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EFFECT OF HOT-IRON BRANDING ON THE CHEEK OR UPPER HIND LIMB ON CORTISOL LEVELS, BEHAVIOUR AND PRODUCTION IN FEEDLOT CALVES

by

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CONTENTS

LIST OF ABBREVIATIONS

ADG   Average daily gain

ACTH   Adrenocorticotropic hormone

ANOVA   Analysis of variance

AUC   Area under the curve

FGM   Faecal glucocorticoid metabolite

CRH   Corticotropin-releasing hormone

HPA axis   Hypothalamic-pituitary-adrenocortical axis

SD   Standard deviation

SNS   Sympathetic nervous system

11,17-DOA   11,17-Dioxoandrostane

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SUMMARY

EFFECT OF HOT-IRON BRANDING ON THE CHEEK OR UPPER HIND LIMB ON CORTISOL LEVELS, BEHAVIOUR AND PRODUCTION IN FEEDLOT CALVES

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Hot-iron branding on the upper hind limb in beef feedlot calves is a usual part of the processing procedure at arrival in many feedlots in South Africa. However, cheek branding is becoming more popular in feedlots due to ease of restraint and better visibility of the brand mark. However, the welfare aspects of cheek branding compared to leg branding have not been investigated. By monitoring physiological and behavioural markers this study was conducted to determine potential stress-related differences between feedlot calves which are branded on the leg, those branded on the cheek and control (sham-branded) animals.

Thirty weaned crossbred beef calves, recently arrived at a commercial feedlot, were habituated to handling in a crush for seven days and then randomly divided into three groups of ten. Group A was branded on the cheek, Group B on the hind leg and Group C was sham-branded with a room temperature iron. Group C was further divided into two groups (n = 5) that were either sham-branded on the cheek or on the leg. Blood was collected at 0, 30, 60, 90 and 120 minutes after branding for serum cortisol determination with a commercial radioimmunoassay kit. In addition, faeces were collected from all animals on the day after

arrival, as well as at day seven, two and one prior to branding, on the day of branding and for seven consecutive days. Faecal glucocorticoid metabolite levels were determined using a group-specific enzyme immunoassay measuring 11,17-dioxoandrostanes (11,17-DOA). Other outcomes monitored included behavioural traits for seven days after branding, individual average daily weight gain (ADG), morbidity and mortality during the feedlot period and histopathological evaluation of the brand mark on the skin after slaughter.

Faecal 11,17-DOA concentrations were higher the day after transport than the day after branding ($P < 0.001$) indicating higher circulating levels of cortisol during transport. Compared to pre-branding levels, serum cortisol was not significantly higher at 30 minutes after branding in all three groups but hormone levels dropped significantly at 60 minutes post-branding. Concentrations at 90 and 120 minutes were not significantly different from pre-branding concentrations in all three groups. The quantifiable rise seen between 60 and 90 minutes post-branding could have been due to the extended time (up to 2.5 hours) spent in the crush. There were no statistically significant differences in blood cortisol or faecal 11,17-DOA between the cheek, leg or sham branded groups at any time. Vocalization occurred more frequently at the time of branding in the cheek branded group than in either the leg branded ($P = 0.030$) or the control group ($P < 0.001$). There were also no significant differences in other behavioural indices between the three groups. Faint brand marks could be seen at the time of slaughter (74 days after branding) on five animals from the leg branded group. No cheek brands were visible. No scarring was seen on histopathological examination. No significant differences were seen in the ADG, and there were no morbidities or mortalities.

In conclusion, using the methods described, there were no obvious differences in serum cortisol levels, faecal 11,17-DOA levels, behavioural indicators of pain or production outcomes between feedlot calves branded on the cheek, the leg, or sham branded.

1   INTRODUCTION

Cattle are an important food source in South Africa as well as on a global scale. In South Africa, beef farming systems include extensive free-range production of weaner calves and breeding stock, intensive feedlot production as well as rural cattle herds. Stock theft is a serious problem in all of these production systems and threatens the livelihood of smaller farms. To help control stock theft South African legislation (Animal Identification Act, 2002 (Act no 6 of 2002)) (National Department of Agriculture, 2009) states that all livestock must be identified using a form of permanent identification that is registered with the relevant authorities (Mogajane, 2010).

1.1   Marking of feedlot cattle

Hot iron branding, freeze branding and tattooing are the most commonly used methods of livestock identification in South Africa (National Department of Agriculture, 2008). Studies have shown that freeze branding leads to a less severe acute pain response (Schwartzkopf-Genswein et al., 1997 a, b, 1998; Lay et al. 1992 a, b, c), but is deemed impractical in South Africa because of the time taken to prepare the site for marking. Most South African cattle feedlots make use of hot-iron branding and it is part of the processing procedure on arrival of the feedlot animals (D. Ford, pers. comm., 2010). Except for the neck area, which is reserved for controlled diseases, branding for identification purposes can be done on any visible part of the body (National Department of Agriculture, 2008; Olivier, 2001; Du Plessis et al., 2012). Many feedlots brand cattle on the upper hind limb, proximal to the hock. However, cheek branding has become popular in many feedlots as restraint of the head is easier, leading to less movement and smudging of the brand (D. Ford, pers. comm., 2010). Other advantages

include ease of access to the area, a decreased loss of hide value with faulty leg branding and easier visibility for identification of individual animals in large groups especially during transportation. On the other hand, cheek branding has been negatively perceived by various stakeholders and has become an emotive issue with some cases resulting in litigation (D. Ford, pers. comm., 2010). Although a few studies attempted to compare the welfare aspects of hot-iron branding to freeze branding (Schwartzkopf-Genswein et al., 1997 a, b, c, 1998; Lay et al. 1992 a, b, c) an objective comparison between the different branding sites with respect to animal welfare has not yet been done. Therefore, an objective comparison between cheek and leg branding with regard to differences in pain was proposed.

1.2   Pain in animals

The behavioural response, as well as physiological and biochemical changes in animals, support the fact that animals experience pain as an unpleasant sensation, just as do humans (Molony and Kent, 1997; Hellebrekers, 2000; Martini et al., 2000; Mellor et al., 2000; Gregory, 2004). Pain can be seen as a stressor and its effect can lead to changes in neural endocrine functions such as cortisol release, but also changes in the autonomous nervous system, the mental state and the behaviour of the animal. When nociceptors of free nerve endings are stimulated, pain results. Nociceptors may be stimulated by chemical, mechanical or thermal injuries. These impulses pass through the spinal cord to the brainstem and thalamus, and perception of pain is then due to activation of areas of the cerebral cortex via the thalamus (Hudson et al., 2008). Pain warns animals that tissue damage might occur, is occurring or has occurred, thereby encouraging avoidance of the cause. Pain-induced stress e.g. caused by husbandry procedures, refers to the physiological responses which reflect the

emotional and physical aspects of the unpleasant experience whether it is actual, potential or perceived (Mellor and Stafford, 1999; Mellor et al., 2000).

1.2.1 The stress response

Stress is the biological response elicited when an individual perceives a threat to its homeostasis (Moberg and Mench, 2000). It is important to note that physiological changes will occur due to a stressful event, whether that event is physical or psychological, real or perceived (Gregory, 2004). A stressful event will lead to the activation of the hypothalamus resulting in secretion of corticotropin-releasing hormone (CRH). Corticotropin-releasing hormone in turn leads to secretion of adrenocorticotropic hormone (ACTH) from the anterior pituitary, which regulates secretion of glucocorticoids (mostly either cortisol or corticosterone) from the adrenal gland. Glucocorticoids will supply substrates needed during repair and recovery by promoting gluconeogenesis and proteolysis in skeletal muscle and the liver, exerting anti-inflammatory effects and suppressing the immune system, and also reducing pain via anti-inflammatory effects (Gregory, 2004). Glucocorticoids also potentiate and support the synthesis of adrenalin (Moberg and Mench, 2000). Chronic elevation of glucocorticoids results in protein catabolism, hyperglycaemia, immune suppression, increased susceptibility to infection and depression (Moberg and Mench, 2000). For this reason prolonged high cortisol levels will result in negative feedback, inhibiting both CRH and ACTH secretion (Dukes, 2004).

The central autonomic network in the brain regulates the autonomic nervous system. This network supplies signals to both vagal and sympathetic neurones which mediate autonomic functions (Gregory, 2004). Activation of the sympathetic nervous system (SNS) after a

stressful event will initiate metabolic changes to aid the animal in either fight or flight (Gregory, 2004). Catecholamines (mainly adrenalin and noradrenalin) are released from the adrenal medulla to aid this process and result in actions such as mobilisation of glycogen from the liver and free fatty acids from adipose tissue, dilation of the pupils, increased heart rate and contractility, vasoconstriction in body regions not necessary for fight or flight and reduced upper intestinal secretions and motility (Gregory, 2004; Stewart et al., 2010).

1.2.2 Assessment of pain in cattle

Several studies have been done on various husbandry procedures with the goal of improving welfare in cattle. This has led to the evaluation of various ways to measure the pain response in cattle.

After injury, local inflammation will result in an acute phase reaction. This reaction will lead to changes in acute phase proteins and lymphocyte numbers (Ceciliani et al., 2012; Coetzee, 2012). These markers can be measured and can give an indication of the pain response due to a painful husbandry procedure. Haptoglobin levels and lymphocyte numbers have been used with varying success to assess the pain after tail docking of dairy cows (Eicher et al., 2000). Other acute phase proteins, e.g. serum amyloid A, α_1-acid glycoprotein, lipopolysaccharide binding protein and fibrinogen, have also been used with success in the past (Mellor et al., 2000; Earley and Crowe, 2002; Coetzee, 2011). Substance P is a neuropeptide that acts like a neurotransmitter and neuromodulator and is associated with inflammatory processes and pain. It has been shown that increases in substance P concentration may be indicative of pain in cattle after surgical castration and dehorning (Coetzee et al., 2008, 2012).

The autonomic nervous system, particularly the sympathetic division, will also respond to a painful stimulus. The sympathetic activity will result in release of catecholamines from the adrenal medulla. Sympathetic nervous system activity can be measured using certain parameters, e.g. heart rate variability, eye pupil diameter, skin resistance, peripheral blood flow and catecholamine concentrations (Stewart et al., 2010). There have not been many studies measuring these effects in cattle, probably due to complicated sample collection, processing and high cost of assays. However it has been shown that catecholamine concentrations increase after branding (Lay et al., 1992a, b), dehorning and castration (Mellor et al., 2002). After activation of the SNS, blood is diverted from cutaneous capillary beds due to vasoconstriction which results in decreased skin temperature. The decrease in temperature can be measured by infrared thermography in the region of the lachrymal caruncle of the eye, and has proved to be a practical, non-invasive measure of SNS activity in cattle (Mellor and Stafford, 1999; Stewart et al. 2010). Variation in intervals between heart beats can be measured as heart rate variability; this variation and not just the rate can be used to assess the balance between sympathetic and parasympathetic tone, thus providing more information about autonomic activity (von Borell et al., 2007). Electrodermal activity is the measurement of the electrical resistance of the skin which will change due to sympathetic stimulation (Coetzee, 2011). Electroencephalography (EEG) has been used to reflect changes in cortical function of the brain. The cerebral cortex has an integral role in the conscious perception of pain and EEG can be used to examine the acute noxiousness of painful events (Ong, et al., 1997; Jongman, et al., 2000; Gibson, et al., 2007; Bergamasco, et al., 2011).

Measuring changes in the hypothalamic-pituitary-adrenocortical (HPA) axis is the standard approach to the study of pain-induced stress and welfare in farm animals (Mormède, et al., 2007). Changes associated with the HPA axis can be measured and assessed as the HPA axis

is activated during the stress response. Increases in plasma CRH, ACTH, or cortisol/corticosterone, determined in various matrices such as blood, saliva, urine, or faeces can be indicative of exposure to an aversive situation (Molony et al., 1995; Morisse et al., 1995; Schwartzkopf-Genswein et al., 1997a; Mellor and Stafford, 1999; Möstl et al., 2002; Negrão et al., 2004; Saco et al., 2008; Heinrich et al., 2009; Coetzee, 2011).

Certain behavioural changes can also be indicative of a painful procedure or experience. Vocalization has been used in the past to assess pain, although it has been found to be unreliable in individual animals but may be useful when assessing a group of animals (Watts and Stookey, 1999). Other behaviours which have been shown to indicate pain in cattle include kicking, tail flicking, falling, bruxism, feed intake and subsequent handling ease (Lay et al., 1992b; Molony and Kent, 1997; Schwartzkopf-Genswein et al., 1998; Hellebrekers, 2000; Coetzee, 2011). However, because most of these measurements are subjective, observer accuracy and consistency are generally poor (Schwartzkopf-Genswein et al., 1998; Coetzee, 2011). Chute exit speed may be indicative of pain but it may also be due to a desire to escape (Coetzee, 2011).

Various production variables have also been used in the past to assess the effects of pain, e.g. weight gain, morbidity, mortality, antibiotic treatment and dry matter intake. However, these are usually used to assess the long term effects of pain and are not good indicators of acute responses to pain (Schwartzkopf-Genswein et al., 1997c; Coetzee, 2011).

1.3   Determination of glucocorticoids in blood

Glucocorticoid concentration can accurately be measured in blood in a variety of species, including cattle (Doornenbal et al., 1988, Morton et al., 1995), and have widely been used as a measurement of pain-induced stress in ruminants (Coetzee, 2011). Baseline cortisol levels in cattle vary between individual animals, with breed, stage of growth, age, reproductive status, lactation stage and diurnal variation due to the circadian rhythm (Grandin, 1997). Normal reference values have a wide variation and normal baseline concentration can vary from a low of 27.6 nmol/l to a high of 67.5 nmol/l (Doornenbal et al., 1988; Smith, 2009). Cortisol levels will increase with the intensity of the stressor in a graded way (Moberg and Mench, 2000), and one may be able to interpret the peak cortisol concentration, duration of peak response, time until levels return to pre-treatment values and the integrated cortisol response by means of a single summary measure, the area under the curve (AUC) (Cunningham and Bradley, 2007; Coetzee et al., 2008; Coetzee, 2011). However, cortisol responses are difficult to interpret at the upper and lower range. At the lower range it may be difficult to see differences between treatment and control group, and at the upper range cortisol does not increase relative to the severity of a stressor (Coetzee, 2011). This is also known as a "ceiling effect", where cortisol levels are elevated to such an extent that relatively small differences between stressors are obscured (Lay et al., 1992b; Molony and Kent, 1997; Stafford and Mellor, 2005; Coetzee et al., 2007, Coetzee, 2011).

Alterations in blood cortisol values can also occur due to natural circadian rhythms or the pulsatile release of cortisol (Hopster et al., 1999; Mellor et al., 2000). The HPA axis is extremely sensitive to environmental changes and when these changes are not controlled in a research study, measurements of adrenocortical response may be obscured. Repeated

sampling, the handling experience, as well as individual differences will affect changes in blood cortisol (Hopster et al., 1999). It is thus important when measuring blood cortisol levels that the method and timing of blood collection should be considered and standardised (Hopster et al., 1999).

1.4   Determination of glucocorticoids in faeces

After a stressful event glucocorticoids released from the adrenal cortex are transported in blood, metabolized in the liver and other organs and then excreted via the urine, faeces and saliva (Von der Ohe and Servheen, 2002). Metabolites then enter the intestine via the bile and combine with digesta as they pass through the intestine (Morrow et al., 2002). 11,17-dioxoandrostane (11,17-DOA), produced after bacterial degradation of these metabolites, is the predominant cortisol excretory product in ruminant faeces and can be an accurate measure of recent adrenal function in ruminants (Möstl et al., 1999; Palme et al., 1999; Möstl et al., 2002).

While blood cortisol levels are affected by circadian variations and other short term fluctuations, faecal cortisol metabolite levels are not, and are considered independent of the time of day that the sample is collected. Faecal glucocorticoid metabolites (FGM) will show a smoothed average of the cortisol released into blood 12-24 hours previously (Palme and Möstl, 1997; Palme et al., 1999; Morrow et al., 2002; Möstl et al., 2002). Faecal glucocorticoid metabolite levels therefore reflect the total amount of cortisol excreted and can thus provide a more stable estimation than a single blood concentration which can change within minutes (Möstl and Palme, 2002). However, the time interval between collection of faecal samples and analysis is critical due to bacterial degradation of metabolites with a

resultant increase in 11,17-DOA concentrations (Möstl et al., 1999). Samples should therefore be collected directly from the rectum or immediately after defaecation (Morrow et al., 2002; Möstl et al., 2002). Collection of faeces does not in itself elicit as great a stress response as blood collection does which may result in an increase in cortisol levels. However, measured concentrations of FGM are, unlike blood cortisol levels, affected by diet (Morrow et al., 2002; Von der Ohe and Servheen, 2002; Millspaugh, 2003), as levels of carbohydrates and proteins are reported to influence the amount of metabolites found. Another important factor which should be taken into account is gastrointestinal transit time. There is a time delay for FGM concentrations to reach a peak due to the time taken for digesta to pass between the bile duct and the rectum and thus will vary with diet and between individuals (Morrow et al., 2002). Diets high in fibre, leading to an increased transit time, will lead to decreased 11,17-DOA levels, whilst diets low in fibre will lead to increased levels of FGM (Von der Ohe and Servheen, 2002). Cows on pasture during lactation have a faster rate of passage and peak FGM levels are reached in approximately 8.6 hours compared to dry cows on a concentrate diet with peak levels reached at 14.8 hours after ACTH injection (Morrow et al., 2002).

1.5   Behavioural measurements of pain in cattle

In cattle the long term effect of branding is difficult to measure and physiological indicators such as blood cortisol levels are generally not regarded as a good indicator of chronic, low grade pain, as the response is short-lived (Molony et al., 1995; Mellor and Stafford, 1999). As an alternative, pain-related behavioural alterations can be quantified, and will be better indicators of the long term effects of a painful procedure. Typical behavioural categories

monitored in this regard are as follows (Lay et al., 1992b; Molony and Kent, 1997; Schwartzkopf-Genswein et al., 1998; Mellor and Stafford, 1999; Hudson et al., 2008):

i)   Automatic responses, i.e. withdrawal, decreased feed intake, bruxism, tail flicking;

ii)   Mechanisms that decrease pain and assist in healing, i.e. lying down, decreased movement, decreased interaction;

iii)   Mechanisms which will stop other animals/persons inflicting pain, i.e. vocalization and defensive actions (biting and kicking);

iv)   Learning mechanisms, i.e. behaviour change to avoid recurrence

The most commonly used behavioural indicators of pain in cattle include amount of movement, interaction with other animals, feed intake, responsiveness to stimuli, change in posture, bruxism and grooming behaviour (Schwartzkopf-Genswein et al., 1998; Hudson et al., 2008). Behaviours recorded in response to hot-iron branding of the hind leg are tail-flicking, kicking, falling and vocalization (Schwartzkopf-Genswein et al., 1998).

Vocalization is thought to be a response to stress which acts as a warning of danger or elicits aid from others (Molony and Kent, 1997). Frequency or occurrence of vocalization can be indicative of pain and distress (Coetzee et al., 2008) but it may also be influenced by stressful environments or other factors such as presence of other animals, social interaction, mother-calf and feeding behaviour (Watts and Stookey, 1999). Vocalization as a measure of pain is thus highly variable and conflicting results have been found in previous studies (Lay et al., 1992b, c; Watts and Stookey, 1999).

1.6 The effect of branding

There have been previous studies done to determine the differences between freeze branding and hot-iron branding, which have used various behavioural and neuro-endocrine parameters to assess the pain response (Schwartzkopf-Genswein et al., 1997 a, b, c, 1998; Lay et al. 1992 a, b, c). It was shown that hot-iron branding leads to a more severe acute pain response compared to freeze branding. Measurements such as cortisol concentrations, head movement, chute exit force, tail flicking, kicking, falling and vocalizing indicated that hot-iron branded animals may have a more acute pain response (Schwartzkopf-Genswein et. al, 1997a,b; Schwartzkopf-Genswein et al., 1998), but that freeze branding may lead to a more lingering pain, since freeze branded animals needed more handling pressure to enter the chute (Schwartzkopf-Genswein et al. 1998). Other studies showed that adrenalin levels, heart rates and escape avoidance behaviours were increased in hot-iron branded cattle, indicating a more severe acute pain response (Lay et al., 1992a, c), but that vocalization frequencies were higher in freeze branded cattle (Lay et al., 1992b).

The anatomical sites used for branding in previous studies were either on the right rib or high on the right hind quarter (Schwartzkopf-Genswein et al., 1997 a, b, 1998; Lay et al. 1992 a, b, c). However, no studies have been done to determine whether the site of branding has any effect on the level of pain experienced.

2 OBJECTIVES OF THE STUDY

The first objective of this study was to determine the effect of branding of calves in a normal feedlot environment by monitoring physiological and behavioural responses as well as production outcomes. The second objective was to determine any differences in the above outcomes between feedlot calves branded at different anatomical sites (cheek or upper hind limb).

Welfare issues related to hot-iron branding have become an emotive issue and have resulted in litigation in certain cases. Although several studies have compared the welfare aspects of hot-iron branding to freeze branding, an objective comparison between branding at different anatomical sites has not been conducted so far.

3 RESEARCH QUESTIONS

a) Does hot-iron branding of the cheek of feedlot calves differ from branding of the upper hind limb in terms of blood cortisol levels?

b) Does hot-iron branding of the cheek of feedlot calves differ from branding of the upper hind limb in terms of faecal 11,17-dioxoandrostane (DOA) concentrations?

c) Does hot-iron branding of the cheek of feedlot calves differ from branding of the upper hind limb in terms of potential pain-related behavioural indicators?

d) Does hot-iron branding of the cheek of feedlot calves differ from branding of the upper hind limb in terms of its effect on average daily gain (ADG), mortality and/or morbidity?

e) Does hot-iron branding of the cheek of feedlot calves differ from branding of the upper hind limb in terms of histopathological and macroscopic scarring of the skin?

f) Does hot-iron branding of feedlot calves differ from sham branding in terms of blood cortisol concentrations?

g) Does hot-iron branding of feedlot calves differ from sham branding in terms of faecal 11,17-DOA levels?

h) Does hot-iron branding of feedlot calves differ from sham branding in terms of potential pain-related behavioural indicators?

i) Does hot-iron branding of feedlot calves differ from sham branding in terms of its effect of ADG, morbidity and/or mortality?

j) Does hot-iron branding of feedlot calves differ from sham branding in terms of histopathological and macroscopic scarring of the skin?

4 MATERIALS AND METHODS

4.1 Experimental animals

All experimental procedures were done at a medium-sized (25,000 head) commercial cattle feedlot (Beefcor feedlot) situated in the eastern highveld of South Africa, using calves that were bought in during the normal course of feedlot operation. After the feeding period the animals were slaughtered at Chamdor abattoir.

Calves were chosen from a single herd of origin, thus sharing the same genetic and environmental background. All animals used were intact males, approximately seven to ten months old, roughly in the same weight (230 – 340 kg) category and were Bonsmara-Angus-Brahman crosses. Animals were fed the standard feedlot ration and had unlimited access to water. The study was approved by the University of Pretoria's Animal Use and Care Committee (Protocol no. V0064/10).

4.2 Experimental design and branding procedure

A prospective, randomized, controlled study was done by comparing three groups of cattle, hot-iron branded either on the cheek or upper hind leg or sham branded with a room temperature iron. The study extended over a period of 97 days. A sample size of ten animals per group was chosen in order to achieve 90% power to detect a difference of 1 standard deviation (SD) in blood cortisol levels between groups, using a linear mixed model with five repeated measurements, with a within-subject correlation of 0.5 and autoregressive covariance structure.

Beef calves were transported from the farm of origin to the feedlot on day -24 (Figure 1). The animals were weighed, identified using an ear tag and processed as per the normal standard operating procedures of the feedlot (excluding branding) on day -23. A more uniform group of 40 animals was selected from the original group of 50 after blocking according to body weight (237 – 339 kg) and removing outliers, and placed in a pen separate from other animals in the feedlot. The animals were then left to recuperate from transport for 15 days (d-22 to d-8). Experienced pen checkers evaluated the animals daily during the trial period, according to their usual protocol, in order to pull any sick animals for further examination and possible treatment.
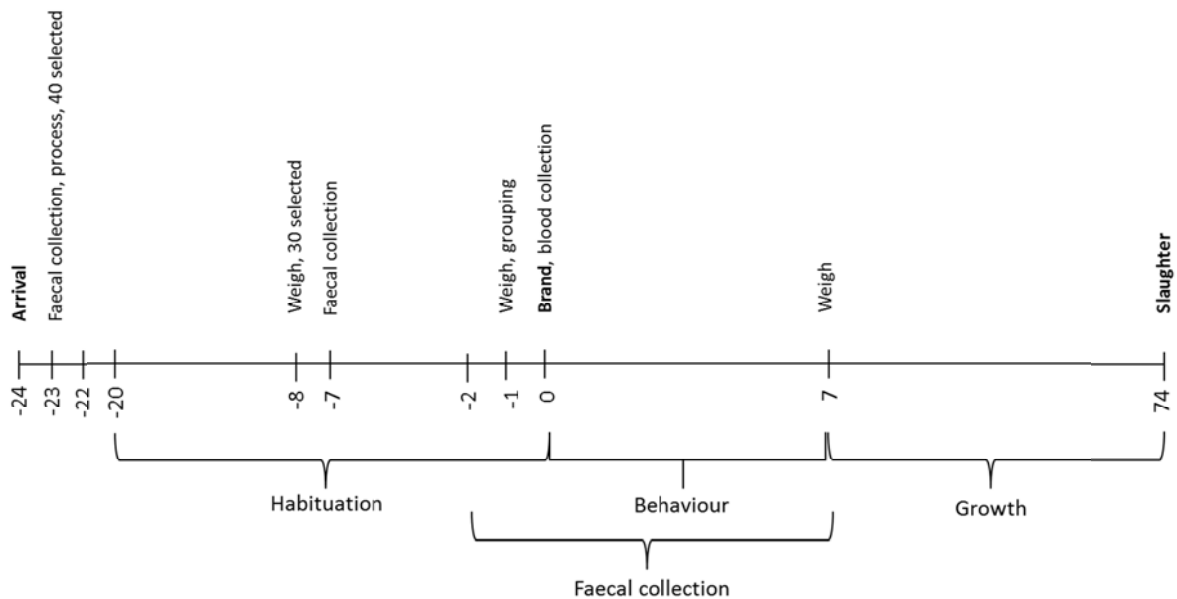
Figure 1. Sequence of events and procedures during the data collection period

On d-8, calves were weighed and 30 healthy animals were selected from the group of 40 by removing outliers. Prior to branding (d-8 to d0), the animals were habituated to the crush by

quietly herding them through at the same time daily for eight days. This was to minimise the effects of handling and restraint on serum cortisol and FGM levels on the day of branding. On the day before branding (d-1) calves were again weighed and were randomly assigned to three groups of ten using block randomisation, after sorting by and blocking on body mass (261 – 330 kg). Group A (n = 10) was to be branded on the cheek and Group B (n = 10) on the upper hind leg. Group C, the control group, was divided into two equal groups (n = 5 each), to be sham branded on the cheek (Group C1) or on the upper hind leg (Group C2).

Branding took place on d0. A single experienced worker at the facility performed all the branding procedures on all animals. Branding was done by placing a cast iron brander, heated on a gas burner until red-hot, on the skin for two seconds at the allocated site. Sham branding was done in the same way, but with a room temperature iron, to adjust for the amount of stress solely related to avoidance of the iron on the cheek as well as restraint. Calves were branded according to the allotted group regardless of their order of entry into the crush. All animals were restrained in exactly the same manner regardless of treatment allocated. Blood samples were collected into serum tubes immediately prior to branding and at 30, 60, 90 and 120 minutes after branding. Blood was sampled from the jugular vein at 0 minutes, just before branding, and from the coccygeal vein at 30, 60, 90 and 120 minutes after branding.

Faecal samples were collected on the day after arrival (d-23), seven days before branding (d-7), for two days before branding, on the day of branding and for seven consecutive days after branding, for FGM determination (Figure 1).

4.3 Behavioural assessment

In addition to physiological markers, behavioural outcomes were monitored during and after branding (days 0-7, Fig. 1). The animals were video-recorded during branding. The videos were analysed and used with data collected during the procedure to report on behaviour during branding. During branding behavioural variables included:

    i)   Presence of vocalisations during restraint with a nose tong

    ii)   Presence of vocalization during branding

    iii)   Presence of vocalization during blood collection

Instantaneous sampling methods, specifically scan sampling, was used to measure behavioural variables after branding. The behaviour of each group was assessed separately by recording the number of animals exhibiting each specific behaviour at set time intervals of 30 seconds. This was repeated 50 times for each group per day. If every animal in the group displayed the specific behaviour it would therefore result in a maximum count of 500 for that day. Each group was scanned every day, at the same time each day, for seven days after branding. An example of the data collection sheet for behavioural variables is shown in Appendix 1.

The behaviours noted included (Table 1):

    i)   Number of animals displaying tail twitches per group

    ii)   Number of animals kicking with the hind leg per group

    iii)   Number of animals ruminating per group

    iv)   Number of animals eating per group

    v)   Number of animals drinking per group

vi)   Number of animals lying down per group

vii)   Number of animals vocalizing per group

Table 1. Description of behaviours recorded to indicate discomfort after branding

Behaviour	Description
Tail twitch	Movement of base of tail to left or right of centre and back again (Schwartzkopf-Genswein et al., 2007a)
Kick	Backwards or sideways movement of one of hind legs (Schwartzkopf-Genswein et al., 2007a)
Rumination	Regurgitation of food into the mouth with re-chewing
Eating	Standing in either hay or feed bunk and actively eating and swallowing
Drinking	Standing at the water trough and actively drinking
Vocalization	Vocalization, irrespective of duration (Schwartzkopf-Genswein et al., 2007a)

4.4   Blood sampling and analysis

A 10 ml evacuated serum collection tube was used to collect the blood for cortisol level determination. After collection the blood was refrigerated and transported to a laboratory facility. The blood was then centrifuged at $1620 \times g$ for 15 minutes at 25°C. The serum was decanted and stored at -18 to -23 °C until analysis. Hydrocortisone, Compound F was measured using a commercially available radioimmunoassay kit (Siemens Coat-A-Count Cortisol) as described by Foster and Dunn (1974). Intra-assay coefficient of variation (CV) was 3.0-5.1% and inter-assay CV was 4.0-6.4% (manufacturer's data). The Coat-A-Count Cortisol antiserum is highly specific for cortisol, with a low cross-reactivity to other steroids present in the blood (Ruder et al., 1972; Farmer and Pierce, 1974; Foster and Dunn, 1974).

4.5 Faecal sampling and analysis

Samples were collected directly from the rectum of the individual animal with a plastic rectal glove. Faeces were placed in plastic faecal vials and immediately placed in a freezer, where they were frozen within approximately 30 minutes. Samples were stored at -20°C until analysis. All samples were analysed together in one batch at the end of the trial period. After storage the samples were lyophilized and pulverized through a mesh strainer to remove any course material. Faecal powder was weighed and approximately 100-110 mg powder was measured off and the exact weight noted. The powder was then extracted with 3 ml 80% ethanol in water by mixing for 15 minutes in an overhead shaker at intermediate speed. The samples were then centrifuged for ten minutes at 1500 × g. The supernatant was decanted into labelled microtiter vials. The supernatant was then stored at -20 °C until analysis. The analysis was done by using an enzyme immunoassay which detects the cortisol metabolite 11,17-DOA (Palme and Möstl, 1997; Möstl et al., 1999; Palme et al., 1999; Möstl et al., 2002). The sensitivity of the assay was 3 pg per well with a linear range of 1.95 – 31.25 pg per well. The intra-assay coefficient of variance was 2.76% - 3.95% and the inter-assay coefficient of variance was 11.70% - 12.06%.

4.6 Histopathology and macroscopic evaluation

Animals were slaughtered at an abattoir on d74, according to normal standard operating procedures of the feedlot and abattoir. At slaughter and dressing of the carcass, any visible macroscopic lesions were noted. Hot iron branding causes second to third degree burns on the skin which leads to a permanent visible mark (O'Toole and Fox, 2003) which can be seen and

graded on histopathology. Samples of skin were taken from the branded area and immediately placed in 10% buffered formalin to fix. Histopathology was then done on the samples to evaluate lesions of the subcutaneous and muscular tissue beneath the branding site (Department of Veterinary Pathology, University of Pretoria). Lesions were subjectively assessed according to the degree of epidermal, dermal, and/or muscular damage and fibrosis.

4.7   Production outcomes

The following production outcomes were monitored during the trial period:

i)   *Average daily gain post-branding.* The weight gained from d-1 to d7 was recorded in order to calculate the average daily weight gain.

ii)   *Morbidity and mortality post-branding.* Experienced feedlot pen checkers observed the cattle twice daily for the duration of the trial, according to the feedlot standard operating procedures. Any sick animals identified by the pen checkers were treated according to the feedlot's standard treatment protocol. The main investigator also checked the animals regularly.

4.8   Data analysis

Data were recorded manually onto data collection sheets (Appendix 1). They were then entered into a computer spreadsheet programme (Microsoft Excel; Microsoft Corporation, Redmond, WA, U.S.A.). Continuous outcomes were assessed for normality using the Shapiro-Wilk test. Data were described by group and time point using mean and SD for continuous outcomes and proportions for categorical outcomes. Statistical analyses described

below were performed using NCSS 2007 (NCSS, Kaysville, UT, U.S.A.) and Stata 12 (StataCorp, College Station, TX, U.S.A.). A significance level of $\alpha = 0.05$ was used throughout.

4.8.1 Faecal 11,17-dioxoandrostane

4.8.1.1 Pre-branding period (d-23 to d0)

The animals were regarded as one group during the pre-branding period, since randomization into groups had not yet occurred. Although there were 40 animals in this group, only data from the 28 animals finally included in the study were included in the analysis. Mean faecal 11,17-DOA was compared between the day after transport (d-23) and each of the other time points (d-7, d-2, d-1 and d0) using a linear mixed model with Bonferroni adjustment for multiple-comparisons.

4.8.1.2 Post-branding period (d0-d7)

From d0 to d7, mean faecal 11,17-DOA concentration was compared between the three groups on each day, and within each group the mean for each day was compared with day 0, using a linear mixed model with Bonferroni adjustment for multiple-comparisons. 11,17-dioxoandrostane concentration-time curves were constructed and the AUC was calculated for each animal using the trapezoidal method, in order to obtain a measure of the overall cortisol response over the 7 day period. Mean 11,17-DOA AUCs were then compared between groups using one-way analysis of variance (ANOVA) with Bonferroni adjustment for multiple comparisons.

4.8.2 Blood cortisol

Mean cortisol concentrations were compared between the three groups on each day, and within each group, the mean for each day was compared with day 0, using a linear mixed model with Bonferroni adjustment for multiple-comparisons. Cortisol concentration-time curves were constructed and the AUC was calculated for each animal using the trapezoidal method, in order to obtain a measure of the overall cortisol response over the 7 day period. Mean cortisol AUCs were then compared between groups using one-way ANOVA with Bonferroni adjustment for multiple comparisons.

4.8.3 Behavioural outcomes

The proportion of animals vocalising during restraint, bleeding and branding was compared between the three groups using Fisher's exact test.

For d0 - d7, the count of each behaviour, in each group, and on each day, was expressed as a proportion of the total possible occurrences of the behaviour to obtain a "behaviour proportion". In the cheek branded group the total possible daily number of occurrences of each behaviour was 500, while in the leg branded and control group it was 450 each, since two animals had been excluded (see below). For each behaviour the median behaviour proportion over the eight day period was compared between groups using Kruskal-Wallis one-way ANOVA with Dunn's multiple comparison test.

4.8.4 Production outcomes

Average daily gain for the first 7 days after branding was compared between groups using one-way ANOVA with Bonferroni adjustment for multiple comparisons.

4.8.5 Exclusions

Two animals were extremely stressed during restraint and as a result went down in the crush. The cortisol levels of these individuals would not have been a true reflection of distress due to branding and they were thus excluded from the trial. The dataset therefore ultimately comprised a total of 28 calves: ten that were cheek branded, nine that were leg branded and nine that were sham branded.

5 RESULTS

5.1 Faecal cortisol

5.1.1 The pre-branding period: d-23 to d0

Figure 2 shows the mean 11,17-DOA concentrations of the 28 trial animals during the pre-branding period. From d-23 to d-7 mean 11,17-DOA levels decreased by 54% to just below 100 ng/g and had dropped slightly further by the day of branding (Table 2). A significant difference was seen when comparing the 11,17-DOA concentration on the day after transport to the concentrations on the other collection days (d-7, d-2, d-1 and d0) ($P < 0.001$ for each).

Table 2. Faecal 11,17-dioxoandrostane concentrations in 28 feedlot calves from the day after arrival to the day of branding

Date of collection	Day	Faecal 11,17-DOA (ng/g DW)		
		Mean	*SD*	*Range*
2011/08/02	-23	206.2	89.5	58 - 413
2011/08/18	-7	95.1	45.3	17 - 208
2011/08/23	-2	91.8	58.6	13 - 251
2011/08/24	-1	76.3	46.6	11 - 205
2011/08/25	0	76.0	39.5	20 - 172
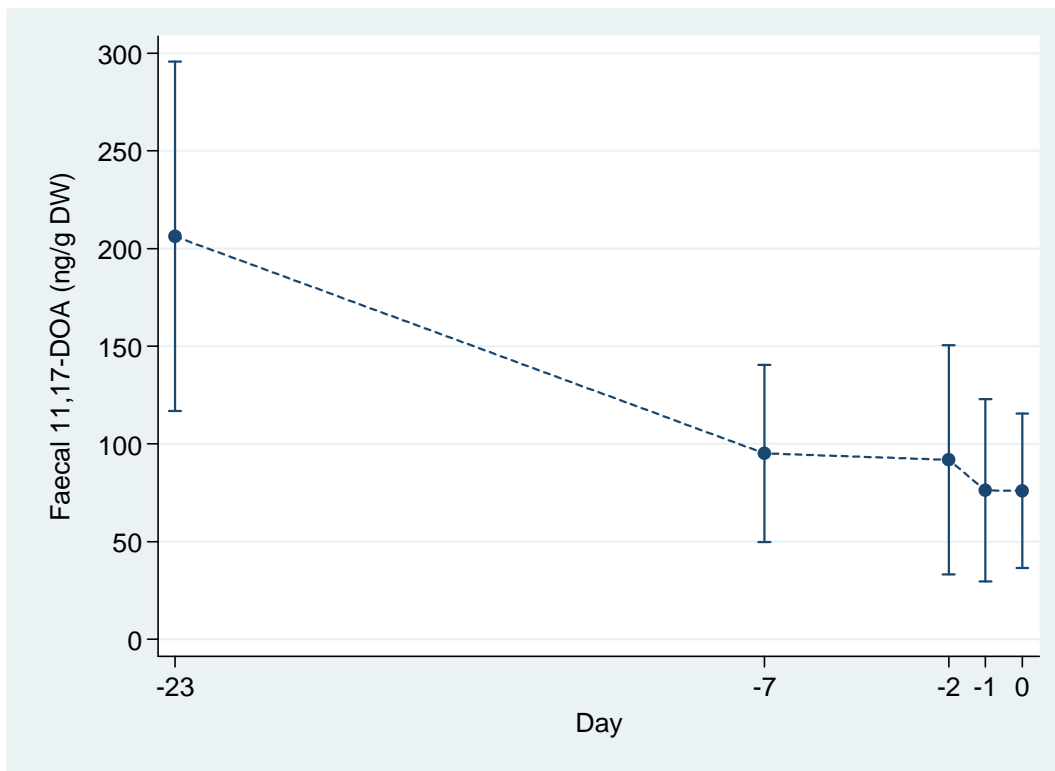
Figure 2. Mean±SD faecal 11,17-dioxoandrostane concentrations in 28 feedlot calves from the day after arrival to the day of branding

5.1.2   The post-branding period: d0 to d7

Overall there were no significant differences in faecal 11,17-DOA concentrations between the three groups during the post-branding period (Cheek branded group vs. control group: $P = 0.513$; Cheek branded groups vs. Leg branded group: $P = 1.000$; Control group vs. Leg branded group: $P = 0.665$), and there was no significant difference between faecal 11,17-DOA on d0 or on any subsequent day. There was a large variation between concentrations within groups as indicated by the SD (Table 3). The mean 11,17-DOA concentration stayed below 100 ng/g in all the groups with no obvious peaks or sudden declines after branding (Figure 2). After branding 11,17-DOA levels remained below the maximum concentrations

reached after transport. There were no significant differences when time points were

compared within groups (P = 1.000 for all three groups).

Table 3. Faecal 11,17-dioxoandrostane concentrations during the post-branding period in feedlot calves hot-iron branded on the cheek (Group A), hot-iron branded on the upper hind leg (Group B), or sham branded (Group C)

Day	Group	Faecal 11,17-DOA (ng/g DW)	
		Mean	*SD*
0 (branding)	A (n = 10)	93.1	49.2
	B (n = 9)	76.3	36.4
	C (n = 9)	56.6	21.0
1	A (n = 10)	85.9	31.4
	B (n = 9)	77.4	22.3
	C (n = 9)	89.8	20.5
2	A (n = 10)	80.0	23.8
	B (n = 9)	70.7	21.7
	C (n = 9)	58.6	32.4
3	A (n = 10)	102.6	39.2
	B (n = 9)	91.4	37.1
	C (n = 9)	75.1	34.0
4	A (n = 10)	79.6	30.8
	B (n = 9)	92.8	36.3
	C (n = 9)	64.6	23.7
5	A (n = 10)	81.9	36.2
	B (n = 9)	87.0	32.1
	C (n = 9)	78.0	30.4
6	A (n = 10)	78.9	38.6
	B (n = 9)	88.6	26.3
	C (n = 9)	80.3	41.7
7	A (n = 10)	78.4	31.3
	B (n = 9)	86.9	31.1
	C (n = 9)	71.1	19.7
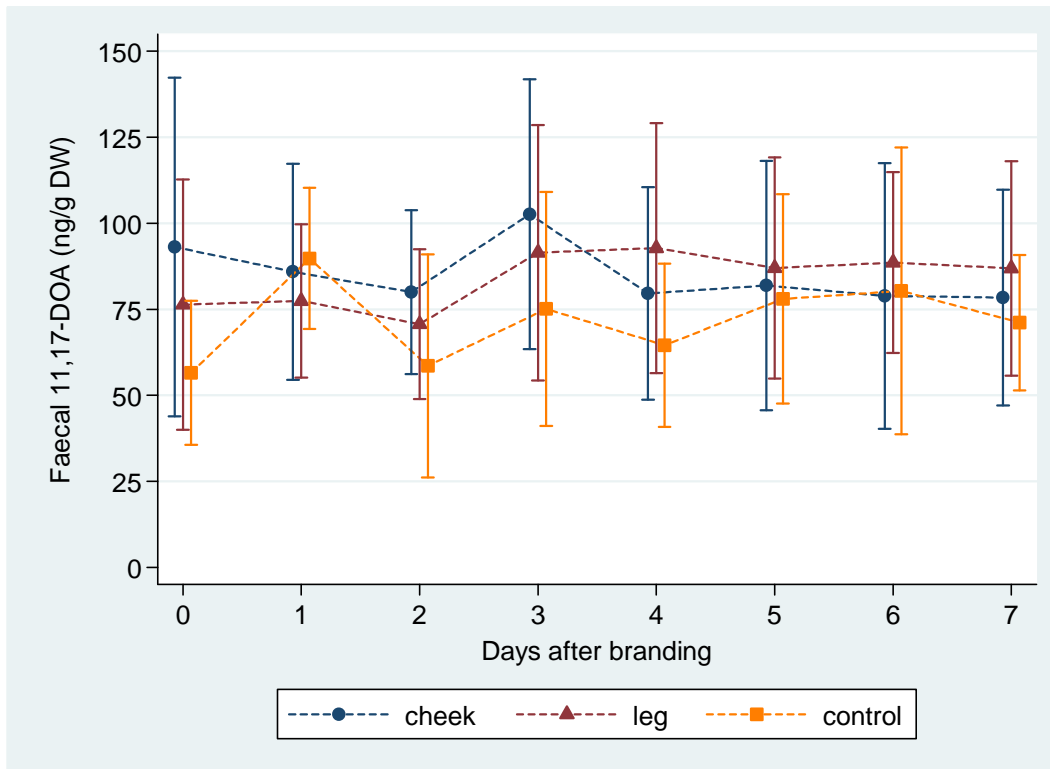
Figure 3. Mean±SD faecal 11,17-dioxoandrostane concentrations during the post-branding period of feedlot calves hot-iron branded on the cheek, hot-iron branded on the leg, or sham branded (control)

The AUC of the faecal 11,17-DOA vs. time curve for d0 to d7 is shown for the three groups in Table 4. There was no significant difference in AUC between groups (P = 0.395).

Table 4. Area under the curve of faecal 11,17-dioxoandrostane concentration vs. time during the post-branding period in feedlot calves hot-iron branded on the cheek (Group A), hot-iron branded on the upper hind leg (Group B), or sham branded (Group C)

Group	AUC of faecal 11,17-DOA (ng/g DW) vs time (day)	
	Mean	*SD*
A (n = 10)	505.1	139.5
B (n = 9)	512.7	132.5
C (n = 9)	436.9	109.3

5.2 Serum cortisol

Serum cortisol concentrations in the three treatment groups are shown in Table 5. There were no significant differences between any groups (control, cheek brand, leg brand) at any of the collection times (0, 30, 60, 90, and 120 minutes). Within each group, serum cortisol did not differ at any time point from pre-branding levels. However, for all groups combined, cortisol levels at 60 minutes after branding were significantly lower compared to the time of branding ($P = 0.009$) (Figure 4).

Table 5. Serum cortisol concentration at 0, 30, 60, 90 and 120 minutes after branding in feedlot calves hot-iron branded on the cheek (Group A), hot-iron branded on the upper hind leg (Group B), or sham branded (Group C).

Group	Time after branding (minutes)	Serum cortisol (nmol/l)	
		Mean	*SD*
A (n = 10)	0	60.4	25.9
	30	68.7	40.4
	60	43.2	28.1
	90	72.7	44.0
	120	57.0	36.3
B (n = 9)	0	71.3	33.3
	30	72.5	23.4
	60	44.3	15.9
	90	63.1	32.2
	120	73.7	35.1
C (n = 9)	0	62.4	32.4
	30	61.8	36.9
	60	43.0	27.5
	90	60.4	53.9
	120	56.8	60.6

The AUC of the serum cortisol vs. time curve at 0, 30, 60 and 120 minutes after branding is shown for the three groups in Table 6. There was no significant difference in AUC between groups (P = 0.827).

Table 6. Area under the curve of serum cortisol concentration vs. time during the post-branding period in feedlot calves hot-iron branded on the cheek (Group A), hot-iron branded on the upper hind leg (Group B), or sham branded (Group C)

Group	AUC of serum cortisol (nmol/l) vs. time (minutes)	
	Mean	*SD*
A (n = 10)	3615.1	1896.8
B (n = 9)	3906.8	1259.7
C (n = 9)	3400.4	1859.4
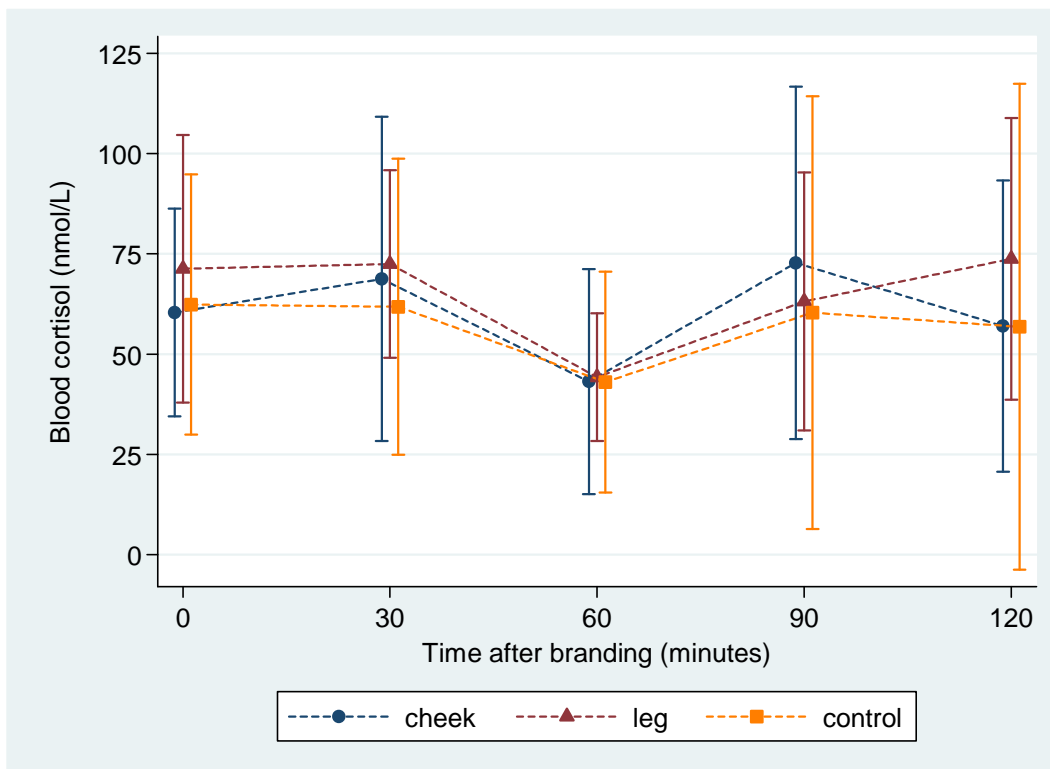
Figure 4. Mean±SD serum cortisol concentrations at 0, 30, 60, 90 and 120 minutes after branding in feedlot calves hot-iron branded on the cheek, hot-iron branded on the upper hind leg, or sham branded (control)

5.3 Behaviour

5.3.1 Vocalization during branding

In Group A (cheek branded), all of the animals vocalized, whereas none of the animals in Group C (sham branded) and 38% of the animals in group B (leg branded) vocalized. There was a significant difference between the cheek branded group compared to the control group (P < 0.001). There was a tendency for the cheek branded group to vocalize more than the leg branded group (P = 0.080). The leg branded group also vocalized significantly more than the control group (P = 0.030). During bleeding there were no significant differences in the proportions of animals vocalizing in the cheek branded group and the leg branded group (P = 1.000), or between the cheek branded group and the control group (P = 0.650), or between the leg branded group and the control group (P = 1.000). During restraint there were no significant differences in the proportions of animals vocalizing between the cheek branded group and the leg branded group (P = 1.000), or between the leg branded and control group (P = 0.615) or between the cheek branded group and the control group (P = 0.370).

5.3.2 Post-branding

The only behaviours frequently recorded were lying down, ruminating and eating, whereas the other behaviours were recorded only in very low numbers (Table 7). There were no significant differences between the groups with regards to the occurrence of tail twitches (P = 0.92), lying down (P = 0.770), vocalization (P = 0.900), kicking (P = 0.090), rumination (P = 0.870), drinking (P = 0.110) and eating (P = 0.790). There was a significant difference in behaviour on the day of branding and one day after branding, compared to all the other days

post-branding (except day 3), where the incidence of eating was decreased while that of rumination and lying down was increased. On day three the same could be observed. The behavioural outcomes are presented graphically in Figure 5.

A

Figure 5. Behavioural proportions indicating occurrence of specific behaviours in feedlot calves over a seven day period after hot-iron branding on the cheek or the upper hind limb, or sham branding. A. Behavioural proportions of animals lying down during the post-branding period

B
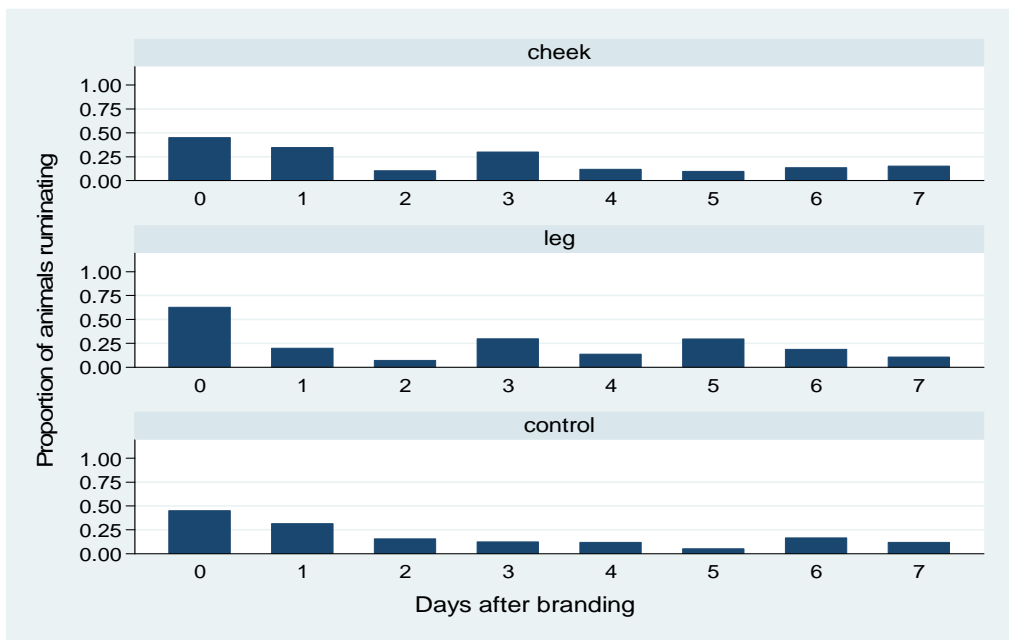
C
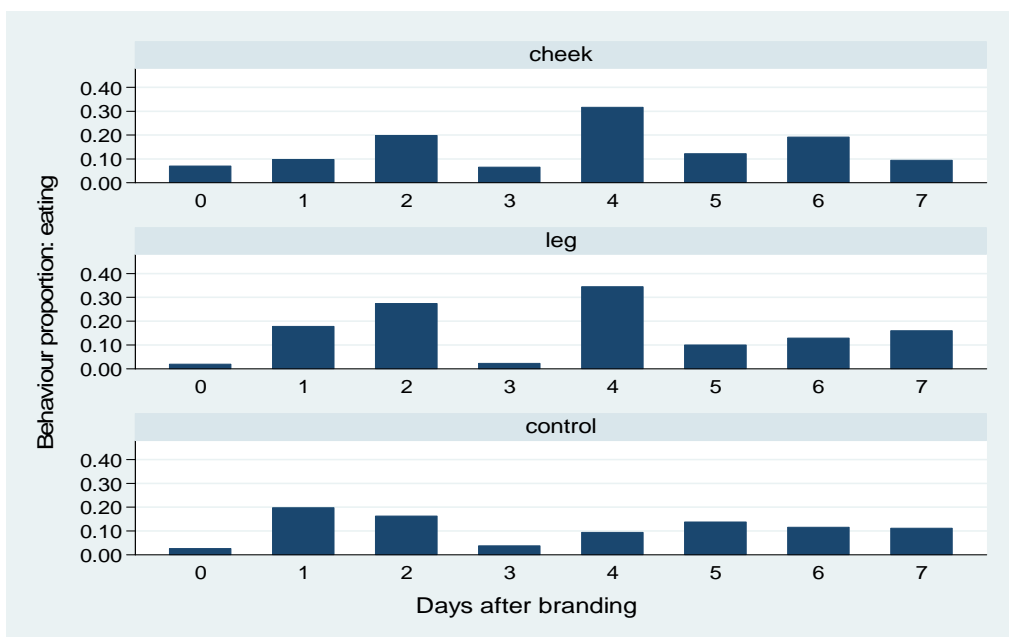
Figure 5. (cont.) Behavioural proportions indicating occurrence of specific behaviours in feedlot calves over a seven day period after hot-iron branding on the cheek or the upper hind limb, or sham branding. B. Behavioural proportions of animals ruminating during the post-branding period. C. Behavioural proportions of animals eating during the post-branding period

D
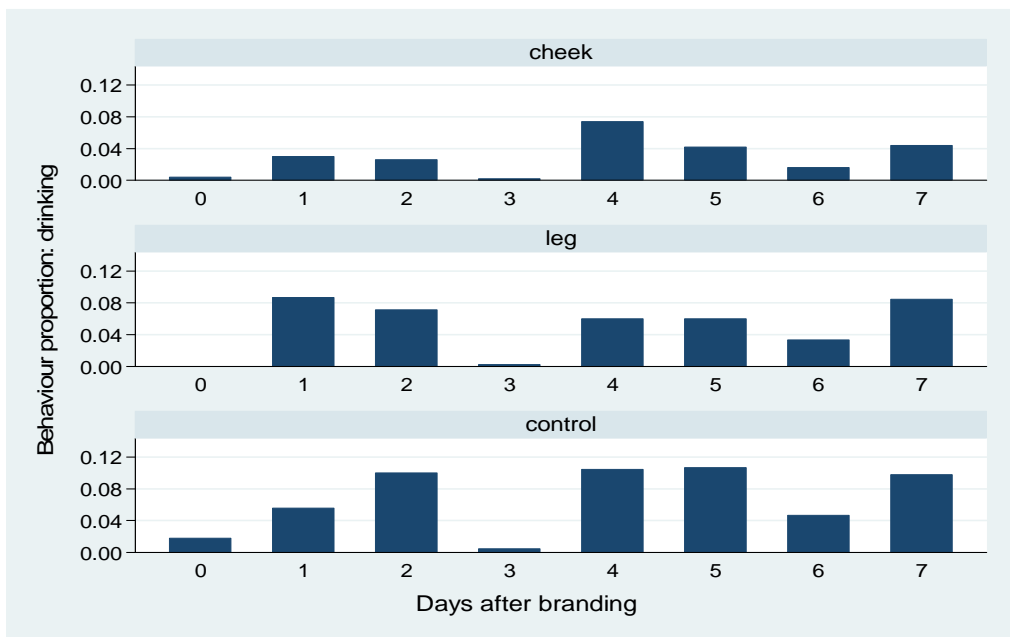
E
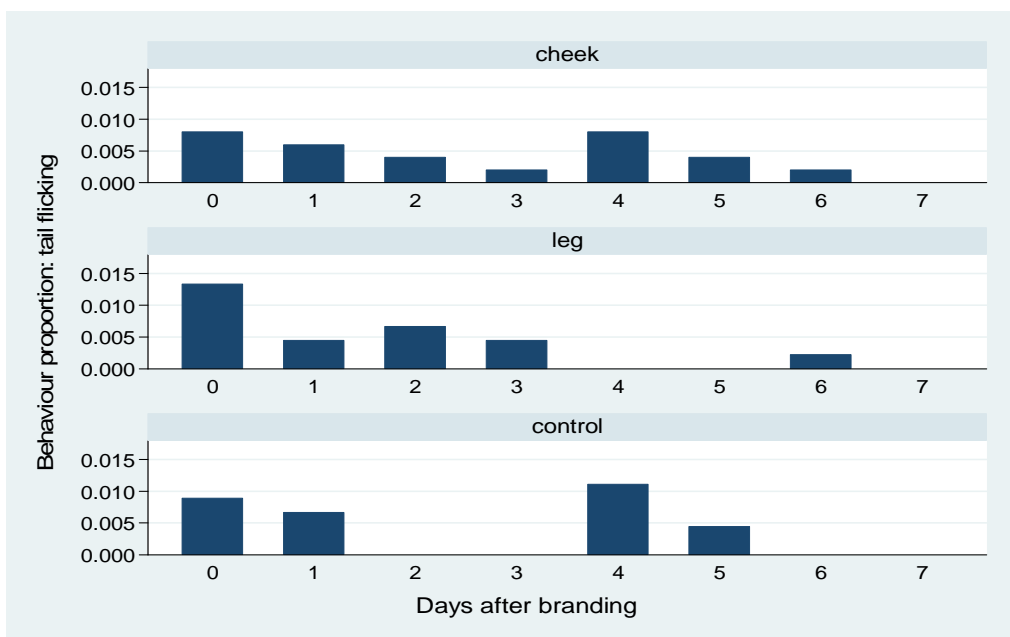
Figure 5. (cont.) Behavioural proportions indicating occurrence of specific behaviours in feedlot calves over a seven day period after hot-iron branding on the cheek or the upper hind limb, or sham branding.. D. Behavioural proportions of animals drinking during the post-branding period E. Behavioural proportions of animals tail flicking during the post-branding period
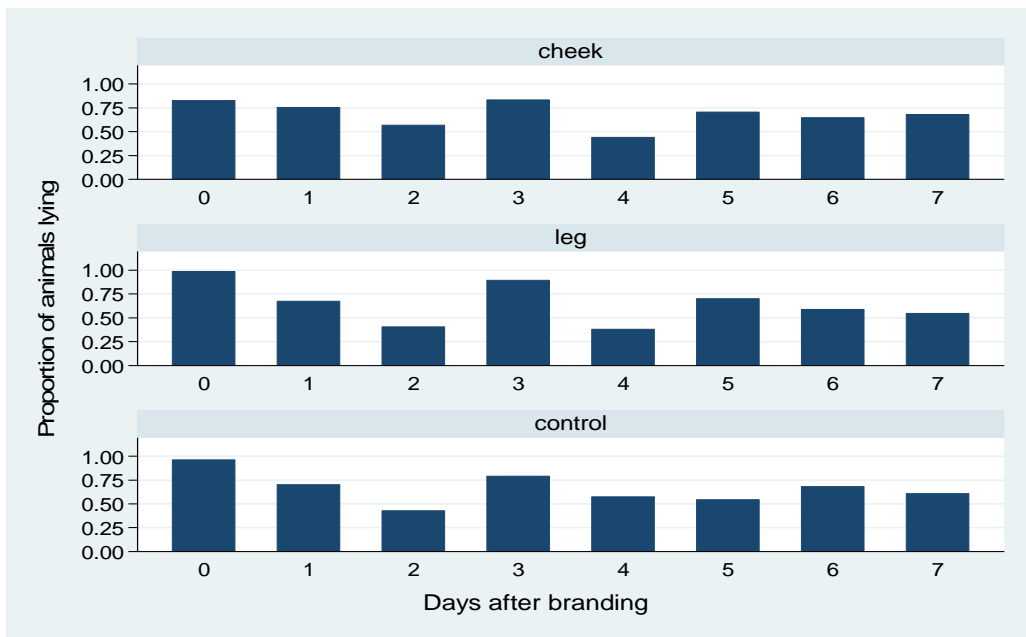
F
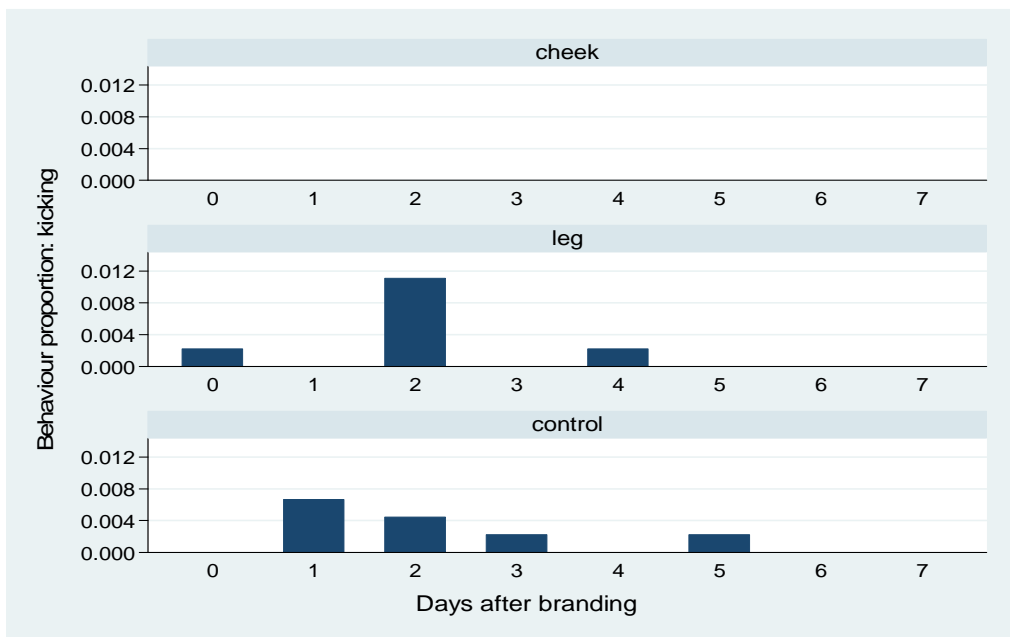
G
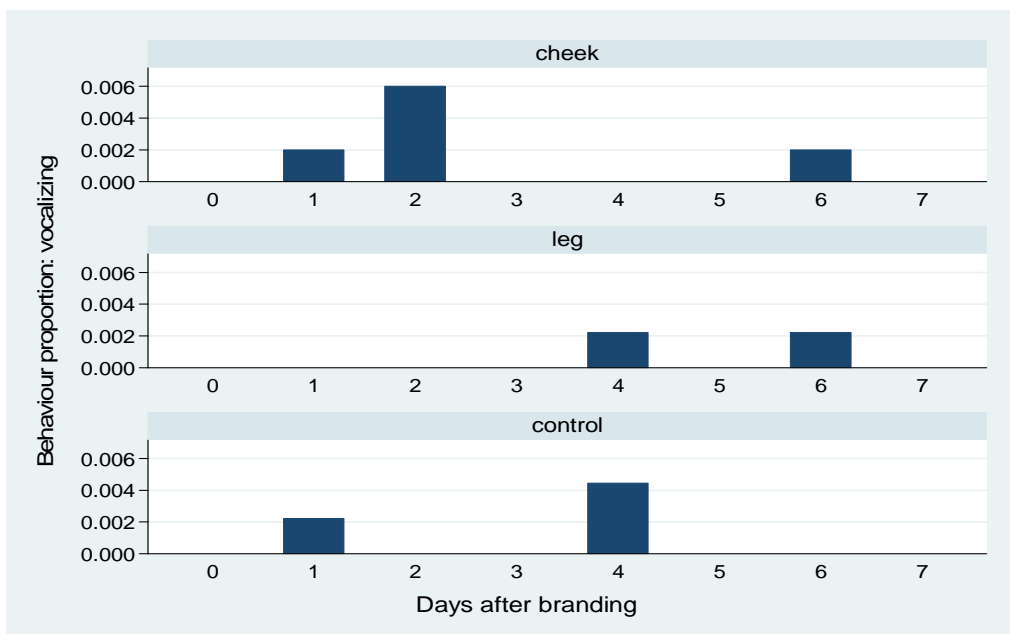
Figure 5. (cont.) Behavioural proportions indicating occurrence of specific behaviours in feedlot calves over a seven day period after hot-iron branding on the cheek or the upper hind limb, or sham branding. F. Behavioural proportions of animals kicking during the post-branding period. G. Behavioural proportions or animals vocalizing during the post-branding period

Table 7. Behavioural proportions of post-branding behavioural outcomes in feedlot calves hot-iron branded on the cheek (Group A), hot-iron branded on the upper hind leg (Group B), or sham branded (Group C)

Day	Group	Behavioural proportion						
		Tail	*Kick*	*Lie*	*Ruminate*	*Eat*	*Drink*	*Vocalize*
0	A	0.0	0.0	0.8	0.5	0.1	0.0	0.0
	B	0.0	0.0	1.0	0.6	0.0	0.0	0.0
	C	0.0	0.0	1.0	0.4	0.0	0.0	0.0
1	A	0.0	0.0	0.8	0.4	0.1	0.0	0.0
	B	0.0	0.0	0.7	0.2	0.2	0.1	0.0
	C	0.0	0.0	0.7	0.3	0.2	0.0	0.0
2	A	0.0	0.0	0.6	0.1	0.2	0.0	0.0
	B	0.0	0.0	0.4	0.1	0.3	0.1	0.0
	C	0.0	0.0	0.4	0.2	0.2	0.1	0.0
3	A	0.0	0.0	0.8	0.3	0.1	0.0	0.0
	B	0.0	0.0	0.9	0.3	0.0	0.0	0.0
	C	0.0	0.0	0.3	0.1	0.0	0.0	0.0
4	A	0.0	0.0	0.4	0.1	0.3	0.1	0.0
	B	0.0	0.0	0.4	0.1	0.3	0.1	0.0
	C	0.0	0.0	0.6	0.1	0.1	0.1	0.0
5	A	0.0	0.0	0.7	0.1	0.1	0.0	0.0
	B	0.0	0.0	0.7	0.3	0.1	0.1	0.0
	C	0.0	0.0	0.5	0.1	0.1	0.1	0.0
6	A	0.0	0.0	0.7	0.1	0.2	0.0	0.0
	B	0.0	0.0	0.6	0.2	0.1	0.0	0.0
	C	0.0	0.0	0.7	0.2	0.1	0.1	0.0
7	A	0.0	0.0	0.7	0.2	0.1	0.0	0.0
	B	0.0	0.0	0.6	0.1	0.2	0.1	0.0
	C	0.0	0.0	0.6	0.1	0.1	0.1	0.0

5.4 Pathology and histopathology

At slaughter, brand lesions could be seen macroscopically only in 5 animals hot-iron branded on the leg and no macroscopic lesions could be seen in animals branded on the cheek. Histopathology done on the skin samples collected at slaughter showed no subcutaneous or muscular tissue damage or fibrosis.

5.5 Production outcomes

Weight gain and ADG for the three groups over the post-branding period are shown in Table 8 and Figure 6. There was large variation in ADG within groups, indicated by the high SD. There were no statistically significant differences between groups (P = 0.252). There were no mortalities or morbidities during the eight days post-branding.

Table 8. Mean±SD average daily gain of feedlot calves hot-iron branded on the cheek (Group A), hot-iron branded on the upper hind leg (Group B), or sham branded (Group C) during the post-branding period (d-1 to d7)

Group	Mean weight gain (kg)	ADG (kg/day)	
		Mean	*SD*
A	9.00	1.13	1.07
B	15.11	1.89	0.97
C	12.11	1.51	0.87
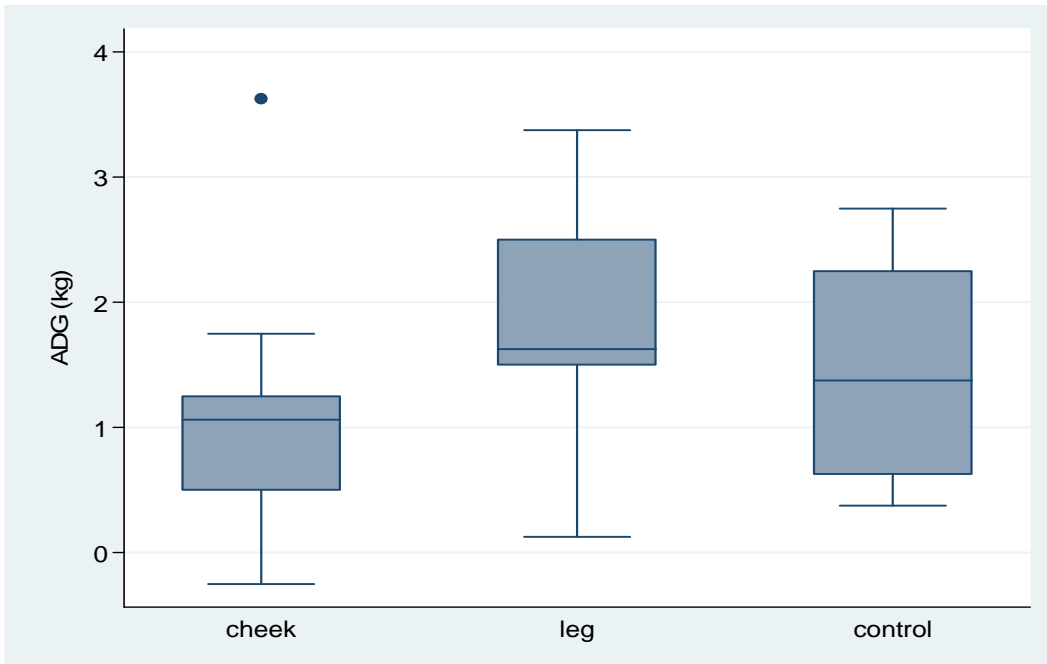
Figure 6. Average daily gain (kg) during the eight day post-branding period in feedlot calves hot-iron branded on the cheek or upper hind leg, or sham branded (control)

6 DISCUSSION

6.1 Faecal glucocorticoid metabolite levels

It is known that transport is highly stressful to cattle (Palme et al., 2000; Burdick et al., 2010). This research revealed similar findings as a study by Palme et al. (2000), where road transport of cattle for two hours led to increased levels of 11,17-DOA in faeces with a peak at 12 hours after transport. The elevated FGM levels determined the day after transport in this study were therefore likely due to the transport the previous day.

The time taken for FGM to return to baseline varies among the type of stressor and also individual animals (Palme et al., 2000), which is why these calves were given an adequate amount of time necessary for cortisol levels to return to normal. Novelty is a very strong stressor in cattle as strange sights or sounds may be a sign of danger (Grandin, 1997). Gradual, peaceful exposure to the crush would have allowed the cattle to become accustomed to the stimulus, resulting in lower cortisol levels. On the day before branding 11,17-DOA concentrations were taken as baseline levels in our study, as sufficient time would have passed between the last stressor (transport) and collection on d-2, as well as habituation to the crush.

6.1.1 Post-branding period

As observed after transport, an increase in the 11,17-DOA concentration the day after branding was also expected, with a return to baseline concentrations gradually over the following 5 days (Möstl et al., 2002). However, no significant increase in FGM was seen

after branding and no significant differences were found between groups. Previous studies have shown similar results where cows were used for teaching invasive procedures and no elevation in FGM or significant differences between the control and treatment group was found (Möstl et al., 2002). This may indicate that the stress response and resulting blood cortisol concentration in our study was not sufficiently increased to elevate the FGM levels the day after branding. Another explanation may be that the cortisol response to the perceived stressor was too short, and subsequently blood levels were not elevated for a long enough period of time for FGM concentrations to be raised high enough for us to see a difference between the different days or even between the different groups.

6.2   Serum cortisol concentrations

The mean baseline value for the calves of this study were 64.67 nmol/l at 0 minutes which is consistent with a previous study (Doornenbal et al., 1988) where bulls of 7.5 months had a mean serum cortisol level of 68 nmol/l with a large SD (SD = 38.1 nmol/l), similar to this study (SD = 30.5 nmol/l). However, a wide variation in individual baseline cortisol concentrations exists, depending on experience, genetic factors and temperament (Grandin, 1997). We assumed that the cortisol concentrations were at baseline levels at 0 minutes before branding, but it might have been that the cortisol levels were already elevated at that time, possibly due to the waiting period in the crush and restraint of the animals. This explanation is supported by the fact that the control group did not differ at any point in time from the branded groups. Another reason to suspect that cortisol was elevated at 0 minutes is because there was a decline in serum cortisol concentrations at 60 minutes in all three groups. Since there is no reason for cortisol to decrease below baseline levels in these animals, we conclude that the serum cortisol levels were not baseline at 0 minutes.

The reason for high serum cortisol concentrations at 0 minutes is likely related to the overall stress experienced by the animals. The calves in this study had been minimally handled prior to the experiment, especially around the face. They also originated from an extensive background and it has been shown that calves in contact with humans whilst reared have lower blood cortisol concentrations when castrated, with a distinct difference seen between treatment and control animals, while animals not exposed to humans showed no difference between control and treatment groups (Stafford and Mellor, 2005). While placement of nose-tongs and restraint of the head on the day of branding may not have caused significant pain it can still be assumed to be a major psychological stressor, especially in cattle raised extensively (Grandin, 1997; Gregory, 2004). Previous studies have shown that restraint in a squeeze chute alone was almost as stressful as being hot-iron branded for extensively reared beef cattle, but dairy cows used to handling had a much higher stress response to hot-iron branding than to handling alone (Lay et al. 1992b, c). The handling and restraint of the calves just before and during the first bleeding thus contributed to the cortisol response and the high levels of cortisol found at 0 minutes.

The reduction in serum cortisol levels found 60 minutes post-branding could possibly have been due to a decrease in perception of the stressor or due to intrinsic negative feedback mechanisms of cortisol (Smith and Dobson, 2002). However, serum cortisol levels increased again at 90 minutes, likely because the calves were not acclimatised to standing in the crush for such a prolonged period of time (up to 2.5 hours) (Grandin, 1997; Gregory, 2004).

Previous branding studies showed increases in plasma cortisol levels up to $100 - 150$ nmol/l with elevations usually occurring between 0 and 20 minutes after branding (Lay et al. 1992 a,

b; Schwartzkopf-Genswein et al. 1997a). In this study there were no significant differences between cortisol concentrations of samples collected at 0 and 30 minutes after branding. Additionally, there were no differences seen between the three groups. This could be because, as discussed previously, cortisol concentrations were already elevated at 0 minutes, and that the effect of branding alone did not further elevate serum cortisol levels when comparing hormone levels 0 and 30 minutes post-branding. Although peak cortisol levels may be associated with the level of pain experienced during a procedure, it is difficult to interpret cortisol values that are at the lower and upper extent of the response range (Coetzee, 2011). It may be that the stress response due to handling and restraint lead to the "ceiling effect" (Coetzee et al., 2007, Coetzee, 2011, Molony and Kent, 1997) which could mask the potential response of the HPA axis due to the pain of branding alone. The pain produced by the branding event was likely not intense enough or prolonged enough to further stimulate adrenocortical activity above levels associated with restraint. These results are consistent with a study done by Lay et al. (1992, b) where, despite habituation, no differences could be seen between calves hot-iron branded, freeze-branded or sham-branded (Lay et al., 1992b).

Successive jugular venipuncture in dairy cows unaccustomed to handling leads to an increase in cortisol concentrations related to the stress of sampling (Hopster et al., 1999). Therefore, repeated blood sampling in this study may have added to the cortisol response seen in this study in all three groups, and contributed to obscuring any possible differences between groups 30 minutes after branding.

Studies have shown that *Bos indicus* cattle breeds exhibit a more pronounced behavioural and physiological reaction to handling than *Bos taurus* breeds (Hearnshaw and Morris, 1984, Fordyce et al., 1988, Zavy et al., 1992, Voisinet et al., 1997, Boissy et al., 2005). *Bos indicus*

cattle breeds show an increased exit velocity and flight speed which is related to fear of handling and is correlated to plasma cortisol concentrations (Curley et al., 2006, Curley et al., 2008). It is therefore likely that a larger between-animal variation in cortisol concentrations will be observed in *B. indicus* breeds. The breed composition of the calves used in this study was at least 50% *B. indicus.* This factor may have contributed to the large variance in serum cortisol concentration observed in this study and reduced the ability to detect significant differences.

6.3   Behavioural measurements

6.3.1   Behavioural outcomes at branding

A significant difference was seen between the control animals and the branded animals in terms of vocalization at branding, but there were no significant differences found between the group branded on the cheek and the leg branded group. There was therefore some indication of a transient difference in level of pain experienced between the three groups. Vocalization has been shown to occur after a painful stimulus in cattle (Schwartzkopf-Genswein et al. 1997b, Watts and Stookey, 1999) but results may be highly variable. It is necessary to analyse vocalization results together with other quantitative measures of pain and results are most valuable when analysed as a proportion of a large group of animals (Lay et al. 1992b; Coetzee et al., 2008). The sample size of this study may thus have been too small to show a significant difference. The result of the vocalization data in this study was also not substantiated by the blood cortisol or FGM data making it less useful as an indicator of stress due to branding.

6.3.2   Behavioural outcomes after branding

No significant differences were seen between the groups with regard to behavioural outcomes measured over the 8-day period after branding. This suggests that branding had no marked effect on behaviour in the longer term. The significant difference seen in drinking and eating behaviours on the day of branding compared to the days following the event might be due to the animals having stood in the crush for a prolonged period of time (Eicher et al. 2000). On day three the peaks in behavioural proportions of rumination and lying down and lower frequency of eating and drinking, seen in all three groups, were likely due to unknown environmental conditions at the feedlot at the time of sampling.

6.4   Histology and macroscopic evaluation

On the day of slaughter only faint hot-iron brand marks were seen in some animals branded on the upper hind leg and no cheek brands were seen. This could have been due to variation in timing and force in the execution of the brand by the brander. Cheek brands are routinely done at the feedlot where the study took place and most other animals at the end of their standing period at the feedlot did not show signs of having been branded.

6.5   Production outcomes

To eliminate the effect of factors not related to branding that would have influenced feed intake in the small sample size in this study, it was decided that ADG would only be

measured over the 8 days after branding. It is possible that this period may have been too short to show any differences between groups and the large variation in recorded weights between individual animals, likely due to transient differences in rumen fill, may have limited the power of the study. However, it is likely that the stress associated with branding was too short lived or not severe enough to affect growth, morbidity or mortality. In a study comparing ADG of groups of cattle either hot-iron, freeze or sham branded, no differences could be seen at 10 or 28 days after branding (Schwartzkopf-Genswein et al., 1997c) and the authors suggested that the stress associated with branding may not have been severe enough to affect weight gain.

6.6   Limitations to the study

Even though the animals in this study were habituated to the crush, they were completely unaccustomed to being touched around the face or further restrained, which could have been highly stressful for them. In the feedlot situation in this study, any potential differences in physiological, behavioural and production outcomes when branded at different anatomical sites, were likely outweighed by other stressors, resulting in large variations between animals and therefore reducing the power to detect statistically significant differences. A similar limitation was encountered in previous studies comparing hot-iron and freeze branding by means of plasma cortisol concentrations (Lay et al., 1992a, b), where the cortisol response to handling and restraint masked the branding stress, although animals in those studies were not acclimatized to handling in a crush. When one wants to compare only the different branding sites all other possible stressors should ideally be eliminated or at least controlled for as far as possible. *Bos taurus* breeds are also less temperamental and using these breeds may lead to

less variability in the results and therefore increased power to detect differences between groups.

In addition, an assessment of the autonomic nervous system may be a better measure when assessing the immediate stress response of a painful husbandry procedure (Moberg and Mench, 2000; Stewart et al. 2008) as cortisol levels respond relatively slowly after a noxious stimulus compared to the SNS which will be activated within seconds after acute pain (Toates, 1995). Measuring SNS activity may thus be a better way to measure the response to branding. Other neuroendocrine changes such as measurement of neuropeptide Substance P may also be considered as a measure of nociception in future studies (Coetzee et al., 2008, Coetzee, 2011).

Average daily gain is a long term indicator of stress in cattle, and the long term effects of different husbandry procedures in different treatment groups have been shown to negatively affect ADG (Cohen, 1990; Goonewardene and Hand, 1991). The 8-day period may therefore have been too short to reliably show differences in weight gain between the groups, since ADG over such a short period of time is prone to short-term fluctuations, e.g. due to differences in rumen fill (Schwartzkopf-Genswein, 1997c). Other studies have shown that production outcomes are often too imprecise to reflect the pain experienced after castration (Stafford and Mellor, 2005).

A further limitation of the study was the fairly small sample size used, which was due to logistic, economic and ethical constraints. This would have resulted in low power of the study to detect small differences in the outcomes between groups, if such differences did exist.

7 CONCLUSION

This study failed to detect a difference in HPA activity determined by changes in cortisol and FGM concentrations between feedlot calves hot-iron branded on the cheek, hot-iron branded on the upper hind leg, and sham-branded. The blood cortisol and faecal 11,17-DOA levels in this study may thus be more indicative of stress associated with handling, restraint and the environment. It could also be that the pain response to branding was too slight or too short lived to detectibly increase cortisol levels. There was no clear difference between groups, but overall blood cortisol concentration may have been raised indicative of a generalized stressful situation at the time of branding and there was a large variation in blood cortisol concentrations between animals within groups. It appeared from the FGM analysis that the transport of the animals was a far more severe stressor than the actual branding event.

The only indication of a difference in response between animals branded and those sham-branded, was the increased occurrence of vocalization in the branded groups, particularly the cheek-branded group. Other behavioural and production outcomes measured after branding did not show differences between groups, suggesting that the acute stress of branding did not affect these cattle in the longer term.

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Appendix 1. Data capture sheet of behaviours on d6

31 Aug Behaviour 08:06

	1A	B	C	2A	B	C	3A	B	C	4A	B	C	5A	B	C	6A	B	C	7A	B	C	8A	B	C
Tail	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Kick	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lie	6	4	7	9	5	7	6	5	7	6	5	7	6	5	7	6	6	7	8	5	7	9	7	7
Rum	1	3	3	1	2	3	1	4	3	0	2	2	0	2	2	0	2	2	0	2	2	2	1	2
Eat	1	0	1	2	0	0	3	0	0	2	0	0	2	0	0	3	0	0	0	0	1	0	0	1
Drink	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Voc	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

(play — under 2A; play — under 7A)

	9A	B	C	10A	B	C	11A	B	C	12A	B	C	13A	B	C	14A	B	C	15A	B	C	16A	B	C
Tail	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Kick	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lie	9	8	7	9	8	7	9	8	8	9	8	8	9	8	8	9	8	8	9	8	8	10	7	8
Rum	0	3	3	0	3	3	0	3	3	0	2	2	0	3	2	0	1	2	0	2	3	0	0	3
Eat	0	0	1	0	0	1	0	1	1	0	1	1	0	1	1	0	1	0	0	0	0	0	0	0
Drink	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Voc	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

	17A	B	C	18A	B	C	19A	B	C	20A	B	C	21A	B	C	22A	B	C	23A	B	C	24A	B	C
Tail	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Kick	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lie	9	5	9	8	5	9	8	5	9	8	5	9	7	5	9	6	5	8	7	5	4	7	5	9
Rum	0	2	2	0	0	3	1	1	2	1	3	2	0	2	2	1	0	2	1	1	2	1	1	1
Eat	0	0	1	0	0	0	0	0	0	0	0	0	2	1	0	3	1	0	3	0	0	2	0	0
Drink	0	1	0	0	1	0	1	3	0	0	2	0	0	0	0	0	2	0	0	0	0	0	0	0
Voc	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0

(play — under 10A)

	25A	B	C	26A	B	C	27A	B	C	28A	B	C	29A	B	C	30A	B	C	31A	B	C	32A	B	C
Tail	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Kick	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lie	5	5	9	5	5	9	6	5	9	6	5	8	6	5	8	6	5	8	6	5	7	6	4	7
Rum	1	2	1	1	2	3	1	2	2	1	2	2	2	2	3	1	0	1	1	3	0	3	4	0
Eat	2	1	0	2	1	0	3	1	0	3	2	0	3	1	0	3	1	0	2	1	1	2	1	1
Drink	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Voc	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

(play — under 29A)

	33A	B	C	34A	B	C	35A	B	C	36A	B	C	37A	B	C	38A	B	C	39A	B	C	40A	B	C
Tail	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Kick	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lie	7	5	4	0	6	0	7	7	5	7	5	6	7	5	4	7	5	5	6	5	5	6	5	4
Rum	3	1	1	0	0	0	4	5	1	4	1	0	2	1	0	4	1	0	3	1	0	4	1	0
Eat	3	4	1	0	3	0	2	2	2	3	2	2	2	2	1	2	2	1	3	2	1	3	2	1
Drink	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	2	0	1	2
Voc	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

	41A	B	C	42A	B	C	43A	B	C	44A	B	C	45A	B	C	46A	B	C	47A	B	C	48A	B	C
Tail	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Kick	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lie	6	4	4	5	3	3	5	4	3	4	4	3	4	4	2	4	4	2	4	4	2	3	4	2
Rum	3	2	0	2	1	1	3	1	1	2	1	0	2	1	0	2	1	0	3	1	1	2	1	1
Eat	3	1	2	3	2	3	3	2	3	3	3	4	3	3	4	4	4	4	4	2	3	4	2	3
Drink	0	1	2	0	1	0	1	0	2	1	1	1	0	1	2	0	0	1	0	0	1	1	0	2
Voc	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

	49A	B	C	50A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
Tail	0	0	0	0	0	0																		
Kick	0	0	0	0	0	0																		
Lie	4	3	2	3	3	2																		
Rum	2	2	0	2	0	0																		
Eat	4	2	3	4	3	3																		
Drink	1	0	2	1	1	2																		
Voc	0	0	0	0	0	0																		