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Mechanical nociceptive threshold testing in *Bos indicus* bull calves.

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Pain assessment in cattle is difficult, but is essential to assess the effect of surgery and analgesic drugs. Nociceptive threshold testing is an objective pain assessment tool that has not been described in *Bos indicus* cattle. A technique for mechanical nociceptive threshold testing was developed for use in *Bos indicus* cattle undergoing surgical castration to evaluate post-operative pain.

Abstract.

The aim of this prospective, controlled, randomised trial was to develop a technique for mechanical nociceptive threshold testing (NTT) to assess pain in *Bos indicus* bull calves undergoing surgical castration. Analgesia was provided by 0.5 mg/kg subcutaneous (SC) meloxicam (M) and/or 2 mg/kg of intra-testicular and SC (at the surgery sites) lidocaine (L). Forty-eight Brahman bull calves at 6-8 months of age were divided into six study groups, each with eight animals: no surgery control; surgical castration (C) without analgesia; C and $M_{pre\text{-}op}$; C and $M_{post\text{-}op}$; C, L and $M_{post\text{-}op}$; C and L. Mechanical NTT was performed the day before surgery (day -1) and on days 1, 2, 6, 10 and 13 after surgery. A handheld manual pneumatic device with a 1 mm (diameter) blunt pin was used to deliver a mechanical stimulus to a maximum of 27 Newtons (N) either side of the most dorsal aspect of the sacrum. The most frequent responses to the mechanical stimulus were lifting or kicking of the leg on the same side as the stimulus (31%) and stepping away from the stimulus (24.9 %). Data were analysed with a mixed effect linear model with the nociceptive threshold (NT) as the response variable and day and analgesic treatment as predictors ($p < 0.05$ was considered significant). For all groups, there was a trend toward decreasing NT over the study period but there were no significant differences between groups. Step down model selection with day, batch and treatment terms revealed a significant effect of day ($p < 0.001$) and batch ($p = 0.007$). Mechanical NTT for assessment of pain in *Bos indicus* bull calves requires further refinement to determine if this is a useful method of pain assessment.

Introduction

In Australia it is standard practice for cattle on extensive farms in remote regions to be subjected to husbandry procedures, including castration, when they are first mustered between six and twelve months of age. These cattle have had virtually no contact with people and can be very difficult to handle. Despite evidence that castration causes pain in cattle (Petherick, Small *et al.* 2014; Stafford and Mellor 2005) it is common for surgical castration to be performed without anaesthesia or analgesia (Coetzee, Nutsch *et al.* 2010; deOliveira, Luna *et al.* 2014; Petherick, Small *et al.* 2014). This approach is consistent with Meat & Livestock Australia's *Best Practice Guidelines for Routine Husbandry Procedures in Beef Cattle* (Newman 2007). Developing a simple and economical analgesic technique for castration in a field environment is problematic as the efficacy of any technique can only be determined if pain assessment tools are reliable. Furthermore, the analgesic technique must be easy to administer, safe for the operator and the animal, efficacious and cost-effective if it is to be adopted by industry.

Extensive pain assessment research has been undertaken in other bovids. In *Bos taurus* cattle, a range of pain assessment tools have been employed: plasma or serum cortisol concentrations, average daily weight gain, feed intake, morbidity and mortality, acute phase protein concentrations, pedometry, heart rate variability, rectal and eye temperatures, and behavioural observations (Coetzee 2013; Stafford and Mellor 2005). None of these measures are considered entirely reliable for the assessment of pain in *Bos taurus* cattle (Coetzee 2013; Stafford and Mellor 2005). *Bos indicus* cattle are less habituated to stockpeople, are more excitable (Grandin 1998) and behaviourally and physiologically more reactive to handling than *Bos taurus* (Zavy, Juniewicz *et al.* 1992). So extrapolating data from *Bos taurus* to *Bos indicus* should be done with caution. A unidimensional composite pain scale for assessing acute post-operative pain in *Bos indicus* cattle has recently been validated (deOliveira, Luna *et al.* 2014). The scale involves observation and assessment of locomotion, interactive behaviours, activity, appetite and nine miscellaneous behaviours (e.g. licking the surgical wound and wagging the tail abruptly and repeatedly). A score of >4 out of 10 was identified as the point at which analgesia should be administered (deOliveira, Luna *et al.* 2014). Although this publication represents significant progress in the assessment of pain in this species, it stands that pain is a complex and multi-dimensional phenomenon and it should be assessed with more than a single method in animals. In a detailed comparison of castration techniques in *Bos indicus* cattle receiving either saline or ketoprofen, the authors concluded that behavioural data should be assessed in addition to non-behavioural parameters in order to effectively assess pain relief especially in mature cattle (Petherick, Small *et al.* 2014).

Nociceptive threshold testing (NTT) involves the application of a potentially painful stimulus to an animal to elicit a specific response (Beecher 1957). The point at which the response occurs is quantified as a number (e.g. Newtons or degrees Celsius) and comparison of thresholds before and after an intervention, such as a painful procedure and/or the administration of analgesic drugs, enable the identification of hyperalgesia in an animal. This method of pain assessment was first described over 50 years ago (Beecher 1957) but has more recently been employed for the investigation of pain and analgesic drug efficacy in a number of species including chickens (Hothersall, Caplen *et al.* 2011), dairy cows (Raundal, Andersen *et al.* 2014), horses (Love, Murrell *et al.* 2011), donkeys (Grint, Whay *et al.* 2014), pigs (Sandercock, Gibson *et al.* 2009), cats (Dixon, Taylor *et al.* 2007; Taylor, Robertson *et al.* 2007) dogs (Bergadano, Andersen *et al.* 2006; Bergadano, Andersen *et al.* 2009) and sheep (Musk, Murdoch *et al.* 2014). Different stimuli have been used for NTT but contemporary literature most commonly refers to the use of thermal and mechanical stimuli (Love, Murrell *et al.* 2011; Musk, Murdoch *et al.* 2014; Raundal, Andersen *et al.* 2014). In dairy cattle, both mechanical and thermal stimuli have been used for NTT but the superiority of one technique over another has not been determined (Rasmussen, Fogsgaard *et al.* 2011; Raundal, Andersen *et al.* 2014).

The aim of this study was to develop a technique for mechanical NTT for pain assessment in *Bos indicus* cattle. This study was part of a larger project investigating pain assessment and analgesia in *Bos indicus* bull calves undergoing surgical castration. It was hypothesised that mechanical NTT would be able to differentiate animals that had been administered analgesia at the time of castration from those that had not. It was expected that the NT would remain unchanged from day -1 in the NC group, decrease in the C group (a hyperalgesic effect) and increase in the C+$M_{pre\text{-}op}$, C+$M_{post\text{-}op}$, C+L and C+L+$M_{post\text{-}op}$ groups (a hypoalgesic effect).

Materials and Methods

Approval for this study was granted by the Animal Ethics Committee of Murdoch University (Permit number R2551/13) within the guidelines of the National Health and Medical Research Council of Australia Code of Practice for the Care and Use of Animals for Scientific Purposes (AustralianGovernment 2004).

Forty eight Brahman bull calves from an extensive cattle station in the north-west of Australia (the Pilbara region) were studied in two batches during two discrete periods of time in winter. The first batch arrived at the Murdoch University farm in the south-west of the country, and consisted of *Bos indicus* animals at an estimated age of eight months and a mean weight of 186 (±18) kg. The second batch arrived 21 days later, and consisted of *Bos indicus* crosses, that were approximately six months of age, with a mean weight of 145 (±17) kg. Each batch was managed in the same way: animals arrived at the University farm eight days prior to surgery to allow for acclimatisation. The cattle had not been handled by the farmer and were not accustomed to contact with humans. Access to oaten hay and water was allowed *ad lib.* and a complete mixed ration was fed daily (EasyBeef pellets, Milne AgriGroup Pty Ltd, Perth, Western Australia) at approximately 3% of bodyweight. As part of the University's biosecurity protocol, the animals were tested for *Bovine viral diarrhoea virus* on arrival at the University farm.

The cattle were randomly divided into six groups of eight animals: no surgery control (NC); surgical castration (C) without analgesia; surgical castration with pre-operative meloxicam (C+$M_{pre\text{-}op}$); surgical castration with post-operative meloxicam (C+$M_{post\text{-}op}$); surgical castration with lidocaine (C+L); and surgical castration with lidocaine and post-operative meloxicam (C+L+$M_{post\text{-}op}$). Lidocaine was administered by injection into each testicle and in the subcutaneous tissue at each incision site on the scrotum five minutes prior to the first incision (2 mg/kg, Ilium Lignocaine 20, 20 mg/mL, Troy Laboratories, Glendenning, NSW, Australia) in the C+L and the C+L+$M_{post\text{-}op}$ groups. Meloxicam was administered by subcutaneous injection over the shoulder (0.5 mg/kg, Ilium Meloxicam 20, 20 mg/mL, Troy Laboratories, Glendenning, NSW, Australia) either 30 minutes prior to castration ($M_{pre\text{-}op}$) in the C+$M_{pre\text{-}op}$ group, or immediately afterwards ($M_{post\text{-}op}$) in the C+$M_{post\text{-}op}$ and C+L+$M_{post\text{-}op}$ groups. Surgical castration was performed with the animal restrained in a crush and head bail. The scrotum was cleaned with 4% chlorhexidine surgical scrub and an open castration with a scalpel blade was performed as described and recommended by Meat & Livestock Australia (Newman 2007).

To develop a technique for mechanical NTT in six- to eight-month-old *Bos indicus* bull calves, the characteristics of the pin contacting the skin, the site of application of the stimulus and a repertoire of responses needed to be defined. In the initial testing stages animals from the first batch were held freestanding in a crush on day -6. A handheld manual pneumatic device (ProdPro, Topcat Metrology Ltd) was used to deliver a mechanical stimulus to a maximum of 27 Newtons (N). The handheld actuator was always positioned so the pin was perpendicular to the skin. Three pin diameters were tested: 1 mm, 3 mm and 6 mm blunt tips.

To determine the site associated with the most consistent response, the stimulus was applied to the scrotum, the skin over the gluteal muscles, the medial aspect of the hock and either side of the most dorsal prominence of the sacrum. It became apparent that many of the animals responded as the pin initially made contact with the skin so these responses were ignored and a preload force of 2-4 N was applied and maintained for approximately three seconds at the beginning of each test. During this preload period, contact with the skin was maintained. As soon as a response was observed, the test was terminated and the force (in Newtons) was recorded. If there was no response to the stimulus and the maximum capacity of the device was reached, the result was recorded as 27 N.

Mechanical NTT was performed the day before surgery (day -1) and on days 1, 2, 6, 10 and 13 after surgery. The operator (THH) stood on a raised platform next to a race which held six animals at a time and a second observer stood a few metres away at ground level. The race was 0.68 metres wide and 6.2 metres long. There was a clear view through the side rails which were 1.65 metres high. Each test was performed five times with at least five minutes between each test. To minimise skin damage, the first, third and fifth test were performed on the left side and the second and fourth were performed on the right side of the sacrum. The mean of the five tests was used for analyses. Results were not included within this set of five tests if they were more than two standard deviations from the mean of the five.

The animals were monitored daily for eight days prior to surgery and for each of 13 post-operative days for general health and wellbeing. Multiple assessments were undertaken in this period: weight, daily activity with pedometry, behavioural observations, appetite, interaction with other animals, plasma cortisol concentration and inspection of the wound. These data are not presented here. If an animal was considered in pain or unwell, independent veterinary attention was sought. Rescue analgesia was meloxicam by subcutaneous injection (0.5 mg/kg, Ilium Meloxicam 20, 20 mg/mL, Troy Laboratories, Glendenning, NSW, Australia).

Data were analysed with a mixed effect linear model with nociceptive threshold (NT) as the response variable and day and analgesic treatment as predictors. A step down model selection with day, batch and treatment terms was also performed. To focus on the acute post-operative period, the difference in NT between day -1 and day 1 and also between day -1 and day 2 was isolated within each study group and each batch of animals, and a one way analysis of variance (ANOVA) was performed. $p < 0.05$ was considered significant. Data are expressed as mean (± SD).

Results

All animals were negative for *Bovine viral diarrhoea virus*. During the technique development phase on day -6, a response could not be repeatedly elicited with the two widest blunt pin tips as the maximum force of 27 N was often reached during those tests. The smaller 1 mm diameter blunt pin tip was associated with the highest response rate (data not shown) so was chosen for the study. The site of application associated with the most consistent response was approximately 3 cm either side of the most dorsal prominence of the midline of the sacrum (Fig. 1). Responses to mark the end-point of the test included stepping away from the stimulus, kicking or lifting the leg closest to the site of the stimulus, flexing the pelvis (‘hunching’), turning the head towards the site of the stimulus, or swishing the tail.

A total of 1440 tests were attempted: five repetitions in each data set on 288 occasions (two batches of 24 animals assessed on six test days). One NT result was excluded in 59 of the 288 data sets (20%) and two NT results were excluded in nine

(3%) of them as they were more than 2 standard deviations from the mean. The most frequent responses to the mechanical stimulus were lifting or kicking of the leg on the same side as the stimulus (31%) and stepping away from the stimulus (24.9%). Tests could not be performed for 4.3% of the 288 data sets because the animal was either agitated and did not stop reacting after the application of the initial preload, or was recumbent in the race. There was no response to 27 N of stimulus in 12.7% of tests (Table 1). The ramp speed was 4.1 (± 1.9) N/second.

There were no statistically significant differences in the NTs between groups on any test day (Table 2 and Fig. 1). Step down model selection with day, batch and treatment terms revealed a significant effect of day (p < 0.001) and batch (p = 0.007). The effect of day is evident as there is a trend for the NT to decrease in all groups over the study period (Fig. 2). In batch 1, the NT on day -1 was higher than in batch 2 (24.4 ± 2 vs. 18.7 ± 5, p < 0.001) although the NT on day 13 was comparable (16.3 ± 5.3 vs. 14.7 ± 2.2, p = 0.573) (Fig. 3). There was no significant difference between study groups or batches of animals when comparing the change in NT from day -1 to either day 1 or day 2 (Table 3).

Veterinary attention was sought for three animals (one in each of the $C+M_{pre\text{-}op}$ (on day 1), $C+M_{post\text{-}op}$ (on day 11) and $C+L+M_{post\text{-}op}$ (on day 10) groups) with local wound infections. Oxytetracycline (20 mg/kg subcutaneous injection, Alamycin LA, 200 mg/mL, Norbrook Laboratories, U.K.) was administered to these animals and the surgical wounds were cleaned with chlorhexidine solution. Rescue analgesia was not administered, as the infections resolved within four days. These animals were not removed from the study.

Discussion

The technique developed for mechanical NTT in this study was not able to distinguish animals that underwent surgery with or without analgesia from those that had not been castrated. It was expected that the NT would remain unchanged from day -1 in the NC group, decrease in the C group (a hyperalgesic effect) and increase in the $C+M_{pre\text{-}op}$, $C+M_{post\text{-}op}$, C+L and $C+L+M_{post\text{-}op}$ groups (a hypoalgesic effect). There are a number of factors that may influence the results of NTT and these include animal, personnel and equipment factors.

The animals in this study were not accustomed to human contact and although an acclimatisation period was incorporated into the study, there were no efforts to accustom the animals to human interactions or to the NTT regime. The aim was to simulate the field environment but it is possible (perhaps likely) that the stress associated with handling was significant enough to alter the responses to NTT. Mechanical NTT has been reported to be more consistent in sheep that are familiar with the testing equipment (Welsh and Nolan 1995) while NTs did not vary with different environmental conditions (including distracters) in donkeys (Grint, Whay *et al.* 2014). The presence of conspecific animals may also impact upon responses to NTT as isolated individuals may become distressed and alter their behaviour. The presence of a companion did not alter the NT in donkeys (Grint, Beths *et al.* 2014) but cattle may be different. In this study it was ensured that companion animals were always in the race during testing to minimise any distress from isolation. For future studies of this nature it would be better to minimise stress for the animals to diminish the impact of this confounding factor on their responses to nociceptive threshold testing. Stress associated with interactions between personnel and the unfamiliar environment may have overridden the animal's ability to respond in a meaningful way to the NTT regime in this study.

There are a number of personnel factors that will impact upon responses to NTT. A handheld prod was used in this study and this meant the operator was standing within one metre of the animal at the time of testing. This proximity may have influenced the response to NTT. A remote position may be more appropriate, especially in a prey species that is not accustomed to humans, although this would necessitate a period of close contact with personnel when equipment was positioned and secured on the animal. It is postulated that if a remote system could be arranged, the animal's responses would be more natural and less influenced by fear. Furthermore, the handheld device was not automated so the ramp speed varied for each test. Although a single operator performed all the tests (THH), and they were guided by real time measurements of the force, the reliance on the operator to generate the applied force meant that it was impossible to standardise it for all animals and all measurements. The mechanical NT was higher when the ramp speed was faster, to a maximum of 1.2 N/sec, in donkeys (Grint, Beths *et al.* 2014) and those authors suggest that the ramp speed must be constant within a study and between studies if valid comparisons of mechanical NTT are to be made. If the ramp speed is too fast, the influence of the reaction time of the operator becomes more significant and if it is too slow, the likelihood of distractions occurring during the test increases (Haussler and Erb 2006). In our study, the ramp speed was 4.1 (± 1.9) N/sec. There are only a few studies that our ramp speed can be compared to and so it is difficult to conclude what impact the ramp speed, that was used in this study, had on the results obtained. The final personnel factor contributing to the results was the ability of the operator of the device to consistently interpret the animals' responses to the stimulus. In this study, the operator stood on a raised platform alongside the race and applied the force from above the sacrum. This meant that at times, it was difficult to see the entire repertoire of responses. For this reason, a second observer was positioned a few metres away. This second person could more readily observe a kick or leg lift. Given this design, it is probable that the latency in observing the response and terminating the stimulus, along with the relatively fast ramp speed, increased the NTs in this study. In addition, this situation exposes the data to operator bias. Bias during NTT is introduced when the operator determines the ramp speed, the duration of stimulation and subjectively determines the end-point of the test (Grigg, Robichaud Ii *et al.* 2007). To overcome bias and to refine the technique for mechanical NTT in *Bos indicus* cattle, using equipment that enabled the delivery of the force at a constant rate would be desirable. Furthermore, fixing the actuator to the animal so the stimulus can be delivered remotely and the observer can be distanced from the animal may also minimise operator bias.

Testing prior to the study proper was performed to determine the most appropriate site for application of the stimulus and to define the end-point of the test. This pilot work is essential when developing a method for NTT in any species (Love, Murrell *et al.* 2011; Sandercock, Gibson *et al.* 2009). Ideally the site of application of the stimulus should be close to the surgical site but we found the site that was associated with the most consistent set of responses was on the skin over the sacrum. The scrotum itself was not a suitable testing site as it was difficult to position the actuator perpendicular to the skin and the tissue tension is relatively low. Other studies emphasise the importance of positioning of the actuator as being perpendicular to the skin with minimal amounts of distensible tissue underneath the actuator, to reduce the spread of pressure across a larger area (Love, Murrell *et al.* 2011). Therefore the sacrum seemed most suitable in this study as the soft tissue tension is high and it was safe for the operator to access the site with the hand held actuator.

The type of stimulus will also impact the response to a test. Previously, thermal NTT in sheep caused second and third degree burns with epidermal and dermal necrosis seven days after testing (Musk, Murdoch *et al.* 2014) so in this study only a mechanical stimulus was used to avoid any tissue damage at the site of application of the stimulus. Furthermore, efforts were made to avoid tissue damage by alternating the site of stimulation between either side of the sacrum. There was no gross evidence of skin damage in the study animals (data not shown). Ideally multiple threshold testing modalities would be used simultaneously to assess pain and analgesic efficacy in animals but this approach increases the complexity of the physical testing and prolongs the time taken to perform a set of tests (Dixon, Robertson *et al.* 2002). Confining the animals for a longer period of time in turn increase the potential for extraneous factors to influence the response repertoire of the animal. Moreover, many nociceptive neurones will respond to more than one type of stimulus. C and the three A fibre nociceptor subtypes all respond to mechanical stimuli, so the stimulus used in this study should have been appropriate to differentiate our study groups (Djouhri and Lawson 2004).

The importance of the result demonstrating a significant effect of batch is unknown. For logistical reasons, the animals were studied in two batches and the intention was that the demographic of the animals in each batch would be comparable. The second batch of cattle was approximately two months younger than the first batch. It is not known if age impacts upon an individual's response to NTT. The significant effect of study day on the results is also of interest. Over time, the NT decreased and ordinarily this trend would be interpreted as evidence of hyperalgesia developing in an animal. As this effect was across all study groups, and not different between study groups, it is possible that the response of the animal was overshadowed by distress at being held in a race and the close presence of humans. Although we did not observe any gross evidence of skin damage, it is also possible that the test sites on either side of the sacrum became hypersensitive and the thresholds decreased during the course of the study for this reason.

The size of each study group was determined by reference to previous publications where n = 7 or 8 is standard (Grint, Beths *et al.* 2014; Musk, Murdoch *et al.* 2014; Rasmussen, Fogsgaard *et al.* 2011). Given the excitable temperament of unhandled and untrained *Bos indicus* cattle, there was a lot of variation in our results and our group size may have been too small to detect a difference. Acclimatising the animals to humans and careful preparation of the animals prior to a study such as this may be beneficial if they become accustomed to the study environment and personnel.

The study was deliberately designed to include two control groups: animals that were not castrated and animals that were castrated without analgesia. For the former control group, the reaction of *Bos indicus* bull calves to the same type and amount of interaction with personnel as animals undergoing castration, was investigated. A no-analgesia control group was included for two reasons. First, without a no-analgesia control group, the various treatment groups can only be compared to animals that have not been surgically castrated. The no-analgesia group serves as a baseline that is essential to answer the fundamental research question of this study which is "can mechanical NTT differentiate *Bos indicus* bull calves who received analgesia for pain associated with castration from those that did not receive any analgesia?" If our study had only included a no-surgery control group, then a tempting conclusion from our results might have been that all of the animals provided with analgesia could not be differentiated from the animals that were not surgically castrated. Or to put it another way, these results would have provided support for a pain relieving effect of the analgesics used in this study on *Bos indicus* calves. By including the no-analgesia control group, our results have instead come to the opposite conclusion, which is that using mechanical NTT as described earlier, the analgesics used in this study, at the doses chosen, provided no measurable benefit to animals that were surgically castrated. The second reason a no-analgesia control group was needed is because surgical castration is commonly performed in the field without any analgesia in extensively-managed Australian *Bos indicus* bull calves. This means that this experimental group serves as a reflection of what is currently (rightly or wrongly) an industry standard for these animals.

To develop a reliable and valid pain assessment tool requires an understanding of the response to a certain test (mechanical NTT) in a pain-free animal and in an animal that has been exposed to a painful stimulus (surgical castration) with and without analgesia (Slingsby 2010). The inclusion of a no-analgesia group was justified on the basis that intervention levels were defined so rescue analgesia could be administered, the animals were closely monitored for 13 post-operative days, and the usefulness of the results of this study should promote reduction and refinement in any future work of this nature in this species. Furthermore, the paucity of information on pain assessment and analgesic efficacy in *Bos indicus* cattle creates a need for well-designed studies with appropriate control groups (Slingsby 2010).

For NTT, the stimulus should be easy to apply and repeatable, the behavioural response should be clear and easily identifiable, and the stimulus should produce no long lasting harm to the animal (Beecher 1957). In this study, the aim was to use mechanical NTT for investigation into the analgesic efficacy of lidocaine and/or meloxicam for surgical castration of *Bos indicus* bull calves. Despite developing a test that met the criteria of Beecher (1957), and that was contextualised for the species in question and the study environment, further refinements are required to investigate analgesic drug efficacy and pain in extensively farmed *Bos indicus* bull calves with mechanical NTT. These refinements should be centred around habituation of the animals to the study environment, personnel and the equipment, standardising the ramp speed through the actuator, identifying the ideal site of application of the stimulus and application of the stimulus remotely by fixing the actuator to the animal. Finally, although the technique developed for mechanical NTT in this study was not able to distinguish animals that underwent surgery with or without analgesia from those that had not been castrated, it is likely that this species is capable of experiencing pain so further work investigating tools for pain assessment is warranted.

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