final report

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Development of a self-medication methodology for pain relief in sheep and cattle

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Abstract

As consumer concern for animal welfare is increasing there is growing interest in the use of analgesic agents to control pain associated with invasive husbandry procedures. There are several factors that can limit sheep and cattle producers' use of analgesics, including registration issues that affect access to drugs and ease of administration of analgesic agents. Non-steroidal anti-inflammatory drugs (NSAIDs) are attractive candidate drugs for pain relief in sheep and cattle as there is a large literature on their efficacy in these species, they are relatively safe, they are cheap, and they are active when administered by a range of routes including orally. There is potential in providing pain relief through feed, furthermore, if we can teach livestock to self-select and self-administer feed containing analgesics, it can provide us with an insight into animal pain states.

The project "Development of a self-medication methodology for pain relief in sheep and cattle" was part of a PhD project which was conducted by the University of New England student, Danila Marini. Due to feasibility and time constraints the self-medication methodology was not able to be tested on cattle, therefore the report will focus on research conducted with sheep only. It was also identified early on that the original experimental plan to achieve the objectives were not achievable and a revised experimental plan was recommended in 2013.

The experiments conducted for this project identified flunixin meglumine as an effective pain relief for sheep and lambs when administered either as a drench or through feed. A method to teach lambs to self-medicate after undergoing castration and tail-docking was also developed and implemented.

Executive summary

The pain associated with most painful husbandry procedures in sheep may last for several days and the effectiveness of analgesics used at the time the procedure is performed is limited by their half-lives. Repeated administration of medications by humans over successive days to livestock experiencing pain associated with husbandry procedures is generally considered not feasible within conventional livestock management practices. If sheep can readily learn to self-medicate on analgesic agents it could provide an opportunity for animals to provide themselves with extended pain relief by repeated self-dosing.

This project identified flunixin, a non-steroidal anti-inflammatory drug (NSAID), as effective at alleviating pain in sheep and lambs when it is administered orally. Flunixin was then tested for palatability and pharmacokinetics for administration through feed, this was required to:

- Identify if sheep may have an aversion to flunixin;
- Determine how long it would take for sheep to obtain levels of flunixin in their blood that would be sufficient to provide pain relief.

Following this flunixin was tested for efficacy at alleviating the pain of surgical castration and tail-docking in lambs when administered through pellets.

The final experiment was an ethological study examining whether we can understand more about pain through a lamb's preference for medicated feed. This was done through the development of a method that would allow lambs to learn the benefit of flunixin at alleviating pain and for lambs to demonstrate self-medication. In order to do this, a pilot study was conducted to develop a model of pain that would provide lambs with a sufficient timeframe to be able to learn to self-medicate. The pilot study involved ring castrating and ring tail-docking lambs on separate days to see if it would be a suitable model of chronic pain, allowing lambs sufficient time to learn the effects of flunixin.

Inducing lameness and testing the efficacy of oral use of NSAIDs

The objective of this experiment was to test the efficacy of three NSAIDs, carprofen, ketoprofen and flunixin at alleviating pain associated with an oil of turpentine injection. The model of pain used in this study was previously designed by Colditz et al. (2011).

The experiment was a randomised design with four treatment groups, three given NSAIDs and one control group (n = 10/group). Treatment was given as an oral drench by syringe at 24 h intervals for 6 days at dose rates that were expected to achieve therapeutic concentrations in sheep: carprofen (8.0 mg/kg), ketoprofen (8.0 mg/kg) and flunixin (4.0 mg/kg). Oil of turpentine (0.1 mL) was injected into a forelimb of each sheep to induce inflammation and pain; responses (force plate pressure, skin temperature, limb circumference, haematology and plasma cortisol) were measured at 0, 3, 6, 9, 12, 24, 36, 48, 72, 96 h post-injection. NSAID concentrations were determined by using ultra-high-pressure liquid chromatography, negative electrospray ionisation and tandem mass spectrometry.

The NSAIDs were detectable in sheep plasma 2 h after oral administration, with average concentrations between 4.5 - 8.4µg/mL for ketoprofen, 2.6 - 4.1µg/mL for flunixin and 30 - 80µg/mL for carprofen. NSAID concentrations dropped 24 h after administration. Pain response to an oil of turpentine injection was assessed using the measures applied but did not see any effect of the NSAIDs. Although this pain model has been previously validated, the responses observed in this study differed from the previous study.

The 3 NSAIDs reached inferred therapeutic concentrations in blood, 2 h after oral administration. Although there was an inconsistent from the sheep that received no pain relief, flunixin was identified as the most appropriate NSAID to use for further studies. This experiment was published in the Australian Veterinary Journal (**93** (2015) p. 265-270) and is presented in Appendix 1.

Testing the efficacy of administering flunixin through feed and voluntary consumption test (Palatability and Pharmacokinetics)

Applying analgesics to feed is a potentially easy method of providing pain-relief to sheep and lambs that undergo painful husbandry procedures. To be effective, the medicated feed needs to be readily accepted by sheep and its consumption needs to result in therapeutic concentrations of the drug. In this experiment pelleted feed was supplemented with flunixin (4.0 mg/kg live weight) and offered to eight sheep. To test the palatability of flunixin, the individually penned sheep were offered normal feed and feed supplemented with flunixin in separate troughs for two consecutive days. A trend for a day by feed-type (control versus flunixin supplemented) interaction suggested that sheep may have had an initial mild aversion to pellets supplemented with flunixin on the first day of exposure, however, by on the second day there was no difference in consumption of normal feed and feed supplemented with flunixin.

To test pharmacokinetics, sheep were offered 800 g of flunixin supplemented feed for a 12 h period. Blood samples were taken over 48 h and plasma drug concentrations were determined using ultra-high-pressure liquid chromatography, negative electrospray ionisation and tandem mass spectrometry. The mean ± S.D. time required to reach maximum concentration was 6.00 ± 4.14 h and ranged from 1 to 12 h. Average maximum plasma concentration was 1.78 ± 0.48 µg/mL and ranged from 1.61 to 2.80 µg/mL. The average half-life of flunixin was 7.95 ± 0.77 h and there was a mean residence time of 13.62 ± 1.17 h.
Free access to flunixin supplemented feed enabled all sheep to obtain inferred therapeutic concentrations of flunixin in plasma within 6 h of starting to consume the feed. Provision of an analgesic in feed may be an alternative practical method for providing pain relief to sheep. This experiment was published in PeerJ (14 March 2016, DOI 10.7717/peerj.1800) and is presented in Appendix 2.

Evaluation of the efficacy of flunixin at alleviating pain in a castration and tail docking

It can be difficult for farmers to provide pain relief to livestock following the application of painful husbandry procedures such as castration and tail-docking. An analgesic incorporated into feed could provide an easy method of giving livestock access to pain relief.

To test the efficacy of this method, sixty-four, singleton, male Merino lambs were randomly allocated to one of four treatment groups. The groups were: sham control (S), castrated + tail-docked + no pain relief (C), castrated + tail-docked + flunixin in feed (4.0 mg/kg, CF) and castrated + tail-docked + flunixin injection (2 mg/kg, CI). Haematology, cortisol, plasma haptoglobin, were measured before and up to 48 h after treatment. Behaviours were recorded by video for 12 h after treatment. Lambs in the CF and CI groups displayed less active pain avoidance behaviours than C lambs in the first hour following treatment. CF and CI lambs also displayed less pain related postures in the 12 h following treatments. All lambs that were castrated and tail-docked had an increase in cortisol 30 min after treatment, however lambs in the CI group had reduced concentrations by 6 h and CF buy 12 h compared to C lambs. Pain relief also reduced inflammation, with CF and CI lambs having lower neutrophil/lymphocyte ratios and lower average wound scores than C lambs.

Provision of flunixin in feed was as effective the flunixin injection, both methods of analgesic administration provided partial analgesia for surgical castration and hot-knife tail-docking.

Investigation of a model of pain to allow lambs to learn to self-medicate

Lambs were monitored for up to 35 days after ring castration and tail-docking to assess chronic pain that may be associated with this procedure. Ring castration and ring tail-docking were performed on separate days to produce two events rather than one, so that the pain effect is more similar to chronic pain. The objective of this study was to establish a chronic pain model through extending the duration of pain with the application of rings for castration and tail docking on separate days to be used for a self-medication experiment.

Overall lambs that were castrated and tail-docked didn't show a significant amount of observable signs of pain (pain related behaviour and postures) after the treatment day but they still exhibited discomfort of the wound site up to 35 days following the procedure. Ring castration and tail-docking can be a suitable chronic pain model for use in the self-medication trail. Lambs that were tail-docked 3 days after castration, again displayed pain related behaviour on that day of treatment, indicating that a secondary acute pain can be achieved.

Can lambs indicate their experience of pain through a preference for medicated feed?

The final experiment explored the potential to train lambs to self-administer flunixin to provide pain relief over a period of several days. If sheep can learn to self-medicate, their voluntary choice to ingest medications that are non-addictive is a strong indicator that the animal feels unwell and is motivated to alleviate that negative affective state. Therefore development of a test procedure to examine the choice of sheep to self-medicate would also provide a valuable indicator of affective state in these animals and help us better understand pain and its impacts in livestock.

The model of chronic pain used was ring castration and tail-docking. Rings are commonly used for castration and tail-docking and are shown to cause an acute pain lasting 4 h as well as causing chronic pain. The objectives of this study was to castrate lambs, and offer them medicated feed during a training period. The lambs were then tail-docked a week later and offered medicated and non-medicated feed again to see if the lamb would have a preference, if lambs have learnt to associate the odour they were trained on with flunixin, they should have a preference for it. During this experiment lambs were monitored for feed consumption and for pain behaviours.

Conclusions

The experiments conducted throughout this project identified flunixin as an effective drug for providing pain relief and ameliorating the inflammatory response (reduced neutrophil/lymphocyte ratios and reduced wound swelling and improved wound appearance) to lambs when administered through feed. Flunixin consumed with pellets is rapidly absorbed into sheep plasma and therapeutic concentrations can be reached within a few hours of ingestion. Sheep had no aversion to consuming medicated feed. Lambs receiving flunixin in feed exhibited fewer pain related behaviours and reduced abnormal postures following marking than lambs with access to un-medicated feed. A preference for lambs to consume medicated feed rather than un-medicated feed when in pain could not be demonstrated.

Key Findings

- For practical provision of pain relief to lambs for painful husbandry procedures such as marking flunixin can be administered as a drench or incorporated into feed.
- Oral or in-feed administration of flunixin 1-2 h prior to painful husbandry procedures would ensure that lambs have therapeutic concentrations in their blood at the time the procedures are performed.
- Medicated feed could be offered before and on the days following marking to provide pain relief for the duration of the pain responses to marking with minimal disturbance to the animals.
- The need to train lambs to accept pellets by feeding ewes and lambs with pellets prior to marking needs to be considered.
- There is potential for flunixin to be incorporated into pellets in its powdered form.
- As a generic drug, flunixin could be taken to market by more than one pharmaceutic company or feed manufacturer. Registration issues for infeed delivery of flunixin have not been addressed in this project.

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1. Background

As consumer concern for animal welfare is increasing there is growing interest in the need for use of analgesic agents to control pain associated with invasive husbandry procedures. There are several factors that can limit sheep and cattle producers' use of analgesics, including registration issues that affect access to drugs and ease of administration of analgesic agents. Non-steroidal anti-inflammatory drugs (NSAIDs) are attractive candidate drugs for pain relief in sheep as there is a large literature on their efficacy in these species, they are relatively safe, they are cheap, and they are active when administered by a range of routes including orally.

We decided to use the potent NSAID flunixin in the experiments for this PhD as flunixin is commonly used in veterinary medicine for its anti-inflammatory, analgesic and antipyretic properties. Like other NSAIDs, flunixin reduces inflammation by inhibiting cyclooxygenase and, in turn, decreasing the production of prostaglandins (Cheng et al., 1998). Flunixin has been shown to be effective at increasing the thresholds to noxious mechanical stimulation, in sheep suffering from footrot (Welsh, 1995). It has also been shown to reduce pain-related behaviours in lambs that have undergone mulesing (Paull et al. 2007). Flunixin has also been shown to be more potent as an analgesic than codeine in rats and has been shown to be a comparable analgesic to morphine in primates, however unlike these analgesics animals do not build a tolerance to the analgesic action of flunixin (Ciofalo et al. 1977). Currently flunixin is registered for use in other livestock in the USA, Europe and Australia. Flunixin is water soluble and is stable under normal temperatures and pressures, however the stability of flunixin has not been fully investigated. Flunixin has been previously manufactured into sheep pellets for a previous PhD (Rennie, 2004).

When pain relief is provided to livestock, it's provided at the time of injury and animals are usually not given a follow up dose as repeated administration of medications is generally considered not feasible within conventional livestock management practices. However only one dose may not provide sufficient pain relief for all animals, as like humans, an animal's sensitivity and experience of pain varies, with factors such as species (Stasiak et al., 2003), age, sex (Robertson et al., 1994; Guesgen et al., 2011) and size (Di Giminiani et al., 2013) having an effect. The effectiveness of analgesics used at the time the procedure is performed is also limited by their half-lives. Providing livestock with the opportunity to learn to self-medicate through food and water could address this need providing the animals with the opportunity to medicate according to the level of pain that they are experiencing and would allow them to return to homeostasis by consuming either feed or a liquid containing analgesic. Control by the animal over the amount of medication it consumes differentiates self-medication from the widespread practice of delivering medicines in formulated feeds for disease control and growth promotion.

2. Project objectives

The original objectives were to be completed through a PhD project, they were:

2.1. Develop methodology for self-selection and self-administration of analgesics to sheep and cattle.

First year using a pain model the capacity for sheep to be trained with the conditioned place preference paradigm and to learn to select medicated feed will be examined. Training methodologies will be refined

2.2. Apply methodology to lambs undergoing marking (castration, tail docking, tagging, and vaccination) to assess efficacy in alleviation of pain.

Second year of the project a cohort of trained lambs will be examined in a range of painful and stressful husbandry procedures to gain knowledge of the impact of these procedures on the affective state of the animals. Performance in the self-medication test protocol will be compared with established physiological and behavioural measures of pain.

2.3. Apply methodology to assess affective states in painful husbandry procedures and models of pain.

Activities in the third year of the project will be to conduct a field trial of the self-administration methodology under commercial farming conditions and a proof of principle trial that methods established in sheep can be adapted for use in cattle, together with completion of the thesis.

However we needed to understand which medication would the best at alleviating pain in sheep when administered orally. We also needed to know which would be the most effective way of administering the medications in a way that sheep would be able to self-medicate. It was recommended that the experiments that were to be conducted were revised, the suggested experiments conducted slightly changed the objectives to the following:

2.4. Objective 1: Determine an NSAID that is effective at alleviating pain in sheep when administered orally

Experiment 1: Inducing lameness and testing the efficacy of oral use of NSAIDs.

Aim: To evaluate the efficacy of non-steroidal anti-inflammatory drugs when administered orally in alleviation of pain and inflammation in a lameness model.

Method: Using the methods described in (Colditz, et al., 2011) lameness will be induced in sheep. During the experiment sheep will be individually penned. The NSAID of choice will be administered orally to treatment sheep; control sheep will not receive pain relief. Blood will also be taken and tested for drug concentration.

Experiment 2: Testing the efficacy of administering NSAID tested in experiment 1 through feed.

Aim: To evaluate if NSAID can be administered to sheep via feed

Method: Pain will not be induced in this experiment. Sheep will be individually penned during the experiment. Blood will be collected from the sheep to test for concentration of drug. Results of blood can be compared to experiment one to determine if sheep are ingesting an adequate dose that would be needed to relieve pain.

Experiment 3: Voluntary consumption test

Aim: To determine if sheep may have an aversion to NSIAD in feed.

Method: Sheep will be individually penned and offered both a feed containing the NSAID and a feed without. Troughs containing the feed with the NSAID will be alternated between sheep. Consumption of the feed will be measured. This experiment will test if the sheep may have an aversion to the feed containing NSAIDs.

2.5. Objective 2: Apply methodology to lambs undergoing marking to assess efficacy in alleviation of pain.

Experiment 4: A pen study where a pain model using knife castration and tail docking will be used and sheep will be offered feed with NSAID.

Aim: To test the efficacy of NSAID in feed to reduce pain associated with the painful husbandry procedures that are done during marking

Method: Using castration and tail docking, pain will be induced in sheep. During the experiment one group of lambs will have access to feed with NSAID.

2.6. Objective 3: Develop methodology for self-selection and self-administration of analgesics to lambs

Experiment 5: A field study to determine a chronic pain model using ring castration

Aim: To establish a chronic pain model through extending the duration of pain with the application of rings for castration and tail docking on separate days to be used for a self-medication experiment.

Methods: Lambs will either sham handled, castrated on day 0 or castrated on day 0 and tail-docked on day 3. Their behaviour, body weight and wounds were monitored for 35 days.

Experiment 6: Experiment with lambs undergoing marking

Aim: To evaluate if sheep can learn to consume feed containing NSAIDs to alleviate pain in a castration and tail docking model. Determine if lambs can indicate their experience of pain through a preference for medicated feed.

Methods: lambs will be exposed to grain/chaff with mothers so they can learn to consume it. At marking lambs will be placed into either a non-medicated or medicated group. NSAIDs will be mixed with the feed that lambs had previously been exposed to. Behaviours associated with pain will be recorded in the lambs.

Objective 1 has been completed and from the experiments two papers have been published. The publication for both experiment 1 (Australian Veterinary Journal; **93** (2015) p. 265-270) and experiment 2 (PeerJ; 14 March 2016, DOI 10.7717/peerj.1800) is presented in the appendix.

3. Objective 2: Apply methodology to lambs undergoing marking to assess efficacy in alleviation of pain

This Paper is currently being written for publication and is currently formatted to the Journal of Applied Animal Behaviour Science.

3.1. Introduction

Providing pain relief to lambs undergoing painful procedures, such as castration, tail-docking and mulesing is a welfare issue of increasing importance (Phillips et al., 2009). There has been extensive research into pain relief for sheep, with local anaesthetics such as lignocaine (Wood et al., 1991; Sutherland et al., 1999) and bupivacaine (Graham et al., 1997; Lomax et al., 2008; 2013), and non-steroidal anti-inflammatory drugs (NSAID), such as carprofen, flunixin (Paull et al., 2007; Paull et al., 2009a) and meloxicam (Paull et al., 2012; Small et al., 2014) shown to be effective at alleviating the pain associated with painful husbandry

procedures. Although NSAIDs are known to be effective, none are registered for use in sheep in the main sheep producing countries in the world. The only pain-relieving drug/substance that is registered for use in sheep in Australia is Tri-Solfen® which is currently restricted for use following mulesing. Tri-Solfen® has been shown to be effective at alleviating pain in lambs that have been surgically castrated and tail-docked (Lomax et al., 2010b) but it is ineffective against ring castration and tail-docking (Paull et al., 2009a), due to local anaesthetics having poor skin penetration.

Currently the lack of registered anaesthetics and analgesics is the biggest limitation in providing sheep with pain relief in Australia but ease of administration is also an obstacle for farmers, as anaesthetics and NSAIDs are commonly administered through injection and can require veterinarian involvement. An alternative option is buccal administration of analgesics which has been shown to be an effective method of providing pain relief to lambs (Small et al., 2014).

Another important consideration is the duration of pain relief provided by local anaesthetics and analgesics. Pain caused by castration and tail-docking lasts several days (Chapman et al., 1994; Melches et al., 2007b) whereas the effects of most local anaesthetics last only a few hours (Morishima et al., 1979; Mather et al., 1994) while NSAIDs have half-lives ranging between 3 and 30 h (Welsh et al., 1992a; Welsh et al., 1993). Therefore, in order to provide adequate pain relief to lambs, it is likely that repeated administration of drugs over several days would be required, which is logistically difficult when large numbers of animals managed in extensive systems are involved.

Administration of analgesics in feed is a potentially practical method for farmers to provide pain relief to lambs over several days. This method of drug administration has been previously explored with NSAIDs in chickens (Danbury et al., 2000) and cattle (Odensvik, 1995). We recently showed that sheep have no aversion to consumption of the NSAID flunixin when it is incorporated into a pelleted complete mixed ration and that inferred therapeutic levels in blood can be reached within 2 h of consumption of the medicated feed (Marini et al., 2016). Flunixin is a potent NSAID and has been shown to reduce temperature, signs of inflammation and improve behaviour in cows with mastitis (Anderson et al., 1986; Zimov et al., 2011). The aim of the current research was to test the analgesic, anti-inflammatory and anti-pyretic efficacy of a flunixin administered in a complete mixed ration pelleted feed following castration and tail-docking of lambs. We also compared the method with intramuscular injection of flunixin which is the current method of administration of this drug for most livestock. We hypothesized that administering flunixin in feed would be as effective at alleviating pain associated with castration and tail-docking as intramuscular injection.

3.2. Method and materials

The experiment was undertaken at CSIRO's FD McMaster Laboratory, Armidale, New South Wales (NSW), Australia. The protocol and conduct of the experiment was approved by The CSIRO Chiswick Animal Ethics Committee under the NSW Animal Research Act, 1985 (approval ARA 14/21).

Sixty-four, singleton, male Merino lambs aged between 4 and 10 weeks were used in this study. Lambs were ear tagged at birth and ewe-lamb pairs allocated to 4 cohorts, cohort being based on birthdate. Animals were group housed with their mothers and acclimatised to indoor housing for 2 weeks, during which time lambs were caught and handled once a day to reduce subsequent handling stress. After the acclimation period, lambs were randomly allocated to a treatment balanced for weight and moved with their mothers to smaller pens (4.4 m × 3.0 m). Each pen had four lambs of the same treatment group and for each cohort the treatments were rotated between pens. Lambs within a pen received the same treatment

to permit administration of flunixin in feed to all animals within the pen. Pervious research has indicated that grouping lambs by castration treatment within a pen, or mixing castration treatments within a pen does not affect the behavioural responses of lambs to castration in this experimental model (Colditz et al., 2012). Castration and tail-docking occurred outside the pen; during the procedure lambs were placed in a restraint cradle for castration and tail-docking which took approximately 1 minute to complete. The treatments were as follows:

1. Sham handling (S): The scrotum was handled to simulate surgical castration and the tail handled to simulate gas-knife tail-docking (for 1 minute)
2. Castration and Tail-docking (C): Knife castration was performed by cutting off the lower half of the scrotum with a knife then pulling out the testes with the aid of a hook on the knife. Tail docking was performed below the third palpable joint with a TePari knife (Scissor action LPG hot knife, TePari Products, New Zealand).
3. Flunixin in feed + Castration and Tail-docking (CF); Castration and tail-docking were performed as for C but lambs were provided with feed containing flunixin (4.0 mg/kg, Bayer, Australia) 24 h and 1 h before the procedure. The dose of flunixin was calculated based on the heaviest lamb in the group and 1.6kg of a standard pelleted ration was assumed to be consumed by each ewe-lamb pair. The feed was mixed with the required amount of liquid flunixin to allow an assumed intake of 4mg/kg bodyweight of the lamb.
4. Flunixin injected + Castrations and Tail-docking (CI); Castration and tail-docking were performed as for C but lambs were given an intramuscular injection of flunixin (2.0 mg/kg, Flunixon Injection, Norbrook, Victoria, Australia) 1 h before the procedure.

3.2.1. Behavioural observations

Behaviours were recorded for 12 h on the day of treatment using two cameras that were mounted at the end of each pen. The cameras were connected to digital video recorders and captured by Smartguard software (Pacific Communications, Australia). To identify the lambs, three lambs from each pen were marked on the left and right side of the body with a different coloured paint and one lamb left unmarked. Across pens, marks were randomly associated with treatments. Assessment of behaviour post-treatment was classified into pain avoidance behaviour and postural behaviour using The Observer XT software package (Noldus Ltd., The Netherlands). The pain avoidance behaviour assessment took place every 5 min for 1 min continuous monitoring during the first hour post castration and tail docking, or sham treatment. Postural behaviours were classified by scan sampling every 15 min for 12 h on the day of treatment and were summed within three periods of 4 h duration for analysis. Observation time points were synchronized to each lamb's individual treatment time. The active pain avoidance behaviours and postural behaviours used in this study (Table 1) were previously validated as indicators of pain in lambs following painful husbandry procedures (Lester et al., 1996; Molony et al., 2002; Paull et al., 2012).

Table 1: Ethogram used for behavioural observations of lambs receiving no pain relief or flunixin treatment after castration and tail docking and sham treated lambs

Behaviour	*Description*
Active pain avoidance	
Restlessness	Number of times lamb stood up and laid down
Kicking/foot stamping	Limb was lifted and forcefully placed on the ground while standing or used to kick
Rolling	Lamb rolled from lying on one side to the other without getting up
Jumping	Lamb moved forward using bunny hops with its hind limbs
Licking/biting wound site	Head moved beyond the shoulder, including looking and touching at wound
Restless hindquarters	Weight was shifted slowly between hindquarters, without walking
Easing Quarters	Abnormally lowers rear quarters or attempts to keep quarters off the ground
Pain behaviours	All active pain avoidance behaviours summed.
Postural behaviours	
Normal ventral lying	Lay on sternum with legs tucked in and head up or down
Abnormal ventral lying	Ventral lying with hind limbs partially or fully extended or keeping scrotal region off the ground (dog sitting).
Ventral lying other	Lamb was lying ventrally but unable to clearly categorise the lying posture.
Lateral lying	Lateral (on side) with one shoulder on ground, extension of hind limbs with head up or down.
Abnormal lying	Abnormal lying categories combined (abnormal ventral lying and lateral lying)
Total lying	All lying categories combined
Normal standing	Standing with no apparent abnormalities
Statue standing	Immobile standing with an obvious withdrawal from interaction with other pen members and outside stimuli. Legs positioned further back than normal. Can show arched back.
Abnormal standing	Standing hunched or unsteadily, often associated with foot stamping, kicking and tail wagging.
Standing other	Lamb was standing but unable to clearly categorise the standing posture.
Normal walking	Walking with no apparent abnormalities
Abnormal walking	Walking unsteadily or stiffly, includes walking backwards, on knees, moving forward with bunny hops, circling, leaning or falling.
Walking other	Lamb was walking but unable to clearly categorise the walking type.
Total Standing	All standing and walking categories combined (including all normal, abnormal and unknown)
Total abnormal behaviours	All abnormal posture categories combined (abnormal ventral lying, lateral lying, abnormal standing, statue standing and abnormal walking)

3.2.2. Blood sampling and cortisol analysis

Blood was collected by jugular venepuncture using 21 gauge needles into 4.5 mL vacutainers containing EDTA. Samples were collected prior to treatment (0 h) and 30 min, 6 h, 12 h, 24 h and 48 h post-treatment. Neutrophil and lymphocyte counts in whole blood were determined with an automated haematology analyser (Cell Dyn 3500R, Abbott Diagnostics, Illinois U.S.A). The blood samples were then centrifuged at 2000 × g for 15 min at 5^{o}C and plasma were separated into three aliquots which were then stored at -20^{o}C until assayed for haptoglobin and cortisol concentration. Plasma cortisol concentrations were determined using a commercial radioimmunoassay (Plasma Cortisol RIA, MP Biomedical, Australia) previously validated for use in sheep in our lab (Paull et al., 2007). Coefficients of variation on the quality control plasma samples (50.3, 101.1, 211.7 nmol/L cortisol) were 14.0, 10.6, 11.7% for intra-assay and 16.0, 8.1 and 7.3% for inter-assay.

3.2.3. Clinical observations

Tail and scrotal wounds were scored and temperature of the wound sites were recorded immediately after every blood sampling time-point. Tail and scrotal wounds were scored individually using a 4-point scale for appearance and swelling (Table 2). The wound temperature was measured using an infrared thermometer (ABW Industries, Australia) with a resolution of 0.1 ºC held 300 mm from the wound surface.

Table 2: Wound score descriptors, score increases when wound condition worsens and then decreases as the wound heals. Wound score is the total of swelling description and wound appearance

Swelling descriptor	Score	Wound appearance	Score
No swelling	1	Edges close together, dry scab	1
Slight swelling along wound edges (up to 5mm either side)	2	Small area (<1cm) wet and oozing, no visible pus	2
Large area swelling, but soft	3	Medium area wet and oozing (1-5cm); small amount pus	3
Large area hard swelling; pitting oedema (thumb impression can be made)	4	Large area wet (>5cm); necrotic; copious pus draining	4
Reducing hard swelling with loose cover (healing phase)	3	Granulation tissue forming, but still oozing (healing); watery exudate	3
Scarring or nodule (healed)	2	New skin evident, shiny, not oozing (healed)	2

3.3. Statistical analysis

All data were analysed using R (R Development Core Team, Boston, Massachusetts) and the packages *nlme* (Pinheiro et al. 2013), *pgirmess* (Giraudoux, 2016), *pscl* (Zeileis 2008) were used. Data were tested for normality using the Shapiro-Wilk test and visual inspection of residual plots and transformed where necessary. $P < 0.05$ was considered statistically significant and $0.1 > P > 0.05$ was considered a statistical tendency.

3.3.1. Behaviour analysis

Active pain avoidance behaviours were analysed using a general linear model using Poisson regression. Restless hindquarters required the use of zero inflated Poisson model due to a high amount of zeros in the data. Postural behaviours were analysed in the same way using a repeated measures model. The frequency of occurrence for individual postures was too low for data analysis, therefore categories had to be combined as outlined in Table 1.

3.3.2. Blood parameters

Cortisol concentrations and neutrophil, lymphocyte ratio were analysed using a repeated measures analysis to determine the relationship between treatment and time-point, fitting pre-treatment values (time 0) as a covariate and animal as a random effect. Cortisol data were log transformed for analysis. All data from animal 8031 were excluded as the base measurement at 0 h was 208.64 nmol/L which was inconsistent with other animals at time 0 and with other time measurements for that animal. The 48 h measurement (260.82 nmol/L) was also removed for animal 8091 as it was inconsistent with previous measurements.

3.3.3. Wound analysis

Wound temperature data was analysed using a repeated measures analysis of the relationship between treatment and time-point. Wound score data could not be normalised by transformation and were analysed using Kruskal-Wallis rank sum test to test the effect of treatment on wound score.

3.4. Results

3.4.1. Behaviour post-castration

Of the active pain avoidance behaviours, restless hindquarters was the only behaviour that had sufficient occurrences to allow for individual analysis. This behaviour was affected by treatment, with lambs in the C group exhibiting this behaviour more (1.29, Z = 9.80, P < 0.05) than CF (-0.49, Z = - 2.05, P = 0.04) and S (-1.49, Z = - 4.84, P <0.05) lambs. All other active pain avoidance behaviours had to be combined for analysis. Treatment had a significant effect (P < 0.001) and cohort tended to have an effect (P = 0.06) on the total number of pain avoidance behaviours displayed in the first hour following castration and tail-docking. Lambs in the S group showed significantly less pain avoidance behaviours (Z = 6.89, P < 0.05, mean = 1.50) than lambs in the other groups. Lambs treated with flunixin in both the CF and CI groups exhibited less pain avoidance behaviours (P < 0.05, mean = 3.06 and 3.75 respectively) compared with lambs receiving no pain relief (mean = 6.06).

Similar results were seen for the postural behaviours where there was an effect of time (P = 0.03) and treatment (P < 0.001) but no interaction. C lambs displayed significantly more abnormal postures than CF (Z = 6.89, P < 0.001), CI (Z = 6.89, P < 0.001) and S lambs (Z = 6.89, P < 0.001). All groups tended to display more abnormal behaviours (Z = 1.72, P = 0.085) at 8 h compared to 4 h post-treatment (Table 3).

Table 3: Postural behaviour values for (S) sham control, (C) castrated + tail-docked + no pain relief, (CF) castrated + tail-docked + flunixin in feed (4.0 mg/kg) and (CI) castrated + tail-docked + flunixin injection (2 mg/kg) in the three observation periods of 4 h duration following treatment. Mean ± SD, [A] Means with a superscript indicate they are significantly different ($P < 0.05$) to S lambs, within that observation time and behaviour category. [B] Means with a superscript indicate they are significantly different ($P < 0.05$) to C lambs, within that observation time and behaviour category.

Behaviours	0 – 4 h				4 – 8 h				8 – 12 h			
	S	C	CF	CI	S	C	CF	CI	S	C	CF	CI
Abnormal lying	1.94 ± 1.44	2.06 ± 2.24	2.63 ± 2.33	1.75 ± 1.95	3.69 ± 2.21	2.94 ± 2.46	3.63 ± 2.90	2.69 ± 2.27	1.81 ± 1.94	2.56 ± 2.73	2.94 ± 2.05	1.44 ± 1.93
Total lying	7.44 ± 2.2^{B}	5.19 ± 3.37^{A}	7.56 ± 2.71^{B}	5.25 ± 2.62^{A}	9.25 ± 2.14^{B}	4.50 ± 3.46^{A}	7.25 ± 2.54^{AB}	6.94 ± 2.43^{AB}	8.19 ± 2.26	4.69 ± 3.70	7.88 ± 3.76	8.94 ± 1.95
Total Standing	5.13 ± 1.96	8.94 ± 3.19	6.13 ± 2.68	8.25 ± 2.77	4.75 ± 1.77^{B}	9.00 ± 2.99^{A}	6.00 ± 2.90^{B}	6.81 ± 2.51^{AB}	6.19 ± 2.26	10.31 ± 3.63	6.00 ± 3.43	6.19 ± 2.10
Total abnormal behaviours	3.69 ± 3.40^{B}	6.94 ± 2.62^{A}	4.13 ± 2.55^{B}	4.25 ± 2.24^{A}	5.00 ± 3.31^{B}	7.44 ± 2.22^{A}	5.06 ± 2.69^{B}	4.25 ± 2.08^{B}	2.63 ± 1.82^{B}	6.88 ± 2.99^{A}	4.81 ± 2.46^{A}	3.38 ± 2.22^{B}

3.4.2. Blood parameters

There was a treatment effect ($F_{3, 57}$ = 21, P < 0.001) as well as a significant interaction of treatment by time ($F_{3, 303}$ = 5, P = 0.003) for cortisol. At 30 min following treatment, groups C, CF and CI all showed significant increases in plasma cortisol concentration of 10.73, 12.72 and 6.56 nmol/L respectively (df = 57, *P* < 0.05 for all) compared to 0 h before treatment. Treatment application had no effect on cortisol response of S lambs (increase of 1.05 nmol/L, *P* = 0.8106), and they had significantly lower cortisol concentrations (17.03 nmol/L) compared to C (t_{57} = 10.91, +10.06, P <0.001), CF (t_{57} = 10.88, +9.74, *P* <0.001) and CI (T = 9.75, +7.62, *P* <0.001) lambs 30 min after treatment application. Lambs in the CF group had lower cortisol concentrations than C lambs at 12 h (t_{57} = 2.17, 25.02 nmol/L vs 44.25 nmol/L, *P* = 0.009) and tended to have lower concentrations at 24 h (t_{57} = 1.76, 33.44 nmol/L vs 48.42 nmol/L, *P* = 0.084). CI lambs had lower cortisol concentrations than C lambs at 6 h (t_{57} = 3.16, 31.81 nmol/L vs 61.55 nmol/L, *P* = 0.002) but were not significantly different from CF lambs (t_{57} = 1.58, 44.25 nmol/L). Lambs in the C, CF and CI groups maintained higher concentrations of cortisol than S lambs until 48 h, where S lambs had a significantly increase concentration compared to their baseline (t_{287} = 3.24, 1.89 nmol/L, *P* = 0.0013, Figure 1).

A significant ($F_{15, 298}$ =13, *P* < 0.001) treatment by time effect was seen for neutrophil/lymphocyte ratio. Animals in the C, CF and CI groups had an increase in neutrophil/lymphocyte ratio at 6 h following castration, which was significantly higher than S animals (*P* < 0.001 for all groups, Figure 2). The administration of flunixin to lambs significantly reduced inflammation (*P* < 0.05) at 6, 12 and 24h following marking compared to lambs that had no pain-relief. Lambs given flunixin through feed had lower neutrophil/lymphocyte ratio than those injected with flunixin at 12 and 24 h (*P* < 0.05).

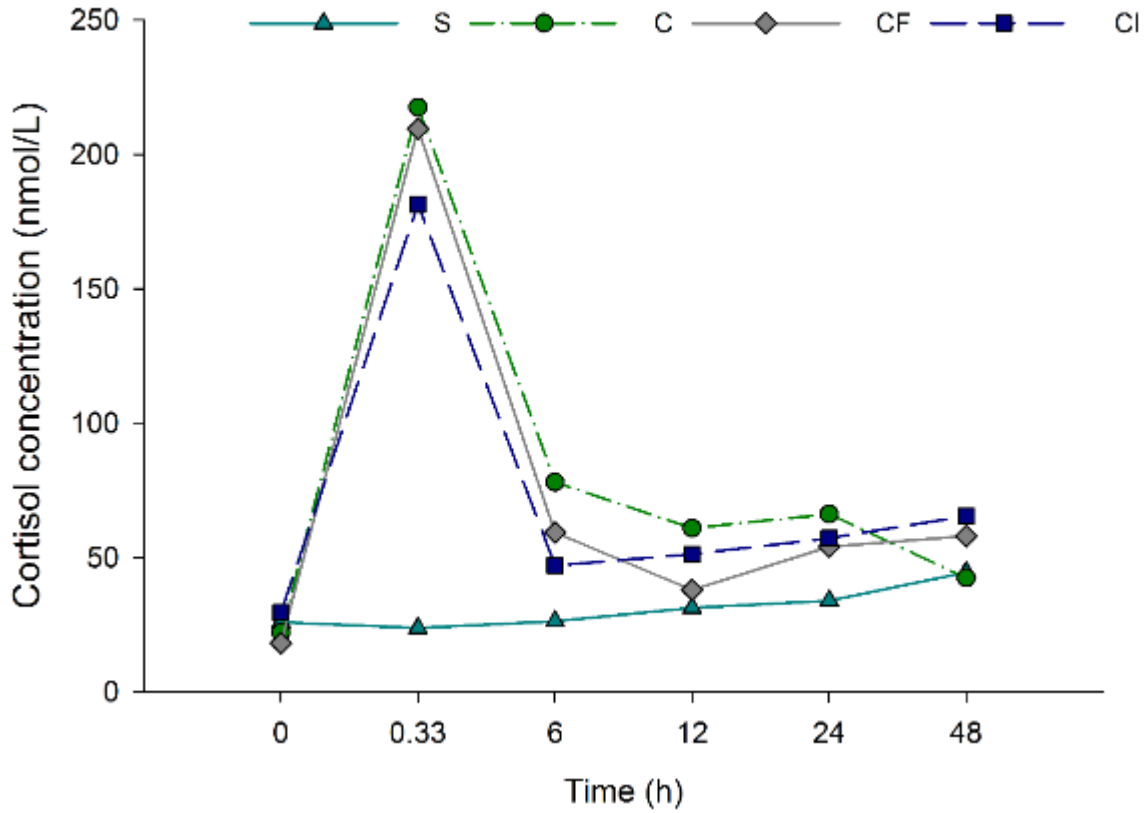

Figure 1: Raw data of the average plasma cortisol concentration for all treatment groups before castration and tail-docking or sham treatments and in the 48h period following. S (sham), C (castration and tail-docking no pain relief), CF (castration and tail- docking, flunixin in feed) and CI (castration and tail-docking, flunixin injected).

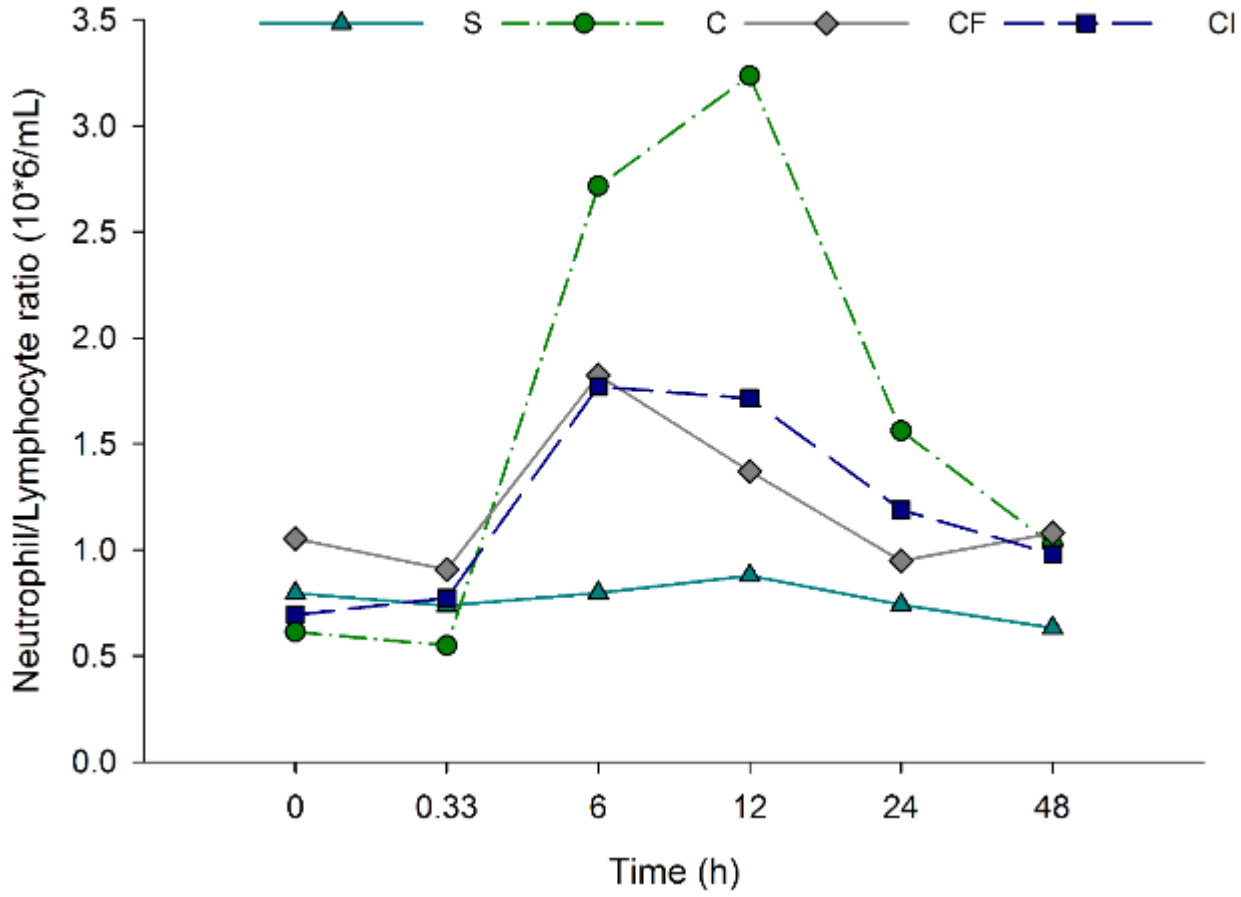

Figure 2: Raw data of the average neutrophil/lymphocyte ratio for all treatment groups before castration and tail-docking or sham treatments and in the 48h period following. S (sham), C (castration and tail-docking no pain relief), CF (castration and tail-docking, flunixin in feed) and CI (castration and tail-docking, flunixin injected).

3.4.3. Wounds

There was no treatment by time interaction and no treatment effect for tail and testes wound temperature but there was a time effect for testes wound temperature ($F_{4,\ 180}$ = 19, P < 0.001). All castrated groups had a significant increase in wound temperature at 6 h (3.53°C for C; 4.34°C for CF; and 5.39 °C for CI) but the increase did not differ between groups. At 12 h and 24 h lambs in the CI group testis wound temperatures were respectively higher (t_{180}= 2.8, +2.64 °C, P = 0.006 and t_{180}= 2.1, +1.96 °C, P = 0.04) compared to temperature at 30 min (26.52 °C). There were no differences in tail wound temperature.

There was no treatment by time interaction; however, an overall treatment effect was seen on wound scores. Testis wound score was significantly affected by treatment, H (2) = 10, P = 0.009. The focused comparisons of the mean ranks between the groups showed that testis wound scores for CF lambs were significantly lower (*difference* = 32.8) compared to C lambs, H (2) = 9, P = 0.009. For tail wound scores both CF (*difference* = 31.1) and CI (*difference* = 31.0) had significantly lower scores than C lambs, H (2) = 10, P = 0.005. In all case the critical difference (α = 0.05) was 24.6.

3.5. Discussion

The results of this study indicated that voluntary consumption of flunixin in a complete mixed ration was as effective as flunixin administration by intramuscular injection at reducing the pain and inflammation associated with castration and tail-docking. The benefits of pain relief for physiological response occurred over the 48 h post-treatment period and for behavioural responses for the 12 h post-treatment period examined in the study. Nonetheless, residual signs of pain were evident in lambs during the period when maximal blood and tissue concentrations of the drug would have occurred.

There was a large increase in cortisol concentrations at 30 minutes in all castrated groups, as previously seen following knife castration (Paull et al., 2008; Paull et al., 2009a). In accord with these previous studies on the effects of NSAIDs on the cortisol response to castration, flunixin treatments had no effect on this cortisol response in the current study. A similar lack of effect of flunixin on cortisol responses has also been seen following mulesing (Paull et al., 2007). Sham treatment induced no immediate cortisol response but as the experiment continued there was a gradual rise in plasma cortisol concentrations by 48 h in this group, which may have been due to the effect of repeated blood sampling. The observation that the acute cortisol increase following mulesing is reduced by a combination of flunixin (2.5 mg/kg) with topical local anaesthetic and is abolished by a combination of the NSAID carprofen (4.0 mg/kg) with topical local anaesthetic (Paull et al., 2007). Similar results have occurred following surgical castration in cattle where the use of ketoprofen or flunixin alone was not as effective at reducing or preventing the cortisol response as the combination of these NSAIDs with a local anaesthetic (Earley and Crowe, 2002; Webster et al., 2013). This indicates that cortisol is an important indicator of the effectiveness of analgesia following surgical husbandry practices and that failure of flunixin to reduce cortisol in the current study is one indicator of residual pain in these lambs.

Lambs that received flunixin also had reduced inflammation compared to C lambs as indicated by the lower testis wound score for CF lambs and tail wound score for both CF and CI lambs. Lambs in the CF also had reduced neutrophil/lymphocyte ratio at 12 and 24 h post-treatment compared with C Lambs and it is possible that these differences can be attributable to the higher dose given to lambs in the flunixin oral group or the effect of continued consumption of flunixin in feed on its blood concentration.

Lambs in CF and CI groups exhibited less pain avoidance behaviours in the hour following treatment compared to C lambs, but their behaviour differed from S lambs. For postural behaviours, lambs in the CF group spent more time lying in the first 4 h following treatment and their behaviour was similar to S lambs, however CF lambs still displayed significantly more abnormal postures compared to S lambs in the first 4 h. In the 4 to 8 h following treatment CF and CI lambs lay down more than C lambs. Normal lying is considered to be a sign of comfort, whereas standing following surgical castration and tail-docking is seen as an attempt to avoid pain (Molony, 1993). Previous pen and field studies have also shown an increased lying in lambs that received NSAIDs following surgical castration (Paull et al., 2009a; Small et al., 2014).

The display of active pain avoidance behaviour by the CF and CI lambs and display of postural pain behaviours by the CI lambs indicates that they were experiencing some residual pain. This is also confirmed by the increase in cortisol in all animals that had been castrated and tail-docked and although the cortisol levels in the CF and CI lambs decreased quicker than the animals without pain relief they still did not return to baseline. Even though flunixin was effective at alleviating pain associated with castration and tail-docking it did not completely ameliorate the pain. In order to improve the effectiveness of analgesics to provide adequate pain-relief to livestock it may be necessary to use a combination of

anaesthetics with NSAIDs prior to the procedures as was demonstrated by Earley and Crowe (2002), Paull (2007) and Webster (2013). It may also be necessary to consider repeated administration of NSAIDs over successive days.

3.6. Conclusions

Following castration and tail-docking, lambs that consumed flunixin voluntarily as a component of a total mixed ratio or received flunixin via injection exhibited less pain-related behaviour and had reduced inflammation compared to animals that received no pain relief,. However, there were some residual behavioural and physiological indications to show that some level of pain remained in these animals. The results indicate that voluntary consumption of flunixin in feed could be a practical and effective method for relieving pain in lambs.

4. Objective 3: Develop methodology for self-selection and self-administration of analgesics to lambs

4.1. Investigation of a model of pain to allow lambs to learn to self-medicate

4.1.1. Objective

Lambs were monitored for up to 35 days after ring castration and tail-docking to assess chronic pain that may be associated with this procedure. Ring castration and ring tail-docking were performed on separate days to produce two events rather than one, so that the pain effect is more similar to chronic pain.

The objective of this study was to establish a chronic pain model through extending the duration of pain with the application of rings for castration and tail docking on separate days to be used for a self-medication experiment.

4.1.2. Methods and materials

There were 30 lambs involved in this experiment. Lambs were castrated at approximately 6-12 weeks of age. Lambs were weighed the day before treatment, individually side branded, and assigned to treatment groups on the basis of weight:

There were three treatment groups, 10 lambs in each treatment group.

- Group 1: Sham control
- Group 2: Elastrator castration day 0 and tail-docking on day 3
- Group 3: Elastrator castration and tail-docking on day 0 as per farm protocol (day 0)

Lambs were kept with their mothers, in a paddock situation. They were separated from their mothers on the morning of treatment, for a maximum of 3 h. The ewes were released into a small paddock (approximately 40 m X 60 m) beside the lamb marking pen. Lambs were caught, restrained in a marking cradle, and treated according to assigned treatment. After castration, the lamb was released into the small paddock containing their mothers.

Behaviour of the lambs was monitored for 2 h by personnel blinded to treatments. A team of 2 observers undertook scan sampling of lamb behaviour every 15 minutes. Behaviour was classified as

- Standing (normal standing, hunched standing, grazing)
- Lying –ventral and lateral
- Suckling
- Walking
- Running/ playing

At day 1, 2, 3, 7, 10, 14, 17 21, 28 and 35 post-castration, behaviours were assessed by 5-min continuous focal animal sampling. Counts of all events were summed for each 5-min observation period. On day 3 lambs had their behaviour observed after group 2 lambs were tail-docked.

On days -1, 7, 14, 21 and 28, ewes and lambs were mustered and lambs weighed.
Blood sampling (10 mL) was taken at 0 h, 4 h, 7 h and day 1, 2, 3, 7, 10, 14, 17 21, 28 and 35 post-castration for analysis of plasma cortisol and haptoglobin. Following blood sampling each lamb had their wound palpated and their response was recorded as a score from 0=no response to 2= struggling.

Observations ceased after day 35.

4.1.3. Results

Lamb weight was not affected by treatment, all lambs gained weight following treatment day. On the day of treatment, there was a significant difference ($P < 0.05$) in the amount of pain related postures exhibited by the three groups. Control animal exhibited less pain related postures than both castrated groups. Lambs in the castrated and docked group exhibited more pain related postures ($P = 0.03$, 1.6 ± 0.71) than lambs that were only castrated on day 0.

On day three which was the day of tail-docking for the castrated only group, this group of lambs exhibited more pain related behaviour then they did on day 1 ($P = 0.04$). They also exhibited more pain related behaviour than the group that was castrated and tail-docked on day 0 ($P < 0.05$). However, overall there was no difference in the cumulative amount of pain related behaviour exhibited by the groups over the 35 day period.

Lambs that were castrated and tail-docked still exhibited a response to the palpation of their wound at 35 days post-castration (Figure 3). Both groups that were castrated and tail-docked had more lambs react to the palpation of their wound site than the control group ($P < 0.001$). There was no difference between the two castration groups in the number of lambs that reacted to palpation.

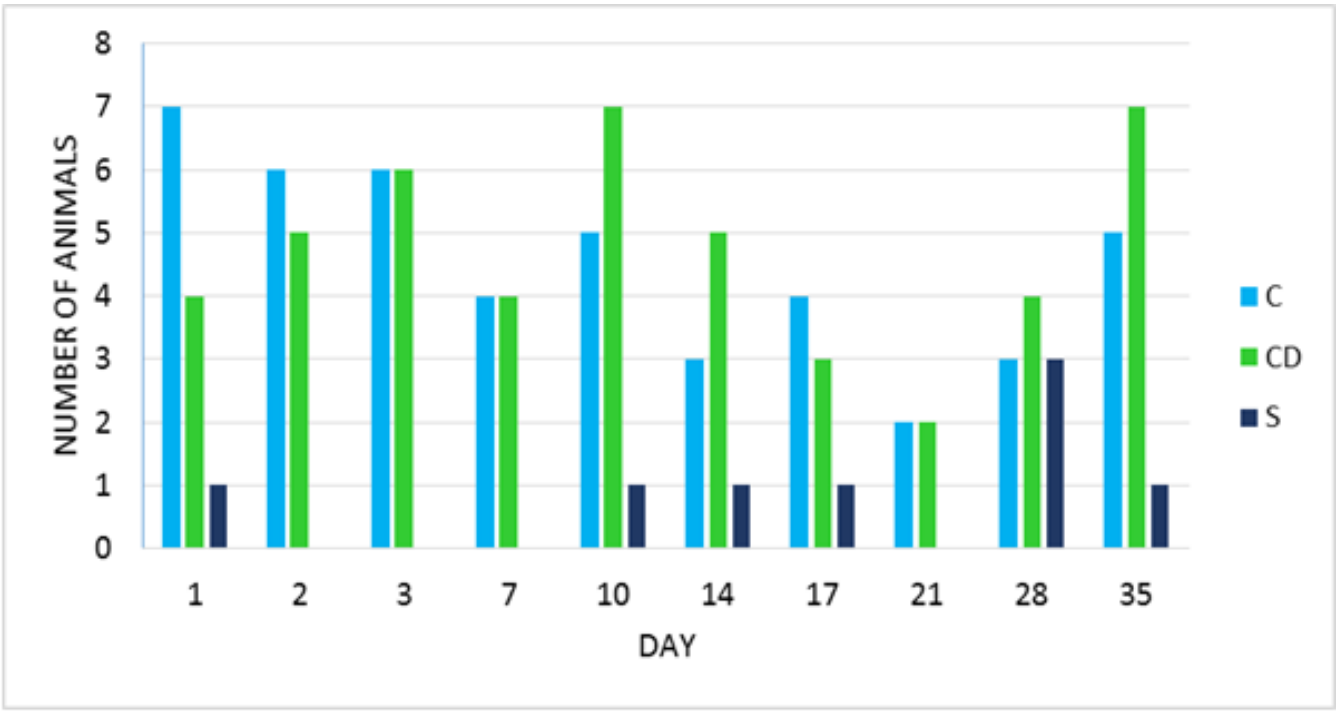

Figure 3: Number of lambs in each group (n=10/group) that exhibited a response to palpating the “wound” site following castration and tail-docking, which occurred on day 0. C is lambs castrated and tail-docking on day 0, CD is lambs that were castrated on day 0 and tail-docking on day 3, S is control lambs that did not undergo the procedures.

4.1.4. Conclusion

Lambs were monitored for up to 35 days after ring castration and tail-docking to assess chronic pain that may be associated with this procedure. Ring castration and ring tail-docking were performed on separate days to produce two events rather than one, so that the pain effect was more likely to induce chronic pain. The objective of this study was to establish a chronic pain model through extending the duration of pain with the application of rings for castration and tail docking on separate days to be used for a self-medication experiment.

Overall lambs that were castrated and tail-docked didn’t show a significant amount of observable signs of pain (pain related behaviour and postures) after the treatment day but they still exhibited discomfort of the wound site up to 35 days following the procedure. This suggests that ring castration and tail-docking can be a suitable chronic pain model for use in the self-medication experiment. Lambs that were tail-docked 3 days after castration, again displayed pain related behaviour on that day of treatment, indicating that a secondary acute pain can be achieved.

4.2. Development of a self-medication methodology for lambs to self-select and administer analgesics

Can lambs indicate their experience of pain through a preference for medicated feed?

4.2.1. Self medicative behaviour

Animals have the ability to balance their nutritional requirements through diet selection for macronutrients and micronutrients. When animals have access to a variety of plants, they have the opportunity to select plants for specific nutrients and they are able to change their preference of plant species based on their current nutritional deficiencies (Provenza, 1995). In contrast to diet selection to balance macro and micro nutrient requirements, self-medication is considered to occur when animals consume plants or non-vegetable substance (e.g. soils) that are not part of their normal diet in order to alleviate the effects of infection. In experimental settings livestock have been shown to have the ability to learn how to self-medicate. There has been extensive research conducted previously with sheep on their ability to learn to self-medicate for parasitic infection as well as other internal pains. For therapeutic self-medication an animal has to first learn that a certain substance can attenuate a state of illness or pain. An individual animal is likely to learn the benefit of a substance through trial-and-error; however, it can be difficult for an animal to make the association if the behaviour and consequence are not paired closely together (Provenza, 1987; Villalba and Provenza, 2007).

Animals often learn by watching and copying close relatives or other animals within their group however for them to gain nutritional wisdom they need to sample plants and learn consequences themselves whether positive or negative through post-ingestive feedback (Villalba and Provenza, 2009). For example in social animals, social facilitation can help animals overcome a food aversion; an animal watching peers eat a food that they originally had an aversion too will resample the food in small amounts, if there are no negative consequences the aversion can be extinguished (Ralphs and Provenza, 1999). Therefore on the occasion that an animal has made an association with a medicinal benefit of a specific plant or substance, maternal influences or social-interactions within the group could be important for faster transmission of the learned behaviour, as socialising can increase learning of individuals (Villalba and Provenza, 2007).

Most instances of livestock self-medication in experimental settings are in response to parasitism. A study conducted by Fishpool (2012) looked at whether sheep could learn to self-medicate with a medicated feed block that contained fenbendazole, when infected with a gastrointestinal nematode. After a seven week training period where sheep were exposed to an un-medicated feed block for five weeks and then a medicated feed block for a total of two weeks. The sheep were split into two groups, a gastrointestinal nematode infected group and an uninfected group. Both groups were then given access to the medicated feed block for one week. During this one week period it was found that infected sheep ate more of the medicated feed block in the first four days and were able to receive a therapeutic dose of fenbendazole which caused a rapid decline in worm egg count by the sixth day. In Fishpool's experiment, self-medication was demonstrated by the nematode infected sheep but could not be confirmed due to the variability in the amount of the medicated feed block consumed by both infected and un-infected sheep during the one week period, and the continuing consumption by infected animals even after their worm egg counts had decreased. There have been other demonstrations by Lisonbee et al. (2009); Villalba et al. (2010); Juhnke et al. (2012) that sheep can learn the benefits of CT in their diets and subsequently preferentially consume CT containing feed whilst parasite burdens are high. Similar to Fishpool's (2012) results, lambs that were parasitized preferentially consumed CT containing supplements compared to uninfected sheep (Lisonbee et al., 2009). This higher preference

for CT feed by parasitized lambs continued for the first 12 days but then reduced as their parasite burden decreased.

One of the methods used to determine if an animal has learnt to self-medicate is through preference testing. In preference testing animals are given the opportunity to show us how they feel. During a preference test the animal is provided with a variety of choices for a particular situation (e.g. a choice of bedding type) and they are allowed to essentially “vote with their feet” (Duncan, 1992). Preference testing also has the potential to be used to detect pain in an animal. If an animal self-selects analgesic when it is given a choice of a normal feed and a feed containing an analgesic, it could be indicative that it is in pain. However for the animal to have the ability to make choices, such as to self-medicate, it must first experience the consequences (whether positive or negative) of the feed options in question. This is usually done by including a conditioning period prior to the preference test.

The objectives of this study are to castrate lambs, and offer them medicated feed in a training period. The lambs will then be tail-docked a week later and offered medicated and non-medicated feed again to see if sheep have a preference. If lambs have learnt to associate the odour they were trained on with flunixin, they should have a preference for it. It is hypothesised that if sheep are no longer in pain after 5 weeks any preference they may have exhibited in the preference test after tail-docking should be gone. During this experiment lambs will be monitored for feed consumption and for pain behaviours. The aim of the experiment is to see if lambs can learn to self-medicate with flunixin that has been added to feed when they are experiencing pain associated with castration and tail-docking. Further, we predict that if sheep are no longer in pain after 5 weeks any preference they may have exhibited in the preference test after tail-docking should not be evident.

4.2.2. Determining an odour cue

To determine which odours to use in the self-medication experiment and to test for any aversions in the sheep, we conducted a pilot trial where five odours: banana, apple, strawberry, coconut and green tea were tested in pelleted feed.

To test the odours, eight sheep were acclimated to the mating yards and reintroduced to eating pellets for a week, after which they were split into two groups of four in two of the yards. They then had the option of 6 feeds placed in separate feed troughs (2.5 kg of each). One feed contained only normal pellets and the remaining five contained one of the odours (banana, apple, strawberry, coconut and green tea). They were offered these feeds over a period of 5 days and their intake of each was recorded 24 h after they were first offered.
The results of this pilot trial indicated that sheep had a higher preference for both banana and strawberry over the other odours.

4.2.3. Self-medication method

The experiment was undertaken at CSIRO’s FD McMaster Laboratory, Armidale, New South Wales (NSW), Australia. The protocol and conduct of the experiment was approved by The CSIRO Chiswick Animal Ethics Committee under the NSW Animal Research Act, 1985 (approval ARA 15/09).

A total of 36 male Merino lambs aged 9 - 10 weeks old went through acclimation to the odours and through the first preference test but only 32 were included in the self-medication training. Lambs were ear tagged at birth and ewe-lamb pairs allocated to 2 cohorts, cohort being based on birthdate. Prior to the study ewes and lambs were exposed to pellets whilst in the paddock. At 7 weeks of age lambs were moved into the animal house with their mothers, where they were acclimatised to indoor housing and to the standard pelleted ration.

After the first week of acclimation ewe-lamb pairs were moved into individual pens (2.5m x 1.5m) and exposed to the odours (strawberry and banana) that were to be used as cues. Lambs were weaned from their mothers after a week of being individually penned, after weaning lambs were handled once a day to reduce subsequent handling stress. Lambs were then tested for their preference of the two odours for a week prior to treatment.

For the self-medication there were two treatment groups;

(1) Ring castrated day 0 and tail-docked day 7

(2) Sham handled day 0 and day 7

The odour cue used for the medicated feed was evenly divided between the treatment groups (Figure 4). Treatments were spaced out to occur every 2 minutes.

All the feed troughs were labelled with the odour that they would contain to avoid any confusion. All feed was prepared in the trough, the pellet and chaff were first added to the trough which was on a scale to ensure each lamb was receiving correct amount of feed. The lambs dose of flunixin was then applied directly to the pellets and then mixed through by hand. Incorporation of flunixin with the pellets was noted by the change in the pellets colour. The corresponding odour was then added to the feed; using a spray bottle 10 sprays (of approximately 10 ml) were applied.

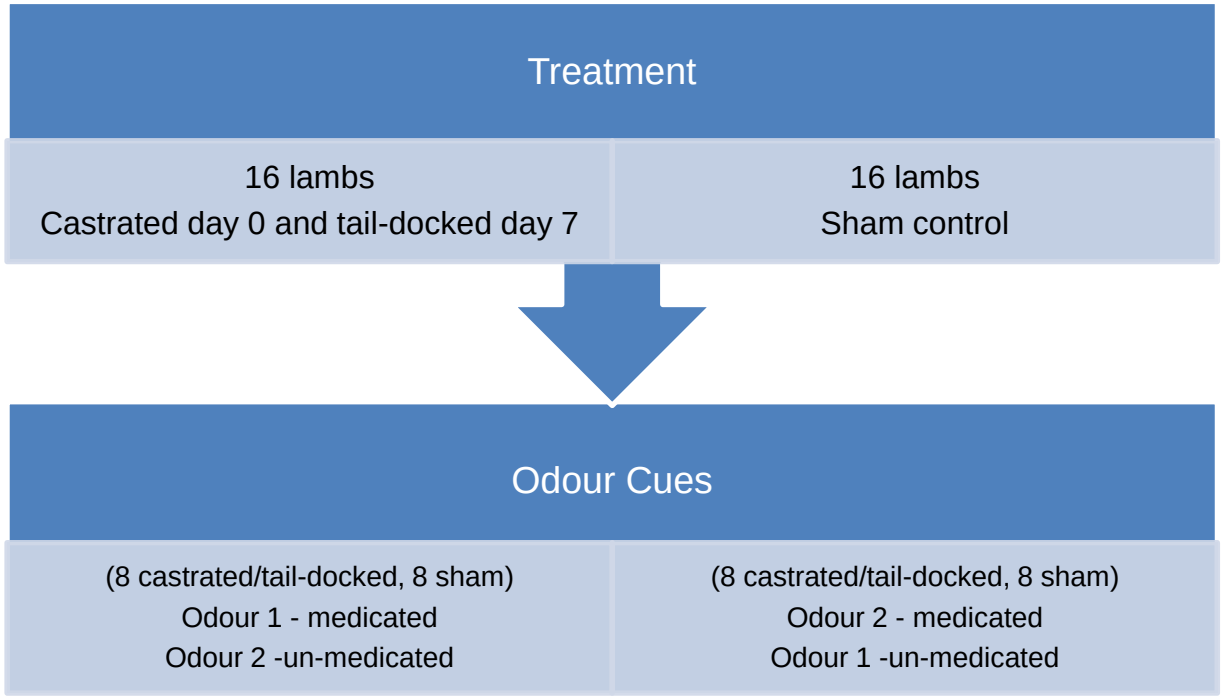

Figure 4: Allocation of odour cue for medicated feed between treatment groups

On treatment day (day 0 - Monday) sham lambs were handled as if they were to be castrated and treated lambs were ring castrated. Thirty minutes following treatment all lambs were offered 600 g of pellets and 200 g of chaff which contained their calculated dose of flunixin (4.0 mg/kg), with the selected odour. Lambs were offered the same preparation of feed from day 1 to 3. On day 4 – 6 were given regular pellets and chaff to ensure that they would not have therapeutic levels of flunixin when they were tail-docked. On day 7 (the following Monday) lambs in the sham group were again handled and the castrated group were ring tail-docked. Thirty minutes following treatment all lambs were then offered a choice

of both the feeds (medicated 1000g and non-medicated 1000g) with the odour cue they were given the week before. Their feed was weighed 1 h after they first were given access and then at 12 h both feed troughs were removed and residual feed recorded, this was done for 5 days.

Figure 5 : A lamb in its individual pen, selecting feed from one of the two troughs on offer. Troughs have been labelled to indicate what odour they contain.

After the self-medication test lambs were removed from the animal house and kept in a paddock for 5 weeks. After 5 weeks lambs re-entered the animal house and retested for their preference for 5 days but this time only using the cues in the feed.

Figure 6: Lambs were placed in individual pens next to other lambs, where they were able to interact with each other

4.2.4. Measurements

Feed preference

Feed intake was recorded daily 1 h and 12 h after being given to the lambs. The preference was calculated by:

$$\frac{(Intake\ of\ medicated\ feed + 5)}{(Intake\ of\ medicated\ feed + 5) + (Intake\ of\ un-\ medicated\ feed + 5)}$$

To account for chance, preference for a particular odour or feed was then determined by calculating two times the standard error either above or below 0.50.

Behaviour

Video cameras were used to continuously record the behaviour of lambs in the study. For each pen, one camera was mounted on roofing rafters at each end of the pen. Each camera provided a view of the entire area available to the lambs. The cameras were connected to digital video recorders and captured by IVMS4200 software from Hangzhou Hikvision Digital Technology Co., Ltd. The behaviour of the lambs in their pens on Day 0 were collated from the digital video records by observation of a replay of the video record on IVMS4200 software from Hangzhou Hikvision Digital Technology Co., Ltd. The behaviours recorded are the same as that for the pen castration (Page 17).

Haematology

Blood was collected by 21 gauge needles into 10 mL vacutainers containing EDTA. Individual blood samples were collected at 0 h, 30 min, 6 h, and 12 h on the day of treatment (day 0 and day 7) and then every morning for up to 72 h post treatment days. Neutrophil and lymphocyte counts in whole blood were determined with an automated haematology analyser (Cell Dyn 3500R, Abbott Diagnostics, Illinois U.S.A). The blood samples were then centrifuged at 2000 × g for 15 min at 5^{o}C and plasma were separated into three aliquots which were then stored at -20^{o}C until assayed for haptoglobin and cortisol concentration. Plasma cortisol concentrations were determined using a commercial radioimmunoassay (Plasma Cortisol RIA, MP Biomedical, Australia).

Anti-Inflammation measures

Lambs had their wounds palpated at the time of blood sampling and their response recorded as a score from 0 = no response to 2 = struggling.

Weights

The lambs were weighed on Day -1, 7, 14, 21, 28 and 35 in relation to day of castration.

Statistics

All data were analysed using R (R Development Core Team, Boston, Massachusetts) and the packages *nlme* (Pinheiro et al. 2013) and *pscl* (Zeileis 2008) was used. Data were tested for normality using the Shapiro-Wilk test and visual inspection of residual plots and transformed where necessary. Active pain avoidance behaviour required the use of zero inflated Poisson model due to a high amount of zeros in the data. Wound palpation scores were analysed using a GLM with a binomial distribution. $P < 0.05$ was considered statistically significant and $0.1 > P > 0.05$ was considered a statistical tendency.

4.2.5. Results and discussion

Week 1 preference

When analysing the feed intake of all animals, there was no flavour (banana vs strawberry) by location interaction (left or right) and no effect of location or flavour on the consumption of pellets. For the calculation of individual lambs preference, a score of 0.53 and above was considered a preference for strawberry and a score 0.47 and below was a preference for banana. For odour preference there was a fairly even split with 15 lambs preferring strawberry and 16 preferring banana. Five lambs did not have a preference.

Weight gain

The lambs weight gain was not effected by treatment (whether they were castrated or not). For the animals that were castrated there was no effect of their preference of feed (medicated, non-medicated or none) on weight gain.

Castration week

In the first hour following treatment, lambs that were castrated did display more active pain behaviours than sham lambs ($P < 0.05$). Therefore we know lambs were experiencing pain before they were given access to feed containing flunixin. Lambs received the feed with pain relief after their 30 minute bleed. From our previous experiment (Marini et al. 2016) we can assume that if lambs had eaten at least 22 g of the feed in 10 minutes then they would have therapeutic concentrations of flunixin in their blood within 2 h.

Following castration there was a time by treatment effect for the neutrophil/lymphocyte ratio between sham and castrated lambs. There was a time effect, with castrated lambs having a higher neutrophil/lymphocyte ratio at 6, 24, 48 and 72 h compared to their baseline ($P < 0.05$ for all). Sham lambs also had an increase in neutrophil/lymphocyte ratio at 12 and 48 h compared to baseline ($P < 0.05$). At 72 h post treatment castrated lambs still had higher neutrophil/lymphocyte ratio than sham lambs ($P = 0.008$).

There was no treatment or time effect, lambs that were castrated reacted more to the palpation of their wound at 6 h following castrate ($P = 0.035$)

Self – medication

One lamb had to be removed from the study 2 days after tail-docking as he became depressed. In the hour following tail-docking lambs that were treated showed significantly more active pain behaviours than sham lambs ($P < 0.001$). On the day of tail-docking lambs did not have access to the food containing pain relief until after their 30 minute bleed. In the 12 h that they had access to the medicated and non-medicated feed there was no interaction between feed location and the choice of feed ($P = 0.77$). There was no difference between the treatments and preferences of the feeds within any of the 4 days following tail-docking. There was also no overall difference between the sham and tail-docked treatment and the choice of medicated or non-medicated feed. For individual preferences, there was a difference between lambs in both groups and their preferred feed. For the calculation of individual lambs preference, a score of 0.56 and above was considered a preference for medicated feed and a score 0.44 and below was a preference for non-medicated feed. Of the lambs that were tail-docked ($n = 15$), 4 had no overall preference over the 5 days, 7 had a strong preference for the medicated feed and 4 had a strong preference for non-medicated feed. For the sham lambs ($n = 16$), 3 had no overall preference, 5 had a strong preference for the medicated feed and 8 had a strong preference for non-medicated (Table 4).

Following tail-docking there was no time by treatment interaction, there was a time effect ($P < 0.0001$) and a treatment trend ($P = 0.064$). Sham lambs experienced an increase in neutrophil/lymphocyte at 24 h ($P = 0.02$). Tail-docked lambs had significantly higher ($P < 0.05$) neutrophil/lymphocyte compared to baseline at 24, 48 and 72 h following treatment.
For the palpation scores there was a significant treatment effect ($P < 0.05$), sham lambs reacted less to the palpation of the wound area than lambs that were tail-docked ($P = 0.0013$).

Week 5 preference

This preference was to test is lambs still had a preference for the odour that they were assigned as the cue of their medicated feed. In this test feed did not contain flunixin. Overall there was no effect of treatment or odour on the lamb's preference. For the calculation of individual lambs preference, a score of 0.55 and above was considered a preference for medicated feed cue and a score 0.46 and below was a preference for the non-medicated feed cue. For the castrated and tail-docked lambs ($n = 15$), 6 did not have a preference for either odour, 4 had a preference for the odour that was the cue for the medicated feed and 5 had a preference for the non-medicated cue. For the sham lambs ($n = 16$), 4 had no preference, 8 had a preference for the medicated cue and 4 had a preference for non-medicated cue.

Table 4: Overall preference of medicated and non-medicated feed in lambs that were either handled or ring castrated and tail-docked, during the self-medication test. Blue indicates the lambs had no change in odour preference, green indicates the lambs had a change in preference.

Lamb ID	Odour used medicated feed	Treatment	Preferred during self-medication	Original Preference
8278	S	Castrate	medicated	S
8279	B	Castrate	non-medicated	N
8286	S	Castrate	medicated	S
8298	S	Castrate	none	B
8311	S	Castrate	none	B
8314	B	Castrate	non-medicated	N
8327	B	Castrate	medicated	B
8343	B	Castrate	non-medicated	S
8456	S	Castrate	medicated	S
8468	S	Castrate	medicated	B
8473	S	Castrate	none	B
8481	B	Castrate	non-medicated	S
8510	S	Castrate	medicated	B
8518	B	Castrate	medicated	B
8531	S	Castrate	none	S
8273	S	Sham	none	S
8295	S	Sham	non-medicated	B
8305	B	Sham	non-medicated	S
8308	S	Sham	none	S
8316	S	Sham	non-medicated	S
8320	B	Sham	medicated	S
8325	B	Sham	non-medicated	S
8330	B	Sham	non-medicated	S
8458	B	Sham	non-medicated	B
8459	B	Sham	medicated	B
8461	B	Sham	none	B
8476	S	Sham	medicated	N
8495	B	Sham	non-medicated	B
8511	S	Sham	medicated	B
8519	S	Sham	medicated	S
8529	B	Sham	non-medicated	N

Conclusion

We know that lambs that were castrated and tail-docked a week later experienced pain on both the applications of the treatment. This was observed by the increased amount of active pain avoidance behaviours displayed by the lambs in the hour following castration and tail-docking compared to sham lambs. Lambs also experience the inflammation associated with the treatments as displayed by the increase in neutrophil/lymphocyte ratio that was maintained in castrated and tail-docked lambs. However lambs were not able to indicate their experience of pain through a preference for medicated feed.

It is known from the pharmacokinetics study that although the lambs would reach therapeutic concentrations that would provide pain relief within 2 h of first eating their medicated feed, the lambs would have had to eat around 800g in the 8-12 h to maintain therapeutic levels. The information from the pharmacokinetics, even though ewes were 12 months old, should be transferable as lambs at 10 weeks would have functioning rumens. Therefore lambs would need to maintain a preference for the medicated feed. After removal of feed at 12 h lambs would have had a decline in the concentration and therefore would need to reselect medicated feed on the following days. While plasma was collected, the drug concentration in plasma was not analysed, so we can only speculate based on our previous study that they achieved therapeutic concentrations of flunixin in their blood.

A majority of the reported self-medication in animals including sheep is in response to a parasitic infection. It is suggested that the animals learn which substance will improve this negative state by trial and error, the animal learns about food and develops preferences through the interactions of the foods characteristics (odour, flavour and texture) and post-ingestive feedback. Perhaps the mechanisms for learning to self-medicate for pain may be different for other negative states such nutritional deficit and parasitism.

The experiments presented in this report demonstrated the potential for delivering pain relief to lambs in medicated feed, but was not able to establish whether lambs could identify a benefit of medicated over un-medicated feed. Supplementing both ewes and lambs with medicated feed as in the castration study is suitable for use by industry in the field without the need to train lambs to associate medicated feed with pain relief.

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6. Appendix

Appendix 1 - Randomised trial of the bioavailability and efficacy of orally administered flunixin, carprofen and ketoprofen in a pain model in sheep

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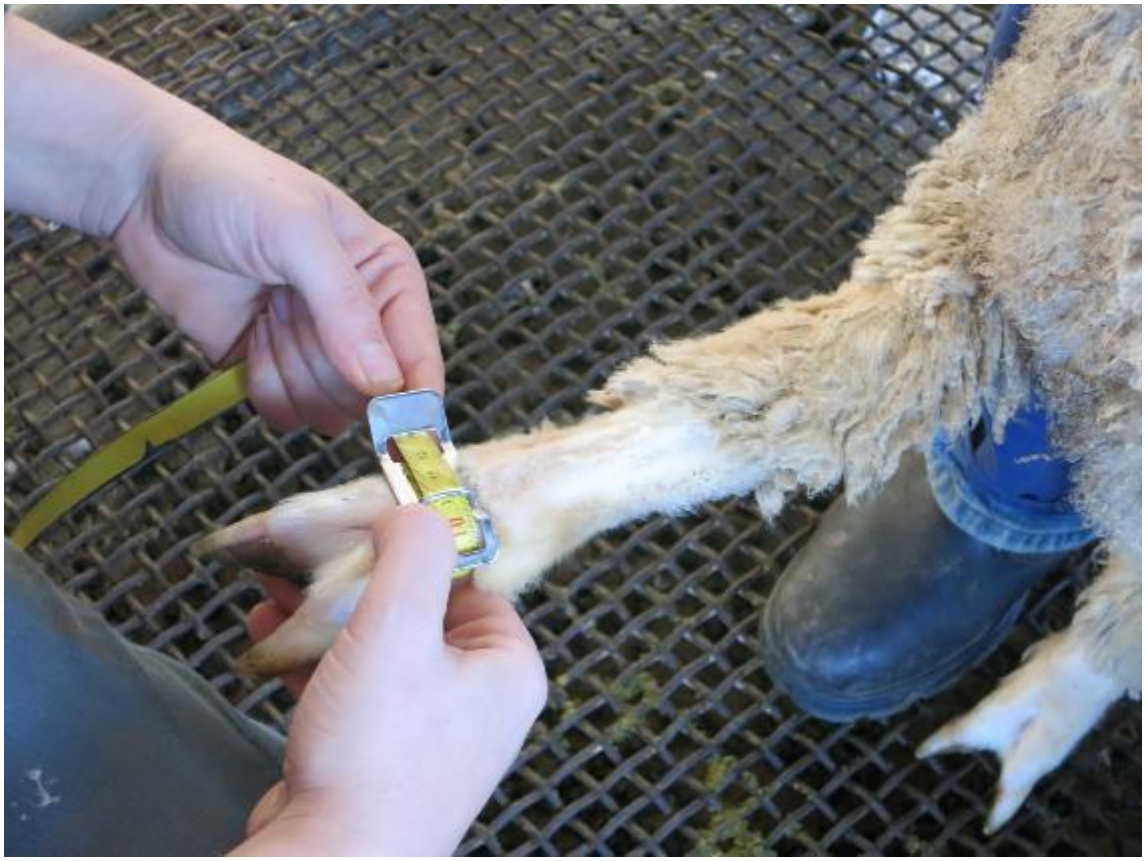

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2.1 Abstract

Objective
To determine the efficacy and bioavailability of non-steroidal anti-inflammatory when administered orally to sheep

Study Design
Randomised experimental design with four treatment groups, three given NSAIDs and one control group (n = 10/group).

Animals
40, 18 month old, Merino ewes, average weight 31.4 ± 0.5 kg

Methods
Treatment was given orally at 24 h intervals for 6 days at dose rates expected to achieve therapeutic levels in sheep: carprofen (8.0 mg/kg), ketoprofen (8.0 mg/kg) and flunixin (4.0 mg/kg). Oil of turpentine (0.1 mL) was injected into a forelimb of each sheep to induce inflammation and pain; responses (force plate pressure, skin temperature, limb circumference, haematology and plasma cortisol) were measured at 0, 3, 6, 9, 12, 24, 36, 48, 72, 96 h post-injection. NSAID concentrations were determined by UHLPC.

Results
The NSAIDs were detectable in ovine plasma 2 h after oral administration, with average concentrations between 4.5 - 8.4µg/mL for ketoprofen, 2.6 - 4.1µg/mL for flunixin and 30 - 80µg/mL for carprofen. NSAID concentrations dropped 24 h after administration. Pain response to an oil of turpentine injection was assessed using the measures applied but did not see any effect of the NSAIDs. Although this pain model has been previously validated, the responses observed in this study differed from the previous study.

Conclusions and Clinical Relevance
The 3 NSAIDs reached inferred therapeutic concentrations in blood, 2 h after oral administration. The oil of turpentine lameness model may need further validation.

Key words (up to 6)
Sheep, pain, non-steroidal anti-inflammatory drugs (NSAIDs), animal welfare.

Abbreviation
NSAID, non-steroidal anti-inflammatory drug; UHPLC, Ultra High Pressure Liquid Chromatography.

2.2 Introduction

Routine husbandry procedures performed on lambs include castration, tail-docking, tagging and mulesing. Pain caused by these procedures can last several days[1, 2]. With the exception of mulesing, for which a topical local anaesthetic formulation is widely used, lambs do not receive therapeutic interventions to provide pain relief for routine husbandry procedures. Research studies have shown that local anaesthetics and analgesics are effective at relieving pain associated with castration, tail-docking and mulesing[3-5], however, the duration of anaesthesia or the period of effectiveness of local anaesthetics is typically less than the period over which lambs experience pain. Factors limiting the use of analgesics by sheep producers include availability of registered drugs, the regulatory environment governing access to registered drugs[6-8], difficulty of administration of injectable drugs and practicality of providing pain relief for the period over which pain is experienced[3, 9].

Attractive candidates for long-acting pain relief are non-steroidal anti-inflammatory drugs (NSAIDs). The pharmacokinetics of NSAIDs such as carprofen, ketoprofen and flunixin have been well documented in cattle[10-12], horses[13, 14] and sheep[15-17]. There are reports describing the efficacy of NSAIDs to alleviate pain in sheep when administered via subcutaneous, intramuscular (I.M.) and intravenous (I.V.) routes[4, 18]. Carprofen is a long-acting analgesic that has a half-life of greater than 30 h in sheep[16] and has been shown to reduce pain-related behaviours in lambs that have undergone mulesing and castration[4, 6, 18]. Flunixin has been shown to be effective at increasing the thresholds to noxious mechanical stimulation on the first day of treatment, in sheep suffering from footrot[19]. It has also been shown to reduce pain-related behaviours in lambs that have undergone mulesing[4]. Ketoprofen has been shown to increase nociceptive thresholds in the absence of inflammation[20] , decrease pain and lameness in horses[21] and reduce cortisol response to castration in cattle[22].

Like anaesthetics, NSAIDs are most commonly administered parenterally, which can be difficult for producers; however some NSAIDs can also be given orally. Literature on the efficacy of orally administered NSAIDs in sheep is limited, although this route has been examined in cattle[23, 24]. Bioavailability of flunixin when administered orally as granules to cattle was 60% of that attained following intravenous injection[25]. This reduced bioavailability is probably due to the influence of the rumen[26] and has led others to double the dose used non-parenterally in studies using orally delivered NSAIDs in cattle[24]. If NSAIDs can be shown to alleviate pain and inflammation when administered orally in sheep, it may be applicable to incorporate them in feed, providing producers with a quick and easy method to deliver extended pain relief to sheep following painful husbandry procedures.

To test the efficacy of NSAIDs, a turpentine model of pain and inflammation that has previously been developed and validated[27] in sheep was used. In the study by Colditz et al. (2011) an injection of 0.1mL oil of turpentine caused local and systemic signs of inflammation as well as increased sensitivity in the limb, with animals decreasing the amount of weight borne on the injected limb. The authors also showed that treatment with meloxicam reduced limb sensitivity following turpentine injection.

The aim of this research was to test the bioavailability and efficacy of the NSAIDs carprofen, ketoprofen and flunixin administered orally to provide pain relief to sheep experiencing limb inflammation and pain associated with an oil of turpentine injection. Sheep receiving NSAIDs were expected to exhibit fewer or less severe signs of pain and inflammation associated with the oil of turpentine injection when compared to placebo-treated sheep.

2.3 Methods and materials

2.3.1. Sheep and housing

The experiment was undertaken at CSIRO's FD McMaster Laboratory, Armidale, New South Wales (NSW). The protocol and conduct of the experiment was approved by The CSIRO Chiswick Animal Ethics Committee under the NSW Animal Research Act, 1985. Fifty 18 month old, Merino ewes (average weight 28.5 ± 0.5 kg) were trained for the study. For 2 weeks prior to the experiment, ewes were kept in yards (44.32 m^2 per animal) where they were supplemented with a complete pelleted ration (Ridley Agriproducts, Australia; 17% crude protein dry matter; 9.04 MJ/kg dry matter). Pellets were given twice a day y to accustom them to the diet and to human contact. The sheep were fed 1.4 kg of complete pelleted ration plus 100 g of oaten chaff, each morning.

The ewes were tested sequentially in two cohorts, 2 weeks apart. One week before each testing periods, the cohort was moved to individual pens in a covered shed. During this week they were weighed for dose calculation (average weight (31.4 ± 0.5 kg)) and were trained to

use a weighing platform twice daily. During training sheep were made to stand on a split weigh bar system with their forefeet, in an enclosed area. Once on the weigh bars a pole was placed behind the sheep to prevent backwards movement, sheep were offered pellets and had a companion sheep in a pen in front of the platform to keep them calm whilst they stood on the platform for 1-2 min.

A total of 50 sheep were trained and subsequently 40 were selected and included in the study. Two animals were excluded for failing to learn to use the platform and a further 8 were excluded based on weight (2kg less or more then the second lightest or heaviest ewe). This gave us a total of 40 sheep during the experimental period, 10 for each treatment group. Four days before the commencement of testing of each cohort, Oster clippers (Thrive, USA) were used to remove wool from the neck of each sheep to facilitate blood sample collection and on the anterior aspect of the forelimbs between the fetlock and coronet to enable limb measurements.).

2.3.2. Treatments

Sheep were randomly allocated to a cohort balanced for weight. Within each cohort ewes were randomly assigned to a pen and treatment group. There were 4 treatment groups which received: carprofen (8.0 mg/kg, PiaPharma, Chatswood West, NSW), ketoprofen (8.0 mg/kg, PiaPharma, Chatswood West, NSW), flunixin (4.0 mg/kg, PiaPharma, Chatswood West, NSW) and saline (Baxter, Australia) daily for the six days of the trial. Dose rates were based on doses of these NSAIDs given to sheep in previous experiments[16-17], the dose was then doubled due to the rumen's potential to reduce bioavailability. All of the treatment groups were given their assigned treatment orally as a solution with a syringe to the back of the tongue. Lameness was induced in one forelimb of every animal irrespective of treatment group on the second day of the experiment, 90 min after treatment dosing by the injection of 0.1 mL of oil of turpentine, subcutaneously between the fetlock and the coronet of the forelimb. Use of this oil of turpentine inflammation model has been described previously[27].

2.3.3. Forelimb measurements

Forelimb measurements were taken for both treated and untreated (control) limbs. The measurements collected were pressure exerted by forelimbs, skin temperature and limb circumference at injection site for both treated and control limbs. Measurements were recorded at -24, 0, 3, 6, 9, 12, 24, 36, 48, 72 and 96 h relative to oil of turpentine injection. The weight exerted on the sheep's forelimb was measured on two weighing platforms using the same method as that used for training the sheep. The forelimbs' surface temperature was measured using an infrared thermometer (ABW Industries,Australia) with a resolution of 0.1 ºC measured at 300 mm from the skin surface. The temperature was recorded at the injection site and on the matching site on the control limb. Circumference of both limbs were measured to the nearest mm, using a scrotal circumference measuring tape.

2.3.4. Lameness observations

Lameness observations were recorded at -24, 0, 3, 6, 9, 12, 24, 36, 48, 72 and 96 h relative to oil of turpentine injection. Video images were recorded for each sheep for 30 seconds and the proportion of time weight was not bourne on each limb during this time recorded (limb lift). The lameness of each sheep was also recorded as it moved voluntarily along a race 1 m wide and 10 m long on its return to its home pen following limb pressure measurements using an unmanned camera positioned at the end of the race. Lameness was scored from 0 to 4 (Table 4).

Table 4: Lameness scoring criteria.

Score	Observation
0	no abnormality in gait
1	head or shoulder drops on 1 or 2 strides
2	head or shoulder drops 3 or more strides
3	limb carried on 1 or 2 strides
4	limb carried on 3 or more strides

2.3.5. Body temperature

Rectal temperature was recorded daily as an assessment of fever, temperature was taken in the morning and was measured rectally using a digital thermometer with a resolution of 0.1 ºC. Ambient temperature was recorded.

2.3.6. Haematology

Blood was collected in 4.5 mL EDTA vacutainers (BD, UK) by jugular venipuncture for haematology at -22 h, 30 min, 4, 8, 24, 48, 72 and 96 h relative to oil of turpentine injection and processed using an automated haematology analyser (Cell Dyn 3500R, Abbott Diagnostics). Parameters measured included white blood cell count, neutrophil, lymphocyte and neutrophil/lymphocyte ratio.

2.3.7. Cortisol

Plasma samples were centrifuged at 2000 × g for 15 min and the plasma transferred to tubes for storage at -20 ºC. The plasma cortisol concentrations were determined using a commercial radioimmunoassay (Plasma Cortisol RIA, MP Biomedical, Australia) validated for ovine plasma. The intra-assay coefficient of variation (CV) for control samples containing 50.3, 101.1, 211.7 nmol/L cortisol, respectively, were 12.9, 11.1, 6.5%, the inter-assay CV's for the same control samples were 15.4, 14.4 and 9.0% respectively.

2.3.8. NSAID assay protocol

Blood was also collected in 10 mL heparin vacutainers for analysis of drug concentrations. These samples were collected 10 min prior to and 2 h after each oral dosage of NSAID or saline solution. These samples were prepared the same as cortisol samples. They were then transported frozen to PiaPharma PTY LTD, Chatswood West, NSW for analysis using Ultra High Pressure Liquid Chromatography.

Determinations were conducted on a 500 µL aliquot of plasma. Deuterated internal standards (2.0µg/mL flunixin-d3, 2.0µg/mL ketoprofen-13Cd3, and 4.0µg/mL carprofen-d3) were added prior to sample extraction with acetonitrile and subsequent clean-up using Solid Phase Extraction (SPE) sorbent (Cleanup® WSHQAX205, UCT).

Following wash steps, analytes and internal standards were eluted from the sorbent with two x 500µL aliquots of 4% acetic acid in methanol. The extracts were mixed gently and plate sealed with a pierceable sealing mat prior to transferring to the autosampler for determination.

An Eksigent® Ekspert™ ultraLC 100-XL Liquid Chromatograph was used for separation of the target analytes from any matrix interferences. A 10µL sample of extract was injected into the system, and separation performed using a Supelco Ascentis® Express 50x2.1mm, 2.7µm analytical column maintained at 50°C. A gradient elution program, using 0.1% formic acid and acetonitrile as mobile phase constituents operating at 0.8 mL min-1, resolved 5-hydroxy flunixin, ketoprofen, flunixin and carprofen from matrix interferences and endogenous sample components within a 4-min run time. The deuterated internal standards eluted at the same retention time as the non-deuterated equivalents.

An AB Sciex API 3200 triple-quadrupole mass spectrometer was interfaced with the liquid chromatograph. The detector was configured with a proprietary turbo V source for desolvation and operated in negative electrospray ionisation (-ve ESI) mode for optimum analyte sensitivity. The mass spectrometer was operated in MS/MS mode, with transition masses identified and optimised for declustering potential, collision energy, and cell entry and exit potentials.

A minimum of six calibration standard solutions were prepared at incremental concentrations spanning the relevant concentration range. Concentrations of the analyte were calculated using the peak area ratio of target analyte detected in each sample to the corresponding internal standard, and the regression equation of the calibration curve. The internal standard for 5-hydroxyflunixin was flunixin-d3.

2.3.9. Statistical analysis

Data was analysed with R (R, USA), with a repeated measures model to perform a linear mixed effects analysis of the relationship between treatment and time-point (the time the sample was taken). As fixed effects, cohort, treatment and time were entered into the model as individual and treatment x time as interaction terms. As random effects, intercepts for sheep were included. Limb circumference, limb lift and plasma cortisol concentrations required a log transformation to normalise the distribution of residuals. Lameness score required the use of Poisson error distribution. Visual inspection of residual plots did not reveal any obvious deviations from homoscedasticity or normality. P-values were obtained by likelihood ratio tests of the full model with the effect in question against the model without the effect in question. $P < 0.05$ was considered statistically significant and $0.1 > P > 0.05$ was considered a statistical tendency.

2.4. Results

2.4.1. NSAID Bioavailability

Carprofen, ketoprofen and flunixin were all detectable in ovine plasma 2 h after oral administration (Fig 7). Placebo animals receiving saline had lower than detectible limits of each drug at each time point (< 10 ng/mL). The average carprofen concentration found 2 h after administration over the 6 days ranged between 30 and 80 µg/mL. Average concentrations for flunixin were between 2.6 and 4.1 µg/mL and between 0.10 and 0.78 µg/mL for the metabolised residue 5-hydroxyflunixin. The average ketoprofen concentrations were between 4.5 and 8.4 µg/mL 2 h after administration. All 3 NSAID concentrations in plasma decreased over the 24 h period post administration with ketoprofen and flunixin returning to levels of < 0.1 µg /mL at 24 h. The concentrations of carprofen in plasma increased over the 6 days of the testing period, with levels not dropping below the minimum 31.0 µg/mL seen at time-point 0 h.

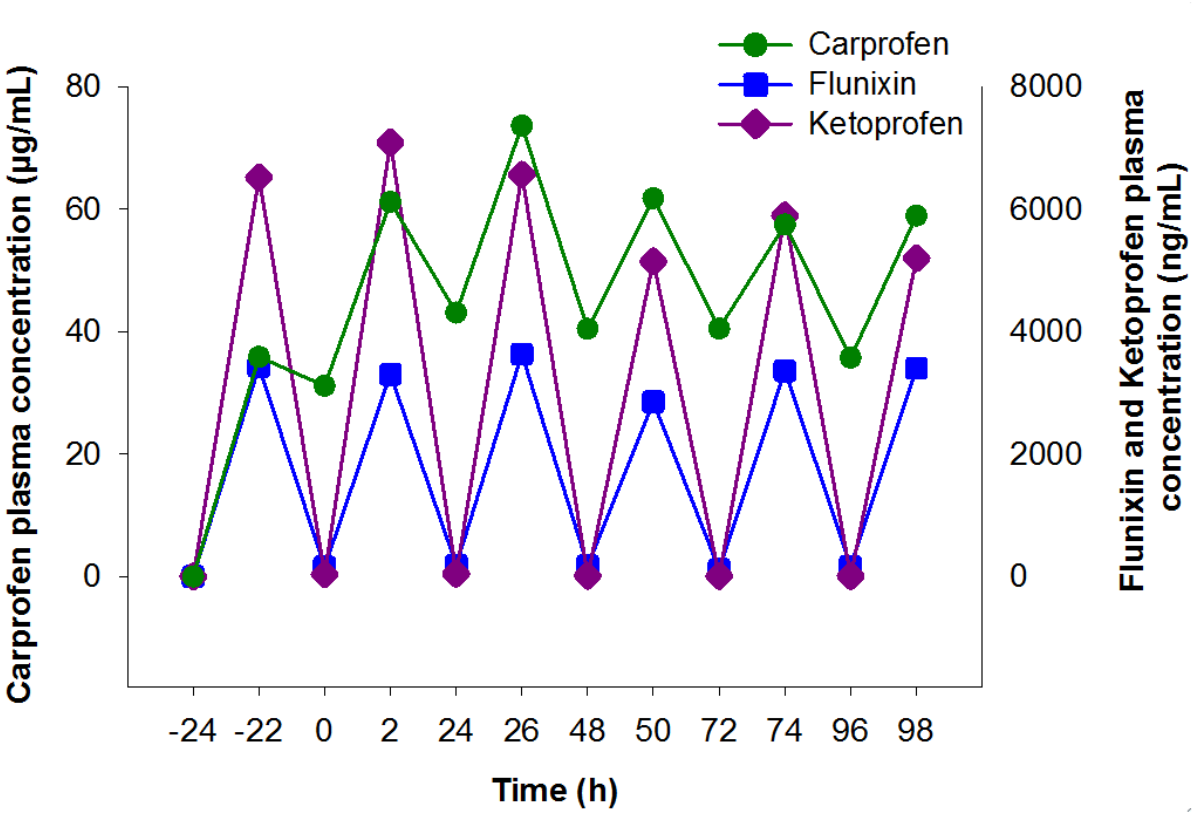

Figure 7: Bioavailability of the NSAIDs Carprofen, Ketoprofen and Flunixin at 10 minutes before and 2 h after daily oral administration over a period of 6 days in 40 sheep.

2.4.2. NSAID efficacy

Forelimb measurements Animals in all treatment groups showed an increase in limb circumference (maximum average increase of 1.8cm at 36 h, P=0.000), limb temperature (average increase of 3.3^{o}C at 3 h, P= 0.0002) and lameness (maximum average score increase of 1.4 at 24 h, P= 0.000) following the oil of turpentine injection. Placebo sheep placed more weight on their oil of turpentine-treated forelimbs at 3 h (P<0.05, 7.56 ±1.26kg) compared with other treatments (carprofen 3.19±1.05kg, ketoprofen 1.08 ±0.48kg, flunixin 4.61 ± 0.97kg). Weight borne on the forelimbs returned to the range seen in saline-treated ewes at 9 h for flunixin-treated sheep. In contrast, ketoprofen-treated animals still placed significantly less weight on their limbs (P=0.02) at 9 h, with weight-bearing returning to normal at 24 h for ketoprofen- and carprofen-treated animals. Limb lifting returned to pre-oil of turpentine injection times at 6 h for flunixin-treated sheep and at 24 h for carprofen- and ketoprofen-treated sheep (Fig 8).

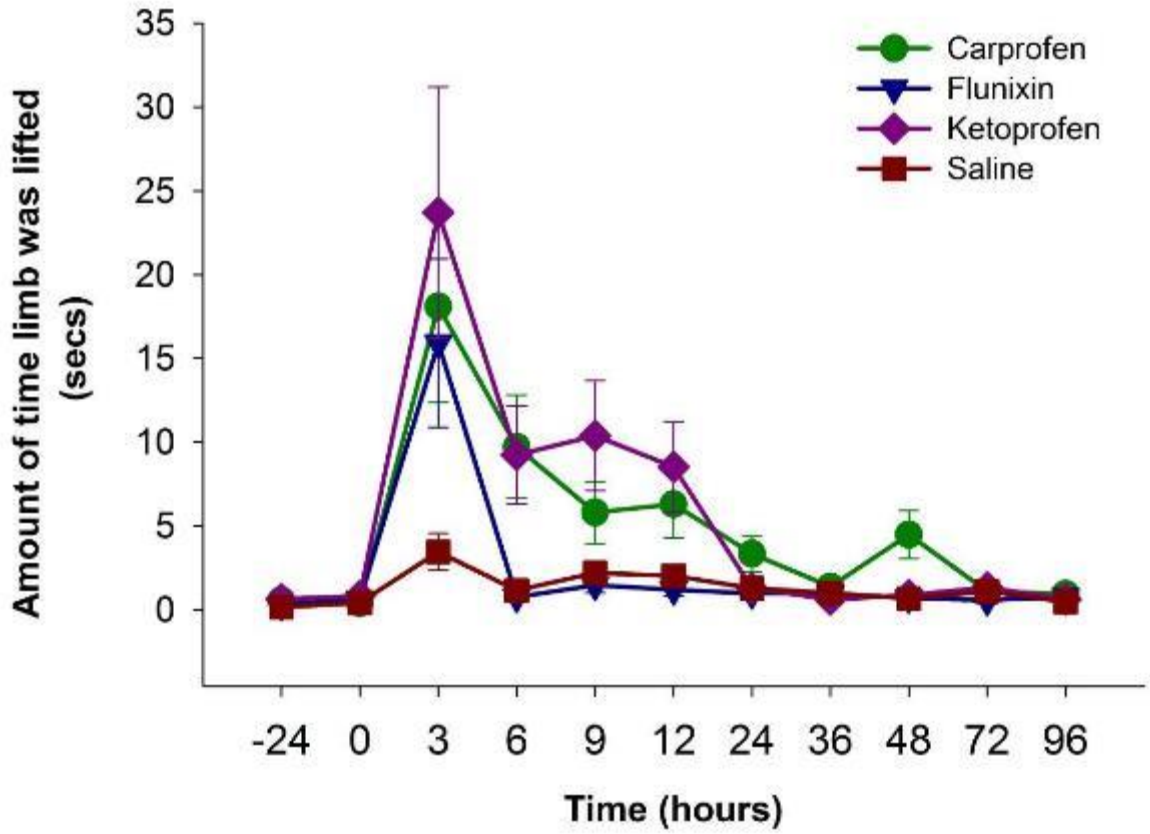

Figure 8: Amount of time the limb injected with oil of turpentine was lifted off the ground. Limb lift measurement involved observing the sheep's forelimbs for 30 seconds and recording how long weight was not borne on each limb during that time.

Lameness observations
Animals receiving flunixin had lower lameness scores than placebo sheep at 12, 24 and 48 h (P= 0.017, 0.029 and 0.025 respectively) and animals receiving ketoprofen had lower lameness scores then placebo animals at 24 and 48 h (P= 0.046 and 0.027 respectively). However at 3 h ketoprofen treated animals exhibited more lameness then placebo animals (P=0.027) and carprofen and flunixin treated animals at 9 h (P= 0.027 and 0.008 respectively).

*Body temperatur*e
Oil of turpentine injection induced moderate fever, with rectal temperature of >40 ºC recorded 24 h after injection. Across time, sheep receiving ketoprofen tended to have higher body temperatures than other groups (P = 0.08). NSAIDs did not limit the fever response, in comparison with the placebo.

Haematology
There were no significant effects of treatment on white blood cell count, neutrophil, lymphocyte and neutrophil/lymphocyte ratio.

Cortisol
Cortisol concentrations were significantly elevated at 30 min after injection for carprofen-treated animals (P=0.0013) compared to other time points. There were no overall differences in cortisol concentrations between the treatment groups; however, the treatment by time

effect (P=0.07) approached significance and a significant time effect (P=<0.0001) was observed.

2.5. Discussion

This study addressed the bioavailability and therapeutic efficacy of orally administered carprofen, ketoprofen and flunixin in sheep subjected to oil of turpentine-induced lameness. The three NSAIDs tested were present in blood at concentrations inferred to be therapeutic from studies in other species by 2 h after administration[13, 28-31].The hypothesis could not be supported as evidence of anti-inflammatory or anti-pyretic activity was not observed for any NSAID administered, this is due to an anomalous response of placebo sheep to oil of turpentine injection.

As there is little information on therapeutic concentrations of carprofen, ketoprofen and flunixin in sheep, assumptions are generally drawn from comparisons with other species. Therapeutic concentrations of carprofen in plasma are 10-17 µg/mL for dogs[28], 7µg/mL for cats[29] and above 1.5 µg/mL for horses[30]. The average carprofen concentrations measured in the current study 2 h after administration were 30 - 80 µg/mL. Minimum carprofen concentrations 24 h after each administration were between 31.0 - 45.9 µg/mL. These relatively high concentrations are in line with the reported long half-life of carprofen in sheep[15] and are likely to have contributed to the high concentration seen each day at the 2 h time point after re-administration of the drug. Reported therapeutic concentrations for flunixin are 0.2-0.9 µg/mL in horses[13]. In the current study, plasma concentrations 2 h after administration were 2.6 - 4.1 µg/mL for flunixin and 0.1 - 0.78 µg/mL for its metabolised residue 5-hydroxy flunixin. Therapeutic levels have been reported for ketoprofen enantiomers in pigs, with concentrations of 26.7 µg/mL for S-ketoprofen and 1.6 µg/mL for R-ketoprofen[31]. Maximum concentrations of 7.42 mg/L for S-ketoprofen and 2.55 mg/L for R-ketoprofen when administered orally in pigs[32]. The concentration of ketoprofen in plasma was 4.5 - 8.4 µg/mL in our sheep 2 h after administration, R-ketoprofen has been recorded as the most prominent enantiomer found in sheep [17]. Based on these results, oral administration of these NSAIDs at twice the standard parental dose shown to be therapeutic in other species was expected to be within the therapeutic range for sheep, however, it is recommended that work should be done to identify the therapeutic levels in sheep.

Therapeutic efficacy of the NSAIDs used in this study has been observed in a number of studies in sheep and cattle. Ketoprofen has been shown to reduce pain-related behaviours when administered orally at a dose of 3 mg/kg through milk in dairy calves that have undergone dehorning[33]. The efficacy of flunixin and carprofen for reducing pain-related behaviours and physiological responses to painful husbandry procedures in sheep has been demonstrated in several studies[4, 6, 18].

The administration of NSAIDs as oral medication at double the I.V. dose may have the potential to relieve pain in sheep, provided they are bioavailable in therapeutic doses, as extrapolated from other animals. However, there may be a need to administer the oral dose prior to painful procedures as previous studies that have recorded the pharmacokinetics of orally administered NSAIDs have found that they take longer to reach their maximum concentrations than when administered I.V. [23, 26, 34]. From the current study it appears that, when given orally at double the standard dose, carprofen and flunixin should be administered 2 h before sheep undergo painful procedures to allow the NSAIDs to be present at the putative therapeutic dose levels. Although carprofen maintained high concentrations in plasma 24 h following administration, both flunixin and ketoprofen had dropped to concentrations of < 0.1 µg /mL by this time. These pharmacokinetics have implications for the use of flunixin and ketoprofen as a single dose drench following painful husbandry procedures. As the pain from castration can last several days[2], repeated

administration of flunixin and ketoprofen would be required in order to provide pain relief for this duration. However further pharmacokinetic studies on oral administration for these NSAIDs are required to determine the maximum plasma concentration levels and the time required to reach them.
The lameness and weight-bearing responses of the sheep receiving saline in the current study were anomalous. In the paper describing development of this model[27], all sheep receiving 0.1 mL of oil of turpentine were reluctant to bear weight on the treated limb, had raised skin temperature and inflammation. In the current study, oil of turpentine again increased limb temperatures, induced fever and caused swelling of the limb, however saline-treated animals continued to place weight on the oil of turpentine-treated limb. In contrast to these animals, and in line with previous observations, NSAID-treated sheep bore less weight on the oil of turpentine-treated limbs than on contralateral control limbs. One possible reason for the different pain responses between the two studies could be differences in the breeds and age of sheep used. Colditz et al. (2011) used mature Merino x Romney ewes whereas 18-month-old Merinos ewes were the subjects in this study. It is recognised that pain response can vary greatly depending on species, sex, age, body size and even between individual animals[35, 36]. Differences in sheep breeds have been observed with the analgesic effects of xylazine in response to limb threshold pressure technique was measured by Ley, Waterman and Livingston [37], with Cluns sheep having a greater response than Welsh sheep and Swaledale sheep having an intermediate response.

The reaction of placebo sheep to the oil of turpentine injection, where they placed weight on the affected limb, may be an alternate pain response to removing weight from the limb. Chronic pain in animals can lead to them keeping the painful region still[38]. Placing weight on the injected limb may also be a means for modulating pain[39]. A further mechanism that might be operating in the placebo group could be an increase in the threshold to noxious stimulation due to stress-induced analgesia[40]. However saline-treated animals did not have the elevated cortisol concentrations that would have been expected as a response to oil of turpentine injection. Due to the anomalous response from the placebo sheep whilst using this model of pain, the model should be further validated. It is important that a model of pain in sheep be developed and validated in order to allow researchers to determine the efficacy of analgesics.

In conclusion, carprofen, flunixin and ketoprofen administered orally achieved putative therapeutic concentrations within 2 h. Although we were able to assess the pain response to an oil of turpentine injection using the measures applied, we saw little evidence of therapeutic efficacy of the NSAIDs. This was due to contrasting results to previous results with this model, the placebo sheep in this study continued to place weight on the oil of turpentine-treated limb despite tissue inflammation. A dose response study using the oil of turpentine injection as well as a more detailed pharmacokinetic data following oral administration would be valuable for each of the NSAIDs in sheep. In view of the importance of developing efficient and practical methods for on-farm management of pain associated with surgical husbandry procedures, further work to determine efficacy of these NSAIDs is warranted.

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Appendix 2 - Palatability and pharmacokinetics of flunixin when administered to sheep through feed

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3.1. Abstract

Applying analgesics to feed is a potentially easy method of providing pain-relief to sheep and lambs that undergo painful husbandry procedures. To be effective, the medicated feed needs to be readily accepted by sheep and its consumption needs to result in therapeutic concentrations of the drug. In the present experiment, pelleted feed was supplemented with flunixin (4.0 mg/kg live weight) and offered to eight sheep. To test the palatability of flunixin, the individually penned sheep were offered normal feed and feed supplemented with flunixin in separate troughs for two consecutive days. A trend for a day by feed-type (control versus flunixin supplemented) interaction suggested that sheep may have had an initial mild aversion to pellets supplemented with flunixin on the first day of exposure, however, by on the second day there was no difference in consumption of normal feed and feed supplemented with flunixin. To test pharmacokinetics, sheep were offered 800 g of flunixin supplemented feed for a 12 h period. Blood samples were taken over 48 h and plasma drug concentrations were determined using ultra-high-pressure liquid chromatography, negative electrospray ionisation and tandem mass spectrometry. The mean ± S.D. time required to reach maximum concentration was 6.00 ± 4.14 h and ranged from 1 to 12 h. Average maximum plasma concentration was 1.78 ± 0.48 µg/mL and ranged from 1.61 to 2.80 µg/mL. The average half-life of flunixin was 7.95 ± 0.77 h and there was a mean residence time of 13.62 ± 1.17 h. Free access to flunixin supplemented feed enabled all sheep to obtain inferred therapeutic concentrations of flunixin in plasma within 6 h of starting to consume the feed. Provision of an analgesic in feed may be an alternative practical method for providing pain relief to sheep.

3.2. Introduction

Flunixin meglumine is a potent non-steroidal anti-inflammatory drug (NSAID) that is commonly used in veterinary medicine for its anti-inflammatory, analgesic and antipyretic properties. Like other NSAIDs, flunixin reduces inflammation by inhibiting cyclooxygenase and, in turn, decreasing the production of prostaglandins (Cheng et al., 1998b), which are important inflammatory mediators. Flunixin is known to be effective at relieving pain in various domesticated species such as horses (Keegan et al., 2008; Toutain et al., 1994) and cattle (Currah et al., 2009) and is currently registered for use for these animals in the USA, Europe and Australia. Although flunixin has also been shown to be effective for pain relief in sheep (Paull et al., 2007; Welsh 1995), there are currently no NSAIDs registered in Australia for use in sheep. Pain relief can be impractical and costly to administer to livestock raised in extensive systems due to the necessity for repeated application over time and the limited availability of registered drugs (Lizarraga & Chambers, 2012). A potential practical method of providing pain relief is through oral administration, allowing farmers to either provide NSAIDs as a drench or through feed in the form of granules or a liquid formulation. It is known that the rumen can decrease the bioavailability of NSAIDs if they are administered orally (Mosher et al., 2012; Odensvik 1995). In previous work to counteract the reduced bioavailability when administering NSAIDs orally to cattle the dose given was double compared with that recommended for parenteral administration (Coetzee et al., 2012).

Incorporation of flunixin to an animal's diet could possibly elicit a neophobic response or reduced feed intake if flunixin is unpalatable. Therefore the objectives of this study were 1) to test the palatability of flunixin and 2) determine the pharmacokinetics of flunixin in sheep plasma when feed supplemented with flunixin was offered. We hypothesised that all sheep would achieve therapeutic concentrations of flunixin in plasma when consuming feed supplemented with flunixin.

3.3. Materials and methods

3.3.1. Experimental animals

Nine, 2-year-old, maiden Merino ewes with mean live weight of 38.8 ± 2.83 kg (mean ± S.D.) were used in this study. Sheep were clinically healthy at the time of the study. Upon entry to the experiment the animals body condition was checked, they were then vaccinated with Glanvac® 6S B12 (Zoetis Animal Health, Australia) and drenched with Firstmectin (Virbac, Milperra, NSW, Australia), Flukazole C (Virbac, Milperra, NSW, Australia) and Rycozole (Novartis, North Ryde, NSW, Australia) at the manufacturers' recommended dose rates. Following vaccination and drenching the sheep were then monitored daily for any signs of ill health, such as behavioural and respiratory changes. There was a month between drenching treatments and the pharmacokinetic experiment. The sheep were housed in individual pens in a covered shed which was open on the North face and were in close proximity to allow visual and social interaction with other experimental animals. Animals were fed a complete pelleted ration (Ridley Agriproducts, Australia; 17 % crude protein dry matter; 9.04 MJ/kg dry matter). During acclimation, sheep were offered a small excess of feed over their previous day's intake (between 800 – 1000 g) supplemented with 100 g of oaten chaff daily so that some residual feed was left at the end of each day. Water was also provided ad-libitum. The experiment was undertaken at CSIRO's FD McMaster Laboratory, Armidale, New South Wales (NSW). The protocol and conduct of the experiment was approved by The CSIRO Armidale Animal Ethics Committee under the NSW Animal Research Act, 1985 (ARA 14/01).

3.3.2. Palatability test

One week prior to the start of the experiment, each animal was acclimatised to eating from two troughs within its pen and daily feed intake was monitored. The palatability test ran over 2 days; in the morning sheep were offered feed in two troughs, one containing 2 kg of the standard pelleted ration and the other containing 2 kg of the same standard pelleted diet supplemented with 20 mL (300 mg) of liquid flunixin (Flunixin Oral solution, 15 mg/mL, Pia Pharma Pty Ltd, Gladesville, NSW, Australia). The amount of flunixin added per kg of feed was equivalent to an approximate single dose for the live weight of each ewes (i.e. eating 1 kg of the supplemented feed would deliver 1 dose at 4 mg/kg body weight). The feed was prepared each morning by putting the liquid flunixin onto the pellets and thoroughly mixing them together in the trough; even incorporation of the liquid was characterised by the change in colour of the pellets. Following flunixin application the trough did not appear to be wet and there was no free liquid present at the bottom of the trough. Both troughs were placed into the pen simultaneously and the location of the trough containing flunixin supplemented feed was alternated for the second day of testing.

3.3.3. Pharmacokinetic protocol

After the palatability test, the ewes were kept in a paddock for a 2-week flush-out period. They were then returned to the same individual pens that were used for the palatability test, 1 week prior to the beginning of the pharmacokinetic experiment. The sheep were again fed the complete pelleted ration ad libitum supplemented with 100 g of oaten chaff once a day. The day prior to supplementation of feed with flunixin, sheep were weighed and had the wool clipped from their necks. To allow for intensive blood sampling, catheters were inserted aseptically in the left jugular vein using a 12 G catheter needle to puncture the vein. A piece of catheter tubing was then threaded through the needle and then, to ensure the catheter was inserted correctly, the line was flushed with heparinised saline and then liquid withdrawn until blood was seen flowing. Catheters were then re-flushed with heparinised saline. The catheter needle was removed and the line was sealed with a three-way tap adaptor containing a luer lock syringe port. The line was secured to the animal at the exit point with

Elastoplast tape, the remaining catheter tubing was then encased in 7.5 cm wide Elastoplast bandage which was gently wrapped around the sheep's neck.

On the day of the study, sheep were offered 800 g of feed containing a dose of flunixin (at a rate of 4.0 mg/kg live weight) adjusted for each animal's body weight. Flunixin was added to feed as described for the palatability test. The first sheep was presented with the flunixin supplemented feed at 0700 h and the remaining sheep were given their feed at 2 min intervals thereafter. Blood samples (10 mL) were collected before the flunixin supplemented feed was offered (0 h) and at 5, 10, 15, 20, 30, 45 min and 1, 2, 4, 6, 8, 12, 24, 36, 48 h relative to the time each sheep was first observed to have consumed some of the supplemented feed. Prior to the collection of each blood sample, 2 mL of blood was withdrawn from the catheter and discarded to ensure that fresh blood was collected. Blood samples were centrifuged (2000 × g) and the separated plasma collected and frozen at -20 ^{o}C. Residual feed remaining in the trough was weighed at each blood sampling time point until 12 h post-initial ingestion.

3.3.4. Plasma flunixin concentration determination

Plasma samples were transported frozen to Pia Pharma Pty Ltd, Gladesville, NSW for flunixin concentration determination using ultra-high-pressure liquid chromatography, negative electrospray ionisation and tandem mass spectrometry (UHPLC/ -ve ESI MS/MS). Each plasma sample was thawed to room temperature on the day of analysis. For determination, a 250 µL aliquot of each plasma sample was dispensed into a 2 mL polypropylene centrifuge tube. Flunixin-d3 internal standard (50 µL of 2.0 µg/mL flunixin-d3) was added and the sample mixed gently prior to the addition of 350 µL acetonitrile. The sample was vortexed (1 min) and centrifuged (13000 rpm/5 min) to remove any sediment. Type 1 water for UHPLC applications (0.5 mL) was then added to the extract and the mixture was filtered through a 0.45 µm filter prior to determination. An aliquot of sample extract (5 µL) was injected into an Eksigent® Ekspert™ ultraLC 100-XL Liquid Chromatograph fitted with a Supelco Ascentis® Express 50 x 2.1 mm, 2.7 µm analytical column maintained at 40 °C. A gradient elution program, based on a combination of 0.1 % formic acid and acetonitrile as mobile phase constituents operating at 0.4 mL min-1, resolved flunixin and flunixin-d3 (retention time of 2.5 min) from matrix interferences and endogenous sample components. The identity of peaks was predicted using an AB Sciex API 3200 triple-quadrupole mass spectrometer interfaced with the liquid chromatograph. The detector was configured with a proprietary turbo V source for desolvation and operated in negative electrospray ionisation mode (-4500 V), desolvation temperature 550 °C, for optimum analyte selectivity and sensitivity. The transitions for flunixin and flunixin-d3 were 295.1→191.0 and 298.2→254.0 respectively.

Matrix matched calibration standard solutions of flunixin were prepared at increasing concentrations between 10 and 4000 ng/mL in plasma from animals prior to treatment. The calibration curve was prepared by plotting the nominal flunixin concentration (x axis) against the determined peak area ratio of flunixin and flunxin-d3 for each calibrator. A correlation co-efficient (r) greater than 0.99 was required for the calibration curve to be used for quantitative purposes. Analyte concentrations were calculated using the peak area ratio of flunixin detected in each sample relative to the corresponding flunixin-d3 internal standard, and the regression equation of the calibration curve.

Method accuracy and precision were monitored with the inclusion of fortified quality control samples. Four plasma samples containing flunixin concentrations of 13.1, 328.5, 1314.1, 3942.3 ng/mL (n=3) were prepared on the day of the analysis. The mean percentage of accuracy was 90.8 % at lower limit of quantification (LLOQ) and 102.9 – 111.6 % at all other

concentrations. The coefficient of variation at LLOQ was 2.9 %, and 1.3-3.1 % at other concentrations. Quality control data were acceptable.

3.3.5. Statistics

Palatability data were analysed with R-Project (R, Boston, Massachusetts) using nlme package (Pinheiro et al., 2015) to perform a linear mixed model analysis. Fixed effects included feed type (flunixin present or absent), day (1 or 2), and location of flunixin supplemented feed trough (left or right) and the interaction of feed type by day. Sheep number was fitted as a random effect. Results are presented as mean ± S.D.. Data were tested for normality using the Shapiro-Wilk test. $P < 0.05$ was considered as statistically significant.

3.3.6. Pharmacokinetic analysis

Pharmacokinetic modelling of flunixin in plasma was performed using an open source pharmacokinetic program (PK Solver, China Pharmaceutical University, Nanjing, Jiangsu, China) (Zang et al., 2010). Using non-compartmental analysis, the maximum flunixin concentration (Cmax) in plasma, the time required to reach Cmax (Tmax), mean residence time (MRT) and elimination half-life (t1/2) were determined for each animal. The area under the concentration vs. time curve (AUC0–t) was calculated using the linear trapezoidal rule. Pharmacokinetic parameters were estimated for each animal from which mean values ± S.D. were calculated.

3.4. Results

3.4.1. Palatability

One ewe was excluded from data analysis as she did not consume any of the feed containing flunixin on either day. Location of the different feeds (left or right trough) had no effect ($P = 0.81$) on the amount of each feed (flunixin supplemented versus control) that was consumed. Although there was no main effect of feed type across days ($P = 0.10$), a trend was observed for the day by feed type interaction ($P = 0.08$). On day 1, animals consumed on average 551.14 ± 446.68 g more of the control feed than the flunixin supplemented feed ($P = 0.02$). Whereas on day 2 there were no differences observed in the consumption of control feed and feed supplemented with flunixin ($P = 0.95$). On day 2, consumption of control feed decreased on average by 489.79 ± 468.53 from the quantity consumed on day 1 ($P = 0.03$). Consumption of feed supplemented with flunixin was comparable on days 1 and 2 ($P = 0.73$, Table 5).

Table 5: Palatability test results (mean ± S.D.) for the effect of interaction of feed type (flunixin supplemented or control) by day (1 or 2) and location (left or right) on feed intake (g) in eight sheep.

	Day 1		Day 2	
Location	Control	Flunixin supplemented	Control	Flunixin supplemented
Left	906.00 ± 426.28	451.75 ± 338.76	707.88 ± 451.40	590.67 ± 518.79
Right	1158.88 ± 330.73	562.83 ± 358.93	364.50 ± 446.95	561.63 ± 309.77
Mean	1050.50 ± 365.42	499.36 ± 348.68[A]	560.71 ± 449.62[A]	574.07 ± 414.19

[A] mean is significantly different to the control feed on day 1 ($P < 0.05$)

3.4.2. Pharmacokinetics

The sheep took between 8 and 12 h to consume the total 800 g of flunixin supplemented feed on offer. Most of the sheep spread meals throughout the day except for ewe 466 who ate 350 g of feed in the first 5 min and ewe 627 who consumed 332.5 g in the last 4 h of the 12 h period. Flunixin was absorbed rapidly, all sheep had detectable plasma concentrations (> 20 ng/mL) at 10 min after initial consumption of supplemented feed with the exception of one animal (ewe 627), who only ate 21.5 g of feed in the first 10 min.

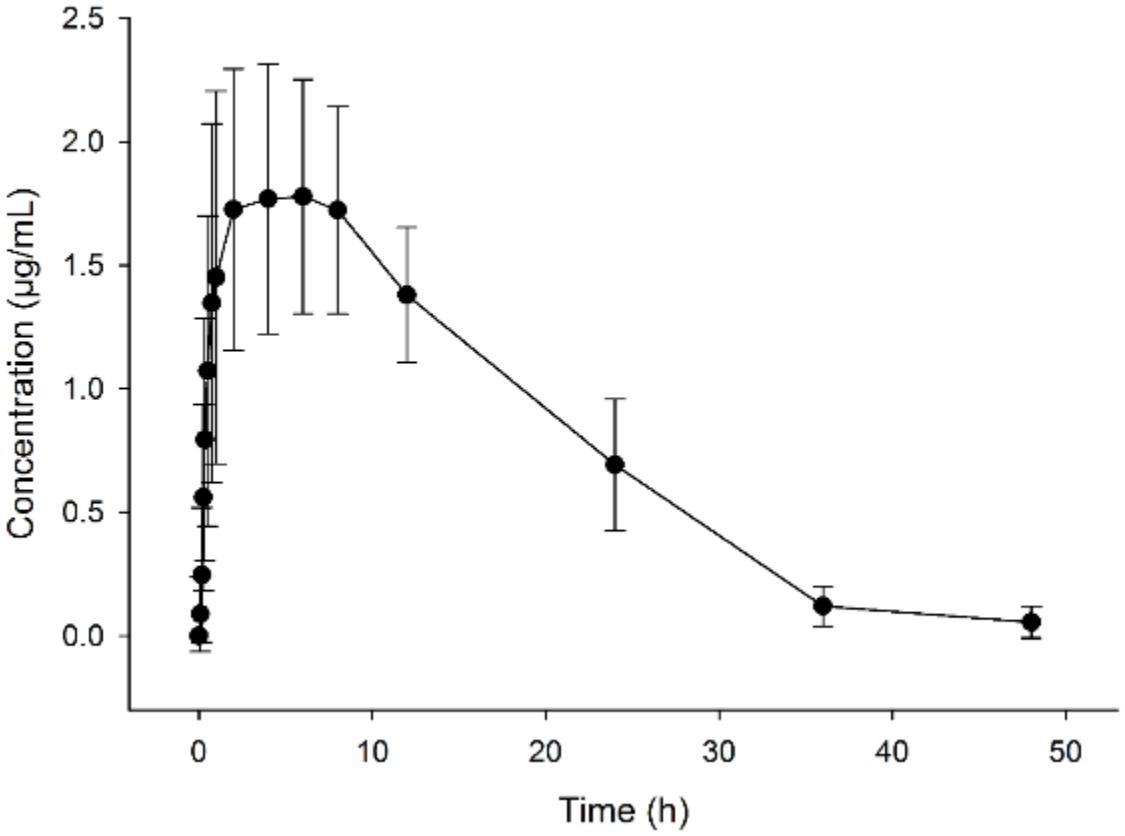

Figure 9: Flunixin in plasma concentration time curve (means ± S.D.) of eight sheep over a 48 h period following administration of flunixin (4.0 mg/kg) through pelleted feed.

All sheep started to eat within a few minutes after the provision of feed. There was large variability between sheep in the amount of feed that was consumed at each time-point (Table 6).

Table 6: Variability in feed intake of eight sheep that were offered 800 g of flunixin supplemented feed for a 12 h period.

Time feed was weighed (h)	Average intake (g) ± S.D.	Median (g)	Range (g)
0.08	174.69 ± 112.12	205.75	21.50 - 357.50
0.17	26.25 ± 29.66	18.50	0.00 - 71.00
0.25	18.63 ± 16.01	15.00	0.00 - 48.00
0.33	6.06 ± 12.27	1.00	0.00 - 36.00
0.50	23.44 ± 17.30	25.00	0.00 - 50.50
0.75	13.13 ± 20.79	6.50	0.00 - 62.50
1	6.19 ± 15.72	0.00	0.00 - 45.00
2	91.38 ± 58.90	75.25	32.00 - 211.00
4	151.63 ± 39.12	148.25	89.00 - 220.00
6	141.56 ± 56.38	149.75	68.00 - 211.00
8	88.38 ± 59.54	71.25	31.00 - 194.00
12	58.69 ± 114.15	8.50	0.00 - 332.50

When animals had free access to feed, the majority of sheep (7 out of 8) achieved plasma flunixin concentrations above 1.0 µg/mL within 2 h of starting to consume the supplemented feed, with maximum concentrations (between 1.33 and 2.80 µg/mL) being reached on average by 6 h. Flunixin concentration time curve (mean ± S.D.) in all sheep plasma over a period of 48 h is shown in Figure 3. This led to a large variability in the Tmax, which ranged from 1 to 12 h. The Cmax average was 1.78 ± 0.48 µg/mL and the flunixin meglumine plasma $t_{1/2}$ was 7.95 ± 2.19 h (Table 7).

Table 7: Flunixin pharmacokinetic parameters following oral administration through 800 g of pelleted feed to eight sheep at a dose rate of 4 mg/kg.

Flunixin non-compartmental pharmacokinetics (PK Solver, China Pharmaceutical University, Nanjing, Jiangsu, China) (Zang et al., 2010), t1/2 = elimination half-life, Cmax = the maximum flunixin concentration in plasma, Tmax = the time required to reach Cmax, AUC0–t = area under the concentration vs. time curve and MRT = mean residence time.

	Sheep ID								
Parameter (units)	305	466	580	612	621	627	648	732	Mean ± S.D.
$t_{1/2}$, h	4.59	5.39	8.23	6.29	7.31	4.85	11.04	5.19	7.95 ± 2.19
Tmax, h	8.00	1.00	6.00	6.00	2.00	12.00	12.00	4.00	6.00 ± 4.14
Cmax, µg/mL	2.39	1.61	2.18	1.89	2.16	1.33	1.63	2.80	1.78 ± 0.48
AUC0-t, µg/mL*h	29.96	38.00	38.21	40.99	42.78	31.84	42.75	36.05	37.68 ± 4.77
MRT, h	9.36	14.34	13.36	13.43	12.98	15.80	19.48	9.32	13.59 ± 3.31

3.5. Discussion

Concentrations measured in this study were somewhat lower compared with those reported in our previous study (Marini et al., 2015) where flunixin concentration in plasma reached values between 2.6 - 4.1 µg/mL 2 h after a single oral dose (4 mg/kg) in sheep. Reports of therapeutic concentrations of flunixin in farm animals are limited, however, Toutain et al., (1994) reported therapeutic effects in horses when plasma concentrations reached 0.2 - 0.9 µg/mL. The results of the current study suggest that the plasma flunixin concentrations achieved following consumption of supplemented feed may be within the therapeutic range for sheep.

Although displaying an initial (day 1) preference for control pelleted feed over flunixin-supplemented feed, there was no overall feed preference effect observed. The initial preference of control pelleted feed may have been due to the novelty of the odour or flavour of flunixin. Odour and flavour help sheep distinguish different types of feed and they are more likely to eat novel feeds that contains some familiar flavours (Hinch et al., 2004; Launchbaugh et al., 1997). Sheep are known to avoid novel feed types for several days before they start to consume it (Chapple et al., 1987). Adding flunixin to a feed with which the ewes were familiar, may have reduced any neophobia. With the exception of one ewe who did not consume any feed supplemented with flunixin over the two days, the intake of supplemented and control feeds was similar on the second day of testing.

In sheep, the pharmacokinetics of flunixin has been investigated following intramuscular and intravenous administration (Cheng et al., 1998a; Welsh et al., 1993). When administered intravenously, the elimination half-life of flunixin meglumine has been reported to be 2.48 h (Cheng et al., 1998a) and 3.83 h (Welsh et al., 1993). The elimination half-life observed in the current study (following oral administration) was longer (7.95 ± 2.19 h). Differences were also observed for the MRT of flunixin following intravenous versus oral administration, with MRT in plasma being 3.20 ± 0.18 h (Cheng et al., 1998a) compared with 13.59 ± 3.31 h in the current study. When flunixin is administered intramuscularly and intravenously it is typically given as a bolus dose, which permits a uniform pattern of absorption and elimination to occur. The longer half-life and mean retention time observed in this study is likely due to animals consuming their dose of flunixin over an extended period of time, rather than as a bolus. The AUC observed in the current study (37.62 ± 4.77 µg/mL*h) was similar to that reported by Cheng et al., (1998a) (30.61 ± 3.41 µg/mL*h). It is probable that our higher AUC was due to the higher dose rate used in our study.

The pharmacokinetics of orally administered flunixin has been studied in goats (Königsson et al. 2003), horses (Pellegrini-Masini et al., 2004; Welsh et al., 1992) and cattle (Odensvik, 1995). Following oral administration of a bolus dose in the absence of feed in these species, flunixin is absorbed rapidly and concentrations can still be detected up to 30 h after administration (Königsson et al., 2003; Odensvik, 1995). Horses that had ad libitum access to hay following the oral administration of flunixin had a slower absorption of flunixin and a lower Cmax although concentrations of flunixin in plasma were maintained for longer when animals had access to feed compared with when they were fasted (Welsh et al. 1992). The AUC was not significantly different when animals were fasted or non-fasted, suggesting that the absorption of flunixin is not affected by the presence of feed. In the current study, flunixin was found to be absorbed rapidly when consumed with feed, with detectable levels present within 10 min in sheep that consumed more than 22 g. Flunixin concentrations remained detectable, but were below inferred therapeutic concentrations, for 36-40 h after consumption of the flunixin supplemented feed ceased (Figure 9). Currently there are no toxicity data for flunixin in sheep, however the animals used in this study did not show any visible side effects as a result of consuming flunixin supplemented feed.

Previous work in cattle by Odensvik, (1995; 1998) showed that oral administration of flunixin (2.2 mg/kg) as a granule inhibited the production of prostaglandin $PGF_2\alpha$ by up to 60 %, which was as effective as the standard therapeutic dose of flunixin (2.2 mg/kg) used parenterally. Although the authors did not directly measure the effectiveness of oral flunixin at reducing inflammation, they concluded that an anti-inflammatory effect was likely to occur due to reduced production of $PGF_2\alpha$ which acts as a pro-inflammatory factor following injury (Ricciotti & FitzGerald, 2011). Although further studies are required it is expected that oral administration of flunixin could provide effective pain relief in sheep.

In conclusion, results of this study demonstrate that when flunixin is administered orally through feed to sheep it is absorbed rapidly into the bloodstream and despite variability in consumption rates of feed, all sheep reached inferred therapeutics concentrations of flunixin within 6 h of starting to consume the feed. Further studies are required to investigate potential binding of flunixin to various feed components and potential impacts that such binding may have on toxicity. The possible mild aversion to feed supplemented with flunixin on day 1 did not persist on day 2 indicating that the medicated feed is readily accepted by sheep. Supplementation of feed with flunixin may provide a practical way to provide pain relief to sheep prior to and after painful husbandry procedures thus eliminating the need for multiple injections, reducing handling stress and minimising labour requirements.

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