x g for 15 minutes within 30 minutes of collection. The plasma portion of each sample was immediately pipetted into three aliquots in micro-centrifuge tubes and snap frozen in liquid nitrogen until transfer into a freezer for storage at - 80°C.

3.1.4.3 Behavioural observations

Behavioural observations were included as a measured outcome in this experiment, as a validated method for assessment of pain in lambs following tail-docking and castration. Video cameras were installed at various angles on the fences of the yards that lambs were held in for 6 hours following treatment. Each lamb had a number painted on its body for identification in the video footage. An ethogram was developed based on behaviours previous studies assessing the pain of tail-docking and castration in lambs (Marini, Colditz, Hinch, Petherick, & Lee, 2017; Paull, Lee, Colditz, & Fisher, 2009; Small et al., 2014) (Table 1). Behaviours assessed were as follows: Restlessness, Normal ventral lying, Abnormal ventral lying, Lateral lying, Abnormal standing other than statue standing, Normal standing, Statue standing, Abnormal walking and Normal walking.

Table 1: Ethogram used for behavioural observations of lambs following sham tail-docking, sham castration, tail-docking, or castration.

Behaviour	Description
Restlessness	Changing from a standing position to a lying position or vice versa at least twice within a 30 second time interval.
Normal ventral lying	Lying on sternum with legs tucked in and head up or down.
Abnormal ventral lying	Lying ventrally with hind limbs partially or fully extended. May be holding scrotal region off the ground.
Lateral lying	Lying laterally with one shoulder on the ground and extension of hind limbs, with head up or down.
Normal standing	Standing with no apparent abnormalities.
Statue standing	Immobile standing with an obvious withdrawal from interaction with other pen members and outside stimuli; Hyperextension of the hind legs; May show arched back; May show head being held below brisket.
Abnormal standing other than statue standing	Standing with an arched back or unsteadily; Often associated with foot stamping, kicking and tail wagging.
Normal walking	Walking with no apparent abnormalities.
Abnormal walking	Walking unsteadily or with a stiff gait; Includes walking backwards, on knees, moving forward with bunny hops, circling, leaning or falling.

3.1.5 Data collation

3.1.5.1 Facial expressions

Still images of lamb faces were captured from videos once every 15 seconds during the 1-minute period prior to treatment and once every minute during the 5-minute period following treatment. This resulted in 4 still images for the pre-treatment period and 5 still images for the post-treatment period. A 'lamb grimace scale' was developed, based on previous literature (Guesgen et al., 2016; Hager et al., 2017). For each image, the presence of three facial action units (orbital tightening, cheek flattening and lip tightening) were scored on a three-point numerical scale of 0 to 2 by three independent observers. A score of '0' indicated that the action unit was absent, a score of '1' indicated that the action unit was present to a moderate degree and a score of '2' indicated that the action unit was present to an obvious degree.

3.1.5.2 Plasma biomarkers

A commercial Substance P ELISA kit (Substance P ELISA kit, Enzo Life Sciences, Inc., NY, USA) was used to analyse plasma samples for concentration of neuropeptide substance P. The percent change in substance P concentrations from before treatment to 1 hour following treatment was calculated for

each animal. Plasma samples are also currently being analysed for concentrations of cytokines TNF-α and IL-1β using commercial Sheep TNF Alpha ELISA kit and Sheep IL-1β ELISA kits (Sheep TNF Alpha ELISA kit and Sheep IL-1β ELISA kit, Lifespan Biosciences, Inc., WA, USA).

3.1.5.3 Behavioural observations

Using the video footage of calves in the holding yard, a single and trained observer who was blinded to treatment conducted hourly instantaneous behavioural observations on each lamb for 6 hours (timepoints 1, 2, 3, 4, 5, 6) following treatment. Behaviours were recorded as present (1) or not present (0). Some behaviours were amalgamated for analysis: Abnormal lying (Abnormal ventral lying and Lateral lying), Abnormal standing (Abnormal standing other than statue standing and statue standing) and Abnormal behaviour (Abnormal ventral lying, Lateral lying, Abnormal standing other than statue standing, statue standing and Abnormal walking).

3.1.6 Statistical analysis

To check that there was no variation in average animal weight between treatment groups, weight data were subjected to a one-way analysis of variance (ANOVA) using the analysis of variance procedure of Genstat 17th Edition statistical software (VSN International Ltd, Hertfordshire, UK). The fixed effect included in the model was treatment.

3.1.6.1 Facial expressions

For each facial action unit, score data were subjected to ordinal logistic regression (OLR) in ASReml 3.0 statistical software (VSN International, Hertfordshire, UK). The fixed effects considered for inclusion in the model were treatment, time-point and their interaction and observer. Animal was included as a random effect in the model. Insignificant terms were dropped from the model using a backwards elimination approach until all terms in the final model were significant. *P* values < 0.05 were considered statistically significant. Post-hoc pair-wise comparisons using least significant differences at a level of $P < 0.05$ were conducted to analyse differences between groups. Data are presented as cumulative probabilities of calves displaying behavioural response scores of Y = 0, 1 and 2.

3.1.6.2 Plasma biomarkers

For percent change in substance P, IL-1β and TNF-α concentrations, data were subjected to restricted maximum likelihood (REML) for repeated measures using the mixed models procedure of Genstat 17th Edition statistical software (VSN International Ltd, Hemel Hempstead, Hertfordshire, UK). The fixed effects considered for inclusion in the model was treatment, time-point and their interaction. Animal was included as a random effect in the model. Treatment was analysed as groups specified above in section 3.1.3 and also as 'pain' and 'no pain' by combining STD and STDC groups (no pain) and TD and TDC (pain). Insignificant terms were dropped from the model using a backwards elimination approach until all terms in the final model were significant or until only one term was tested. *P* values < 0.05 were considered statistically significant.

3.1.6.3 Behavioural observations

Statistical analyses on behaviours were conducted in RStudio© (R Core Team, 2020). Logistic regression models of each animal behaviour were constructed on treatment, timepoint and the interaction between the two. *P*-values ≤ 0.05 were considered significant. Pseudo R-squared values

were generated to signify the proportion of variation in the animal behaviours explained by the explanatory variables in the model (treatment and timepoint). A correlation plot was generated to demonstrate the degree of correlation between the individual behaviours.

3.2 Investigation into a 'calf grimace scale' for assessment of pain in calves

3.2.1 Animals

Twenty male calves (2 to 4 months of age) requiring routine castration were sourced from the University of Sydney owned farm, 'Arthursleigh' in Marulan, NSW. The experiment was approved by the University of Sydney Animal Ethics Committee (approval number 2018/1447).

3.2.2 Experimental design

Prior to the experimental period, calves were kept with their mothers in a nearby paddock. On the day of experimentation, calves and their mothers were quietly moved into the cattle handling yards. Calves were then drafted from their mothers immediately prior to commencement of experimental activities. Calves were separated from their mothers for a maximum period of 3 hours. For treatment (outlined below) and video recording of facial expressions (outlined below), calves were restrained in a standing position using a cattle crush squeeze chute and a head bale. Following treatment and video recording of facial expressions, calves were held in a yard (15m x 10m) adjacent to the handling facilities. Calves were returned to the paddock with their mothers immediately following completion of experimental activities.

3.2.3 Treatments

Calves were randomly allocated to one of two treatment groups: (1) sham castration/control (CON) (n = 10) and (2) surgical castration (SC) (n = 10).

3.2.3.1 Control

The testicular area was gently physically manipulated for 15 seconds without any physical injury occurring.

3.2.3.2 Surgical castration

Surgical castration was performed using a clean, sharp surgical knife and involved the following steps:

1. Excising the distal skin of the scrotum to expose the testes.
2. Extracting each individual testicle from the scrotum by traction, exposing the spermatic cord.
3. Transecting the spermatic cord approximately 10 cm proximal to the head of the epididymis.
4. Cleaning the surgical knife with disinfectant between lambs to ensure its sterility.

3.2.4 Data collection

3.2.4.1 Facial expressions

The face of each calf was video recorded from the front angle using hand-held video cameras for 1 minute prior and 5 minutes following treatment.

3.2.4.2 Behavioural observations

Behavioural observations were included as a measured outcome in this experiment, as a validated method for assessment of pain in calves following castration. Video cameras were installed at various angles on the fences of the yard that calves were held in for 1 hour following treatment. Each calf had a number painted on its body for identification in the video footage. An ethogram was developed based on previously studies examining the pain of castration in calves (Petherick et al., 2014; Van der Saag et al., 2018) Table 2). Behaviours assessed were as follows: Relaxed walking, Walking with a stiff gait, Relaxed standing, Statue standing, Normal lying, Abnormal lying, Arching back, Licking, Licking wound, Stamping, Kicking and Flicking tail.

Table 2: Ethogram used for behavioural observations of calves following sham castration or castration.

Behaviour	Description
Relaxed walking	Walking with relaxed muscles
Walking with a stiff gait	Walking with stiff muscles at slow pace
Relaxed standing	Standing with head and muscles relaxed
Statue standing	Immobile standing with legs hyperextended and head held below brisket
Normal lying	Normal lying posture (ventral position without limbs extending)
Abnormal lying	Abnormal lying posture (lateral recumbency, extended forelimbs, one or both hindlimbs extended)
Arching back	Spine curving
Licking	Head turning back to lick body with tongue or lips, or both
Licking wound	Licking scrotal area while lifting a hind limb
Stamping	Lifting front and hind foot and strenuously returning it to the ground
Kicking	Kicking forwards or backwards with a hind limb
Flicking tail	Moving the tail sideways back and forth

3.2.5 Data collation

3.2.5.1 Facial expressions

Still images of calf faces were captured from videos once every 15 seconds during the 1-minute period prior to treatment and once every minute during the 5-minute period following treatment. This resulted in 4 still images for the pre-treatment period and 5 still images for the post-treatment period. A 'calf grimace scale' was developed, based on previous literature (Gleerup, Andersen, Munksgaard, & Forkman, 2015; Guesgen et al., 2016; Hager et al., 2017). For each image, the presence of three facial action units (orbital tightening, nostril tightening and tension of the muscles above the eye) were scored on a three-point numerical scale of 0 to 2 by three independent observers. A score of '0' indicated that the action unit was absent, a score of '1' indicated that the action unit was present to a moderate degree and a score of '2' indicated that the action unit was present to an obvious degree.

3.2.5.2 Behavioural observations

Using the video footage of calves in the holding yard, a single and trained observer who was blinded to treatment conducted instantaneous behavioural observations on each calf at 10-minute intervals for 1 hour (timepoints 1, 2, 3, 4, 5, 6) following treatment. Behaviours were recorded as present (1) or not present (0).

3.2.6 Statistical analysis

3.2.6.1 Facial expressions

For each facial action unit, score data were subjected to ordinal logistic regression (OLR) in ASReml 3.0 statistical software (VSN International, Hertfordshire, UK). The fixed effects considered for inclusion in the model were treatment, time-point and their interaction and observer. Animal was included as a random effect in the model. Insignificant terms were dropped from the model using a backwards elimination approach until all terms in the final model were significant. P values < 0.05 were considered statistically significant. Post-hoc pair-wise comparisons using least significant differences at a level of $P < 0.05$ were conducted to analyse differences between groups. Data are presented as cumulative probabilities of calves displaying behavioural response scores of Y = 0, 1 and 2.

3.2.6.2 Behavioural observations

All behavioural data were analysed using Genstat® 17th Edition statistical software (VSN International Ltd., Hertfordshire, UK). A generalised linear mixed models (GLMM) procedure with a binomial distribution was used to analyse data on all behaviours. For each observed behaviour, fixed effects were treatment (castration or control) and timepoint (pre-castration or post-castration) and the random effect was calf number. P-values ≤ 0.05 were considered to have statistical significance. Data on observed behaviours is presented as predicted means.

3.3 Assessing the pain of different methods for castrating and tail docking lambs

3.3.1 Animals

A total of 72 Merino first cross lambs (8 weeks old) requiring routine marking, that is tail docking and castration, were used in this study. These lambs were sourced from a commercial property in Goulburn NSW. The experimental protocol was approved by the University of Sydney Animal Ethics Committee (approval number 2019/1577).

3.3.2 Experimental Design

The experiment was conducted over 14 days, with all lambs treated on day 0, and data collected on days 0, 1, 2, 4, 7 and 14 following treatment. Video cameras were attached in a vertical position above four pens within the sheep handling shed where all experimental activities occurred. For treatment, data collection and attachment of accelerometers, lambs were restrained in a lamb marking cradle.

3.3.3 Treatments

Lambs were randomly allocated to one of six treatment groups: (1) Control / no marking (CON, *n* = 6 males and 7 females); (2) Cautery tail-docking (CTD, *n* = 12 females); (3) Surgical tail-docking (STD, *n* = 12 females); (4) Rubber ring tail-docking (RRTD, *n* = 12 females); (5) Surgical castration (SC, *n* = 12 males); and (6) Rubber ring castration (RRC, *n* = 11 males). At the end of the experiment, the lambs in the CON treatment group were immediately castrated with a rubber ring, and hot-iron tail docked, as per normal practice at this property.

Cautery tail-docking: This was performed using a gas-heated tail docking knife after heating the knife for 10-20 minutes prior to use. The cut was made in the area between the second and third coccygeal joints of the tail, after the area was palpated for accuracy. To avoid injury to the anus or vulva, the tail was held at 90˚ to the lamb's body. A standard cleaning technique for the knife was used between each lamb by brushing the cutting edge of the knife with a wire brush. This was to ensure that no residual foreign material was remaining on the knife, affecting the knife temperature and sterility.

Rubber ring tail-docking: This was performed using elastrator pliers and elastrator rubber rings. The ring was stretched with the elastrator pliers and placed in the area between the second and third coccygeal joints of the tail, after being palpated for accuracy. During placement of the ring, the tail was held at 90˚ from the body of the lamb.

Surgical tail-docking: A clean, sharp surgical knife was used to dock the tail at the area between the second and third coccygeal joint, after palpation of the joint for accuracy. The cut was made in a quick motion with the tail held out 90˚ from the body of the lamb. The knife was cleaned with disinfectant between each lamb to maintain sterility.

Surgical castration: A clean, sharp surgical knife was used to first excise the distal skin of the scrotum to expose the testes. Each testicle was exteriorised, and the spermatic cord was cut approximately 10cm proximal to the head of the epididymis. The surgical knife was cleaned with disinfectant between each lamb to maintain sterility.

Rubber ring castration: This was performed using elastrator pliers and elastrator rubber rings. After palpation to locate both testicles, the rubber ring was stretched with elastrator pliers and placed above the testes around the neck of the scrotum.

3.3.4 Data collection and collation

3.3.4.1 Plasma biomarker (Interleukin-10)

Blood samples (10mL) were collected into EDTA vacutainers via jugular venepuncture using a 21G needle from each lamb at each timepoint. Blood samples were stored on ice immediately following collection and centrifuged within 30 min at 1500g for 10 min. Plasma was collected and stored on dry ice until transfer to a -80°C freezer at the end of each data collection day. Samples from days 1, 4, 7 and 14 were analysed for measurement of IL-10 concentrations using a commercial Sheep Interleukin 10 (IL10) ELISA kit (Sheep Interleukin 10 (IL10) ELISA kit, My BioSource, CA, USA).

3.3.4.2 Wound temperature

To measure the surface temperature of the scrotum or tail, infrared photographs of the affected area were captured from all treated and controlled lambs on days 0, 1, 2, 4, 7 and 14 when the lambs were restrained in a lamb marking cradle. Infrared (IR) images were captured using a handheld IR camera, FLIRE50 (FLIR Systems Australia Pty Ltd, VIC, Australia), with a thermal range of -20˚C to 120˚C and a sensitivity of 0.045˚C. Consistency was maintained in images by using a 10cm × 10cm cardboard frame to standardise the image area. The affected area was centred in the frame and the camera frame aligned with the cardboard frame. This helped to ensure a consistent distance from the camera lens to the affected area of the lamb and maximise accuracy of temperature. Ambient temperature and humidity were also recorded as each photograph was taken, and the IR camera was calibrated with this data every 30 minutes for maximum accuracy. The IR images obtained were later analysed by FLIR Tools Software (FLIR Systems Australia Pty Ltd) for maximum, minimum and mean temperature strictly within the selected boundaries of the cardboard frame.

3.3.4.3 Wound morphology

To measure the visual appearance of the treatment site digital photographs were taken of the tail or scrotum using a digital camera on days 0, 1, 2, 4, 7 and 14. Each lamb was restrained in a lamb marking cradle on the treatment day and all observation days to maximise image quality and consistency of positioning. Consistency was also maintained in images by using a 10cm × 10cm cardboard frame to standardise the size of the area imaged and assessed. The photo number and lamb number were recorded onto a spreadsheet and analysis was later conducted using a customised scale. in the frame, with the camera frame aligned with the cardboard frame and the lens in focus.

3.3.4.4 Facial expression

The face of each lamb was video recorded from the side angle using hand-held video cameras for 3 minutes on days 0, 1, 2, 4, 7 and 14. Screenshots of the lamb's faces were captured at 30 second intervals over each 3-minute video. A modified Sheep Grimace Scale (SGS) was then formulated using some of the images captured for each facial action unit: (1) orbital tightening (2) lip tightening and (3) nose tightening. For orbital tightening, partially closed eyes were suggestive of a moderate pain response (SGS 1), whereas completely closed eyes were suggestive of a marked pain response (SGS 2). Lip tightening scales were created such that a straight, flat lip angle was suggestive of a moderate pain response (SGS 1) and a lip with a downward curl was indicative of a marked pain

response (SGS 2). Similar to this, an increased nose angle was suggestive of a greater pain response (SGS 2), whereas a flattened nose angle was suggestive of less nose tightening and hence a lower degree of pain. Each facial action unit was assessed on an individual basis using the modified SGS by two trained observers.

3.3.5 Statistical analysis

3.3.5.1 Plasma biomarker (Interleukin-10)

Data were subjected to restricted maximum likelihood (REML) for repeated measures using the mixed models procedure of Genstat 17th Edition statistical software (VSN International Ltd, Hertfordshire, UK). The fixed effects considered for inclusion in the model was treatment, time-point and their interaction, and body weight. Animal was included as a random effect in the model. Insignificant terms were dropped from the model using a backwards elimination approach until all terms in the final model were significant or until only one term was tested. *P* values < 0.05 were considered statistically significant.

3.3.5.2 Wound temperature

For the wound temperature data, the maximum temperatures were analysed. However, some extreme low temperatures (under 30°C) were removed prior to analysis. The data for tail-docking and castration were each analysed using a linear mixed model with fixed effects for treatment, Day and Treatment × Day interaction, and body weight (covariate), and a random effect for animal. Some additional filtering of data was made by removing observations with absolute values of standardised residuals in excess of 3. Model fitting was also conducted using the lme4 package in RStudio© (R Core Team, 2020), with model-based means estimated using the emmeans package and cld function in the multcomp package used for pairwise comparison of treatment means within the same day.

3.3.5.3 Wound morphology

The wound score data for tail-docking and castration procedures, specifically for swelling. exudate and healing, were recorded on an ordinal scale, namely swelling: 0-3; exudate:1-5; and healing: 1-4. However, due to small frequencies of several of these scores, they were collapsed to binary measures for both tail docking and castration, namely swelling: 0 (no) vs 1-3 (yes); exudate: 1 (no) vs 2-5 (yes); healing stage (knife and hot knife) 1-2 (no scab) vs 3-4 (scab); and healing stage (rings):1-2 (no) vs 3 (yes). For analysis, logistic generalised linear mixed models were fitted to the score data, with separate analyses for the tail docking and castration data, and separately for each treatment as the scales were not comparable between them. The 'yes' and 'no' were converted to 1 and 0 respectively to facilitate analysis. Day was a fixed effect and animal was the random effect. Model fitting was conducted using the lme4 package in RStudio© (R Core Team, 2020). Fitted models were evaluated as the probability of a "yes" for Day using the emmeans package in R.

3.3.5.4 Facial expression

The eye, lip and nose facial grimace score data of lambs are recorded on an ordinal scale (0, 1, 2) and for their analysis, ordinal mixed models were fitted to the score data, with separate analyses for the tail docking and castration data. Fixed effects were Treatment, Day, Observer, together with their two- and three-way interactions as well as bodyweight (covariate). Random effects in the models were Lamb and Day nested within Lamb. Where interactions with Observer were non-significant, these were dropped from the models. Model fitting was conducted using the ordinal package in RStudio©

(R Core Team, 2020). Fitted models were evaluated as the probability distributions (for score of 0, 1 and 2) for each combination of Treatment × Day, or Treatment × Day × Observer if required, using the emmeans and RVAideMemoire packages in R, and displayed as cumulative bar charts. Formal comparison between treatments within a day were conducted using the cld function in the multcomp package in R.

Inter-rater reliability of the Observers was evaluated by a cross-tabulation of scores from the two observers, followed by estimation of the polychoric correlation between the scores using the polycor package in R. This form of correlation is applicable to ordinal data.

3.4 Determining current 'best practice' pain mitigation for castration and dehorning cattle

3.4.1 Animals

762 mixed breed weaner calves (155 ± 18kg) due to undergo routine ‘marking’ (that is castration and dehorning) were used. All animals were sourced from industry partner Consolidated Pastoral Company’s breeding property ‘Isis Downs’ in Qld, where the experiment was conducted. The protocol was approved by the University of Sydney’s Animal Ethics Committee (approval number 2019/1584).

3.4.2 Experimental design

Calves were housed in large yards and were fed a total mixed ration once daily during the experiment, as per normal practice at the property. Calves were processed through a cattle race and restrained in a weigh box and weaner cradle for all experimental procedures. Fitting of ear tag sensors, treatment, blood sampling, and photographing wounds required calves to be processed through a cattle race and restrained in a weaner calf cradle (designed for calves between 6 and 12 months of age).

3.4.3 Treatments

All polled female and polled stud male cattle were allocated to: (1) positive control group (no castration or dehorning) (PC; n = 98). All other calves were blocked by sex and breed and randomly allocated to one of four treatment groups: (2) negative control group (castration and / or dehorning with no pain mitigation) (NC; n = 97), (3) castration and / or dehorning with pre-operative meloxicam (M; n = 155); (4) castration and / or dehorning with intra-operative topical anaesthetic (TA; n = 258), and (5) castration and / or dehorning with pre-operative meloxicam and intra-operative topical anaesthetic (MTA; n = 154). All polled male cattle were castrated only and all female cattle were dehorned only. The inclusion of a treatment group that did not receive any analgesia was necessary to be able to assess and compare the efficacy of each analgesic protocol. However, the numbers in this treatment group were reduced to the minimum number thought to provide statistical significance. Availability of meloxicam was unexpectedly limited, hence the reduced number of cattle in the M and MTA treatment groups. Table 3 outlines the treatment allocations and numbers.

Table 3: Treatment allocation. Numbers of animals that were castrated, dehorned or both, with and without topical anaesthetic, meloxicam or both.

Treatments	Castration	Dehorning	Castration and dehorning	No procedure	Total
No procedure				98	98
No pain mitigation	3	54	40		97
Topical anaesthetic	12	139	107		258
Meloxicam	4	89	62		155
Topical anaesthetic and meloxicam	3	87	64		154
Total	22	369	273		762

3.4.3.1 Castration and dehorning

Castration and dehorning was performed as per normal operation by experienced technicians employed at the commercial beef company where the trial was conducted. Castration was performed surgically, by pushing the testicles to the distal end of the scrotum and incising the scrotal skin and tunica dartos from the base and up each side with a scalpel blade and then the tunica vaginalis to expose the testes. Each testicle was then extruded through the openings to expose and sever the spermatic cords approximately 10 cm proximal to the head of the epididymis using the scalpel blade. Dehorning was performed using a Yearling Cup dehorner. Dehorning was conducted by opening the cup, placing it over the horn, applying downward pressure and closing the handles to remove the horn tissue and immediate surrounding skin. The scalpel blade and the cup dehorner were chemically sterilised between use on each animal.

3.4.3.2 Analgesic products

Analgesic products, topical anaesthetic (Tri-Solfen®, Dechra Pharmaceuticals Australia Pty Ltd, NSW, Australia) and meloxicam (Metacam20®, Boehringer Ingelheim, NSW, Australia) were administered by experienced technicians. Both products were administered as per the instructions. Meloxicam was subcutaneously injected using an injecting gun at a dose rate of 0.5 mg/ kg body weight immediately prior to castration and / or dehorning. Topical anaesthetic was applied topically via a spray applicator, with approximately 4 mL used on castration wounds and approximately 4 mL used for dehorning wounds. For castration, topical anaesthetic was applied following extrusion of the testes and prior to severing the spermatic cords, by inserting the nozzle into the tunica vaginalis and delivering the product into the inguinal canal. For dehorning, Tri-Solfen® was applied directly onto the wounds immediately following the procedure.

3.4.4 Data collection

3.4.4.1 Weight gain

All calves were weighed at the following time-points: 7 days before treatment, immediately before treatment, 7 days post treatment and 35 days post treatment. Percent change in body weight was calculated for each animal at each time-point.

3.4.4.2 Behaviour

Allflex® ear tag sensors (Allflex Australia Pty Ltd, Qld, Australia) were fitted 7 days before treatment and remained fitted for the duration of the experiment. All details for this component of the experiment are described in P.PSH.0819 'Objective measures of welfare' milestone 7 report.

3.4.4.3 Wound temperature and healing

Infrared photographs and digital photographs were collected from a subset of calves (n = 15) from each of the marked treatment groups at the following time-points: immediately prior to treatment, 7 days following treatment and 35 days following treatment. Infrared images were captured and analysed using the same method as described in section 3.3.4.2**.** Digital images of all wounds were captured and wound healing was scored on a numerical rating scale of 1 to 4. For castration, a score of 1 was assigned to open wounds, a score of 2 was assigned to scabbed wounds, a score of 3 was assigned to wounds with fibrous tissue and a score of 4 was assigned to wounds with complete sealing and mature skin. For dehorning, a score of 1 was assigned to wounds that consisted of a hole exposing the sinus, a score of 2 was assigned to wounds with a scab at bone level, a score of 3 was assigned to wounds with a scab at skin level and a score of 4 was assigned to wounds with fibrous tissue.

3.4.5 Statistical analysis

Weight gain and wound temperature data were subjected to the linear mixed models procedure for repeated measures in Genstat (VSN International, Herfordshire, UK) and wound morphology data were subjected to the ordinal logistic regression model in ASReml VSN International, Herfordshire, UK), with treatment (positive control, negative control, topical anaesthetic, meloxicam, topical anaesthetic and meloxicam) or procedure (positive control, castration, dehorning, castration and dehorning) and timepoint (7 days, 35 days) as fixed effects and animal as a random effect in the models.

3.5 Pharmacokinetics of a sustained release meloxicam formulation in cattle

3.5.1 Animals

Six Holstein Friesian heifer calves (age 5 – 7 months, mean weight ± SD, 171.1 ± 14.7 kg) were obtained from the University's commercial replacement heifer herd at 'Corstorphine' dairy farm. All procedures were approved by the Animal Ethics Committee of the University of Sydney (approval number 2019/1645).

3.5.2 Experimental Design

The experimental design was adapted from (Coetzee, KuKanich, Mosher, & Allen, 2009). A randomized cross-over treatment design was conducted, across two 14-day blocks, with a 10-day wash out period between blocks. Heifers were moved from their residing paddock into a general holding area and then individually through the cattle crush (PG40 Crush, Clipex, Qld, Australia). Heifers were restrained in the crush using the head bale to undergo treatment administration and blood collection and were then released back into the holding area. At 0h, heifers were weighed using electronic weigh scales (Gallagher TW-1, Australia) as they entered the crush, and randomly assigned to one of two meloxicam treatments (n=3 calves per treatment): 1.0mg/kg of SC injection with SR Meloxicam (SR-M) (Australian Custom Pharmaceuticals Pty Ltd., NSW, Australia) or, 0.5mg/kg of subcutaneous injection with conventional meloxicam (Metacam20®, Boehringer Ingelheim, NSW, Australia) (CM). Blood collection via jugular venepuncture were taken pre-treatment (0h), at 2h, 4h, 6h, 8h, 10h, 12h and 24h (+/- 4 min) relative to treatment, and on days 2, 4, 5, 6, 7, 10, 14 (+/- 10 min relative to original treatment time

3.5.3 Data collection

Calves were moved from residing paddocks to a general holding area, before being guided through the crush, individually restrained for blood collection in the head bale, and then released back into the holding pen. Blood samples (9mL) for determination of plasma concentration of meloxicam were collected via jugular venepuncture into lithium heparin anti-coagulant tubes at the previously discussed time points. All blood tubes were inverted several times and stored on ice until processing within 30mins of sampling. Blood samples were spun using an Elmi centrifuge (model CM-6MT) at 1500g for 10 minutes (Coetzee et al., 2009). Plasma from each sample was isolated into a separate plasma cryovile using disposable pipettes and frozen at -20 degrees Celsius until laboratory analysis which occurred 45 days after the final day of sample collection.

3.5.4 Quantification of plasma meloxicam concentrations

Plasma meloxicam concentrations were quantified by validated reversed phase high-performance liquid chromatography coupled with UV detection (HPLC-UV), as described by (Woodland et al., 2019). Briefly, the HPLC system consisted of a Shimadzu CBM-20A module (Shimadzu, Kyoto, Japan) equipped with a LC-20AT delivery unit with DGU-20As degassing solvent delivery unit and SIL-20AC auto injector. Chromatographic separation was performed using a Synergi C18 column (MAX-RP 80A, 150 x 4.6mm, 4µm Phenomenex, Lane Cove, NSW) attached to a 1 mm Opti-guard C-18 column (Optimize Technologies, Alpha Resources, Thornleigh, Australia) with separation performed under ambient temperature (Shimadzu, Kyoto, Japan). The isocratic mobile phase composed of 50 mm potassium phosphate buffer (pH 2.15) and acetonitrile (55: 45, v/v), and the flow rate was 1mL/min. The eluent was monitored at 355 nm via SPD-20A UV detector (Shimadzu Kyoto, Japan). The calibration standards of blank plasma spiked with meloxicam (Sigma-Aldrich, St. Louis, MO, USA) [0.048-25µg/mL], QC samples (0.2, 2 and 20 ug/mL) and unknown plasma samples were extracted using protein precipitation method. Briefly, 100 uL of plasma sample was mixed and vortexed with 200 uL of acetonitrile (containing 3.3ug/mL of internal standard; piroxicam (Sigma-Aldrich, St. Louis, MO, USA); followed by centrifugation at 14,000g for 10min. After the centrifugation, 20uL of the supernatant was injected for HPLC analysis. Precision and accuracy of the LOQ (0.048 µg/mL) were <15% [coefficient of

variation (CV)] and within 20% of nominal concentration, respectively. For quality control (QC) samples (0.2, 2 and 20 µg/mL), the intra- (n = 3) and inter-day (3 days) precision ranged from 1.24 to 9.23% and 2.09 to 3.91% (CV), respectively. Intra-day and inter-day accuracy of QC samples expressed as a percentage of the bias ranged from 0.99 to 9.23 and 3.48 to 7.08, respectively. The retention times of meloxicam and the internal standard were approximately at 8.29 and 5.12 min, respectively.

3.5.5 Pharmacokinetic analysis

The pharmacokinetic (PK) profiles for all plasma concentrations were computationally manipulated and analysed using a non-compartmental model in Microsoft Excel (Version 16.37). The PK values calculated include Cmax, the maximum drug concentration in the plasma and Tmax, the time taken to reach Cmax, measured by visual observation of the data. The elimination constant (kel) was determined by the negative gradient of the slope during the elimination phase of the natural log of drug concentration versus time (Woodland et al., 2019), and the area under the concentration time curve, from 0 to the last measurable time point (AUC0-t) was calculated using the linear trapezoidal method in GraphPad Prism (Version 8.4.2 (464)). The half-life, terminal AUC (AUCt-λ), AUC to infinity (AUC0-λ), apparent clearance (CL/F) and apparent volume of distribution (V/F) were determined as seen below.

$$t1/2 = ln\,2/kel$$

$$AUCt - l = Clast/kel$$

$$where\ Clast = the\ last\ measurable\ plasma\ concentration$$

$$AUC0 - l = AUC0 - t + AUCt - l$$

$$Cl/F = total\ dose\ administered\ to\ animal/\ AUC0 - l$$

$$V/F = Cl/kel$$

3.5.6 Statistical analysis

Phase, drug and time were analysed in RStudio© (R Core Team, 2020) (Version 0.99.891) using a restricted maximum likelihood linear mixed model (REML) and packages emmeans, nlme, lme4 to determine any significant differences. The fixed effects included phase, drug and time, while the random effect was animal. A univariant analysis was undertaken where all fixed effects were classified as significant, with $P < 0.05$. Therefore, all factors were included in the linear mixed model where a significant three-way interaction was seen. Pairwise comparisons were also conducted on phase and drug treatment to identify the time points at which significant differences occurred.

3.6 Pharmacokinetics of a novel modified release meloxicam formulation in sheep

3.6.1 Animals and Housing

The experiment was approved by The University of Sydney Animal Care and Ethics Committee (approval number 2017/1215). The study utilised six Merino ewe hoggets sourced from The University of Sydney's property, Mayfarm, in Camden, New South Wales, Australia. The sheep had a mean (±SD)

weight of 41.5 kg (±4.6 kg). During the experimental period, sheep were housed in a sheltered yard (20 m × 10 m) with dirt flooring and straw bedding. All sheep were provided ad libitum access to lucerne hay and water. Sheep were returned to Mayfarm at the conclusion of the study.

3.6.2 Treatment

All sheep were injected with a novel SRMF (60 mg/mL) (Australian Custom Pharmaceuticals Pty Ltd., NSW, Australia) at a dose rate of 2 mg/kg bodyweight. The novel formulation, administered via a single subcutaneous (SC) injection using an 18 g needle, had been specifically formulated for the sustained release of meloxicam. The SRMF consisted of a biodegradable polymer and 60 mg/mL of meloxicam in a water-miscible organic solvent. This formulation forms an in situ solid bolus after subcutaneous injection, releasing meloxicam slowly from the polymer. Although it is common practice for SC injections to be administered into the neck of sheep, in this study, the SRMF was injected subcutaneously under the left forelimb. This was performed to allow physical distancing between the ultrafiltration sampling probes positioned in the dorsal neck and the injection site.

3.6.3 Sample Collection

Blood samples (10 mL) were collected into lithium heparin vacutainers via jugular venepuncture using an 18 g needle. Blood samples were collected immediately prior to treatment (0 h), then at 2, 4, 6, 8, 10, 12, 24, 48, 96, 144, 168, 192, and 336 h following treatment. Following blood collection at each time point, samples were centrifuged at 1700× *g* for 7 min. Plasma was extracted and stored at −20°C until analysed.

Interstitial fluid (ISF) samples were collected from sheep using in vivo ultrafiltration sampling probes (RUF-3-12 Reinforced In Vivo Ultrafiltration Sampling Probe, BASI Research Products, Lafayette, IN, USA) implanted subcutaneously in the neck by insertion using a 14 g canula as a guide and then suturing in place. Vacutainers, attached to the probes for sample collection, were housed in a pouch on a collar placed around the sheep's neck. Sampling probes were inserted immediately prior to treatment. Interstitial fluid was collected from vacutainers at 8 to 12 h, 12 to 24 h, 24 to 48 h, 48 to 52 h, and 92 to 96 h and was stored at −20°C until analysis. The maximum ISF collected at each interval was approximately 1 mL per sheep. These time periods were utilised to allow adequate time between sampling for collection of a sufficient volume of fluid for analysis.

3.6.4 Plasma and ISF Meloxicam Analysis

High-pressure liquid chromatography (HPLC) analysis with ultraviolet detection was utilised to determine the concentration of meloxicam in the plasma and ISF samples as previously described (Woodland et al., 2019).

2.5. Pharmacokinetic Analysis

The PK profile was established through a noncompartmental model using PK Solver [16]. The indices of maximum observed plasma concentration (C_{max}) and time taken to reach maximum plasma concentration (T_{max}) were determined through visual comparison of the plasma concentration and time curve. The elimination rate constant (k_{el}) was established through a semi-log regression of the terminal slope. The terminal half-life ($t_{1/2}$) was determined as ln $2/k_{el}$.

The area under the concentration-time curve (AUC0-t last) was calculated to the last measurable concentration (t_{last}) using the trapezoidal method. The AUC and AUMC from the last observed concentration to infinity were determined by:

$$AUCt{-}\infty = Clast/kel$$

$$AUMCt{-}\infty = (Clast \times tlast/kel) + (Clast/kel2)$$

The mean residence time (MRT) was determined by:

$$MRT = AUMC0{-}\infty/AUC0{-}\infty$$

The apparent volume of distribution was determined by:

$$V/F = (Dose \times AUMC)/AUC2$$

The apparent clearance was determined using:

$$CL/F = Dose/AUC$$

The amount of unbound meloxicam in the ISF was also quantified.

3.7 Plasma concentrations of meloxicam in calves fed meloxicam pellets

3.7.1 Animals

Twelve Holstein Friesian calves (age 6 – 8 weeks, mean weight ± SD, 85kg ± 18.7 kg) were selected from the University's commercial replacement heifer herd at "Corstorphine" dairy farm.

3.7.2 Experimental design

All procedures were approved by the Animal Ethics Committee of The University of Sydney under approval number 2020/1784.

Calves were moved from their home pen to the holding yards (approximately 80m) in the morning of the first day of the experiment where they were individually weighed in a standard cattle race using a weigh platform (Thunderbird CS-2P2 Platform, Mudgee NSW). Calves were then manually restrained in the head bale and sprayed with an individual number (1-12) using tail paint marker on each flank for ease of identification. Calves were released into the holding area and moved back to their home pen. To accurately formulate the medicated pellets at the correct dosage rate, the average daily consumption of non-medicated calf starter pellets (Vella Stock Feeds, Glendenning NSW) by calves was determined over 7 days. One kg per calf of 22% calf grower pellets (Vella Stock Feeds, Glendenning NSW) were fed to calves in two feed troughs (~2m x 60cm) each morning between 8-9am for 7 days. Calves consumed the entire ration of pellets each day so there was no requirement to weigh remaining pellets. At the conclusion of the 7 days of pre-feeding, calves were re-weighed and returned to routine farm practice for an additional 4 days to allow for medicated pellet formulation.

On day 12 the calves were moved to the holding yards, and individually restrained in the head bale for blood collection of a baseline sample. Calves were then released into the holding area and moved

back to their home pen, where 1kg per calf of meloxicam medicated 22% calf grower pellets (USYD Poultry Unit, Camden, NSW) were fed to calves following the same procedure and timeline as the pre-feeding period. Blood samples for meloxicam-medicated pellet were collected pre-treatment (0h) and between 8am and 10am daily for 9 days. Samples were processed as described under sample processing below (Section 2.3).

3.7.3 Pellet formulation

Meloxicam-medicated pellets were formulated by the University of Sydney's Poultry Unit. 100kg of non-medicated calf starter pellets (Vella Stock Feeds, Glendenning NSW) were ground and sieved. Ground pellets were then dosed with meloxicam at a rate of 1mg/kg of bodyweight, based on a 120kg calf, where the final amount of meloxicam added to the ground pellet mix was 12.053g in 100kg of pellets. 3% water was added was also added to the mix prior to pelleting. Ground pellets were then converted back to solid pellets using a cold pelleting compaction technique, run at 50OC. Steel rollers compressed the pellet mix into a steel mould and cut to the standard die dimensions of 4mm.

3.7.4 Blood collection and processing

Calves were moved from residing paddocks to a general holding area, before guided through the crush, individually restrained for blood collection in the head bale, and then released back into the holding pen. Blood samples (9mL) for determination of plasma concentration of meloxicam were collected via jugular venepuncture into lithium heparin anti-coagulant tubes at the previously discussed time points. All blood tubes were inverted several times and stored on ice until processing within 30mins of sampling. Blood samples were spun using an Elmi centrifuge (model CM-6MT) at 1500g for 10 minutes (Coetzee et al., 2009). Plasma from each sample was isolated into a separate plasma cryovile using disposable pipettes and frozen at -20 degrees until laboratory analysis occurred 8 days after the final day of collection.

3.7.5 Quantification of plasma meloxicam concentrations

Meloxicam concentrations in all plasma samples were quantified by validated reversed phase high-performance liquid chromatography coupled with UV detection (HPLC-UV) (Woodland et al., 2019). Briefly, the HPLC system consisted of a Shimadzu CBM-20A module (Shimadzu, Kyoto, Japan) equipped with a LC-20AT delivery unit with DGU-20As degassing solvent delivery unit and SIL-20AC auto injector. Chromatographic separation was performed using a Synergi C18 column (MAX-RP 80A, 150 x 4.6mm, 4µm Phenomenex, Lane Cove, NSW) attached to a 1 mm Opti-guard C-18 column (Optimize Technologies, Alpha Resources, Thornleigh, Australia) with separation performed under ambient temperature (Shimadzu, Kyoto, Japan). The isocratic mobile phase composed of 50 mm potassium phosphate buffer (pH 2.15) and acetonitrile (55: 45, v/v), and the flow rate was 1mL/min. The eluent was monitored at 355 nm via SPD-20A UV detector (Shimadzu Kyoto, Japan). The calibration standards of blank plasma spiked with meloxicam (Sigma-Aldrich, St. Louis, MO, USA) [0.048-25µg/mL], QC samples (0.2, 2 and 20 ug/mL) and unknown plasma samples were extracted using protein precipitation method. Briefly, 100 uL of plasma sample was mixed and vortexed with 200 uL of acetonitrile (containing 3.3ug/mL of internal standard; piroxicam (Sigma-Aldrich, St. Louis, MO, USA); followed by centrifuged at 14,000g for 10min. After the centrifuged, 20uL of the supernatant was injected for HPLC analysis. Precision and accuracy of the LOQ (0.048 µg/mL) were <15% [coefficient of

variation (CV)] and within 20% of nominal concentration, respectively. For quality control (QC) samples (0.2, 2 and 20 µg/mL), the intra- (n = 3) and inter-day (3 days) precision ranged from 1.24 to 9.23% and 2.09 to 3.91% (CV), respectively. Intra-day and inter-day accuracy of QC samples expressed as a percentage of the bias ranged from 0.99 to 9.23 and 3.48 to 7.08, respectively. The retention times of meloxicam and the internal standard were approximately at 8.29 and 5.12 min, respectively.

3.7.6 Pharmacokinetic analysis

The pharmacokinetic (PK) indices for all plasma concentrations were computationally manipulated and analysed using a non-compartmental model in Microsoft Excel (Version 16.37). The PK values calculated include, the elimination constant (kel), determined by the negative gradient of the slope during the elimination phase of the natural log of drug concentration versus time, and the half-life determined as 1/2 = ln 2/kel.

3.8 Investigating the efficacy of medicated meloxicam pellets in calves

3.8.1 Animals

Forty Holstein-Friesian heifer calves (approximately 1 to 2 months old) requiring routine disbudding were sourced from the University of Sydney property 'Corstorphine'. The protocol was approved by the University of Sydney's Animal Ethics Committee (2020/1780). During the experimental period, calves were fed milk and calf pellets (1kg/ head/ day) as per normal practice at this property.

3.8.2 Experimental design

As Holstein-Friesian heifer calves requiring routine disbudding are consistently available at the University of Sydney, we utilised these animals and this husbandry procedure to assess the efficacy of medicated meloxicam pellets. The experiment was conducted in two experimental blocks, with 20 calves per block (5 calves per treatment group). Within each experimental block, treatment occurred on day 0 and data collection occurred on days -1, 0 (4 hours), 1, 2, 3, 6, 9 and 12 relative to treatment. Prior to and during the experimental period, calves were held in housing paddocks in their treatment groups of 5 animals with access to shelter, water, and feed, as per normal practice at this property. On all experimental days, calves were quietly moved into the nearby handling yards for treatment and data collection. On all other days during the experimental period, calves remained in the housing paddocks. For treatment and data collection, calves were restrained in a head bale within a cattle crush. For video recording of animal behaviour, calves remained in the housing paddocks. Calves were returned to the housing paddocks following completion of experimental activities each day. During the experimental period, calves were fed milk and calf pellets as per normal practice at this property.

3.8.3 Treatments

Ten minutes prior to treatment, all calves were sedated with xylazine (0.04mL/kg) and 5 minutes prior to treatment, all a lignocaine cornual nerve block (2% Lignocaine, 5mL/horn bud) was administered to both horn buds. Calves were randomly allocated to the following treatment groups:

1) Positive control – Sham disbudding (gentle manual palpation of the horn buds).

2) Negative control - Cautery disbudding.

3) Conventional meloxicam – Conventional meloxicam (Metacam® 20mg/mL, Boehringer Ingelheim) administered subcutaneously using an 18G needle and syringe at a dose rate of 0.5mg/kg body weight 1 hour prior to cautery disbudding.

4) Medicated meloxicam pellets - Medicated meloxicam pellets (1kg/ head/ day), formulated at a dose rate of 120 mg/kg pellets (to achieve an approximate dose rate of 1mg/kg BW), fed daily for one day prior to and 7 days following cautery disbudding, after which point calves were given unmedicated calf pellets (1 kg/ head/ day) as per normal practice. Meloxicam pellets were formulated as described in section 3.7.3. Calves were fed medicated meloxicam pellets individually in separated pens. If the medicated pellets were not entirely consumed after 2 hours, calves were released from the separated pens and the remainder of pellets were combined and left in a shared trough for calves to access *ad libitum* as a group.

3.8.4 Data collection

Data was collected 1 day prior to (day -1) and at 4 hours (day 0), and 1, 2, 3, 6, 9 and 12 days following treatment. One day prior to treatment, calves were weighed, spray painted with individual identification numbers on both flanks and the back, and accelerometers were attached as described in section 3.8.4.2.

3.8.4.1 Plasma meloxicam

Blood samples (10 mL) were collected into lithium heparin vacutainers for analysis of plasma meloxicam concentration, as described in section 3.7.4. Meloxicam concentration was quantified as described in section 3.7.5. Pharmacokinetic analysis was conducted as described in section 3.7.6.

3.8.4.2 Behavioural observations

Behaviour was continuously recorded using a CCTV system (Swann Smart Security System®, Wi-Fi NVM-490) set up in the housing paddocks. Each animal was identified in the video by its individual spray-painted number. The video footage was used to record instantaneous behavioural samples using an ethogram (Table 4) outlining normal and pain-related behaviours as informed by our previous research and the literature (Faulkner & Weary, 2000; Gleerup et al., 2015; Graf & Senn, 1999; Stafford & Mellor, 2005; Winder et al., 2018). Four trained blinded observers conducted instantaneous behavioural observations during the afternoon of each experimental day (-1, 0, 1, 2, 3, 6, 9 and 12). Dependent on calf visibility, one observation was taken every 15 minutes for approximately 3 to 3.5 hours, resulting in 12 to 13 observations for each calf per day.

Table 4: Ethogram used for behavioural observations of calves following sham disbudding or disbudding.

Behaviour	Description
Ear flicking (EF)	Calf rapidly moves one or both ears independent of head movement or external stimuli e.g. flies.
Head shaking (HS)	Calf rapidly moves whole head side to side or up and down, independent of external stimuli.
Rearing (R)	Transfer of calf bodyweight to hind legs with both fore legs raised simultaneously.

Behaviour	Description
Tripping (T)	Rapid alternate lifting of two or more fore or hind legs, independent of other motive e.g. regaining balance. Event noted each time calf starts lifting legs from having all four on ground.
Pawing (P)	Calf lifts hind leg and arches neck to scratch or attempt to scratch top of their head with foot.
Head rubbing (HR)	Calf rubs head against another object e.g. pen wall, feeder.
Tail flicking (TF)	Calf rapidly moves tail from side to side ~2-3 times.
Foot stamping (FS)	Calf raises hoof and firmly brings it back down.
Vocalisation (V)	Pronounced vocal noises by calf independent of external stimuli e.g. vocalisation in response to other calf vocalisation doesn't count.
Grooming (G)	Calf licks any part of self for more than 5 seconds.
Drinking (D)	Sustained uptake of water from provided trough/container e.g. licking the surface does not count.
Eating (E)	Feed uptake from provided container.
Standing/Lying Transition (S/LT)	Transition of calf from being upright on all four limbs to lying with lower flank in contact with floor. Vice versa applies.
Locomotion (L)	General walking activity around pen.
Standing: Normal (NS)	Calf is stationary and upright on all four limbs with relaxed features and head level with topline.
Standing: Abnormal (AS)	Calf independently assumes standing position that deviates from normal calf posture for more than 10 seconds e.g. extremely raised head. Note nature of abnormal posture.
Lying: Normal (NL)	Calf is stationary with lower flank in contact with floor. Limbs may or may not be tucked in under or close to flank.
Lying: Abnormal (AL)	Calf independently assumes lying position that deviates from normal calf posture for more than 10 seconds e.g. lying on whole side flank with limbs extended. Note nature of abnormal posture.
Running(R)	Any gait faster than a walk e.g. trot.
Head-head Interaction (HHI)	Heads and/or neck of two calves touch for 1+ seconds. Includes sniffing & licking.

Behaviour	Description
Head-body Interaction (HBI)	Any part of the calf's head contacts part of another calf for 1+ seconds. Includes sniffing & licking.
Bucking (B)	Calf's bodyweight shifted from front to back, with both hind hooves lifted off the ground.
Aggression (A)	Interaction with other calves that involves pushing/shoving and attempts to ram another calf with their head.
Isolation (I)	Calf stays away from main group independently of external factors.

3.8.4.3 Accelerometer derived behaviour states

Tri-axial accelerometers (Hobo Pendant® G, Onset) were attached to the left rear hind limb of all calves using sponge, vetwrap and tape, with the x-axis perpendicular to the ground when the calves were in a standing position. The accelerometers were programmed to start recording on the x and y-axis every 20 seconds for 1 day prior to treatment (day -1) and up until 12 days following treatment. On day 6, accelerometers were removed and replaced with new accelerometers to allow sufficient memory for capturing data on days 6 to 12 of the experiment. Data captured from the accelerometers was used for assessment of the proportion of each day that each calf spent lying (as opposed to standing or walking). To validate lying behaviour as categorised by the accelerometers, 1 hour of video footage of all calves from the first experimental block was observed at same time and day and lying behaviour was recorded against time and then time matched to data generated from the accelerometers. Accelerometers were removed from calves at the end of the experiment at the last sampling time-point.

3.8.4.4 Mechanical nociceptive threshold testing

Calves were blindfolded prior to mechanical nociceptive testing to avoid animal responses to visual stimuli. Mechanical nociceptive testing (MNT) was conducted using an algometer (Wagner FDIX, Force One) to apply pressure to two sites on the outer intact tissue of each disbudding wound (2 cm distance from the edge of the wound, or horn bud for POS calves) (left and right horn sites 1), and two sites on the cut skin edge of each disbudding wound, or on the edge of the horn bud for POS calves (left and right horn sites 2). This resulted in a total of eight sites tested on each animal at each sampling time-point. Horn sites were measured in the following order: right horn site 1, right horn site 2, left horn site 1, left horn site 2. The algometer measured the maximum amount of pressure applied before an animal withdrawal response occurred. Two experienced operators conducted all mechanical nociceptive testing throughout the experiment.

3.8.4.5 Horn site temperature

Horn site temperature was taken using a handheld infrared laser thermometer. The distance between the thermometer and the horn site was kept consistent throughout the experiment using a 15cm zip tie connected to the thermometer. Horn site temperature was taken at the same horn sites as those for MNT and sites were tested in the same order as that for MNT.

3.8.4.6 Plasma biomarker (Tumour necrosis factor alpha)

Blood samples (10 mL) from days -1, 6 and 12 were collected into serum vacutainers for analysis of serum TNF-a. Samples were left at room temperature for 2 hours to allow clotting, following which they were centrifuged at 1600 x g for 15 minutes. The serum portion of each sample was immediately pipetted into micro-centrifuge tubes and transferred into a freezer for storage at - 80°C. A commercial Bovine Tumour Necrosis Factor Alpha ELISA kit (Bovine Tumour Necrosis Factor Alpha ELISA kit, MyBioSource, Inc., CA, USA) was used to analyse serum samples for concentration of TNF-α.

3.8.5 Statistical analysis

3.8.5.1 Behaviour

There was a total of 23 behaviour states recorded (presence = 1, absence = 0). For each of these variables where at least 50 observations of the behaviour were recorded (14 behaviour variables), the data were analysed using a logistic regression model, with fixed effects of Treatment, Day and their interaction. Note that initially logistic mixed models were attempted by inclusion of Block and Calf within Block random effects (using the lme4 packager on RStudio© (R Core Team, 2020)), but in most cases, estimated variance components were zero, or model-fitting did not converge, and hence fixed-effect-only models were used. These were fitted using the glm function in R. Model-based probabilities of the behaviour being expressed were calculated using the emmeans package.

To derive a behavioural index which might be used to provide an overall behaviour profile to pain and treatment responses, a behavioural score (BS) was derived by using those behaviours that changed significantly between treatments, or where there was a significant Treatment × Day interaction for that behaviour. The behaviours that are hypothesised would increase in response to pain are: ear flicking (EF), head shaking (HS), head rubbing (HR), tail flicking (TF), foot stamping (FS), locomotion (L), standing/normal standing (NS) and isolation (I). The behaviours that are hypothesised would decrease in response to pain are: grooming (G), eating (E), normal lying (NL), head-to-head interaction (HHI) and head to body interaction (HBI). From each of these binary (0-absence; 1-presncde) variables, the following behaviour score (BS) was constructed. Firstly, the sum EF + HS + HR + TF + FS + L + NS + I – G – E – NL – HHI – HBI was calculated with positive values applied to behaviours with hypothesised increased expression and negative values to those with reduced expression as a result of pain. The resulting frequency distributions of these was as follows:

Sum	-2	-1	0	1	2	3	4	5	6
Frequency	37	811	595	581	432	234	76	27	6

Due to small frequencies, values of '-2' were placed in the '-1' group, and values of '5' and '6' were placed in the '4' group creating a six-point scale. Finally for convenience, '2' was added to add creating a behavioural score (BS) with values from 1 to 6 indicating increasing hypothesised pain. The frequency distribution was therefore as follows:

BS	1	2	3	4	5	6
Frequency	848	595	581	432	234	109

Next the BS data were analysed by using an ordinal mixed model. Fixed effects for the model were Treatment, Day, and Treatment × Day, with random effects of Block and Calf nested within Block. The model was fitted using the ordinal package in RStudio© (R Core Team, 2020). Model-base probability distributions of the scores, i.e. probability for each of scores 1, 2, 3, 4 and 5, were calculated using the emmeans and RVAideMemoire packages in R. Pairwise comparisons of score distributions was made using the cld function in the multcomp package.

3.8.5.2 Accelerometer derived behaviour states

10,746 records of acceleration in the x- and y-directions and corresponding lying versus standing behaviour observations was used to develop various classification methods. These were based on records of 20 animals. A generalised additive mixed model (GAMM) technique was selected to model the probability of each animal lying as a smooth function of the two acceleration predictor variables. This was achieved using 'thin plate splines' which allow the probability of lying versus standing to vary in a smooth way over the two variables. The form of the model is

$$log_e\left(\frac{\pi}{1-\pi}\right)=\text{constant+s(x,y)+Animal}$$

where π is the probability of an animal being classified as lying (with 1 - π being the probability of standing), s(x,y) is a smooth two-dimensional thin-plate spline function of the acceleration measures in the x- and y-directions, and Animal is a random effect, to allow for the repeated measures in the same animal over time. Model-based probabilities of lying were classified as lying whenever the probability exceeded 0.5, otherwise classified as standing. The gamm4 package in RStudio© (R Core Team, 2020) was used to fit the GAMMs.

Based on the GAMM method for classifying behaviour as lying, the proportion of time each calf was lying over each experimental day was calculated. Note that accelerometers were switched on Day 6, resulting in cows having two sets of accelerometer readings that day (AM and PM). The proportion data were analysed by fitting a linear mixed model using the lme4 package in R. Fixed effects for the model were Treatment, Day and Treatment × Day interaction. The random effect for the model was Calf number. Model-based means were estimated using the emmeans package and pairwise treatment comparisons within each study day were done using the cld function of the multcomp package in R. Note that that several repeat analyses were conducted, after identifying potentially problematic days and calves.

3.8.5.3 Mechanical nociceptive threshold

A linear mixed model was fitted to the algometer (kg force) data, with fixed effects for Treatment, Day, Site, together with all two and three-way interactions. Random effects in the model to allow for clustering were Block, Calf nested within Block, and Day nested within the Calf. Because of the positive skew and unstable residual variance, the force data were log-transformed prior to model fitting. The mixed model was fitted using the lme4 package in RStudio© (R Core Team, 2020) and model-based means calculated using the emmeans package in R. Pairwise comparisons of means was conducted using the cld function in the multcomp package in R.

3.8.5.4 Horn site temperature

As the temperature data recorded at the horn sites may be affected by the ambient temperature, Camden Airport (nearest meteorological station) temperature records form the Bureau of

Meteorology were obtained for the corresponding experimental period, on a half-hourly basis. The air temperature on or at the most recent half hour periods were taken as the relevant air temperature. Then a linear mixed model was fitted to the horn site temperature data with the same form of that for the algometer data, with the addition of a fixed effect term for air temperature. No data transformation was required. To allow for a possible nonlinear effect, air temperature was fitted as a spline term, using the splines package in RStudio© (R Core Team, 2020) together with lme4. Model-based means and pairwise comparisons were made, as in the algometer data analysis.

3.8.5.5 Plasma biomarker (Tumour necrosis factor alpha)

Data were subjected to restricted maximum likelihood (REML) for repeated measures using the mixed models procedure of Genstat 22nd Edition statistical software (VSN International Ltd, Hertfordshire, UK). The fixed effects considered for inclusion in the model was treatment, time-point and their interaction, and experimental block. Animal was included as a random effect in the model. Insignificant terms were dropped from the model using a backwards elimination approach until all terms in the final model were significant or until only one term was tested. *P* values < 0.05 were considered statistically significant.

3.8.5.6 Association between algometer force and behaviours

In an attempt to define an objective measure of pain, the algometer force was used (as an inverse measure), and the best set of behaviours that predict that force was investigated. As there were four algometer measurements taken on a calf at a particular time, these values were averaged using a geometric mean (due to the highly positively skewed distribution mentioned in the previous analysis). Similarly, each of the behaviours (0-1 data) was averaged over all the observations on the calf of that study day (the proportion of times on the day the behaviour was observed). However, rare behaviours (under 50 occurrences in the data set) were not considered as they would not be reliable or have much utility as predictors. The set of daily averages for the algometer and behaviour data were then match-merged, on Block, Calf and Day to produce a data set for the analysis.

A linear mixed model was fitted to the algometer (kg force) data, with a fixed effect for the specific behaviour, and random effects for Block, and Calf nested within Block. Because of the positive skew and unstable residual variance, the force data were log-transformed prior to model fitting. Each behaviour was tested separately and any behaviour with a slight association with the algometer force ($P < 0.2$) was identified. These behaviour variables were then entered into an initial multivariable model and a process of backward elimination applied to determine a subset of predictive behaviours. All mixed model fitting was conducted using the lme4 package in RStudio© (R Core Team, 2020).

3.9 Investigation of farmer perceptions of and attitude toward pain in cattle and sheep industries

3.9.1 Sampling

In July and August 2020, a survey composed of 18 multiple choice, 2 ranking questions and 1 Likert-question was distributed to Australian agricultural organisations to identify those who are willing to distribute the survey on behalf of the researchers. The organisations that aided in the distribution of the survey were Cattle Council Australia, Sheep Producers Australia, Tasmanian Farmers and Graziers Association, Australian Wool Innovative, Woolmark Company and Dairy Australia. In September 2020

following human ethics approval through the University of Sydney ethics committee (the project number is 2020/594), organisations circulated a link to the online survey to their members. This allowed for all participants in the study to complete the survey voluntarily and maintain their anonymity. All participants were provided with a link to the survey produced using the Qualtrics program and a participant information sheet detailing the study and survey end-date. By the end of October 2020, 71 responses were retrieved from the online survey and utilised in the statistical analysis for the study.

3.9.2 Survey design

The survey was designed and based on previous studies examining farmer, veterinarian and contract worker (including hoof and claw trimmers) perception and attitudes towards pain relief, animal welfare and pain experienced by livestock (Becker, et. al 2013; Wikman, et. al 2016; Becker, Reist and Steiner 2014; Larrondo et al, 2018; Kilic and Bozkurt 2013). The survey developed for this study was composed of five sections. The first section included four questions focusing on sociodemographic factors including gender, age, experience with livestock and education. The second section was composed of four questions examining farmer perception and attitudes towards livestock. This section examined degree of human-animal interactions (this is referring to the contact between the farmer and their herd/flock), farmer views on animal ethics and attitude towards when pain relief should be used. The third section was comprised of four questions focused on providing an overview of the farm enterprises. This section examined the type of farm system (mixed crop-livestock or solely livestock system), farm enterprise (for example dairy and wool), number of employees and herd/flock size. The fourth section included six questions focused on the farmer's perception and attitude towards pain relief. This section determined when/if pain relief is administered to their herd/flock, type of pain relief being administered, benefits of pain relief administration and limitations to the use of pain relief. The last section included three questions focusing on examining the farmer's belief regarding how much pain is experienced by animals. This section included Likert-scale and ranking questions to determine which procedures and diseases experienced by livestock were believed to induce the greatest degree of pain and the amount of pain animals can experience in comparison to humans.

3.9.3 Data analysis

The online survey data was exported into Excel 2016 (Microsoft) to be processed and refined to ensure the responses collected were suitable for analysis in R studio. Initially, any gaps in the data collected (where no responses were collected) were assigned with N/A (not available). Once this was completed multi word responses were shortened to one word as R studio is unable to analyse responses with more than one word. For questions that allowed for multiple answers these were allocated a number to allow for easier analysis in R studio. Once the data was refined it was then imported into R studio to be statistically analysed.

Ordinal logistic regression was employed for analysis purposes in R studio. The ordinal logistic regression analysis has been employed in this study to determine the odds ratio within the multiple independent variables examined in the survey. By determining the odds ratio within two or more independent variables it would be beneficial for determining if there is a statistical significance. For example, in this study the participants gender was examined in association with their ethical views on the use of animals.

Chi-square test of independence will also be performed to determine if the participant responses in one question had influenced their responses noted in separate questions. For example, the farmers response to the importance of pain relief administration and their ability to recognise pain in their herd/flock will be examined in association with their beliefs regarding the degree of pain experienced by humans, sheep, cattle, dogs and cats. That is, does the farmer's perception of pain relief and ability to recognise pain affect their beliefs towards pain experienced by different species.

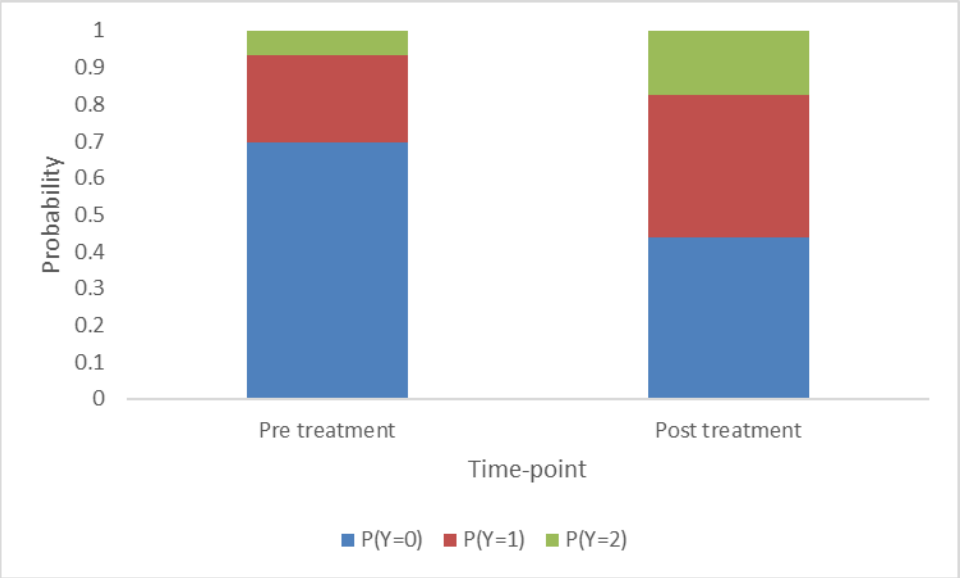
4. Results

4.1 Investigation into novel markers of pain in lambs

4.1.1 Facial expressions

There was no significant effect of treatment (P = 0.439) or observer (P = 0.669) on scores for orbital tightening. There was a significant effect of time-point (P < 0.001) (Figure 1) on scores for orbital tightening.

Figure 1: Probability of all lambs at each time-point displaying orbital tightening scores (Y; 0 = orbital tightening absent, 1 = orbital tightening present to a moderate degree, 2 = orbital tightening present to an obvious degree).



A significant effect was found (P < 0.001). Time-points differ significantly at P < 0.05.

There was no significant effect of treatment (P = 0.532) or time-point (P = 0.095) on scores for lip tightening. There was a significant effect of observer (P < 0.001) on scores for lip tightening.

There was no significant effect of treatment (P = 0.474) or time-point (P = 0.115) on scores for cheek flattening. There was a significant effect of observer (P < 0.001) on scores for cheek flattening.

The lack of an effect of treatment for all facial action units, and the significant effect of time-point on orbital tightening, suggests that facial expressions of lambs may change in response to other factors

independent of pain, perhaps due to the animals being restrained. The significant effect of observer on lip tightening and cheek flattening suggests that there is some degree of subjectivity associated with assessment of these facial action units in lambs. Further training of observers and the inclusion of additional observers may improve inter-observer reliability. Further exploration of lamb facial expression over multiple time-points in both the acute and chronic periods following husbandry procedures is suggested.

4.1.2 Plasma biomarkers

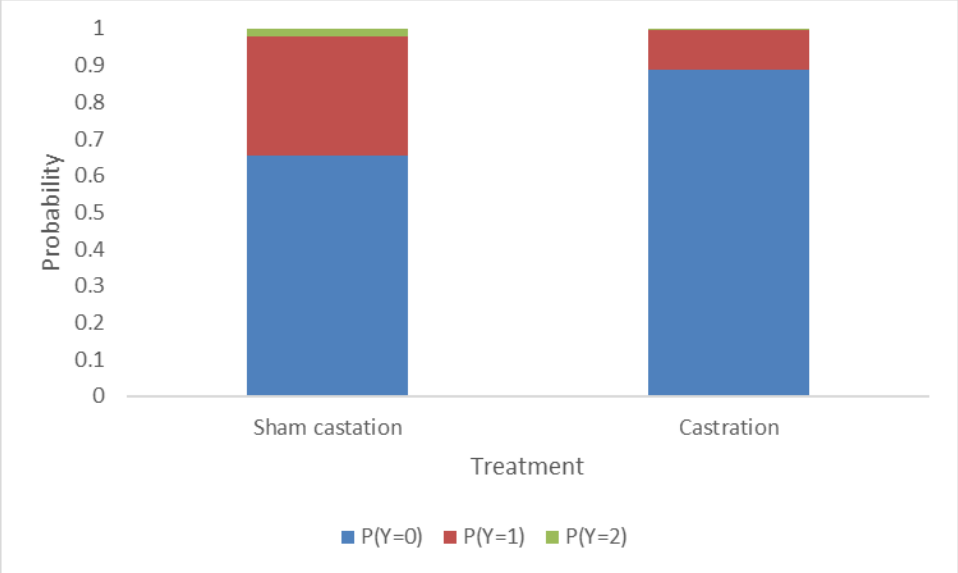
There was no significant effect of treatment as analysed as groups specified above in section 3.1.3 and also as 'pain' and 'no pain' on percent change in substance P ($P = 0.515$; $P = 0.212$, respectively) or IL-1β ($P = 0.718$; $P = 0.626$, respectively) concentrations. This suggests that substance P and IL-1β concentrations did not indicate pain in the acute period (within 1 hour) following castration and/or tail docking of lambs. There was no significant effect of treatment as analysed as groups specified above in section 3.1.3 ($P = 0.201$). There was a significant effect of treatment as analysed as 'pain' and 'no pain' on percent change in TNF-α ($P = 0.036$). There was a 14.07% decrease in TNF-α in the 'no pain' treatment group and a 23.68% increase in TNF-α in the 'pain' treatment group.

4.2 Investigation into a 'calf grimace scale' for assessment of pain in calves

4.2.1 Facial expression

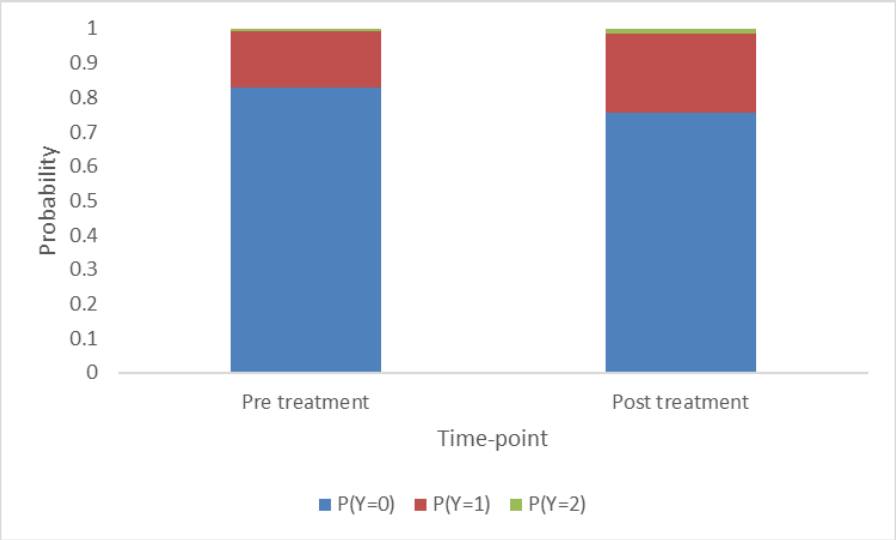
There was a significant effect of treatment ($P = 0.024$) (Figure 2), time-point ($P = 0.041$) (Figure 3) and observer ($P < 0.001$) on scores for orbital tightening.

Figure 2: Probability of calves in each treatment group at all time-points displaying orbital tightening scores (Y; 0 = orbital tightening absent, 1 = orbital tightening present to a moderate degree, 2 = orbital tightening present to an obvious degree).



A significant effect was found ($P = 0.024$). Treatments differ significantly at $P < 0.05$.

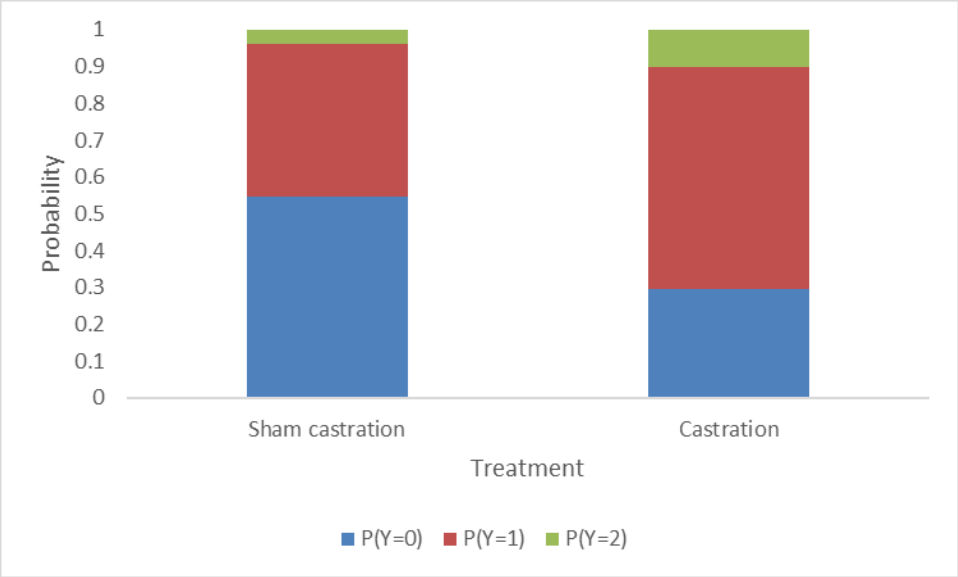
Figure 3: Probability of all calves at each time-point displaying orbital tightening scores (Y; 0 = orbital tightening absent, 1 = orbital tightening present to a moderate degree, 2 = orbital tightening present to an obvious degree).



A significant effect was found ($P = 0.041$). Time-points differ significantly at $P < 0.05$.

There was no significant effect of time-point ($P = 0.391$) on scores for tension of the muscles above the eye. There was a significant effect of treatment ($P = 0.047$) (Figure 4) and observer ($P < 0.001$) on scores for tension of the muscles above the eye.

Figure 4: Probability of calves in each treatment group at all time-points displaying tension of the muscles above the eye scores (Y; 0 = orbital tightening absent, 1 = orbital tightening present to a moderate degree, 2 = orbital tightening present to an obvious degree).



A significant effect was found ($P = 0.047$). Treatments differ significantly at $P < 0.05$.

There was no significant effect of treatment ($P = 0.886$) or time-point ($P = 0.345$) on scores for nostril tightening. There was a significant effect of observer ($P < 0.001$) on scores for nostril tightening.

The lack of a significant interaction between time-point and treatment for all facial action units, and the significant effect of time-point on orbital tightening and nostril tightening, suggests that facial expressions of calves may change in response to other factors independent of pain, perhaps due to the animals being restrained. The significant effect of observer on all facial action units suggests that there is some degree of subjectivity associated with assessment of facial expressions in calves. Further training of observers and the inclusion of additional observers may improve inter-observer reliability. The significant effect of treatment on orbital tightening and tension of the muscles above the eye suggests that these facial action units may be useful for identification of pain in calves. Further exploration of calf facial expression over multiple time-points in both the acute and chronic periods following husbandry procedures is suggested.

4.2.2 Behaviour

There were two missing behavioural observations due to the calf being unable to be identified in the video footage. The two missing samples were second and third observations of the same calf (sham castration group). Behaviours including stamping, licking, kicking, back arching, lying both normally and abnormally occurred infrequently, so these were not Table 5.

Table 5: Mean proportion of time frequency of tail flicking, licking and standing relaxed displayed by calves in each treatment group.

Behaviour	Castration (*n* = 10)	Sham castration (*n* = 10)	*P*-value
Tail flicking	0.85	0.39	<0.001
Licking	0.01	0.02	0.0362
Stand relaxed	0.21	0.46	0.038

4.3 Assessing the pain of different methods for castrating and tail docking lambs

4.3.1 Plasma biomarker (Interleukin-10)

There was no effect of body weight (*P* = 0.925) and there was no significant Treatment x Day (*P* = 0.069) or Treatment effect (P = 0.089). There was a significant effect of Day (*P* < 0.001), with IL-10 concentration significantly greatest on day 14 (56.15 ng/mL) compared to all other days and significantly lowest on day 7 (38.36 ng/mL) compared to all other days. The means for days 1 and 4 were 48.19 and 48.20 ng/mL, respectively.

4.3.2 Wound morphology

4.3.2.1 Castration

Table 6 shows the significance of changes in swelling, exudate and healing across experimental days, for each castration treatment. The blank entries are due to insufficient data for testing. However, all possible comparisons indicated highly significant differences for these treatments over the study days.

Table 6: Significance of changes in swelling, exudate and healing for all experimental days for each castration treatment (RRC = rubber ring castration; SC = surgical castration).

Treatment	Score	DF	Chi-square	*P*-value
RRC	Swelling	5	27.19	5.2E-05
RRC	Exudate	-	-	-
RRC	Healing	-	-	-
SC	Swelling	5	23.10	3.2E-04
SC	Exudate	5	37.71	4.3E-07
SC	Healing	-	-	-

Table 7 shows the model-based probabilities for each outcome separately for each treatment over the study days.

Table 7: Model-based probabilities for swelling and exudate for each treatment over the study days (SC = surgical castration; RRC = rubber ring castration).

		SC		RRC	
	Day	Probability	SE	Probability	SE
Swelling	0	0.00	0.00	0.00	0.00
	1	0.34	0.20	0.13	0.12
	2	0.74	0.14	0.42	0.14
	4	0.21	0.14	0.00	0.00
	7	0.00	0.00	0.00	0.00
	14	0.00	0.00	0.00	0.00
Exudate	0			0.97	0.04
	1			0.29	0.23
	2			0.07	0.08
	4			0.07	0.08
	7			0.00	0.00
	14			0.00	0.00

Incidence of swelling initially increased then reduced for both SC and RRC, and incidence of exudate was initially high on Day 0, but then reduced over the study period.

4.3.2.1 Tail docking

Table 8 shows the significance of changes in swelling, exudate and healing across experimental days, for each tail docking treatment. As is seen, there are highly significant differences for each.

Table 8: Significance of changes in swelling, exudate and healing across experimental days, for each tail docking treatment (CTD = cautery tail-docking; RRTD = rubber ring tail-docking; STD = surgical tail-docking).

Treatment	Score	DF	Chi-square	*P*-value
CTD	Swelling	5	44.60	1.8E-08
CTD	Exudate	5	25.95	9.1E-05
CTD	Healing	5	45.51	1.1E-08
RRTD	Swelling	5	57.44	4.1E-11
RRTD	Exudate	5	47.64	4.2E-09
RRTD	Healing	5	59.34	1.7E-11
STD	Swelling	5	21.59	6.3E-04
STD	Exudate	5	61.81	5.1E-12
STD	Healing	5	66.05	6.8E-13

Table 9 shows the model-based probabilities for each outcome separately for each treatment over the study days.

The probability of swelling initially increased but then decreased over time for CTD and STD but remained high for RRTD. The probability of an exudate was high initially for CTD and STD and then declined while it increased over time for RRTD. For healing, high probabilities for this are seen as time elapses for CTD and STD, whereas RRTD tissue is intact in the acute period following treatment and begins to become damaged by the rings from a few days following treatment.

Table 9: Model-based probabilities for swellng and exudate for each treatment over the study days (CTD = cautery tail-docking; RRTD = rubber ring tail-docking; STD = surgical tail-docking).

		CTD		RRTD		STD	
	Day	Probability	SE	Probability	SE	Probability	SE
Swelling	0	0.00	0.00	0.00	0.00	0.00	0.00

	1	0.51	0.21	1.00	0.00	0.20	0.17
	2	0.94	0.07	1.00	0.00	0.63	0.20
	4	0.20	0.13	1.00	0.00	0.04	0.06
	7	0.00	0.00	0.91	0.09	0.10	0.10
	14	0.00	0.00	1.00	0.00	0.00	0.00
Exudate	0	0.76	0.13	0.00	0.00	1.00	0.00
	1	0.76	0.15	0.00	0.00	0.63	0.17
	2	0.36	0.15	0.00	0.00	0.92	0.08
	4	0.00	0.00	0.09	0.09	0.91	0.09
	7	0.16	0.11	0.73	0.13	0.08	0.08
	14	0.27	0.14	0.80	0.13	0.00	0.00
Healing	0	0.00	0.00	1.00	0.00	0.00	0.00
	1	0.00	0.00	1.00	0.00	0.00	0.00
	2	0.55	0.15	1.00	0.00	0.08	0.08
	4	0.83	0.11	0.82	0.12	0.09	0.09
	7	0.92	0.08	0.18	0.12	0.92	0.08
	14	0.73	0.13	0.00	0.00	1.00	0.00

4.3.3 Wound temperature

4.3.3.1 Castration

There was no significant treatment effect on tissue temperature (*P* = 0.31), nor was there a significant Treatment × Day interaction (*P* = 0.22). However, tissue temperature changed significantly over the study days (*P* = 0.00006), as shown in Table 10.

Table 10: Significance of changes in wound temperature according to Treatment and Day.

Factor	NumDF	DenDF	*F*-value	*P*-value
Treatment	2	23.9	1.24	0.31
Day	5	117.3	5.99	5.7E-05
Treatment × Day	10	117.4	1.34	0.22
Body weight	1	23.8	6.03	0.022

Figure 5 is a plot of the model-based mean temperature over the study period for each treatment. The main feature is the rise then fall in temperature, across all treatments.

Figure 5: Model-based mean tissue infrared (IR) temperature over the study period for each treatment (CON = control; RRC = rubber ring castration; SC = surgical castration).

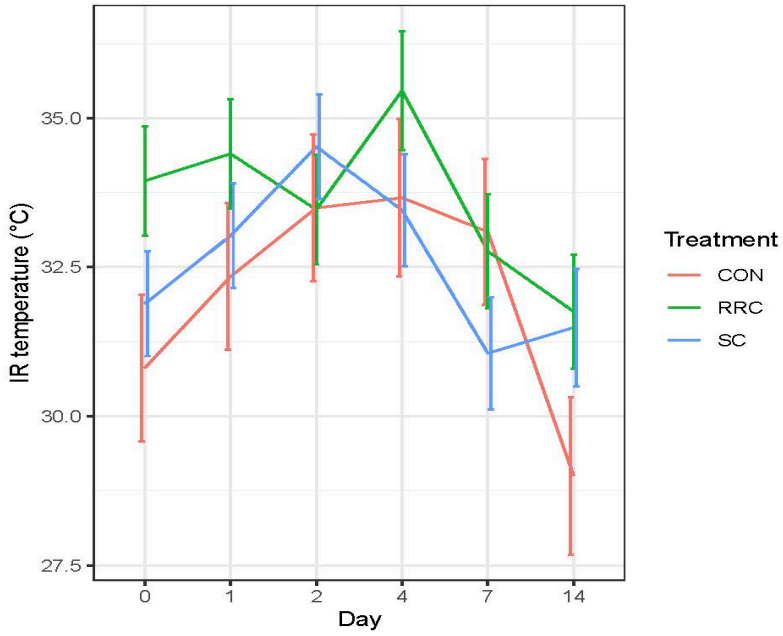


Table 11 shows the means, and all with the exception of Day 0 show no difference between treatment means, however the "significant" difference should not be over-interpreted, given the non-significance of Treatment and Treatment × Day.

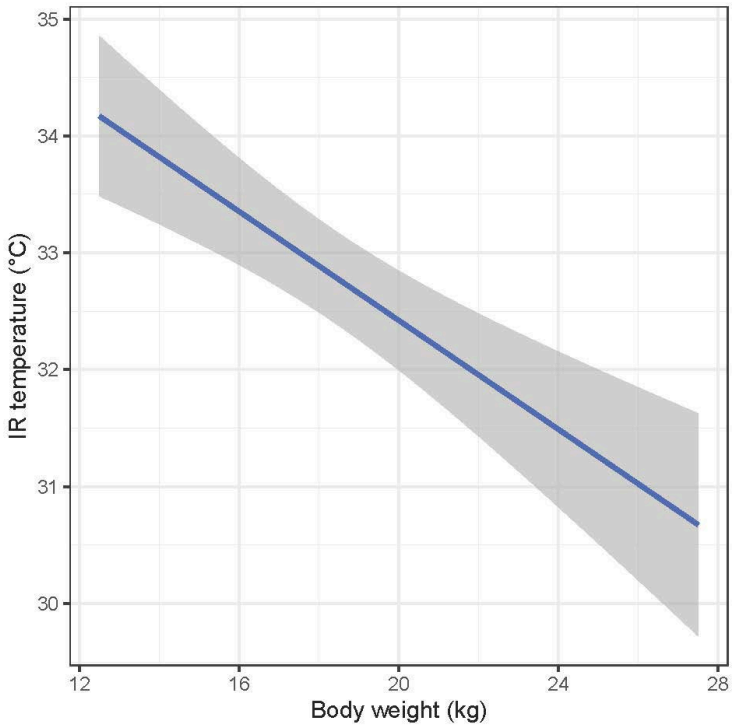
Note there was also a significant effect of body weight ($P = 0.022$), with a decline in tissue temperature as body weight increases (regression slope = –0.233 ± 0.095 °C/kg). This is shown visually in Figure 6 (model-based means ± SE).

Table 11: Mean tissue temperature (°C) (±SE) of all treatments across all study days (CON = control; RRC = rubber ring castration; SC = surgical castration). Means with different superscripts (a, b) are significantly different.

Day	Treatment	Mean	SE	Group
0	CON	30.8	1.23	a
	RRC	33.9	0.92	b
	SC	31.9	0.88	ab
1	CON	32.3	1.23	a
	RRC	34.4	0.92	a
	SC	33.0	0.88	a
2	CON	33.5	1.23	a
	RRC	33.5	0.92	a

Day	Treatment	Mean	SE	Group
	SC	34.5	0.88	a
4	CON	33.7	1.32	a
	RRC	35.5	1.00	a
	SC	33.5	0.94	a
7	CON	33.1	1.23	a
	RRC	32.8	0.96	a
	SC	31.1	0.94	a
14	CON	29.0	1.32	a
	RRC	31.8	0.96	a
	SC	31.5	0.98	a

Figure 6: Model-based means (±SE) of tissue infrared (IR) temperature (°C) in relation to body weight.



4.3.3.2 Tail docking

There was a highly significant treatment effect on tissue temperature (P = 0.0001), and study day (P = 0.00003) although there was no significant interaction between these two factors, as seen in Table 12.

Table 13 shows the model-based mean temperatures for all combinations of treatment and day, together with pairwise comparisons of treatment means within the day.

Table 12: Significance of changes in wound temperature according to Treatment and Day.

Factor	Num DF	DenDF	*F*-value	*P*-value
Treatment	3	36.4	9.36	1.0E-04
Day	5	166.8	6.20	2.7E-05
Treatment × Day	15	166.7	0.98	0.48
Body weight	1	37.7	1.60	0.21

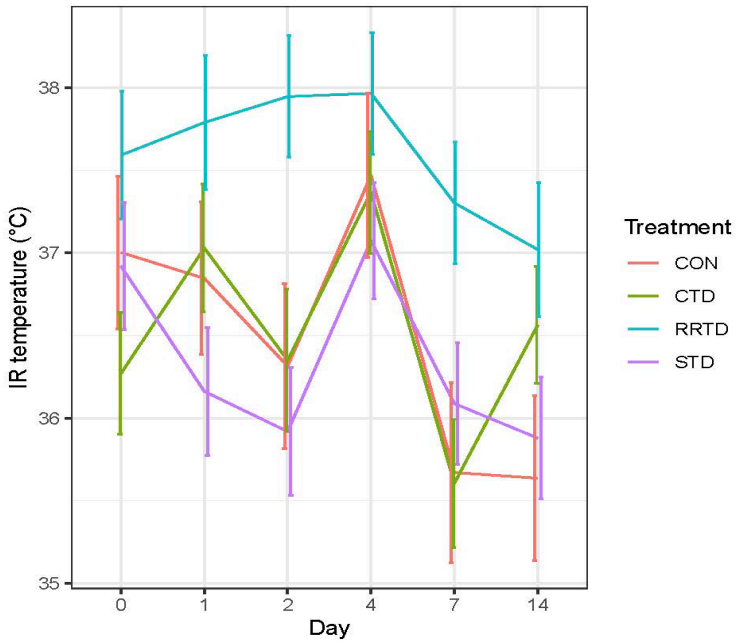
Table 13: Mean tissue temperature (°C) (±SE) of all treatments across all study days (CON = control; CTD = cautery tail-docking; RRTD = rubber ring tail-docking; STD = surgical tail-docking). Means with different superscripts (a, b) are significantly different.

Day	Treatment	Mean	SE	Group
0	CON	37.0	0.46	ab
	CTD	36.3	0.37	a
	RRTD	37.6	0.39	b
	STD	36.9	0.39	ab
1	CON	36.8	0.46	ab
	CTD	37.0	0.39	ab
	RRTD	37.8	0.41	a
	STD	36.2	0.39	b
2	CON	36.3	0.50	a
	CTD	36.4	0.43	a
	RRTD	37.9	0.37	b
	STD	35.9	0.39	a
4	CON	37.5	0.50	a
	CTD	37.4	0.37	a

Day	Treatment	Mean	SE	Group
	RRTD	38.0	0.37	a
	STD	37.1	0.35	a
7	CON	35.7	0.55	a
	CTD	35.6	0.39	a
	RRTD	37.3	0.37	b
	STD	36.1	0.37	a
14	CON	35.6	0.50	a
	CTD	36.6	0.35	ab
	RRTD	37.0	0.41	b
	STD	35.9	0.37	a

Figure 7 is a plot of the treatment means ± SE over the study period.

Figure 7: Mean tissue temperature ± SE for all treatments over the study period (CON = control; CTD = cautery tail-docking; RRTD = rubber ring tail-docking; STD = surgical tail-docking).



The overall trend shown in Figure 7 together with the overall treatment means shown in Table 14 (average over study days), is that wound temperature is higher in the RRTD treatment group compared with the others.

Table 14: Mean tissue temperature (°C) (±SE) for each treatment for all study days.

Treatment	Mean	SE	Group
CON	36.5	0.24	a
CTD	36.5	0.19	a
RRTD	37.6	0.19	b
STD	36.3	0.19	a

4.3.4 Facial expressions

4.3.4.1 Castration

4.3.4.1.1 Eye scores

Table 15 shows no significant Treatment × Day × Observer interaction (*P* = 0.55), allowing an overall time course of each treatment to be considered.

Table 15: Significance of changes in eye scores according to Treatment, Day and Observer.

	Chi-square	Df	*P*-value
Treatment	0.11	2	0.95
Day	14.53	5	0.013
Observer	261.29	1	$< 2.2\times10^{-16}$
Body.weight	1.09	1	0.30
Treatment × Day	12.77	10	0.24
Treatment × Observer	0.19	2	0.91
Day × Observer	74.56	5	1.5×10^{-12}
Treatment × Day × Observer	8.78	10	0.55

Figure 8 is a visual of the fitted model, with cumulative bar charts of model-based probabilities.

Figure 8: Model-based probabilities for eye scores 0, 1 and 2 across study days for each treatment (CON = control; RRC = rubber ring castration' SC = surgical castration).

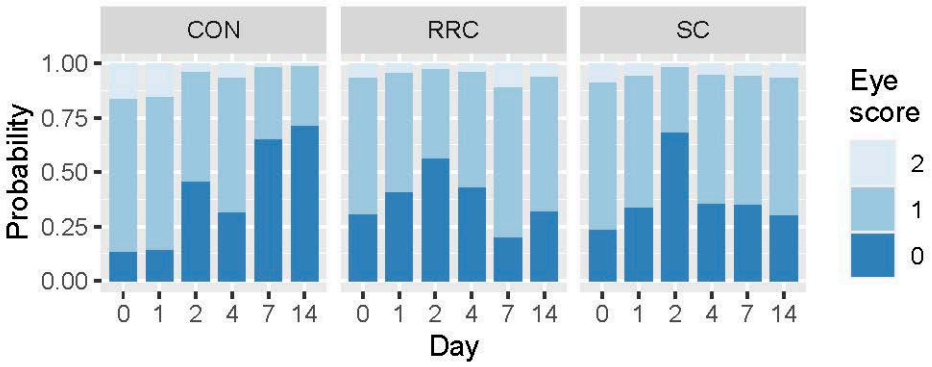


Table 16 shows comparisons between treatments within the same Day averaged over Observer. Elements sharing the same letter are not significantly different ($P > 0.05$).

Table 16: Comparisons between treatment groups for eye scores (CON = control; RRC = rubber ring castration; SC = surgical castration). Means with different superscripts (a, b) are significantly different.

	Day					
Treatment	0	1	2	4	7	14
CON	a	a	a	a	a	a
RRC	a	a	a	a	b	a
SC	a	a	a	a	ab	a

On Day 7, eye scores in the RRC treatment group were significantly higher than that for the control.

4.3.4.1.2 Lip scores

Table 17 shows a significant Treatment × Day × Observer interaction ($P = 9.2 \times 10–4$) indicating different treatment response time courses for the two observers.

Figure 9 is a visual of the fitted model, with cumulative bar charts of model-based probabilities.

Table 17: Significance of changes in lip scores according to Treatment, Day and Observer.

	Chi-square	Df	*P*-value
Treatment	0.45	2	0.80
Day	17.58	5	0.0035
Observer	423.13	1	$< 2.2 \times 10^{-16}$
Body.weight	0.62	1	0.43
Treatment × Day	12.51	10	0.25

Treatment × Observer	10.36	2	0.0056
Day × Observer	117.56	5	$< 2.2\times10^{-16}$
Treatment × Day × Observer	29.82	10	9.2×10^{-4}

Figure 9: Model-based probabilities for lip scores 0, 1 and 2 across study days for each treatment (CON = control; RRC = rubber ring castration' SC = surgical castration).

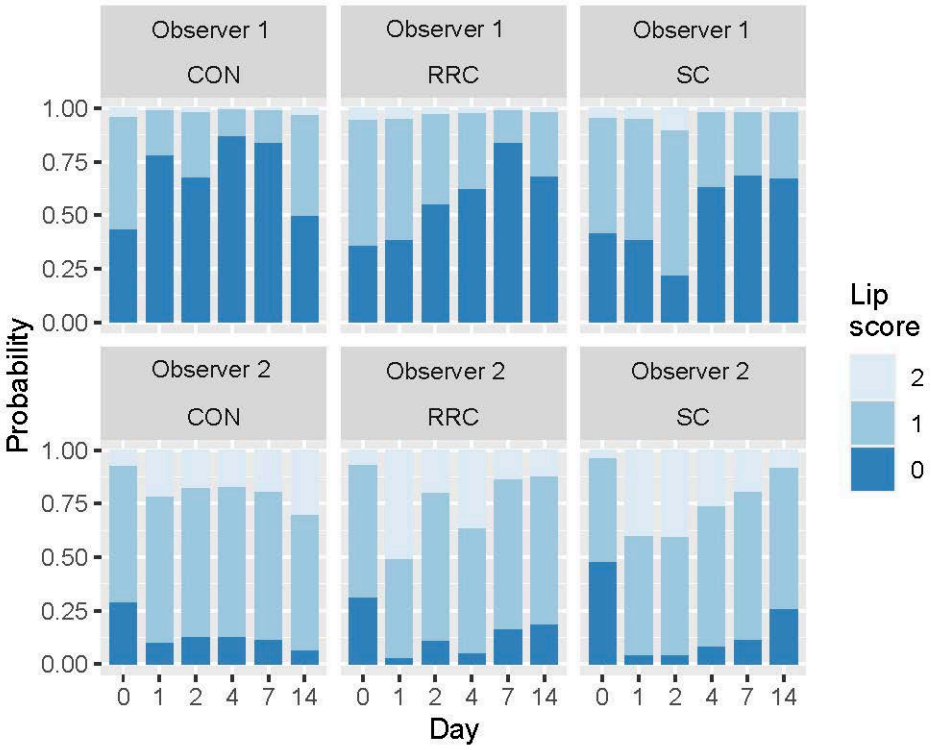


Table 18 shows comparisons between treatments within the same Day and same Observer. Elements sharing the same letter are not significantly different ($P > 0.05$).

Table 18: Comparisons between treatment groups for lip scores (CON = control; RRC = rubber ring castration; SC = surgical castration). Means with different superscripts (a, b) are significantly different.

		Day					
Observer	Treatment	0	1	2	4	7	14

1	CON	a	a	a	a	a	a
	RRC	a	a	a	a	a	a
	SC	a	a	b	a	a	A
2	CON	a	a	a	a	a	a
	RRC	a	a	a	a	a	a
	SC	a	a	a	a	a	a

The only observed difference was for Observer 1 scores on Day 2, with treatment SC having significantly higher scores than CON or RRC.

4.3.4.1.3 Nose scores

Table 19 shows a significant Treatment × Day × Observer interaction (P = 7.6 × 10^{-12}) indicating different treatment response time courses for the two observers.

Table 19: Significance of changes in nose scores according to Treatment, Day and Observer.

	Chi-square	Df	*P*-value
Treatment	0.65	2	0.72
Day	23.33	5	2.9×10^{-4}
Observer	265.02	1	$< 2.2\times10^{-16}$
Body.weight	0.02	1	0.88
Treatment × Day	3.02	10	0.98
Treatment × Observer	11.02	2	0.0041
Day × Observer	75.80	5	6.3×10^{-15}
Treatment × Day × Observer	73.96	10	7.6×10^{-12}

Figure 10 is a visual of the fitted model, with cumulative bar charts of model-based probabilities.

Figure 10: Model-based probabilities for nose scores 0, 1 and 2 across study days for each treatment (CON = control; RRC = rubber ring castration' SC = surgical castration).

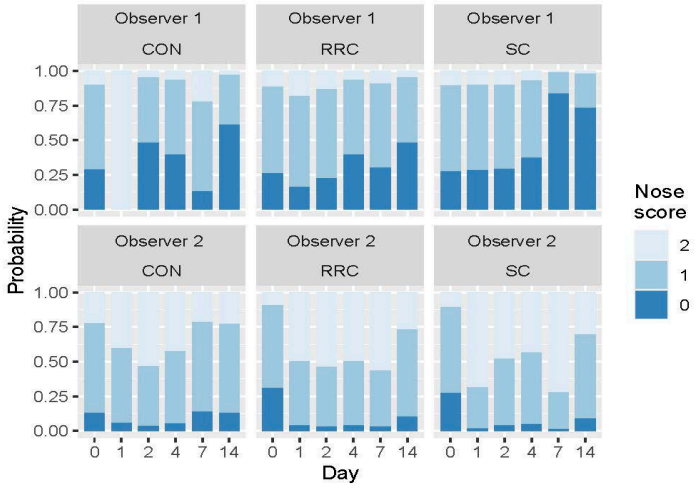


Table 20 shows comparisons between treatments within the same Day and same Observer. Elements sharing the same letter are not significantly different ($P > 0.05$).

Table 20: Comparisons between treatment groups for nose scores (CON = control; RRC = rubber ring castration; SC = surgical castration). Means with different superscripts (a, b) are significantly different.

		Day					
Observer	Treatment	0	1	2	4	7	14
1	CON	a	a	a	a	a	a
	RRC	a	a	a	a	a	a
	SC	a	a	a	a	b	a
2	CON	a	a	a	a	a	a
	RRC	a	a	a	a	ab	a
	SC	a	a	a	a	b	a

The only observed difference was for Observer 2 scores on Day 7, with treatment SC having significantly higher scores than CON.

4.3.4.2 Tail Docking

4.3.4.2.1 Eye scores

Table 21 shows a significant Treatment × Day × Observer interaction ($P = 0.0084$) indicating different treatment response time courses for the two observers.

Table 21: Significance of changes in eye scores according to Treatment, Day and Observer.

	Chi-square	Df	*P*-value
Treatment	3.57	3	0.31
Day	9.60	5	0.087
Observer	409.97	1	$< 2.2\times10^{-16}$
Body.weight	1.15	1	0.28
Treatment × Day	13.85	15	0.54
Treatment × Observer	0.78	3	0.85
Day × Observer	92.90	5	$< 2.2\times10^{-16}$
Treatment × Day × Observer	31.16	15	0.0084

Figure 11 is a visual of the fitted model, with cumulative bar charts of model-based probabilities.

Figure 11: Model-based probabilities for eye scores 0, 1 and 2 across study days for each treatment (CON = control; CTD = cautery tail-docking; RRTD = rubber ring tail-docking; STD = surgical tail-docking).

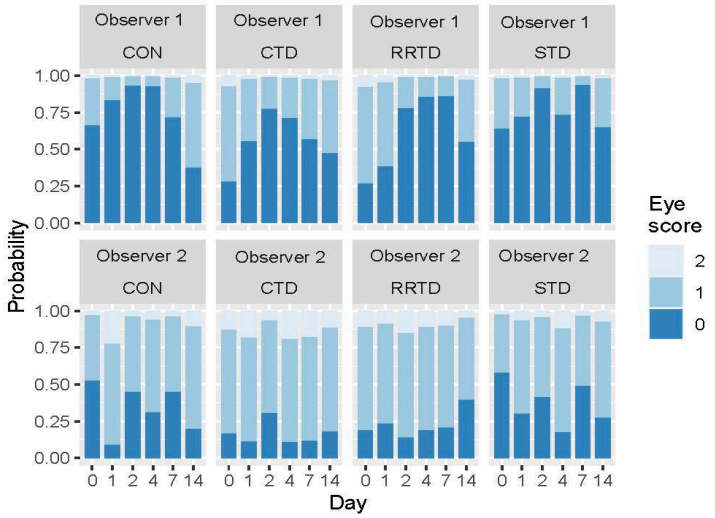


Table 22 shows comparisons between treatments within the same Day and same Observer. Elements sharing the same letter are not significantly different ($P > 0.05$).

Table 22: Comparisons between treatment groups for eye scores (CON = control; CTD = cautery tail-docking; RRTD = rubber ring tail-docking; STD = surgical tail-docking). Means with different superscripts (a, b) are significantly different.

		Day					
Observer	Treatment	0	1	2	4	7	14

1	CON	a	a	a	a	a	ab
	CTD	a	a	a	a	a	a
	RRTD	a	a	a	a	a	ab
	STD	a	a	a	a	a	b
2	CON	ab	a	a	a	ab	a
	CTD	a	a	a	a	a	a
	RRTD	a	a	a	a	ab	a
	STD	b	a	a	a	b	a

Significant differences between treatments were observed on Day 14 only for Observer 1, and Days 0 and 7 for Observer 2.

4.3.4.2.2 Lip scores

Table 23 shows a significant Treatment × Day × Observer interaction ($P = 2.6 \times 10^{-5}$) indicating different treatment response time courses for the two observers.

Table 23: Significance of changes in lip scores according to Treatment, Day and Observer.

	Chi-square	Df	*P*-value
Treatment	5.87	3	0.12
Day	24.95	5	1.4×10^{-4}
Observer	449.97	1	$< 2.2\times10^{-16}$
Body.weight	0.64	1	0.42
Treatment × Day	9.21	15	0.87
Treatment × Observer	21.27	3	9.3×10^{-5}
Day × Observer	114.26	5	$< 2.2\times10^{-16}$
Treatment × Day × Observer	47.92	15	2.6×10^{-5}

Figure 12 is a visual of the fitted model, with cumulative bar charts of model-based probabilities.

Figure 12: Model-based probabilities for lip scores 0, 1 and 2 across study days for each treatment (CON = control; CTD = cautery tail-docking; RRTD = rubber ring tail-docking; STD = surgical tail-docking).

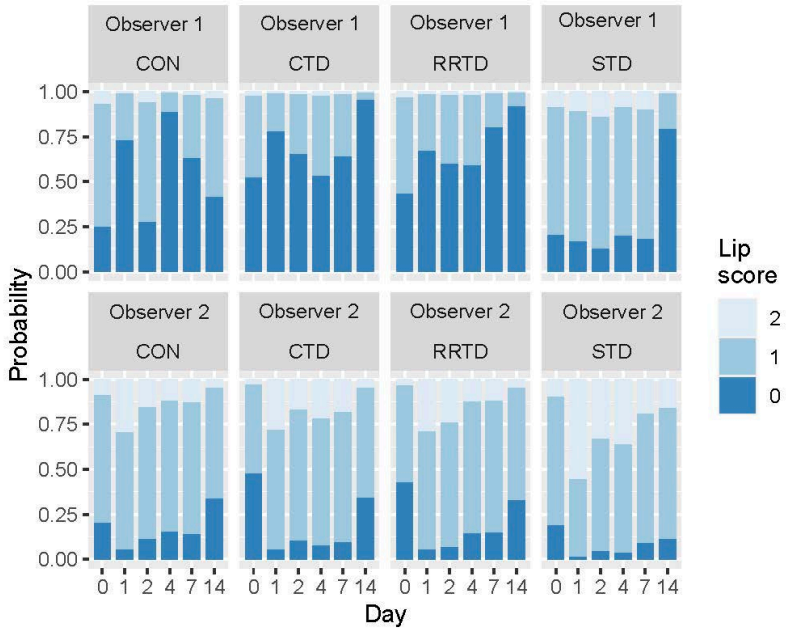


Table 24 shows comparisons between treatments within the same Day and same Observer. Elements sharing the same letter are not significantly different ($P > 0.05$).

Table 24: Comparisons between treatment groups for lip scores (CON = control; CTD = cautery tail-docking; RRTD = rubber ring tail-docking; STD = surgical tail-docking). Means with different superscripts (a, b) are significantly different.

		Day					
Observer	Treatment	0	1	2	4	7	14
1	CON	a	a	ab	a	ab	a
	CTD	a	a	a	ab	a	b
	RRTD	a	a	a	ab	a	b
	STD	a	b	b	b	b	ab
2	CON	a	a	a	a	a	a
	CTD	a	a	a	a	a	a
	RRTD	a	a	a	a	a	a
	STD	a	a	a	a	a	a

While no differences were observed within any one Day for Observer 2, Treatment differences were observed on Days 2, 4, 7 and 14 for Observer 1.

4.3.4.2.3 Nose scores

Table 25 Shows a significant Treatment × Day × Observer interaction (P = 3.6 × 10–9) indicating different treatment response time courses for the two observers.

Table 25: Significance of changes in nose scores according to Treatment, Day and Observer.

	Chi-square	Df	*P*-value
Treatment	0.13	3	0.99
Day	19.99	5	0.0013
Observer	157.44	1	$< 2.2\times10^{-16}$
Body.weight	0.01	1	0.91
Treatment × Day	32.21	15	0.0060
Treatment × Observer	76.52	3	$< 2.2\times10^{-16}$
Day × Observer	102.92	5	$< 2.2\times10^{-16}$
Treatment × Day × Observer	70.53	15	3.6×10^{-9}

Figure 13 is a visual of the fitted model, with cumulative bar charts of model-based probabilities.

Figure 13: Model-based probabilities for nose scores 0, 1 and 2 across study days for each treatment (CON = control; CTD = cautery tail-docking; RRTD = rubber ring tail-docking; STD = surgical tail-docking).

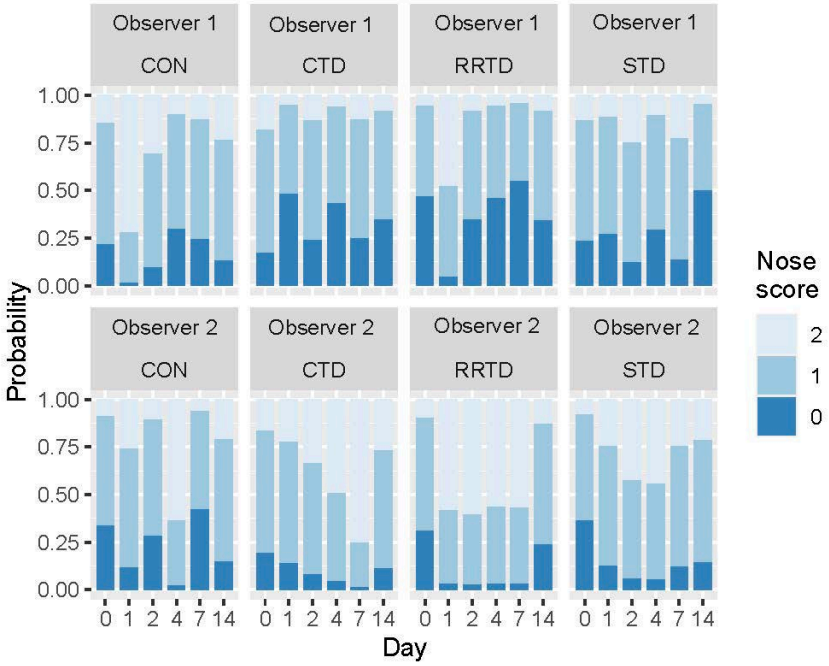


Table 26 shows comparisons between treatments within the same Day and same Observer. Elements sharing the same letter are not significantly different ($P > 0.05$).

Table 26: Comparisons between treatment groups for lip scores (CON = control; CTD = cautery tail-docking; RRTD = rubber ring tail-docking; STD = surgical tail-docking). Means with different superscripts (a, b) are significantly different.

		Day					
Observer	Treatment	0	1	2	4	7	14
1	CON	ab	a	a	a	ab	a
	CTD	a	b	ab	a	ab	ab
	RRTD	b	a	b	a	a	ab
	STD	ab	b	a	a	b	b
2	CON	a	ab	a	a	a	a
	CTD	a	a	b	a	b	a
	RRTD	a	b	b	a	b	a
	STD	a	a	b	a	a	a

4.3.5 Inter-rater reliability

Table 27 shows a cross-tabulation of the two observers scores (Eye, Lip and Nose scores), separately for tail docking and castration procedures.

Table 27: Cross-tabulation of facial grimace scores for Observers 1 and 2 for castration and tail-docking.

	Tail docking				Castration			
Eye score		Observer 2				Observer 2		
	Observer 1	0	1	2	Observer 1	0	1	2
	0	412	359	22	0	195	254	8
	1	12	237	112	1	6	183	63
	2	0	0	91	2	0	0	55
Lip score		Observer 2				Observer 2		
	Observer 1	0	1	2	Observer 1	0	1	2
	0	193	506	60	0	95	367	27

	1	7	222	30	1	5	134	18
	2	1	107	105	2	1	40	70

Nose score		Observer 2				Observer 2	
	Observer 1	0	1	2	Observer 1	0	1	2
	0	75	129	137	0	32	114	115
	1	81	267	153	1	27	154	106
	2	15	93	97	2	7	47	36

As can be seen, the best agreement is seen for eye scores (concentration of frequencies on the diagonal), with poorer agreement for lip and nose scores. This is further quantified by Table 28 that shows polychoric correlation coefficients, separately for each score type and procedure type.

Table 28: Polychoric correlation coefficients for facial grimace scores for castration and tail-docking.

	Tail docking	Castration
Eye score	0.861	0.858
Lip score	0.623	0.673
Nose score	0.114	-0.016

Highest agreement is for eye scores, and moderate for lip scores, but quite low for nose scores. Notably, very similar levels of agreement are seen in tail docking and castration.

4.4 Determining current 'best practice' pain mitigation for castration and dehorning cattle

This experiment was conducted in linkage with P.PSH.0819 ‘Objective measures of welfare’.

4.4.1 Weight gain

There was a significant effect of treatment on percent change in body weight from baseline (immediately before marking on day 0) to day 7 ($P < 0.001$) and to day 35 ($P < 0.001$) (Table 29). There was also a significant effect of procedure (castration, dehorning, castration and dehorning) on percent change from baseline to day 7 ($P < 0.001$) and to day 35 ($P < 0.001$) (Table 30).

All weaners, including those in the positive control group, lost weight over the first 7 days following treatment. The positive control group lost the least weight over days 0 to 7, and gained the most weight over days 0 to 35. There was no difference in percent change in body weight between any of the pain mitigation treatments or the negative control group (Table 29). Dehorned animals lost the greatest amount of weight over days 0 to 7, and gained the least amount of weight over days 0 to 35 (Table 30).

Table 29: Percent change in body weight of calves undergoing various pain mitigation therapies for castration and / or dehorning from immediately prior to treatment to 7 and 35 days following treatment. Means with different superscripts (a, b) are significantly different.

	Positive control	Negative control	Topical anaesthetic	Meloxicam	Topical anaesthetic + Meloxicam
Percent change in body weight from immediately prior to treatment to 7 days following treatment	-0.08[a]	-3.8[b]	-4.1[b]	-4.2[b]	-4.7[b]
Percent change in body weight from immediately prior to treatment to 35 days following treatment	10.2[a]	7.0[b]	6.6[b]	6.2[b]	5.9[b]

Table 30: Percent change in body weight of calves undergoing castration and / or dehorning from immediately prior to treatment to 7 and 35 days following treatment. Means with different superscripts (a, b) are significantly different.

	Castration	Dehorning	Castration + dehorning	Positive control
Percent change in body weight from immediately prior to treatment to 7 days following treatment	-2.0[a]	-4.1[b]	-4.5[b]	-0.1[c]
Percent change in body weight from immediately prior to treatment to 35 days following treatment	13.5[a]	4.5[d]	8.5[c]	10.1[b]

4.4.2 Behaviour

Findings are reported in P.PSH.0819 Milestone 7.

4.4.3 Wound healing

4.4.3.1 Castration

There was no significant effect of treatment on stage of wound healing ($P = 0.217$). There was a significant effect of time on stage of wound healing ($P < 0.001$) (Figure 14).

Figure 14: Probability of various stages of healing of castration wounds at 7 and 35 days following the procedure (Y; 1 = open wound, 2 = scabbed wound, 3 = wound with fibrous tissue, 4 = wound with complete sealing and mature skin).

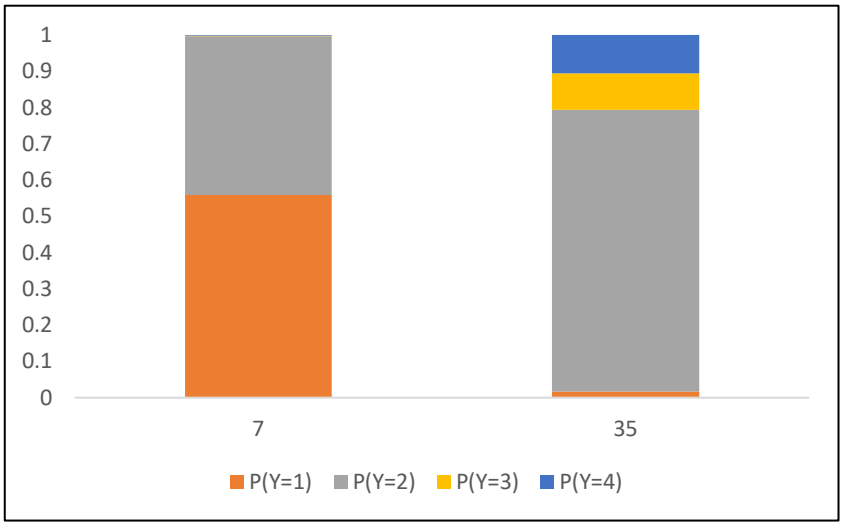
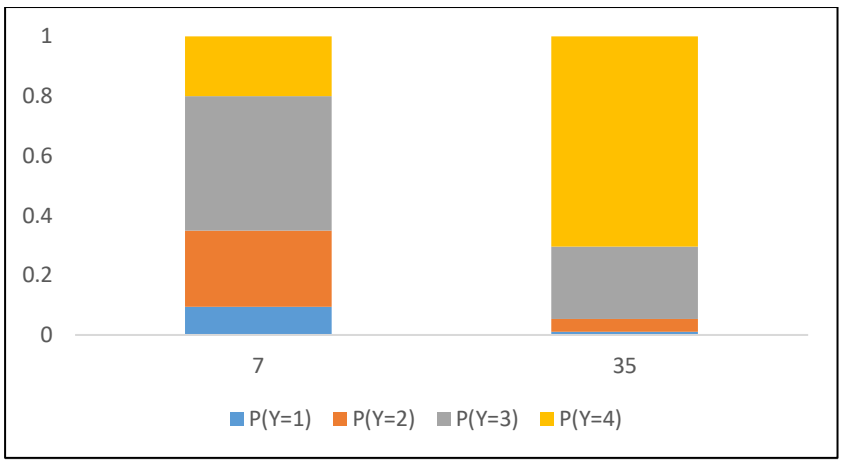


Figure 15: Probability of various stages of healing of dehorning wounds at 7 and 35 days following the procedure (Y; 1= wound with a hole exposing the sinus, 2 = wound with a scab at bone level, 3 = wound with a scab at skin level, 4 = wound with fibrous tissue).



4.4.3.2 Dehorning

There was no significant effect of treatment on stage of wound healing ($P = 0.991$). There was a significant effect of time on stage of wound healing ($P < 0.001$) (Figure 15).

4.4.4 Wound temperature

4.4.4.1 Castration

There was no significant effect of treatment on maximum temperature of castration wounds (*P* = 0.4). There was a significant effect of time on maximum temperature of castration wounds (P < 0.001), with maximum temperature increased at 35 days as compared to 7 days (40.46°C and 37.29°C, respectively).

4.4.4.2 Dehorning

There was no significant effect of treatment on maximum temperature of dehorning wounds (*P* = 0.294). There was a significant effect of time on maximum temperature of castration wounds (P < 0.001), with maximum temperature increased at 35 days as compared to 7 days (42.38°C and 39.29°C, respectively).

4.5 Pharmacokinetics of a sustained release meloxicam formulation in cattle

4.5.1 Pharmacokinetic Results

The average pharmacokinetic (PK) values for Phase 1 and Phase 2 are presented in Table 31 and Table 32, respectively. The SR-M formulation had a mean $t_{1/2}$ of 25.43 h (±5.00 h) in Phase 1 and a mean $t_{1/2}$ of 55.54 h (±3.15 h) within Phase 2, exhibiting variation. Mean plasma concentrations are presented in Figure 16. There was a mean C_{max} of 0.61 ug/mL (±0.21 ug/mL) at an average T_{max} of 3.33 h (±1.15 h) for the SR-M formulation in Phase 1, while in Phase 2 mean C_{max} was 0.76 ug/mL (±0.12 ug/mL) at an average T_{max} of 10.67 h (±2.31 h).

3.2. Statistical Results

There was a 3-way interaction between treatment, time and phase (P< 0.0006). The SR-M concentrations were different for Phase 1 and Phase 2 at all time-points between 6 h and 48 h (P<0.05). More specifically between drugs, CM concentrations in Phase 1 were significantly higher than SR-M concentrations at time points 2h, 4h, 6h and 8h (P<0.05), while in Phase 2, meloxicam concentrations were significantly greater than SR-M concentrations at all time points between 2h and 12 h and at 48 h (P<0.05).

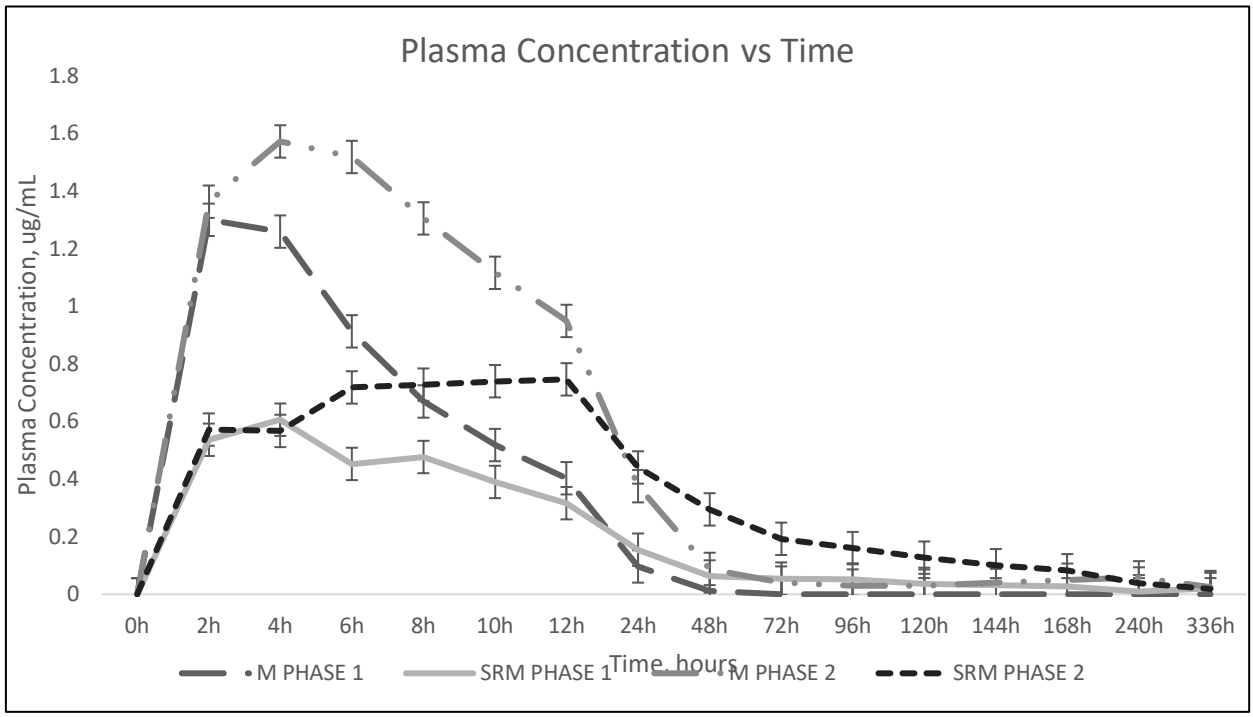
Table 31: Mean Phase 1 pharmacokinetic indices for 0.5mg/kg M and 1.0mg/kg SR-M

		0.5mg/kg M SC injection (Phase 1)			1.0mg/kg SR-M SC injection (Phase 1)		
Parameter	**Unit**	**Ave**	**CV**	**SD±**	**Ave**	**CV**	**SD**
t1/2	h	5.41	0.07	0.39	25.43	0.20	5.00
Tmax	h	2.67	0.43	1.15	3.33	0.35	1.15
Cmax	µg/ml	1.36	0.12	0.16	0.61	0.34	0.21
k_{el}	h^{-1}	0.13	0.07	0.01	0.03	0.19	0.01
AUC 0-t	µg/ml*h	12.71	0.07	0.86	13.17	0.35	4.55
AUC 0-λ	µg/ml*h	13.47	0.05	0.74	15.15	0.32	4.84
Vz	(mg/kg)/(µg/ml)	0.29	0.13	0.04	1.98	0.14	0.28
Cl	(mg/kg)/(µg/ml)/h	0.04	0.06	0.00	0.05	0.11	0.01

Table 32: Mean Phase 2 pharmacokinetic indices for 0.5mg/kg M and 1.0mg/kg SR-M

		0.5mg/kg M SC injection (Phase 2)			1.0mg/kg SR-M SC injection (Phase 2)		
Parameter	**Unit**	**Ave**	**CV**	**SD±**	**Ave**	**CV**	**SD**
t1/2	h	11.01	0.22	2.47	55.54	0.06	3.15
Tmax	h	4.67	0.25	1.15	10.67	0.22	2.31
Cmax	µg/ml	1.65	0.06	0.11	0.76	0.16	0.12
k_{el}	h^{-1}	0.06	0.20	0.01	0.01	0.06	0.00
AUC 0-t	µg/ml*h	28.94	0.17	5.02	41.81	0.13	5.45
AUC 0-λ	µg/ml*h	29.98	0.17	5.17	48.51	0.10	4.89
Vz	(mg/kg)/(µg/ml)	0.26	0.08	0.02	1.67	0.13	0.22
Cl	(mg/kg)/(µg/ml)/h	0.02	0.16	0.00	0.02	0.10	0.00

Figure 16: Plasma concentrations over time of conventional and sustained release meloxicam for Phase 1 and Phase 2



4.6 Pharmacokinetics of a novel modified release meloxicam formulation in Sheep

4.6.1 Pharmacokinetic Results

The plasma PK indices are presented in Table 33 Plasma SRMF concentrations detected in each sheep over 96 h and over time are presented in Figure 17 and Figure 18, respectively.

Table 33: Plasma pharmacokinetic indices following subcutaneous administration of 2 mg/kg sustained-release meloxicam formulation (SRMF) in sheep (n = 6).

PK Indices	Sheep 1	Sheep 2	Sheep 3	Sheep 4	Sheep 5	Sheep 6	Mean	SD
$t_{1/2}$ (h)	17.96	19.48	39.53	25.6	33.43	52.39	31.4	13.17
T_{max} (h)	8.00	10.00	12.00	8.00	12.00	10.00	10	1.79
C_{max} (µg/mL)	2.80	2.41	1.4	1.06	0.85	0.95	1.58	0.82
$AUC_{0\text{-}t}$ (µg/mL×h)	97.01	105.71	74.4	40.02	38.39	64.21	69.96	28.13
$AUC_{0\text{-}inf}$ (µg/mL×h)	99.74	109.89	82.3	43.7	44.78	76.74	76.19	27.46
$AUC_{0\text{-}t}/AUC_{0\text{-}\infty}$ %	97%	96%	90%	92%	86%	84%	91%	97%
$AUMC_{0\text{-}inf}$ (µg/mL×h^2)	2764.99	3520.26	4764.42	1660.06	2173.35	5930.36	3468.91	1623.93
MRT (h)	27.72	32.04	57.89	37.98	48.53	77.28	40.83	12.32
V/F (L/kg)	0.52	0.51	1.39	1.69	2.15	1.97	1.25	0.73
Cl/F (L/kg/h)	0.02	0.02	0.02	0.05	0.05	0.03	0.03	0.01

Figure 17: Plasma concentrations (ug/mL) of 2 mg/kg sustained-release meloxicam formulation (SRMF) following subcutaneous administration in sheep (n = 6) over 96 h. The dotted line at y = 0.1 µg/mL is the lower limit of quantification of the assay (Woodland et al., 2019). The dotted line at y = 0.4 µg/mL is a theoretical plasma analgesic concentration.

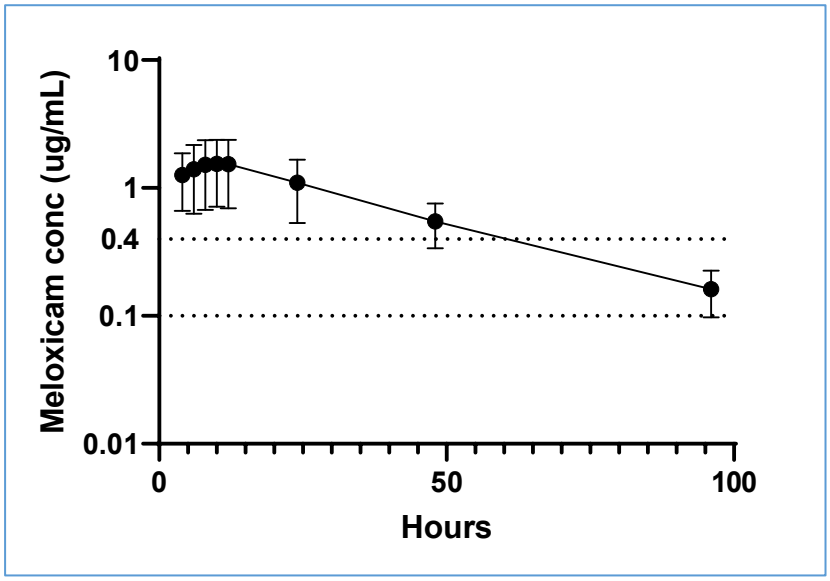
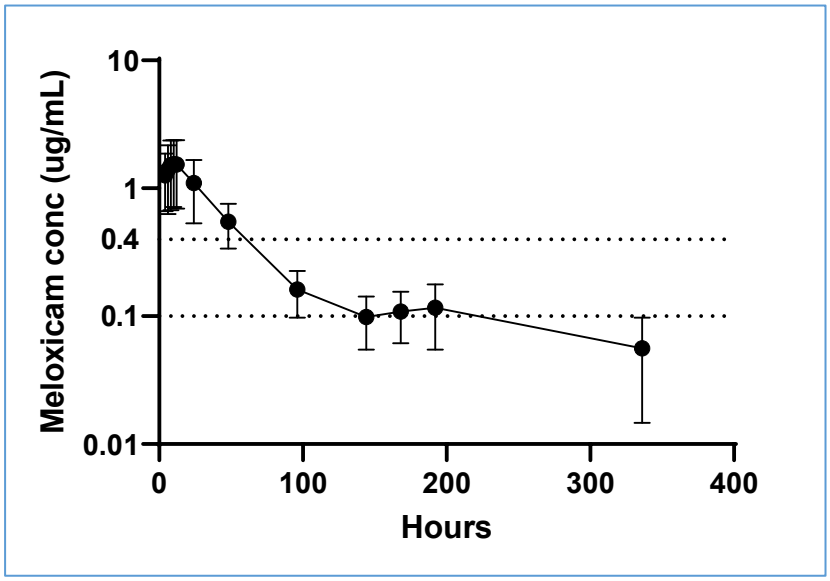


Figure 18: Plasma concentrations (ug/mL) of 2 mg/kg sustained-release meloxicam formulation (SRMF) following subcutaneous administration in sheep (n = 6) over time. The dotted line at y = 0.1 µg/mL is the lower limit of quantification of the assay (Woodland et al., 2019). The dotted line at y = 0.4 µg/mL is a theoretical plasma analgesic concentration

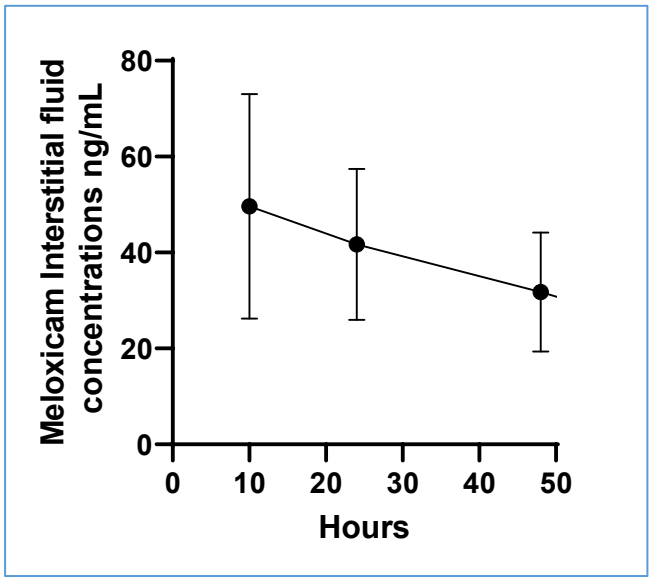


The ISF concentrations of meloxicam are presented in Table 34. The ISF samples from sheep 1 and 6 were not collected and only one fluid sample was collected from sheep 2 at 48 to 52 h due to failure of the ultrafiltration probe. Fluid samples were successfully collected from sheep 3, 4, and 5, which showed a decrease in meloxicam concentration in the fluid samples over time. Meloxicam was not detectable in ISF from sheep 4 and 5 at 24 to 48 h and 48 to 52 h, respectively. The ISF meloxicam concentrations of sheep 2, 3, 4, and 5 are presented in Figure 19.

Table 34: Interstitial fluid meloxicam concentrations (ng/mL) following subcutaneous administration of 2 mg/kg sustained-release meloxicam formulation in sheep (n = 6).

Time Period (h)	Sheep 1	Sheep 2	Sheep 3	Sheep 4	Sheep 5	Sheep 6
8–12	No Sample	No Sample	42.27	30.8	75.8	No Sample
12–24	No Sample	No Sample	31.8	33.5	50.9	No Sample
24–48	No Sample	No Sample	27.2	No Peak Detected	46.2	No Sample
48–52	No Sample	40.52	23	No Peak Detected	No Peak Detected	No Sample
92–96	No Sample	No Sample	9	No Peak Detected	No Peak Detected	No Sample

Figure 19: Interstitial fluid concentrations (ng/mL) of 2 mg/kg sustained-release meloxicam formulation (SRMF) following subcutaneous administration in sheep (n = 4).



4.7 Plasma concentrations of meloxicam in calves fed meloxicam pellets

The mean plasma meloxicam concentrations from Day 0 to Day 9 are presented in Table 35. Plasma concentrations reached a maximum of 3.81 ug/mL (avg 2.53 ug/mL ± 1.05 ug/mL SD) within 24 h and increased daily, reaching a maximum concentration of 6.95 ug/mL (avg 4.07 ug/mL ± 2.17 ug/mL SD) on Day 6. From Day 7 to Day 9, the mean elimination $t_{1/2}$ was 15.97 h (±6.09 h) with an elimination rate (k_{el}) of 0.04 h^{-1} (±0.01 h^{-1}).

Table 35: Average plasma concentrations of 1.0mg/kg meloxicam-medicated pellets from Day 1 to Day 9

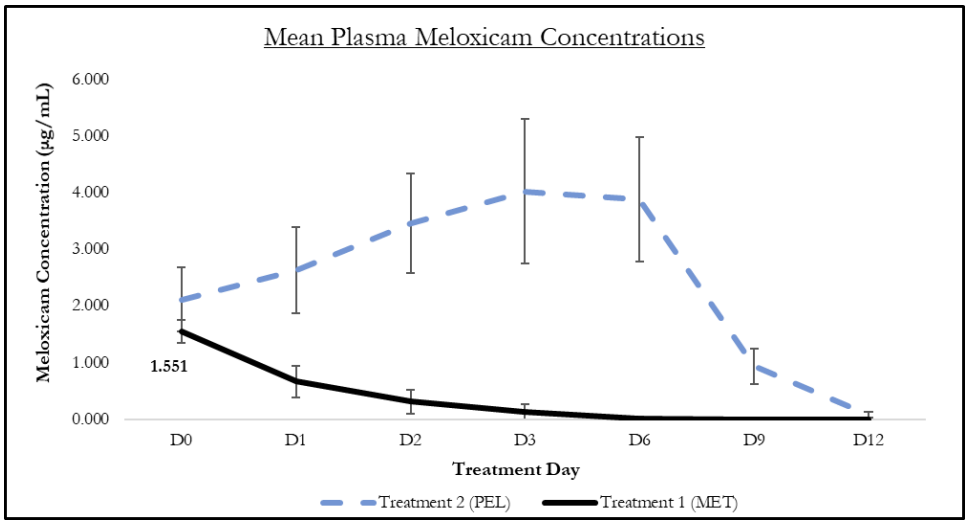
	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9
Average	0	2.53	3.16	3.12	3.52	4.05	4.07	4.19	1.79	0.68
Standard Deviation	0	1.05	1.22	1.18	1.56	1.63	2.17	1.70	0.96	0.46
Min	0	0.99	1.12	1.55	1.32	1.25	0.61	0.97	0.36	0.12
Max	0	3.81	4.99	5.00	6.39	6.47	6.95	6.44	1.62	1.62
Range	0	2.82	3.87	3.45	5.08	5.22	6.34	5.48	1.26	1.50

4.8 Investigating the efficacy of medicated meloxicam pellets for calves

4.8.1 Plasma meloxicam concentration

Plasma meloxicam concentration for the two treatment groups that received meloxicam (MET and PEL) are shown in Figure 20.

Figure 20: Mean plasma meloxicam concentrations (±SD) of calves that received medicated meloxicam pellets (PEL) and subcutaneous meloxicam (Metacam20®, Boehringer Ingelheim) (MET).



4.8.2 Plasma biomarker (Tumour necrosis factor alpha)

There were four missing samples in experimental block 1 due to unsuccessful blood collections. These samples were from 1x POS calf and 1x MET calf on day -1, 1x POS calf on day 6 and 1x POS calf on day 12. There was no effect of experimental block (P = 0.493) and there was no significant Treatment x Time-point (P = 0.212) or Treatment (P = 0.912) or Time (P = 0.249) effect.

4.8.3 Behaviour

Table 36 shows the significance levels (*P*-values) for each of the 14 behaviour traits analysed.

Table 36: Significance of changes in behaviour according to Day and Treatment (EF = ear flicking; HS = head shaking; HR = head rubbing; TF = tail flicking; FS = foot stamping; G = grooming; D = drinking; E = eating; L = locomotion; NS = normal standing; NL = normal lying; HHI = head to head interaction; HBI = head to body interaction; I = isolation).

Behaviour	Treatment	Day	Treatment x Day
EF	2.4E-09	4.5E-12	< 2.2E-16
HS	1.5E-04	5.2E-05	< 2.2E-16
HR	0.82	3.0E-07	2.0E-03
TF	0.045	< 2.2E-16	< 2.2E-16
FS	0.16	6.6E-04	3.5E-16
G	0.012	0.026	0.64
D	0.59	0.44	0.151
E	0.41	< 2.2E-16	6.0E-07
L	0.052	1.00	2.9E-06
NS	9.0E-05	5.2E-05	2.0E-05
NL	3.6E-04	7.1E-07	5.5E-06
HHI	0.011	1.6E-03	0.13
HBI	0.074	1.8E-03	0.013
I	2.9E-05	2.3E-04	7.2E-09

For every trait except ‘D’, there was either a significant effect of Treatment (G, HHI) or a significant Treatment × Day interaction (all remaining traits).

4.8.4 Behaviour score

Based on the ordinal mixed model, the six-point BS showed a significant Treatment × Day interaction ($P = 4.1 \times 10^{-14}$). Table 37 shows the model-based probabilities for each Treatment × Day combination. Note that the probabilities across each row sum to 1, as required for a probability distribution.

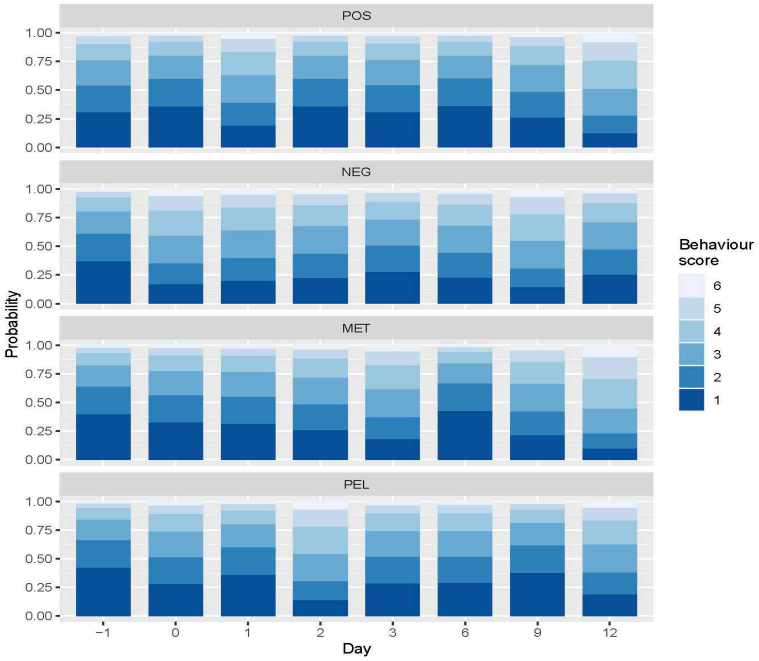
Table 37: Model-based probabilities of behaviour scores 1-6 for each Treatment x Day interaction (CON = control; NEG = disbudding; MET = disbudding with subcutaneous meloxicam (Metacam20®, Boehringer Ingelheim); PEL = disbudding with meloxicam pellets). Treatments within each day that share a superscript (a,b,c) do not differ significantly.

Treatment	Day	BS = 1	BS = 2	BS = 3	BS = 4	BS = 5	BS = 6	Group
POS	-1	0.307	0.235	0.219	0.143	0.067	0.028	a
NEG	-1	0.369	0.241	0.199	0.118	0.053	0.022	a
MET	-1	0.399	0.240	0.188	0.107	0.047	0.019	a
PEL	-1	0.425	0.239	0.178	0.099	0.042	0.017	a
POS	0	0.360	0.240	0.202	0.121	0.054	0.022	a
NEG	0	0.170	0.184	0.243	0.218	0.127	0.059	b
MET	0	0.327	0.238	0.213	0.134	0.062	0.026	a
PEL	0	0.283	0.230	0.227	0.154	0.075	0.032	ab
POS	1	0.193	0.197	0.243	0.204	0.113	0.051	a
NEG	1	0.199	0.200	0.243	0.200	0.109	0.049	ab
MET	1	0.316	0.236	0.217	0.139	0.065	0.027	bc
PEL	1	0.360	0.240	0.202	0.121	0.054	0.022	c
POS	2	0.361	0.240	0.202	0.121	0.054	0.022	a
NEG	2	0.225	0.212	0.240	0.185	0.097	0.042	bc
MET	2	0.262	0.225	0.232	0.165	0.081	0.035	ab
PEL	2	0.142	0.165	0.238	0.236	0.148	0.072	c
POS	3	0.310	0.235	0.219	0.142	0.067	0.028	a
NEG	3	0.278	0.229	0.228	0.157	0.076	0.032	ab
MET	3	0.183	0.192	0.243	0.210	0.118	0.054	b
PEL	3	0.288	0.231	0.225	0.152	0.073	0.031	ab
POS	6	0.363	0.240	0.201	0.120	0.054	0.022	a
NEG	6	0.230	0.214	0.239	0.182	0.094	0.041	b
MET	6	0.429	0.238	0.177	0.097	0.042	0.017	a
PEL	6	0.288	0.231	0.225	0.152	0.073	0.031	ab

Treatment	Day	BS = 1	BS = 2	BS = 3	BS = 4	BS = 5	BS = 6	Group
POS	9	0.263	0.225	0.232	0.164	0.081	0.035	ab
NEG	9	0.144	0.166	0.238	0.235	0.147	0.071	c
MET	9	0.215	0.208	0.241	0.190	0.101	0.045	a
PEL	9	0.377	0.241	0.196	0.115	0.051	0.021	b
POS	12	0.128	0.153	0.232	0.245	0.161	0.081	a
NEG	12	0.253	0.222	0.234	0.169	0.085	0.037	b
MET	12	0.101	0.130	0.217	0.259	0.191	0.103	a
PEL	12	0.189	0.194	0.243	0.206	0.115	0.052	ab

While at Day = -1, there were no significant differences in behaviour scores, the NEG treatment had significantly higher scores than the other treatments on the day of treatment (Day 0). **Figure 21** is a visualisation of these BS probabilities.

Figure 21: Model-based probabilities of behaviour scores 1-6 for each Treatment x Day interaction (CON = control; NEG = disbudding; MET = disbudding with subcutaneous meloxicam (Metacam20®, Boehringer Ingelheim); PEL = disbudding with meloxicam pellets).



4.8.5 Accelerometer derived behaviour states

When all the data were included, there was a marginal Treatment × Day interaction (P = 0.052), indicating the treatments had different time courses in lying. However, a plot of the model-based

means indicates increased proportions across all treatments on Day 6 [AM]: this may be due to reduced monitoring time, and only over the night when lying might be expected to be more frequent. A subsequent analysis excluded these Day 6 [AM] data; from this the Treatment × Day interaction was significant (*P* = 0.031). During generation of the raw data, problems were identified with the accelerometer profiles for calves 29 and 39. In another pair of analyses, data from these two calves was dropped. Using all time points, the Treatment × Day interaction was significant (*P* = 0.020), and after excluding the data on Day 6 [AM], the significance of the Treatment × Day interaction increased (*P* = 0.00085). **Figure** 22 is a plot of the model-based means for the Treatment x Day combination.

Figure 22: Model-based means of proportion of time spent lying for all treatments (CON = control; NEG = disbudding; MET = disbudding with subcutaneous meloxicam (Metacam20®, Boehringer Ingelheim); PEL = disbudding with meloxicam pellets) across experimental days.

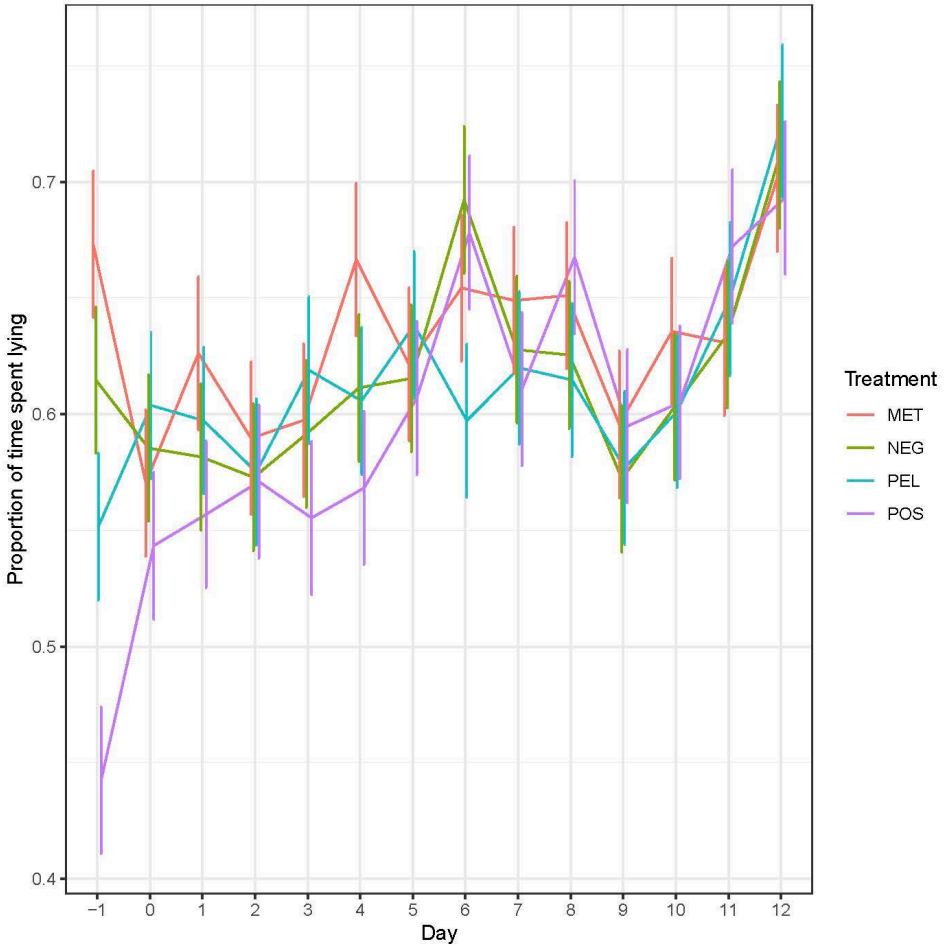


Table 38 shows the model-based means for the Treatment x Day combination from the last analysis (two calves excluded, and AM on Day 6 excluded). Treatment means sharing the same group letter within the same study day are not significantly different (*P* ≥ 0.05).

Table 38: Model-based means of proportion of time spent lying for all treatments (CON = control; NEG = disbudding; MET = disbudding with subcutaneous meloxicam (Metacam20®, Boehringer Ingelheim); PEL = disbudding with meloxicam pellets) across experimental days. Treatment means

sharing the same superscript (a,b,c) within the same study day are not significantly different (*P* ≥ 0.05).

	MET			NEG			PEL			POS		
Day	Mean	SE	group	Mean	SE	group	Mean	SE	group	Mean	SE	group
-1	0.673	0.029	a	0.652	0.031	a	0.552	0.029	b	0.462	0.031	c
0	0.570	0.029	a	0.613	0.031	a	0.604	0.029	a	0.560	0.031	a
1	0.626	0.031	a	0.623	0.031	a	0.597	0.029	a	0.557	0.031	a
2	0.590	0.031	a	0.613	0.031	a	0.575	0.029	a	0.559	0.032	a
3	0.597	0.031	a	0.624	0.031	a	0.619	0.029	a	0.540	0.032	a
4	0.667	0.031	a	0.643	0.031	ab	0.606	0.029	ab	0.555	0.032	b
5	0.621	0.031	a	0.643	0.031	a	0.638	0.029	a	0.596	0.032	a
6	0.654	0.029	ab	0.690	0.031	a	0.597	0.031	b	0.671	0.033	ab
7	0.649	0.029	a	0.626	0.031	a	0.620	0.031	a	0.609	0.033	a
8	0.651	0.029	a	0.627	0.031	a	0.615	0.031	a	0.663	0.033	a
9	0.596	0.029	a	0.574	0.031	a	0.577	0.031	a	0.593	0.033	a
10	0.636	0.029	a	0.601	0.031	a	0.601	0.031	a	0.604	0.033	a
11	0.631	0.029	a	0.624	0.031	a	0.650	0.031	a	0.678	0.033	a
12	0.702	0.029	a	0.702	0.031	a	0.726	0.031	a	0.681	0.033	a

From the table, differences in proportion of time lying across treatments were detected only on Days -1, 4 and 6. However, regardless of the treatment, there was a decline in the proportion of time lying from Days 6-9, followed by a general increase in the remaining days.

4.8.6 Mechanical nociceptive threshold

There were significant Treatment × Day (*P* = 1.9 ×10–9), Treatment × Site (*P* = 4.6 ×10–7) and Day × Site (*P* = 0.044) interactions, but the three-way interaction was not significant (*P* = 0.30). The nature of each interaction is explored through graphs of model-based means, and formal comparisons.

Table 39 shows the means with standard errors for each Treatment x Day combination. Means sharing the same Group letter are not significantly different (*P* > 0.05).

Table 39: Mean mechanical nociceptive threshold (kgf) (±SE) for each treatment across experimental days. (CON = control; NEG = disbudding; MET = disbudding with subcutaneous meloxicam (Metacam20®, Boehringer Ingelheim); PEL = disbudding with meloxicam pellets) across experimental days. Treatment means sharing the same superscript (a,b,c) within the same study day are not significantly different (*P* ≥ 0.05).

Day	Treatment	Mean	SE	Group
-1	POS	3.190	0.639	a
	NEG	4.034	0.815	a
	MET	3.843	0.802	a
	PEL	3.260	0.648	a
0	POS	2.630	0.523	a
	NEG	2.065	0.411	ab
	MET	1.958	0.401	ab
	PEL	1.401	0.278	b
1	POS	2.813	0.559	a
	NEG	1.373	0.273	b
	MET	0.839	0.167	bc
	PEL	0.792	0.158	c
2	POS	4.208	0.844	a
	NEG	1.383	0.276	b
	MET	1.493	0.297	b
	PEL	1.667	0.331	b
3	POS	2.774	0.551	a
	NEG	0.692	0.138	b
	MET	0.471	0.094	b
	PEL	0.564	0.112	b
6	POS	2.529	0.503	a
	NEG	0.886	0.176	b
	MET	0.685	0.136	b
	PEL	0.644	0.128	b
9	POS	1.942	0.386	a
	NEG	0.596	0.118	b
	MET	0.589	0.117	b
	PEL	0.511	0.102	b
12	POS	2.347	0.467	a
	NEG	0.635	0.126	b

Day	Treatment	Mean	SE	Group
	MET	0.697	0.139	b
	PEL	0.636	0.126	b

Prior to treatment (Day -1), as expected there were no significant differences between the treatment means. On the day of treatment (Day 0), the algometer force for PEL was significantly lower than that for POS and continued to be for each subsequent day. From Day 1 onwards all treatments had significantly lower force than POS. Figure 23 is a plot of means, the error bars represent ± 1 SE.

Figure 23: Model-based means of mechanical nociceptive threshold (kgf) (±SE) for all treatments (CON = control; NEG = disbudding; MET = disbudding with subcutaneous meloxicam (Metacam20®, Boehringer Ingelheim); PEL = disbudding with meloxicam pellets) across experimental days.

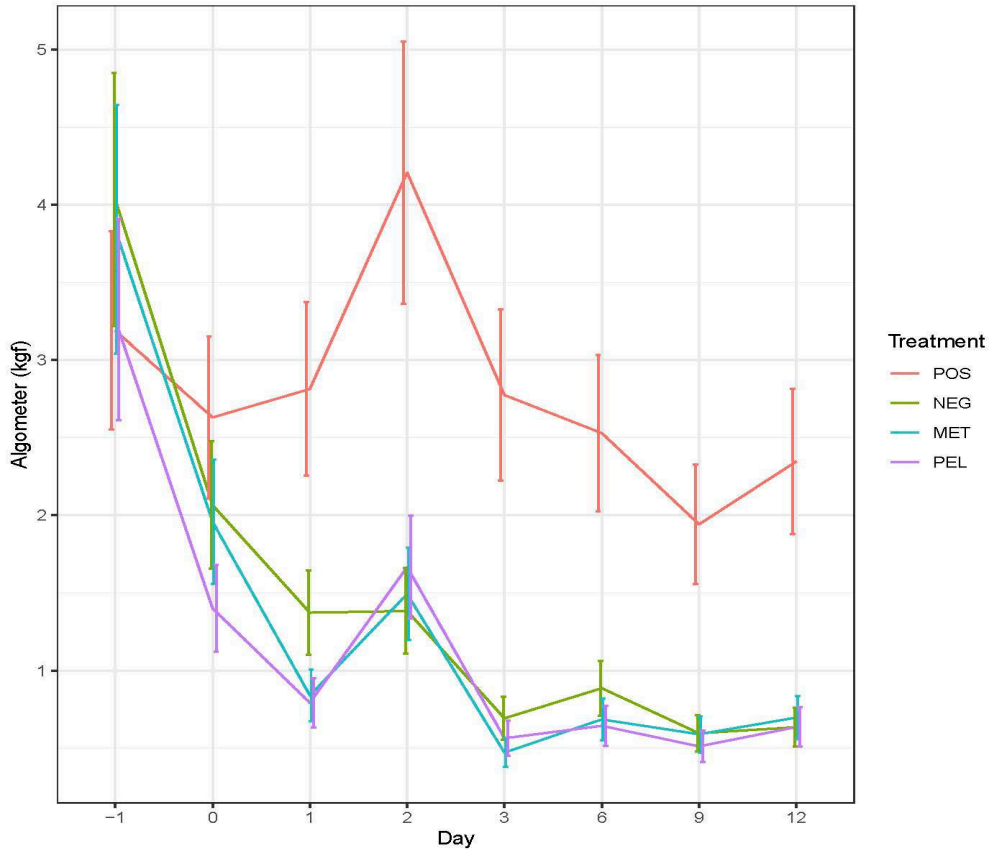


Table 40 shows the model-based means with standard errors for the Treatment x Site combination. Means sharing the same Group letter are not significantly different ($P > 0.05$).

Table 40: Model-based means of mechanical nociceptive threshold (kgf) (±SE) for all treatments (CON = control; NEG = disbudding; MET = disbudding with subcutaneous meloxicam (Metacam20®, Boehringer Ingelheim); PEL = disbudding with meloxicam pellets) for left and right horn sites 1 and 2.

Site	Treatment	Mean	SE	Group
Left horn site 1	POS	2.537	0.406	a
	NEG	1.643	0.263	b
	MET	1.381	0.222	b
	PEL	1.216	0.194	b
Left horn site 2	POS	2.731	0.437	a
	NEG	0.974	0.156	b
	MET	0.849	0.137	b
	PEL	0.866	0.138	b
Right horn site 1	POS	2.825	0.453	a
	NEG	1.309	0.210	b
	MET	1.245	0.200	b
	PEL	1.108	0.177	b
Right horn site 2	POS	2.880	0.461	a
	NEG	0.914	0.147	b
	MET	0.766	0.123	b
	PEL	0.723	0.116	b

POS shows a consistently higher mean in all horn sites, though the difference is more pronounced for left and right horn sites 2. **Figure 24** is a plot of means, the error bars represent ± 1 SE.

Figure 24: Model-based means of mechanical nociceptive threshold (kgf) (± 1 SE) for all treatments (CON = control; NEG = disbudding; MET = disbudding with subcutaneous meloxicam (Metacam20®, Boehringer Ingelheim); PEL = disbudding with meloxicam pellets) for left and right horn sites 1 and 2.

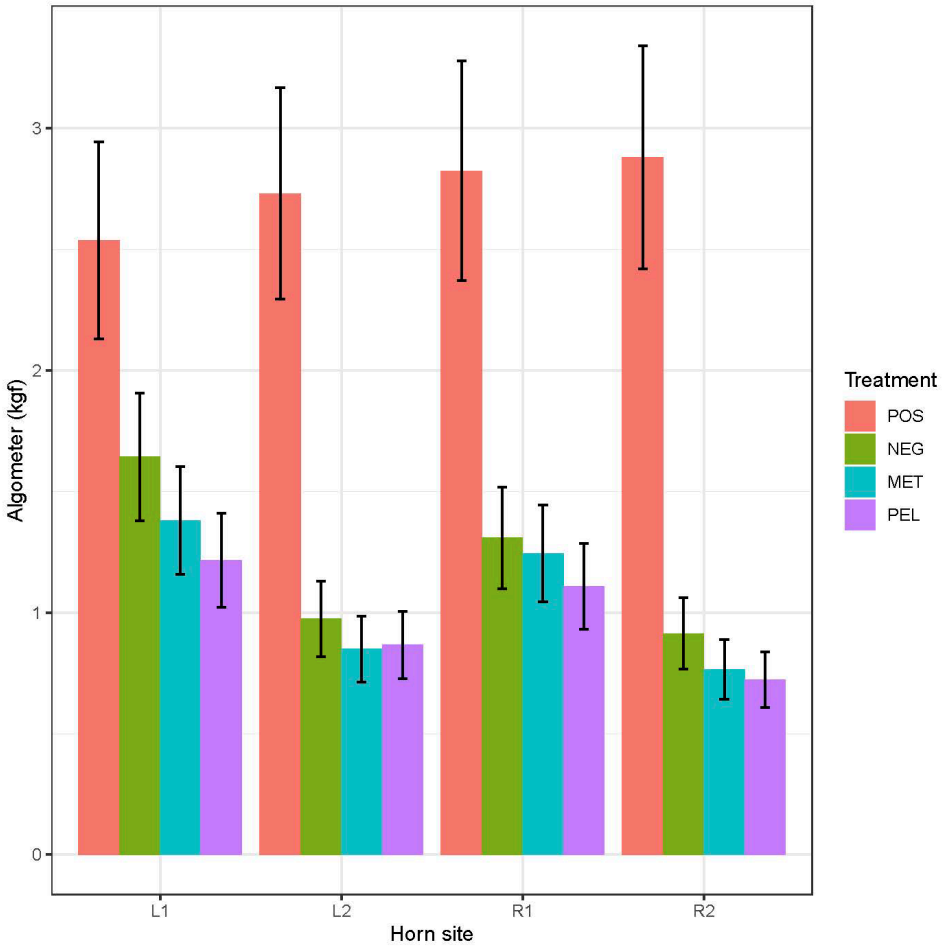


Table 41 shows the model-based means with standard errors for the Day x Site combination. Means sharing the same Group letter are not significantly different ($P > 0.05$).

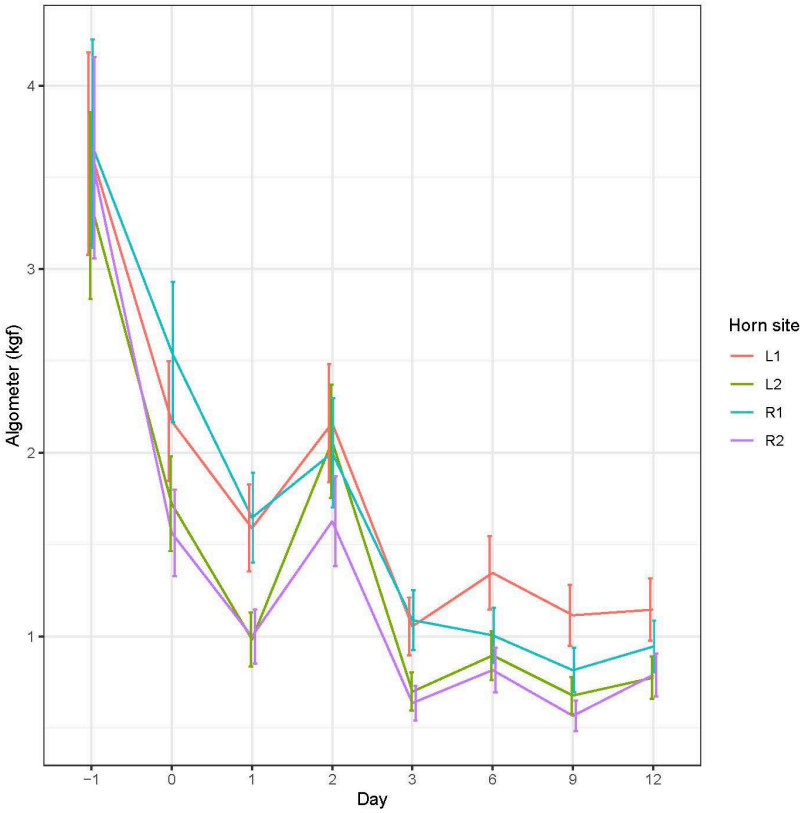
Table 41: Model-based means of mechanical nociceptive threshold (kgf) (±SE) across all study days for left and right horn sites 1 and 2. Sites sharing a similar superscript (a,b,c) are not significantly different.

Day	Site	Mean	SE	Group
-1	L1	3.628	0.552	a
	L2	3.345	0.509	a
	R1	3.683	0.568	a
	R2	3.607	0.548	a
0	L1	2.172	0.326	ab
	L2	1.722	0.258	ac
	R1	2.549	0.382	b
	R2	1.563	0.234	c
1	L1	1.589	0.237	a
	L2	0.983	0.146	b
	R1	1.646	0.245	a
	R2	0.999	0.149	b
2	L1	2.162	0.322	a
	L2	2.062	0.309	ab
	R1	1.999	0.298	ab
	R2	1.626	0.245	b
3	L1	1.054	0.157	a
	L2	0.698	0.104	b
	R1	1.088	0.162	a
	R2	0.636	0.095	b
6	L1	1.345	0.200	a
	L2	0.895	0.133	b
	R1	1.005	0.150	b
	R2	0.816	0.121	b
9	L1	1.114	0.166	a
	L2	0.677	0.101	bc
	R1	0.815	0.121	b
	R2	0.567	0.084	c

Day	Site	Mean	SE	Group
12	L1	1.145	0.170	a
	L2	0.774	0.115	b
	R1	0.944	0.141	ab
	R2	0.789	0.117	b

From Day 0, right horn site 2 seemed to have a lower force (be more sensitive) than other sites. Figure 25 is a plot of means, the error bars represent ± 1 SE.

Figure 25: Model-based means of mechanical nociceptive threshold (kgf) (± 1 SE) across all study days for left and right horn sites 1 and 2.



4.8.7 Horn site temperature

4.8.7.1 Ambient air temperature

Figure 26 shows a plot of the air temperature recorded at Camden airport over the experimental period: a clear 24-hr cycle is evident, as well as a longer-term trend. Figure 27 shows a plot of IR temperature versus air temperature: as the ambient air temperature rose, so did the temperature recorded on the horn site. However, this plot suggests a slight nonlinearity. Figure 28 shows the fitted relationship, indicating a somewhat greater sensitivity to high and possibly low air temperatures, and more moderate in the middle zone (the shaded area represents ± 1 SE). Note that the nonlinear effect

of air temperature, as modelled by the spline, was a better fitting model than as a linear effect (*P* = 0.0076).

Figure 26: Ambient air temperature recorded at Camden airport over the experimental period.

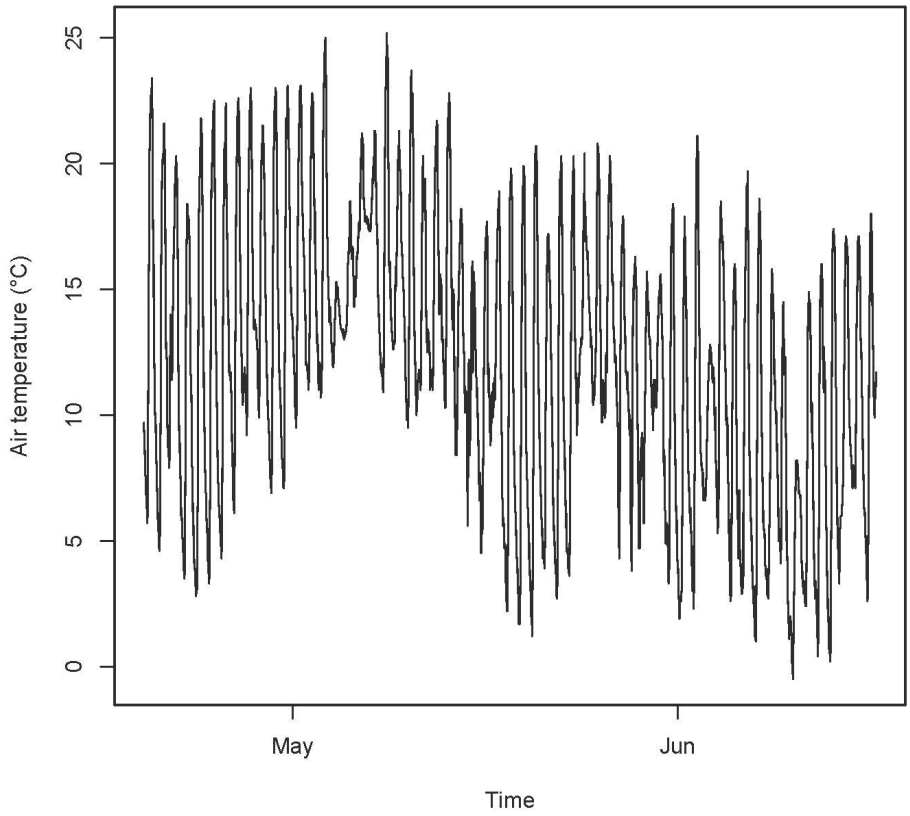


Figure 27: Plot of ambient air temperature and horn site infrared (IR) temperature.

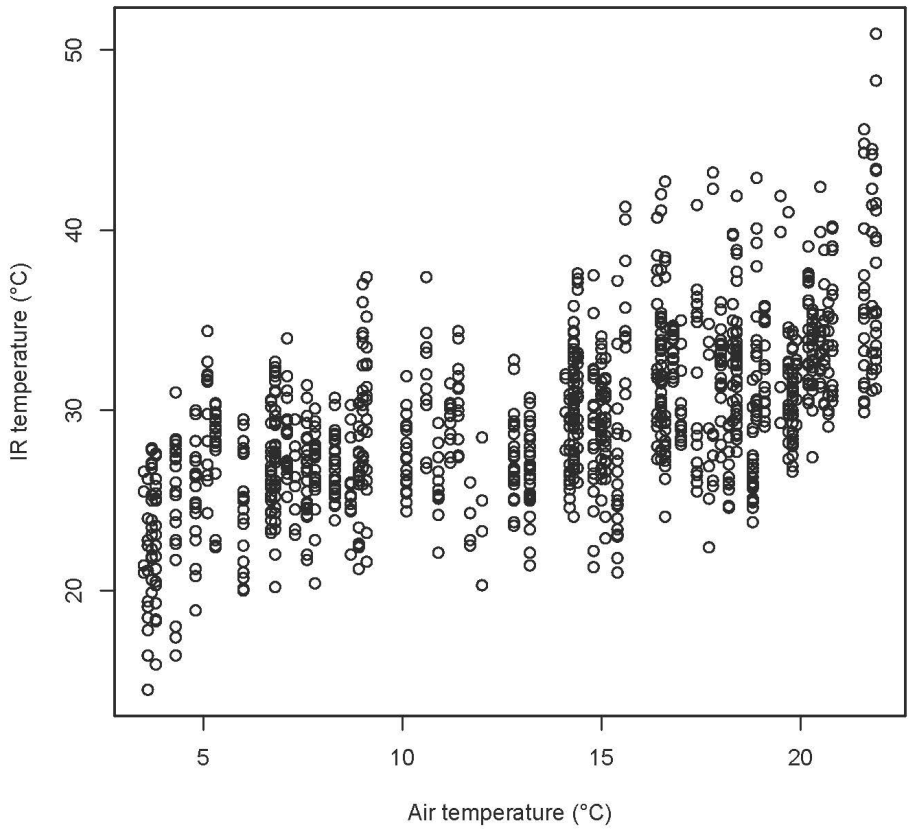
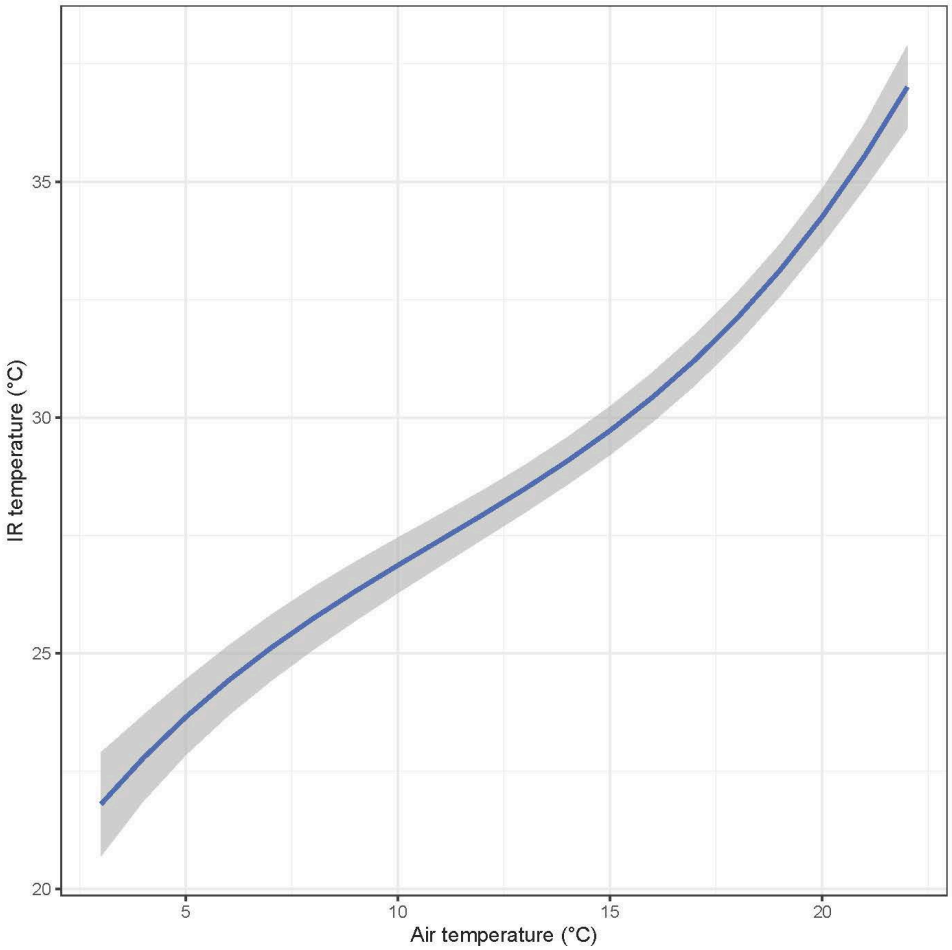


Figure 28: Fitted relationship between ambient air temperature and horn site infrared temperature. Shaded area represents ± 1 SE.



4.8.7.2 Horn site temperature

There were significant Treatment × Day ($P = 0.0050$), Treatment × Site ($P = 1.5 \times 10^{-9}$) and Day × Site ($P = 3.1 \times 10^{-6}$) interactions, but the three-way interaction was not significant ($P = 0.30$). In addition, there was a significant (nonlinear) effect of air temperature ($P < 2.2 \times 10^{-16}$). Each interaction is explored through graphs of model-based means, and formal comparisons.

Table 42 shows the model-based mean temperatures with standard errors for the Treatment x Day combination.

Table 42 Model-based mean temperatures (°C) with standard errors for each treatment (CON = control; NEG = disbudding; MET = disbudding with subcutaneous meloxicam (Metacam20®, Boehringer Ingelheim); PEL = disbudding with meloxicam pellets) across experimental days. Treatment groups sharing a similar superscript (a,b) do not differ significantly.

Day	Treatment	Mean	SE	Group
-1	POS	28.3	1.18	a
	NEG	23.3	1.22	b

Day	Treatment	Mean	SE	Group
	MET	23.8	1.22	b
	PEL	24.7	1.14	b
0	POS	27.4	0.98	a
	NEG	27.0	0.99	a
	MET	27.5	0.92	a
	PEL	26.6	0.91	a
1	POS	28.1	0.83	a
	NEG	28.5	0.83	a
	MET	27.7	0.81	a
	PEL	26.6	0.81	a
2	POS	29.4	0.84	a
	NEG	31.8	0.84	b
	MET	32.2	0.87	b
	PEL	31.4	0.92	b
3	POS	29.9	0.86	a
	NEG	31.9	0.84	b
	MET	30.6	0.88	ab
	PEL	29.0	0.99	a
6	POS	30.5	0.83	ab
	NEG	31.2	0.81	a
	MET	31.1	0.87	a
	PEL	28.8	0.95	b
9	POS	28.9	0.94	a
	NEG	29.4	0.85	a
	MET	29.7	0.83	a
	PEL	29.6	0.83	a
12	POS	30.3	0.98	a
	NEG	28.9	0.93	ab
	MET	30.3	0.85	a
	PEL	28.0	0.92	b

Temperature generally increased consistently from Day 1, but no patterns between treatments were immediately obvious. Figure 29 is a plot of means, the error bars represent ± 1 SE.

Figure 29: Model-based mean temperatures (°C) ±1 SE for each treatment (CON = control; NEG = disbudding; MET = disbudding with subcutaneous meloxicam (Metacam20®, Boehringer Ingelheim); PEL = disbudding with meloxicam pellets) across experimental days.

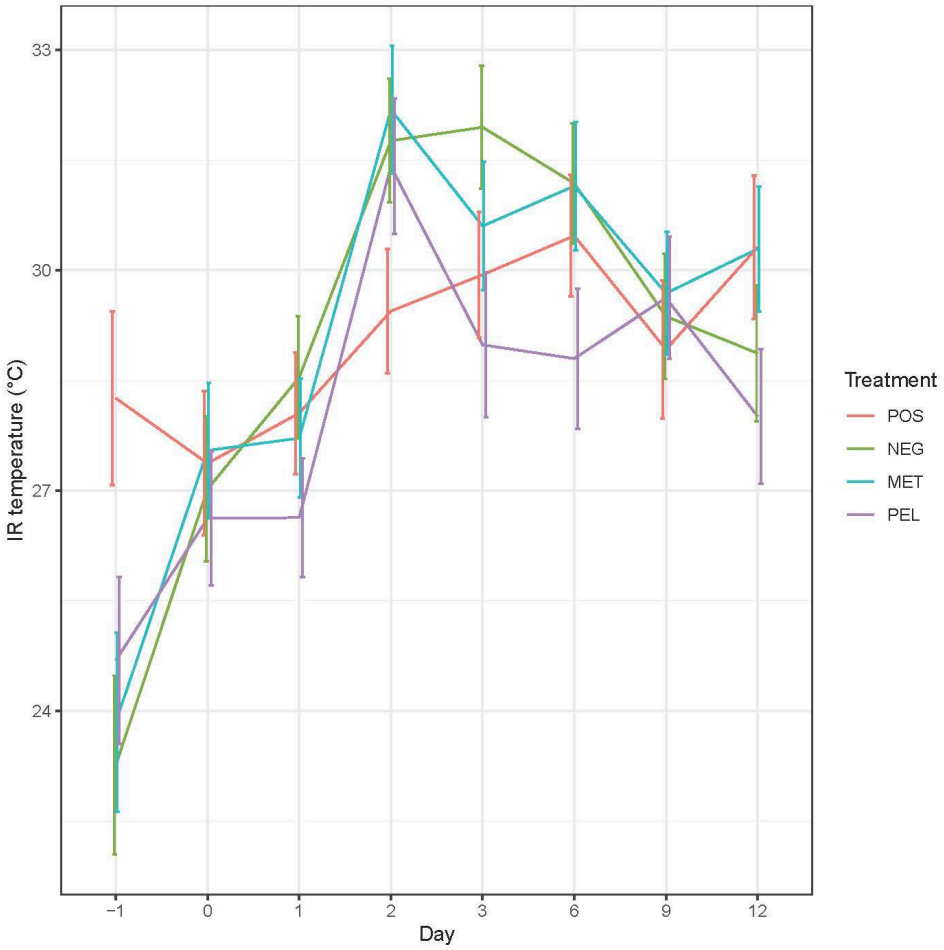


Table 43 shows the model-based mean temperatures with standard errors for the Treatment x Site combination.

Table 43: Model-based means of horn site temperature (°C) (±SE) for each treatment (CON = control; NEG = disbudding; MET = disbudding with subcutaneous meloxicam (Metacam20®, Boehringer Ingelheim); PEL = disbudding with meloxicam pellets) for left and right horn sites 1 and 2. Treatments sharing a similar superscript (a,b,c) are not significantly different.

Site	Treatment	Mean	SE	Group
L1	POS	27.0	0.63	a
	NEG	27.4	0.61	a
	MET	27.5	0.60	a
	PEL	26.9	0.61	a

L2	POS	27.8	0.63	a
	NEG	28.3	0.61	a
	MET	28.6	0.60	a
	PEL	28.6	0.61	a
R1	POS	30.3	0.63	a
	NEG	29.6	0.61	a
	MET	29.7	0.60	a
	PEL	27.3	0.61	b
R2	POS	31.3	0.63	a
	NEG	30.7	0.61	ab
	MET	30.7	0.60	a
	PEL	29.6	0.61	b

Overall, treatment differences within a site were only observed for right horn sites. Figure 30 is a plot of means, the error bars represent ± 1 SE.

Figure 30: Model-based means of horn site temperature (°C) (± 1 SE) for each treatment group (CON = control; NEG = disbudding; MET = disbudding with subcutaneous meloxicam (Metacam20®, Boehringer Ingelheim); PEL = disbudding with meloxicam pellets) for left and right horn sites 1 and 2.

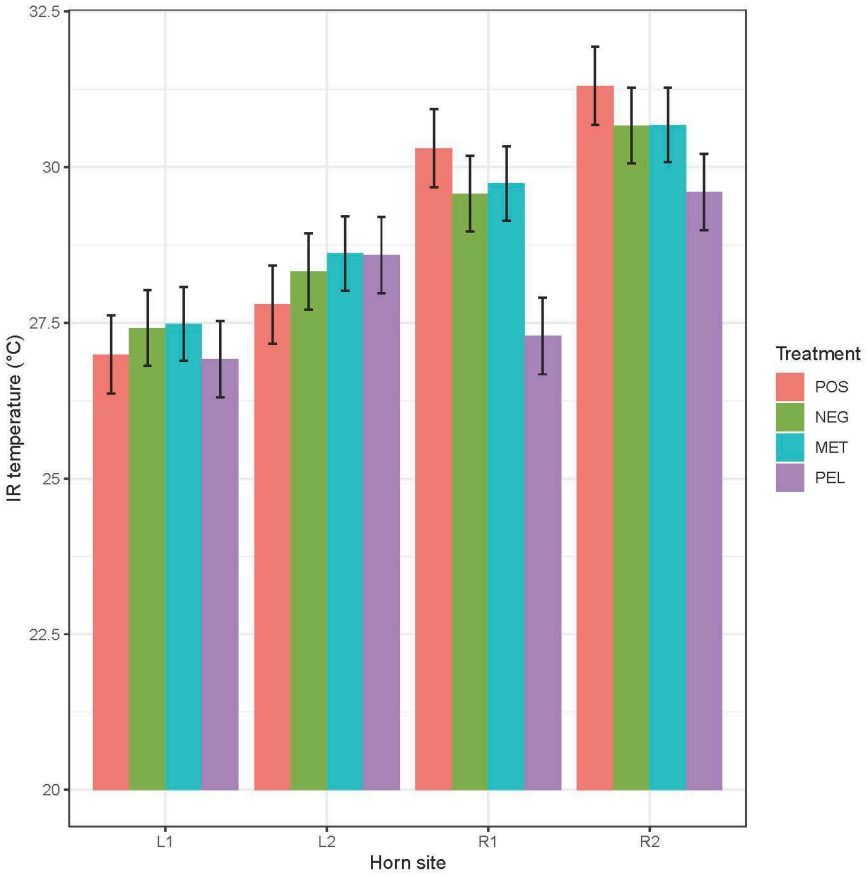


Table 44 shows the model-based mean temperatures with standard errors for the Day x Site combination.

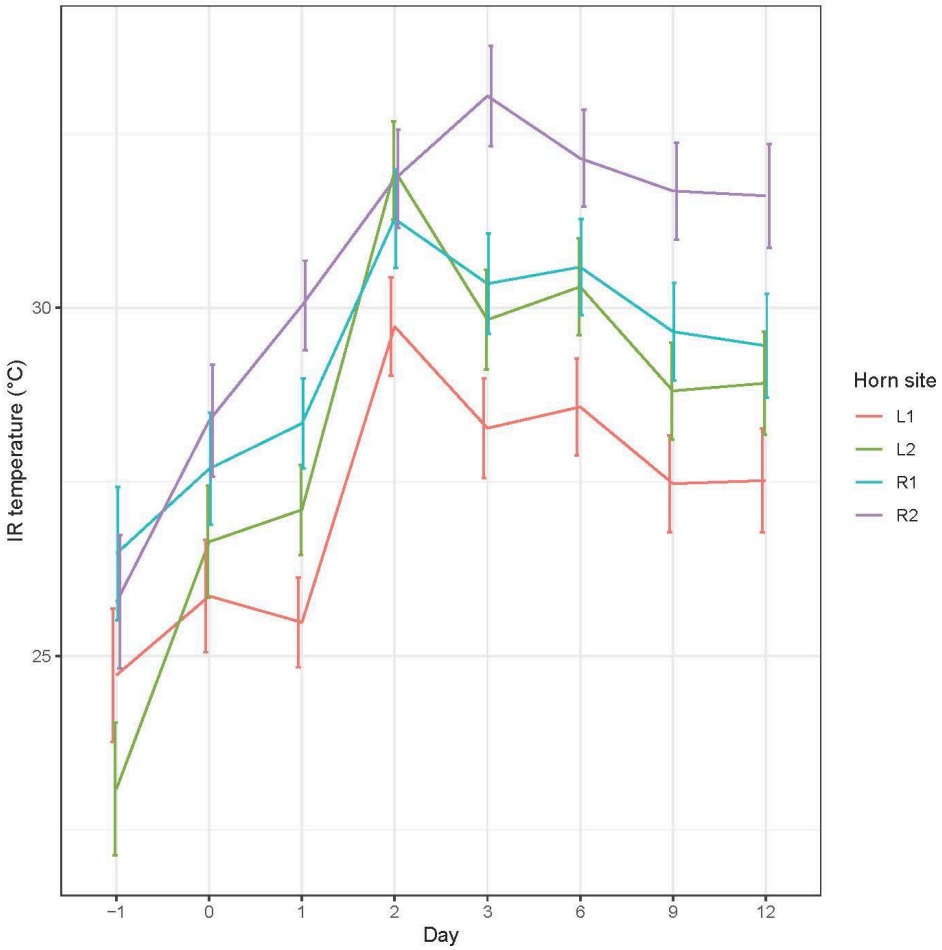
Table 44: Model-based means of horn site temperature (°C) (±SE) for across experimental days for left and right horn sites 1 and 2. Sites sharing a similar superscript (a,b,c) are not significantly different.

Day	Site	Mean	SE	Group
-1	L1	24.7	0.96	a
	L2	23.1	0.96	b
	R1	26.5	0.96	c
	R2	25.8	0.96	ac
0	L1	25.9	0.81	a
	L2	26.6	0.81	a
	R1	27.7	0.81	b

Day	Site	Mean	SE	Group
	R2	28.4	0.81	b
1	L1	25.5	0.65	a
	L2	27.1	0.65	b
	R1	28.3	0.65	c
	R2	30.0	0.65	d
2	L1	29.7	0.71	a
	L2	32.0	0.71	b
	R1	31.3	0.71	b
	R2	31.8	0.71	b
3	L1	28.3	0.72	a
	L2	29.8	0.72	b
	R1	30.3	0.72	b
	R2	33.0	0.72	c
6	L1	28.6	0.69	a
	L2	30.3	0.69	b
	R1	30.6	0.69	b
	R2	32.1	0.69	c
9	L1	27.5	0.70	a
	L2	28.8	0.70	b
	R1	29.7	0.70	b
	R2	31.7	0.70	c
12	L1	27.5	0.74	a
	L2	28.9	0.74	b
	R1	29.5	0.74	b
	R2	31.6	0.74	c

As the experiment progressed, left horn site 1 tended to have a lower temperature, whereas right horn site 2 had a higher temperature. Figure 31 is a plot of means, the error bars represent ± 1 SE.

Figure 31: Model-based mean temperatures (°C) ±1 SE for left and right horn sites 1 and 2 across experimental days.



4.8.8 Association between mechanical nociceptive threshold and behaviours

Table 45 shows the association between each behaviour and log-transformed algometer force, expressed as regression coefficients and standard errors, and their significance levels. None of the variables reached threshold significance (all P > 0.05). Head rubbing (HR) had a marginal non-significant (P = 0.078) negative association with algometer force (b = -1.39 ± 0.74) implying the presence of this behaviour is likely associated with increased pain response. Similarly eating (E) had a marginal non-significant association (P = 0.078). Its positive regression coefficient (0.486 ± 0.274) indicates that the presence of this behaviour is associated with higher algometer force, and therefore its absence is likely associated with increased pain. When these two variables along with drinking (D), backwards elimination failed to leaver any variable in the model with P < 0.05.

Table 45: Association between each behaviour (EF = ear flicking; HS = head shaking; HR = head rubbing; TF = tail flicking; FS = foot stamping; G = grooming; D = drinking; E = eating; L = locomotion; NS = normal standing; NL = normal lying; HHI = head to head interaction; HBI = head to body interaction; I = isolation) and log-transformed algometer force.

Behaviour	*b*	SE	*P*-value

EF	-0.046	0.249	0.854
HS	0.240	0.371	0.518
HR	**-1.386**	**0.737**	**0.061**
TF	0.086	0.215	0.690
FS	-0.391	0.529	0.460
G	-0.130	0.442	0.768
D	**-1.379**	**0.929**	**0.139**
E	**0.486**	**0.274**	**0.078**
L	0.253	0.398	0.526
NS	-0.150	0.226	0.506
NL	0.231	0.231	0.317
HHI	1.036	0.894	0.248
HBI	0.141	0.870	0.872
I	-0.467	0.570	0.413

4.9 Investigation of farmer perceptions of and attitude toward pain in cattle and sheep industries

4.9.1 Sociodemographic factors

Information regarding farmer age, gender, education and degree of experience working with livestock was collected. Most farmers who participated in this study were aged between 56-65 (23.94%). The gender of participants in this study was also found to be skewed predominantly towards males (60.56%) compared to females (39.44%). There were significantly more farmers educated regarding animal welfare and pain relief notions (67.61%) included in this study compared to those who were not (23.94%). Amongst the farmers who participated in this study 46.48% completed a university degree with part of their study encompassing animal welfare and management concepts. When farmers were asked to identify the degree of experience working with livestock, the largest group farmers identified as was the 25+ years of experience working with livestock (46.48%).

4.9.2 Belief and attitude toward livestock

Farmer belief and attitudes towards livestock were examined. From the results obtained most farmers perceived their ability to recognise pain as very good (38.02%) and excellent (25.35%). When farmers were asked if they believe pain relief should be administered most farmers agreed with this notion (86.11%). Farmer agreeance towards the use of pain relief could be linked to the degree of interactions they have with their herd/flock. From the responses obtained in this study most farmers reported the

degree of human-animal interactions was medium (42.25%). That is, livestock are monitored by workers on a herd/flock level somewhat regularly approximately once weekly on average. However, there is limited close contact between livestock and workers. Farmer views on animal ethics were hypothesized to influence farmer perception of pain relief importance. In the study most participants identified themselves as utilitarian (67.61%).

4.9.3 Overview of enterprise

The farm enterprises were examined based on the type of system, livestock kept on the farm, herd/flock size and number of employees. In this study it was found that 59.15% of farmers included in this study conducted solely livestock production systems. When the type of livestock enterprise was further examined it became apparent that beef (25.71%) and veal (25.14%) were the most common.

The herd/flock size and number of employees were examined in this project to determine if they significantly influence farmer ability to recognise pain in their herd/flock and farmer beliefs towards pain relief administration. These factors were quite variable overall, and farmers were asked to provide a numerical value instead of being given a range. From the 71 responses obtained in this study the average herd/flock size was 13 579 and the average employee size was 12.

4.9.4 Perception and attitude toward pain

In this study it was found that most farmers showed support for the use of pain relief and a lack of similarity to the responses obtained in published studies. Cost was a common limiting factor identified in multiple studies including Ison and Rutherford (2014). When this factor was examined as a potential barrier to pain relief administration rates on Australian farms it was found through the responses obtained that 71.43% disagreed with this notion. This may have been due to 42.25% of farmers agreeing market access and 57.47% agreeing farm productivity was improved following pain relief administration. When these factors were examined in accordance to each other no statistical significance was identified. Despite the lack of statistical significance identified between these factors there was unanimous support for the benefits of pain relief outweighing assumed barriers examined in this study including labour, time and training requirements and cost.

4.9.5 Impact of veterinary and professional services on farms

The influence of veterinarian and contractor services on farm procedures was examined in this study to determine how farmers perceive the importance of these services. The results in this study highlight that of the 57 farmers who employ veterinary or contractor services 33 agreed their beliefs and attitudes were significantly influenced by their recommendations.

In general farmers indicated that castration is the most common condition/procedure when pain relief is administered on-farms. When the form of pain relief commonly administered was examined it was evident that the majority opted for the application of a topical anaesthetic (42.67%). Topical anaesthetic may be more commonly employed on-farms as it is cheaper, requires less training for administration and reduced risks of handler injury (for example needle injury) (Van der Saag et. al, 2018).

4.9.6 Farmer beliefs regarding animal pain

This study showed farmers perceptions of the most painful procedure/condition experienced by cattle was ear tagging, notching and natural calving, but for sheep it was mulesing. The least painful procedure/condition experienced by cattle was unanimously identified as artificial insemination and for sheep it was ear tagging and notching.

When farmers were asked to indicate how much pain humans, cattle, sheep, dogs and cats are capable of experiencing it was found that the majority indicated severe pain.

5. Conclusion

A large focus of this project was to evaluate the pain of husbandry procedures in a commercial context. Practicality and commercial adoption of pain relief were the focus of investigations into novel pain alleviation strategies. Animal pain is challenging to assess, with no direct measure and multiple limitations associated with the numerous indirect measures. Likewise, pain in extensively managed livestock is difficult to ameliorate. An important finding from this project regarding pain assessment is that within a commercial context, remote and continuous measurement of animal behaviour is a useful tool for evaluating welfare following husbandry procedures. An important finding from this project regarding practical pain amelioration is that the duration of analgesic action can potentially be extended through novel formulations or delivery methods of an existing drug. Specific key findings from this project are outlined below.

5.1 Key findings

This project highlights the impact of aversive husbandry procedures on animal welfare, supporting previous research findings. Furthermore, results of the producer survey demonstrated that most producers support the use of pain relief for husbandry procedures. Providing producers with a choice of cost-effective forms of pain relief should be prioritised in future research.

5.1.1 Pain Assessment

- Physiological biomarkers (IL-1β, IL-10, SP, TNF- α), facial 'grimace' scores and ear-tag based accelerometer behaviour profiles were developed and investigated throughout this project as 'novel' measures of pain. These measures were analysed over various periods of time following husbandry procedures in sheep and cattle. The studies conducted throughout this project demonstrated that:
    - There were some trends for facial features to change in response to husbandry procedures. However, overall, facial 'grimace' scores did not appear to be useful measures of pain. Further, the level of agreement on scores assigned to facial features was not consistent between various observers, suggesting that this method of assessment was somewhat unreliable. It is likely that restraint of animals affected facial expression.
    - Changes in most individual biomarkers examined in this project did not appear to reflect pain.
    - In linkage with P.PSH.0819, the experiment *Determining current 'best practice' pain mitigation for castration and dehorning of cattle* was the first to report remotely and

continuously recorded behavioural data across 35 days following dehorning and castration of cattle using ear-tag based accelerometers (Allflex eSense). Importantly, this study was a large-scale experiment conducted under commercial settings. The continuous behaviour profiles generated using the ear-tag based accelerometers were investigated as a 'novel' means of assessing pain. The behaviour profiles generated from this technology appeared to reflect animal pain and welfare states over both acute and longer-term periods. It is likely that the significant amount of data obtained through regular sampling and large sample sizes contributed to the apparent success of this method for assessing pain and welfare. The study demonstrated the long-term impacts of amputation dehorning and the limited efficacy of commercially available pain relief options for this procedure. This highlights the need for further work investigating longer term pain and wound management options, and the potential for remote sensing technologies to provide objective measures of pain across time. Further details are reported in P.PSH.0819 'Objective measures of welfare' milestone 7 report.

- Some routine variables for measurement of animal pain (behaviour, mechanical nociceptive threshold, wound temperature, wound morphology and average daily gain) were investigated throughout this project. These measures were analysed over various periods of time following husbandry procedures in sheep and cattle. The studies conducted throughout this project demonstrated that:
    - Individual behaviours related to the presence and absence of pain appear indicative of pain states. However, their frequency when observed instantaneously is often low, making it difficult to delineate smaller differences between treatments. Therefore, a common approach for analysis of behaviours is to group categories of behaviours. This project used a statistical approach to do this which demonstrated some usefulness for assessment of pain.
    - Continuous, remote measurement of lying behaviour using accelerometers has previously been demonstrated as a useful means of measuring postural changes related to pain. However, this is variable, and in this project, there were no clear trends relating to pain. As mentioned above, the novel ear-tag based accelerometers appear to fill these gaps in behavioural information.
    - Mechanical nociceptive threshold, as obtained using a pressure algometer, appears to be a useful, objective measure for assessment of local pain. However, in this project, the validity of this measure for assessment of analgesic efficacy was unclear.
    - Wound temperature and morphology appear to be useful measures for assessment of pain, as indirectly inferred from their relationship with inflammation. These outcomes are generally practical to collate and analyse. However, the indirectness of the relationship between these measures and pain should be considered.
    - Average daily gain appears to be a useful measure for assessment of pain in a commercial context and provides an industry relevant quantifiable outcome. As other physiological measures of pain, the indirectness of the relationship between this measure and pain should be considered. In addition, large sample sizes are required to account for a large degree of individual variability.

- Overall, this project reinforces the need for multiple and varied measures of pain. There has not yet been a single measure identified that appears to be direct, objective and discrete, especially when the aim is to determine differences between surgical procedural methods and pain relief therapies. Statistical methods for demonstrating some pain measurement variables to be predictors of other pain measurement variables could be an option for selection of most appropriate variables for measurement in future research studies.
- Despite variable results, based on the extensive research conducted in this field, husbandry procedures cause long lasting pain and welfare implications. Improved assessment of pain relief efficacy is still needed.

5.1.2 Pain amelioration

- This project has added to the knowledge base on the duration of pain following husbandry procedures and the adequateness of analgesic options regarding this. In summary:
  - It is difficult to draw conclusions on 'best practice' regarding the various methods of husbandry methods. However, this project demonstrates that the inflammatory and healing process is ongoing for several weeks, particularly for methods utilising rubber rings, and resulting in additional welfare impacts in some instances, such as wound infection.
  - Current analgesic options do not appear to have any longer-term animal welfare or production benefits.
  - As outlined above, the large-scale experiment, *Determining current 'best practice' pain mitigation for castration and dehorning of cattle,* conducted in linkage with P.PSH.0819, demonstrates the duration of impact that amputation dehorning of weaner cattle has on core behaviours and production outcomes.
- This project has proved the following concepts in sheep and cattle:
  - The half-life of meloxicam is extended via a slow-release injectable formulation of the drug.
  - Multiple doses of meloxicam can be successfully delivered via ingestion of medicated feed.
- The efficacy of these novel analgesic therapies remains unclear. However, this project has demonstrated the feasibility of delivering longer lasting analgesia at a presumed 'therapeutic' dose which could vastly improve animal welfare.

5.2 Benefits to industry

Pain has long been a challenge for the red meat industry, with various contributing complexities associated with measuring and mitigating pain in sheep and cattle. This broad research program addressed a spectrum of challenges and highlights opportunities for R,D and E into the future. The project insights lead to some key implications for the red meat industry regarding our understanding of the impacts of husbandry procedures and the suggested pathways forward. It is important that informed technical advice on current options for pain relief continues to be disseminated to producers for correct and effective use of available products, whilst recognising the limitations of these options for older animals, and specific procedures and procedural methods. This project emphasises that the impacts on animal welfare following husbandry procedures go beyond acute pain. Hence, the recommendations for future research and development described in section 6 propose opportunities

for continued improvement to pain relief therapies and holistic approaches for managing animal welfare and production impacts following husbandry procedures.

6. Future research and recommendations

This project addressed multiple aspects of the animal welfare issue of pain, that remains a challenging priority area for the red meat industry. Overall, this project provides a perceptive overview of the various areas that could be progressed, with project insights leading to some valuable recommendations for focused future research and development on pain in sheep and cattle.

6.1 Pain assessment

This project investigated practical, cost-effective methods for pain assessment. However, the study findings demonstrate that pain assessment in sheep and cattle remains a challenge, particularly for evaluating and comparing the effectiveness of pain relief therapies. It is suggested that discovery and development of accurate and objective measures of pain, requires focused research input to improved methods that move beyond preliminary analyses and that utilise advanced and emerging technology in both the laboratory and the field.

The study conducted in conjunction with P.PSH.0819 'Objective measures of welfare', demonstrates the potential for remote monitoring of behavioural changes via ear-tag based accelerometers to be a robust method for assessment of pain. Future research should consider the use of this form of technology as a feasible and non-invasive means of assessing animal pain in real time and continuously. This one study allowed the assessment of animal pain over a long duration of time and with a large sample size. On that point, it is worth conducting more of these types of studies for monitoring behaviour of large numbers of animals. Larger sample sizes may account for probable significant individual variation that is contributing to the limitations in understanding pain and analgesic efficacy in livestock.

The studies in this project have highlighted some limitations associated with preliminary analysis of individual biomarkers using traditional immunoassay techniques. It is recommended that the use of advancing technology, such as mass spectrometry, and multiplex assays, is utilised for future research and development on pain biomarkers, as it allows for the simultaneous detection and quantification of a broad range of biomarkers, and their inter-related changes, offering the potential for an enhanced understanding of responses to pain.

The studies in this project have highlighted some limitations to assessing facial 'grimace' scores, particularly under experimental conditions where animals are restrained. Remote, automated detection and analysis of facial expression is suggested as a means of overcoming some of the limitations highlighted in this project. Additionally, automating the process may allow for practical application on-farm for detection of pain.

Lastly, engaging high level statistical expertise in animal pain assessment studies should be considered as important in future research and development. Novel statistical approaches to study design and data analysis could simplify the experimental process and generate models that can better discriminate between degrees of pain.

6.2 Pain amelioration

This project investigated practical methods for improving pain relief in sheep and cattle, with a focus on extending analgesic duration of action for longer lasting efficacy. The project findings demonstrate long lasting impacts on animal welfare following invasive husbandry procedures, that are not adequately addressed with current pain relief options. The issue of pain is one alongside haemorrhage, infection, inflammation, prolonged healing and weight loss, with some procedures and procedural methods implicated in the severity of animal welfare outcomes and the effectiveness of current pain relief therapies. It is recommended that more large-scale studies conducted in commercial farm settings are completed, perhaps through utilisation of Producer Demonstration Sites (PDS), for evaluation of analgesic efficacy in a range of animal ages and production contexts. It is recommended that future research and development activities are holistically focused on animal pain and welfare throughout the entire healing process following husbandry procedures. The project proved the concepts of administering meloxicam as a 'once-off' slow-release injectable formulation and through daily medicated feed for prolonged systemic concentrations at a presumed 'therapeutic' level. It is recommended that future research and development of these concepts involves strategic partnerships and study design to ensure an efficient pathway to adoption of successful products. A stepwise methodology involving efficacy, safety and residue studies, with expert regulatory input, should therefore be considered for future research and development of such concepts.

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8. Appendix

8.1 Metadata Storage

All metadata associated with this project is digitally stored by the University of Sydney on a password protected drive.