Non-invasive measurement of stress and pain in cattle using infrared thermography

A thesis submitted in partial fulfilment of the requirements for the degree of

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This thesis is dedicated to my late mother; my inspiration

Mrs Ann Stewart

who, alongside my father Don and brother Sean, provided the encouragement and inspiration to succeed

Abstract

The aim of this thesis was to validate the use of infrared thermography (IRT) to non-invasively measure stress and/or pain in cattle. The main approach was to measure changes in heat emitted from superficial capillaries around the eye (referred to as eye temperature) in response to various aversive husbandry procedures used routinely on farms. In addition, various exogenous challenges were given to investigate the role of the hypothalamic-pituitary-adrenal (HPA) axis and the autonomic nervous system (ANS) in regulating the eye temperature response. No evidence was found to support the hypothesis that an increase in eye temperature was due to HPA activity in cattle. A rapid drop in eye temperature occurred immediately after disbudding, an electric prod, startling and shouting. It is suggested that this was caused by the redirection of blood from the capillary beds via sympathetically-mediated vasoconstriction. Therefore, the role of the ANS was tested by measuring eye temperature, heart rate variability (HRV) and plasma catecholamine responses simultaneously. Somatic pain from disbudding and initial responses to surgical castration included a synchronised drop in eye temperature, increases in catecholamines and changes in HRV indicative of increased sympathetic activity. The role of the sympathetic nervous system was further confirmed by a drop in eye temperature that occurred following an epinephrine challenge. In contrast, deeper visceral pain from castration caused a more marked increase in eye temperature and changes in HRV indicative of increased parasympathetic tone. The underlying mechanism driving the increase in eye temperature is unknown; however, it is possible that it may be caused by vasodilation due to increased parasympathetic activity. These differences in ANS responses to different procedures, detected by IRT and HRV, may be due to the nature of the pain and the relative fear associated with the procedure. In summary, this research showed that during stress or pain, the heat emitted from superficial capillaries around the eye changes as blood flow is regulated under ANS control and these changes can be quantified using IRT. A combination of IRT and HRV is a non-invasive way to measure ANS activity and assess acute welfare impacts of husbandry practices in cattle. Further research using pharmacological inhibition and stimulation of the ANS activity would be beneficial to fully understand the underlying regulatory mechanisms of the eye temperature and HRV responses in cattle and other species during stress and/or pain. The full capability of IRT and HRV for detection of disease and emotional states and the effects of different intensities of pain, individual traits and previous experience also deserve attention.

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The calves themselves deserve special mention. Although some were subjected to pain, this can only be justified by the hope that this and future research will contribute to the knowledge required to improve the welfare of animals during routine husbandry practices and help to influence animal welfare standards that will encourage changes in the future. Thanks to the continuous harassment of Dr Tony Day who made my life as hard as he possibly could while applying for ethics approvals, very few calves suffered during this research. Thank you to Dr Day also for advice on veterinary matters and permission to cancel days and go home.

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Table of Contents

Declaration

Each chapter in this thesis is set out as a paper in the style and format required of the journal to which it has been published or is in the process of being submitted to at the date of submitting this thesis. Consequently, there is some repetition, particularly in the methods, and there are inconsistencies with the style and format between the chapters. Contributions to the research have been made by co-authors indicated in each chapter, however, my input was greatest, as I designed the research, undertook the fieldwork, analysed the data and wrote the manuscripts.

Chapter One

General Introduction

This chapter includes background information relevant to the overall aims of the experimental work and an overview of the structure of the thesis. Chapter 2 is a literature review of the topic and each of the following five chapters describe experiments and include a comprehensive introduction to the area that was examined. Similarly, each chapter includes a discussion of the results from each experiment. The final chapter (Chapter 8) is a brief discussion of the main findings of the thesis and integrates the results from each experimental study.

1.1. Aim

The aim of this thesis was to validate the use of infrared thermography (IRT) to non-invasively measure stress and/or pain in cattle.

1.2 Background

1.2.1 Issues

The assessment and alleviation of pain and stress during and following routine husbandry procedures used on farms (e.g., disbudding and castration of calves) are important components of farm animal welfare. In farm animals, pain has traditionally been overlooked and pain management has not progressed to the same degree as in companion animals. This is due to many factors including availability of effective analgesics, the attitudes of farmers and veterinarians, and ethical issues regarding the use of farm animals in research. However, there is now a higher level of public awareness and concern for animal welfare on-farm, and in recent years consumer demand has placed pressure on agricultural industries to provide 'welfare friendly' products that also meet high standards of food quality and safety. Despite evidence which demonstrates the welfare benefits of using analgesics, in most countries it is still common practice and legal to conduct procedures such as disbudding, dehorning and castration of young calves without pain relief. The reasons which are used to explain the lack of use of analgesics in farm animals include practical and economical factors, difficulty in administering the drugs, low value of the animal, high cost of the treatment, scarcity of licensed analgesic agents for use in animals intended for human consumption and concerns over drug residues in food (Vinuela-Fernandez et al., 2007).

The assessment and management of pain in farm animals have been reviewed previously (Mellor et al., 2000; Rutherford, 2002; Anil et al., 2005; Vinuela-Fernandez et al., 2007). A major issue for animal welfare research is that currently most of the methods we use to measure stress or pain are invasive (e.g., blood sampling) and there is a the lack of reliable, non-invasive tools (Stewart et al., 2005). Limitations of available non-invasive measures of stress and remote sampling are reviewed in Chapter 2. In order to improve animal welfare, new technologies and tools to evaluate the welfare impact of different husbandry practices are necessary.

1.2.2 Definitions

Throughout the following chapters, terms such as fear, stress and pain or a combination of these have been used to describe the responses of cattle to various husbandry practices. There are no standard, universally accepted scientific definitions for stress, fear or pain in animals. This lack of consensus regarding these definitions reflects the complexity and difficulty in assessing animal welfare. Fear has been defined as an emotional/anticipatory response to perceived danger, which plays a crucial role in motivating animals to avoid potentially harmful situations (Rushen et al., 1999b). Stress has been defined as *"a state that occurs when an animal is required to make abnormal or extreme adjustments in its physiology or behaviour in order to cope with adverse aspects of its environment and management"* (Fraser et al., 1975). Therefore, fear is the primary emotion that motivates an animal to flee and stress is the mechanism that equips an animal to react and flee more rapidly. Rushen (1986) suggested that 'stress' is just a convenient term to indicate a general topic of discussion and in some cases it may be best to avoid using the term. Mellor et al. (2000) suggested that the term 'distress' be used *"to acknowledge the emotional component of a noxious experience that elicit physiological stress responses in animals, whether the experience is predominantly emotional (e.g., fear), predominantly physical (e.g., exercise) or a combination of both (e.g., pain)"*.

Pain is a complex phenomenon, with sensory, cognitive and emotional components (Vinuela-Fernandez et al., 2007). Pain has been defined by the International Association for the Study of Pain as *"an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage"* (ISAP,

1979). However, this definition has not been accepted as being relevant to non-verbal animals as it relies on self-report. Therefore, Molony and Kent (1997) defined pain as *"an aversive sensory and emotional experience representing an awareness by the animal of damage or threat to the integrity of its tissues; it changes the animal's physiology and behaviour to reduce or avoid damage, to reduce the likelihood of recurrence and to promote recovery"*. From an evolutionary perspective, pain is advantageous and is considered to be crucial for survival. Although there is some disagreement regarding a definition of pain, there is much agreement among researchers that pain assessment in animals is a complex and difficult task.

1.2.3 Measurements of stress and pain

Most assessments of stress and pain in animals have used a combination of physiological (e.g., endocrine, autonomic and immune system responses) and behavioural responses. Following a noxious stimulus, the hypothalamic-pituitary-adrenal (HPA) axis and the autonomic nervous system (ANS) are the two main systems that co-ordinate the stress response and are primarily involved in metabolic homeostasis. Activation of the sympathetic division of the ANS and the resultant release of catecholamines (e.g., adrenaline and noradrenaline) from the adrenal medulla occur extremely rapidly to help the animal prepare for the 'fight or flight' reaction. Measurement of plasma cortisol concentrations to determine HPA activity has been the standard approach for studying stress and pain in farm animals. Cortisol affects carbohydrate metabolism by promoting gluconeogenesis, which provides energy to support the stress response (Matteri et al., 2000). In contrast to the rapid sympathetic nervous system (SNS) response to stress, the cortisol response is slower, more persistent and more easily measured. The functioning of the HPA axis and its response to stress have been well-defined and extensively studied in farm animals (see Mormede et al. (2007) for the most recent review) and cortisol responses to painful stimuli during dehorning (Stafford and Mellor, 2005a) and castration (Stafford and Mellor, 2005b) of cattle have been well documented.

Cortisol concentrations in blood are used widely to study HPA activity. This presents some problems related to the invasiveness of blood sampling techniques (Stewart et al., 2005) and alternatives have been used (e.g., urine, saliva, faeces, milk). In addition, the

cortisol response to a noxious stimulus may not persist for the duration of the effects of the stressor and thus may not reflect the overall impact of the stressor on the animal. There is also little evidence that cortisol concentrations vary with the severity of the stressor, as the HPA axis is highly sensitive to a range of stimuli that may not be harmful to the animal (Mormede et al., 2007). Therefore, to accurately evaluate the intensity of the response to noxious stimuli, complementary measures, such as behaviour and ANS activity, are necessary.

Physiological changes elicited by the ANS (e.g., increased heart rate and plasma catecholamines) may be more useful than cortisol for assessing acute responses to pain or stress because of their quicker response time. The ANS, which is under direct control of the central nervous system, is made up of two branches, the parasympathetic nervous system (PNS) and the SNS. Parasympathetic pathways predominate in the relaxed state and their primary function is to restore energy reserves. The effects of dominant parasympathetic activity include decreased heart rate, increased visceral activity (e.g., digestion) and decreased metabolic rate. Sympathetic pathways predominate when an animal is threatened. These stimulate the release of catecholamines from the adrenal medulla and function to mobilise the body's energy stores required for the 'fight or flight' reaction, a response that occurs in a matter of seconds. This involves increased heart rate and blood pressure, allowing more oxygen to be pumped around the body more rapidly; contraction of the spleen, releasing stored red blood cells to carry additional oxygen; release of stored sugar from the liver for muscles; deepening of respiration and dilation of bronchioles in order to take in more oxygen; dilation of the pupils, possibly to increase visual acuity; increase in the bloods ability to coagulate for sealing wounds; increase in lymphocytes to help repair damage to tissues and vasoconstriction. However, relatively few studies have measured sympathetic-adrenomedullary responses, such as plasma catecholamines, in farm animals. This may be due to the high cost of assays and practical difficulties in collection and measurement of catecholamines, as a result of their low concentrations and short half life (1-2 min) in plasma (Hjemdahl, 1993).

Behavioural responses to a noxious experience have an advantage in that they occur immediately, provide a good indication of the duration and different phases of a painful experience (Mellor et al., 2000) and can be measured non-invasively. Animals typically

withdraw from noxious stimuli or situations and if injury is inflicted, the resulting pain triggers the onset of protective behaviours that prevent further damage. There are many factors that influence behavioural responses to pain, such as the location of the pain and the type of tissue involved, the intensity of pain and whether it is an acute or chronic pain. However, the measurement and interpretation of behavioural responses can be difficult and the variation in responses can be misleading (see review by Rushen, 2000). Responses may vary depending on the individual's characteristics, previous experience, breed and species. Prey species show fewer outward signs of pain (e.g., vocalisations) compared to species with few or no natural predators. For example, following a painful husbandry procedure, such as castration, calves and lambs may adopt an immobile stance (statue standing), which could be misinterpreted as the animal not experiencing any pain. Therefore, knowledge of selective pressures affecting the species is required for accurate interpretation of behavioural responses. On the other hand, physiological responses to pain vary little among species (Broom, 2001) and may be more useful than behavioural responses for assessing the maximal intensity of a noxious experience (Mellor et al., 2000). Therefore, it is clear that a multidisciplinary approach is necessary to assess the welfare status of farm animals, which means integrating data from behaviour observations, physiology, immunology and production.

1.2.4 Infrared thermography and heart rate variability as non-invasive measures of autonomic nervous system activity

The potential of two methods, IRT and heart rate variability (HRV), as non-invasive measures of ANS activity that may complement other indexes of welfare assessment are investigated in this thesis. Traditional measures of sympathetic activity include plasma catecholamine concentrations, pupillary diameter, skin resistance and peripheral blood flow. Measures of heart rate in cattle have been recorded in response to many different procedures such as branding (Lay et al., 1992), transport (Kenny and Tarrant, 1987), human handling (Rushen et al., 1999a; Hemsworth, 2003), shouting (Waynert et al., 1999) and electric shock (Lefcourt et al., 1986). Interpretations have mainly been based on the assumption that increased heart rate reflects sympathetic activity, however, heart rate can only be interpreted as the net effects of both divisions of the ANS and is of limited use for assessing sympathovagal regulation. On the other hand, HRV provides a more accurate measure of ANS activity. Methods of HRV analysis use the cardiac

interbeat interval (IBI or R-R interval), which is calculated as the time interval between successive R waves of the electrocardiograph (ECG) (Figure 1). By using the IBI to calculate HRV parameters in time, frequency and non-linear domains, it is possible to

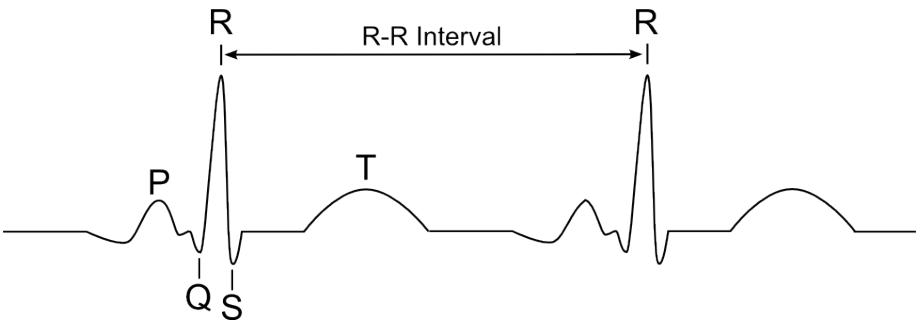

Figure 1. Three components of the electocardiograph (ECG); P wave, QRS complex and T wave. The interbeat interval is calculated as the time interval between successive R waves.

measure the balance between the SNS and the PNS. Time domain measures are the simplest parameters used to analyse HRV and reflect various aspects of statistical variability in the IBI data sets. In this thesis, the main time domain measurement used was the root mean square of successive differences (RMSSD), which is determined by calculating the difference between consecutive IBIs before squaring and summing them, the values are then averaged and the square root is obtained (von Borell et al., 2007). The RMSSD is the main time domain parameter used to estimate the high frequency beat-to-beat variations that represent parasympathetic activity (Malik and Camm, 1995). In the frequency domain, HRV is typically analysed using Fast Fourier transformation (FFT) and was used to calculate all HRV parameters in the experiments described in this thesis. FFT has the ability to assign the power of different bands to different underlying physiological functions. It is widely accepted that the high frequency (HF) band represents parasympathetic activity. The HF is influenced by respiratory rate, therefore, species-specific respiratory rates must be taken into account when locating the HF band during HRV analysis (von Borell et al., 2007). The power in the low frequency (LF) band and the LF/HF ratio have been suggested to indicate the level of

sympathetic activity or as an indicator of sympathovagal balance; however, their physiological meaning has been the topic of much debate (Ahmed et al., 1994; Goldberger, 1999). Table 1 defines the HRV parameters used in this thesis. The frequency band width of 0.30 to 0.80 Hz used for the HF power corresponds to range of 18 to 49 breaths per minute, which we measured in calves (see Chapter 5). See von Borell et al. (2007) for a recent review of the use of HRV measurements as an assessment of farm animal welfare.

Table 1. Classification and definition of the heart rate variability parameters used in this thesis.

Type of analysis	Parameter	Definition
Time domain	HR (bpm)	Mean heart rate
Frequency domain	RMSSD (ms)	The square root of the mean of the sum of the squares of differences between successive IBIs
	HFnu*	Power of the high frequency band (0.30-0.80 Hz)
	LFnu	Power of the low frequency band (0.04-0.30 Hz)
	LF/HF ratio	Power of the low frequency band divided by the power of the high frequency band

* The power of the HF and LF bands was presented as normalised units (nu), which represent the relative value of each power component in proportion to the total power minus the very low frequency component (frequencies lower than the lower limit of the LF band).

IRT is a non-invasive approach to indirectly measure blood flow changes by detecting small changes in skin temperature that are related to alterations in emotional state. Sympathetically-mediated vasoconstriction that occurs during the 'fight or flight' reaction functions as a protective mechanism to minimise blood loss from vulnerable areas (such as the skin) during injury. However, because short-term requirements of the skin are not crucial during attack or a painful stimulus, blood can be diverted from the cutaneous bed and redirected to organs (e.g., muscles and brain) with more urgent metabolic requirements (Blessing, 2003). IRT has been used to measure blood flow changes in humans during different emotional states. For example, Pavlidis et al. (2002)

used IRT as a lie detection tool and found that eye temperature increased in subjects that were lying, and Levine et al. (2001) found that eye temperature increased and cheek temperature decreased in response to a fright. However, few studies have investigated such responses in animals (see Chapter 2 for a review). In the following experimental chapters, IRT was used to measure the area within the medial posterior palpebral border of the lower eyelid and the lacrimal caruncle (see Figure 1, Chapter 5) in cattle. For simplicity, this measurement will be referred to from hereafter as 'eye temperature'.

1.3 Thesis structure

This thesis consists of a series of novel experiments that have been designed to validate IRT as a non-invasive measure of stress and/or pain. Chapter 2 is a literature review of recent advances and current research in the use of IRT as a non-invasive measure of stress in farm animals. Chapters 2, 3, 4 and 5 have been published in peer-reviewed international journals and Chapter 6 and 7 are in the process of being formatted for submission to peer-reviewed journals. A short paper, that investigated fear responses of sheep was published in the 2007 Proceedings of the New Zealand Society of Animal Production and is included in Appendix 1. Appendix 2 is a series of abstracts that were presented at various international conferences during the course of this PhD and Appendix 3 presents results that show that the two different methods of IRT analysis used during the experiments are highly compatible.

The first experiment (Chapter 3) investigated the possibility that the underlying mechanism responsible for driving eye temperature responses was the HPA axis, as suggested by Cook et al. (2001). A series of exogenous challenges (e.g., adrenocorticotrophic hormone (ACTH) and bovine corticotrophin-releasing hormone) in cattle were used to investigate which level of the HPA axis might be involved. There was no evidence that the HPA axis was driving the eye temperature response, and it was suggested that there may need to be a cognitive component for a response in eye temperature to occur.

Chapter 4 investigated eye temperature responses of cattle to various aversive handling procedures. During this study, eye temperature was recorded at a very high frequency for the first time and a rapid drop in eye temperature was detected following the use of

an electric prod, shouting and startling. This was the first time that this response had been recorded and the possibility that this may be due to sympathetically-mediated vasoconstriction is discussed.

The study in Chapter 5 investigated eye temperature responses to pain associated with disbudding of dairy calves and a similar drop in eye temperature occurred. Measurements of HRV were taken to investigate the role of the SNS in the eye temperature response. In addition, an ACTH challenge in these calves confirmed that HPA axis is not involved in mediating the eye temperature response.

Chapter 6 describes a study designed to evaluate the effects of using a non-steroidal anti-inflammatory agent (NSAID) in combination with local anaesthetic to alleviate pain following disbudding of calves using a combination of eye temperature, HRV and behavioural responses. All three parameters measured responded to the onset of pain when the effect of the local anaesthetic wore off. The final experiment (Chapter 7) investigated the possible role of the ANS in mediating the eye temperature response during painful procedures by measuring eye temperature, HR, HRV and catecholamine responses to castration of calves. The results show that IRT and HRV respond differently to castration compared to disbudding. Disbudding caused a more pronounced sympathetic response and castration caused a more pronounced parasympathetic response. In addition, further evidence for the drop in eye temperature being driven by sympathetic activity was confirmed following an epinephrine challenge.

The possibility that IRT and HRV may be able to detect responses to different types of pain stimuli is discussed in more detail in Chapter 8. Chapter 8 is a general discussion, integrating the results from the different experimental studies in order to draw overall conclusions and to discuss the limitations of IRT and how it can be used as a non-invasive method for assessing welfare. It includes a discussion of future areas of research that have arisen from this research. A list of references is provided at the end of each chapter.

1.3.1 Ethical statement

As stated in each chapter, the protocol and conduct of each study was approved by the Ruakura Animal Ethics Committee, Ruakura, Hamilton, New Zealand. Measures were taken to reduce the number of animals that were subjected to painful treatments and steps were taken to limit unnecessary suffering wherever possible. A veterinarian was always available during trials for advice and drug administration. The studies were approved by the committee and justified by the authors because the animals would normally be subjected to various procedures which are accepted husbandry practices used routinely on commercial farms. In addition, the studies contribute to the knowledge required to make such practices more humane and ultimately improve animal welfare.

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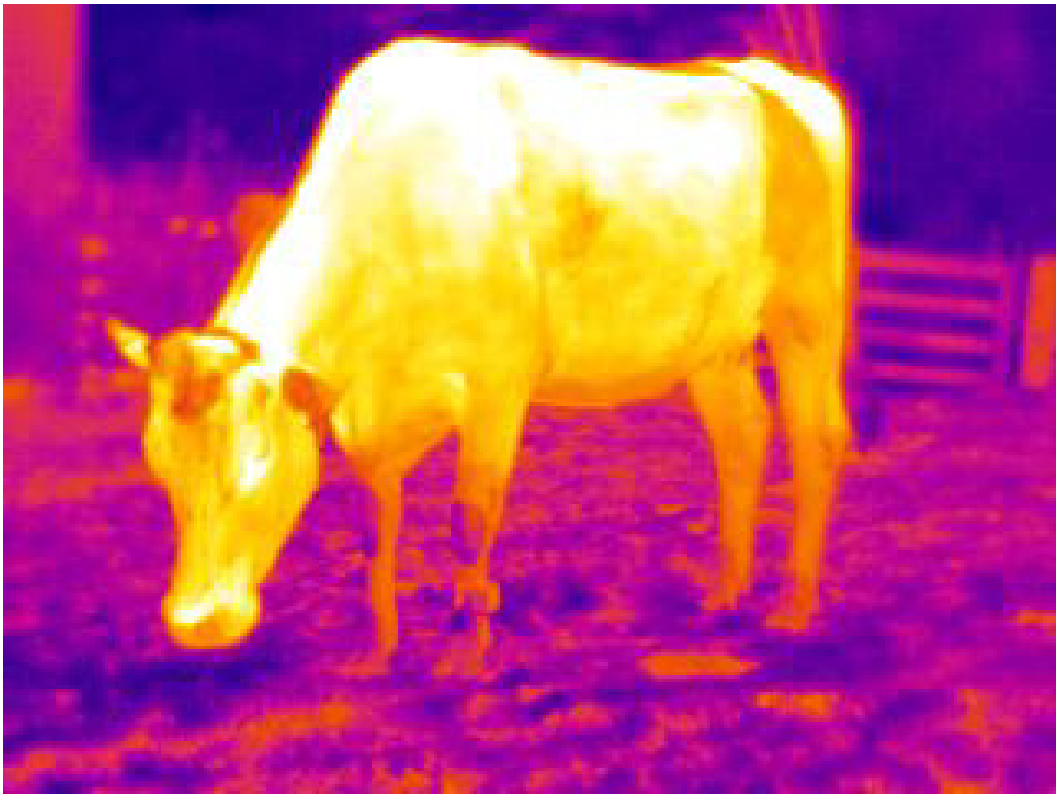
Chapter Two

Infrared thermography as a non-invasive tool to study animal welfare



Authors note: Chapter two is presented in the style of the journal Animal Welfare where it was published as:

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Abstract

Growing public concern about animal welfare and consumer demand for humanely produced products have placed pressure on the meat, wool and dairy industries to improve and confirm the welfare status of their animals. This has increased the need for ways to reliably assess animal welfare during commercial farm practices. Measurement of the stress caused by these practices is a major component of animal welfare assessment. However, a major issue for animal welfare science is that many of the techniques used to measure stress involve invasive procedures, such as blood sampling, that may cause a stress response themselves and thus perturb the measurement of interest. To reduce this problem, a number of non-invasive or minimally invasive ways to measure stress have been developed. These include measurement of cortisol concentrations in saliva and faeces, and remote devices for recording body temperature, heart rate and collection of blood samples. This review describes the benefits and limitations of some of these methods for measuring stress. In particular, the review focuses on recent advances and current research in the use of infrared thermography (IRT) for this purpose. Specific applications for IRT in the dairy and beef industries are described including an automated, non-invasive system for early diagnosis of infection in cattle. It is essential that non-invasive measures of acute and chronic stress are developed for reliable assessment of animal welfare during standard farm management practices and IRT may be a useful tool for this purpose. IRT may offer advantages over many other non-invasive systems as it appears to be capable of measuring different components of the stress axis, including acute sympathetic and hypothalamic-pituitary-adrenocortical (HPA) responses.

Keywords: animal welfare, infrared thermography, non-invasive techniques, remote sampling, stress measurement

Introduction

Stress has been defined as a state that occurs when an animal is required to make abnormal or extreme adjustments in its physiology or behaviour in order to cope with adverse aspects of its environment and management (Fraser *et al* 1975). Measuring stress is therefore central to the assessment of animal welfare. In recent years there has been a dramatic rise in public awareness of animal welfare during routine farm animal management practices and concern about whether animals suffer when they are exposed

to such situations. Some of these practices are coming under increasing public scrutiny, such as live animal export trades, where high mortality rates occur, and intensive herd management of dairy cows, where the animals' normal behaviours may be compromised.

Poor welfare standards are not only undesirable on ethical grounds, but they also have negative effects on animal health, meat quality and food safety. During transport and pre-slaughter handling, poor welfare can also affect meat quality by carcass damage from bruising and can result in dark-cutting beef, reduced tenderness (Schaefer *et al* 1988) and susceptibility to bacterial spoilage. Transport stress has been shown to cause increases in shedding of *Salmonella* spp. in cattle, which leads to increased hide contamination (Barham *et al* 2002). Consumer demand has placed pressure on the dairy, meat and wool industries to provide 'welfare friendly' products that also meet high standards of food quality and safety. In order to improve animal welfare, safety and overall productivity, development of new technologies and tools to evaluate the welfare impact of different management systems, particularly on-farm or pre-slaughter, are necessary.

Current measures of stress and their limitations

Behavioural and physiological systems are both involved in the response to stress and are often used in combination to assess stress (Broom & Johnson 1993). While many researchers have focussed on physiological measures, the importance of behavioural responses in this process has been highlighted by Jensen & Toates (1997) who suggested that stress is primarily a behavioural/psychological phenomenon. Behavioural responses to aversive stimuli often act to remove the animal from a potentially harmful situation as a first line of defence (Clark *et al* 1997). Factors that may influence a behavioural response include the individual's characteristics, species and breed, previous experience and the nature and severity of the stressor (Clark *et al* 1997). However, the measurement and interpretation of some behaviours can be subjective and the variation in responses can be misleading. For example, one animal may act aggressively in defence while another may withdraw in response to the same stressor. In addition, behavioural observations can require a large number of resources, sometimes over a long period of time, such as during 24 hr observations. Adequately trained personnel (ie to define behaviours and set objective criteria) who have a good

knowledge of the animal's species-specific behaviour are essential and in some cases lighting, video and digital analysis equipment may also be required. Psychological factors, including an animal's prior experience, may also influence its response to a particular situation and are hard to control and determine. The more an animal can predict and/or control its situation, the lower the stress levels (Weiss 1972; Jensen & Toates 1997). For example, as well as the physical stress of being restrained, an animal might also experience psychological stress due to the inability to avoid or escape from what it perceives to be a harmful or threatening situation.

Monitoring physiological responses such as hypothalamic-pituitary-adrenocortical (HPA) activity, and changes in plasma cortisol concentrations in particular, are frequently used to study stress responses in farm animals (Broom & Johnson 1993). Depending on the type of stressor being investigated, there may be some limitations on the usefulness of hormones as a measure of stress. These include difficulties in obtaining true baselines, inadequate sampling frequency that may miss rapid response times, and circadian and ultradian rhythms in hormone levels. A major problem is that methods such as blood sampling, that are used to measure some physiological indicators of stress, require restraint of the animal and handling procedures that can themselves cause a stress response that may lead to confounding results. Collection of a single blood sample via jugular venipuncture or jugular vein catheterisation can increase cortisol concentrations in dairy cows for up to 60 and 130 min, respectively (Alam & Dobson 1986). Hopster *et al* (1999) found that successive blood samples collected via jugular venipuncture from loose-housed dairy cows caused an increase in cortisol concentrations, however, the increase was less for cows that were accustomed to handling and restraint. Therefore, by acclimatising cows to sampling procedures, increased plasma cortisol concentrations due to handling associated stress can be avoided (Hopster *et al* 1999). However, this may not be true in all situations as De Silva *et al* (1986) found that the stress response of sheep to blood sampling was not reduced by previous handling, and cortisol and prolactin levels were lower when a remote blood sampling procedure was used. Ultradian and circadian rhythms also need to be accounted for when analysing physiological responses to stress as they can alter background hormonal levels. Strong ultradian rhythms of cortisol concentrations, with a period of around 120 min, have been found in lactating cows (Lefcourt *et al* 1993).

Cortisol levels may also be affected by other factors, such as social status (Mulleder *et al* 2003), reproductive state (Bell *et al* 1991) and activity levels (Fisher *et al* 2002).

Other physiological indicators of stress that have been used are changes in immune function (Kelley 1985) and sympathetic responses, such as increased heart rate, respiratory rate, body temperature and secretion of catecholamines (eg epinephrine) (Broom & Johnson 1993). Problems arise when using heart rate as a measure of stress, because changes due to metabolic activity are hard to distinguish from those due to emotional responses (Broom & Johnson 1993). The mean heart rate can only be interpreted as the net effects of interactions between both divisions of the nervous system, whereas measurement of heart rate variability (HRV) allows more detailed interpretation of cardiac activity in terms of the autonomic nervous system (Marchant-Forde *et al* 2004). It has therefore been suggested that HRV is a more accurate measure of stress in animals (Mohr *et al* 2002; Marchant-Forde *et al* 2004). One of the limitations is the lack of remote equipment that is capable of storing large amounts of data required for HRV analysis, therefore, few studies have investigated HRV in farm animals. However, technologies for storage and data analysis are improving (Marchant-Forde *et al* 2004). To conclude, there is no simple definitive method by which stress can be measured. It has been agreed by most researchers that a combination of physiological and behavioural measures may provide the best assessment of animal welfare (Clark *et al* 1997) and that there is a need for more reliable, non-invasive ways to measure stress.

Developments in non-invasive measures of stress and remote sampling

Remote devices have been developed to record physiological responses that may be useful for measuring stress, such as heart rate (Lefcourt *et al* 1999), respiratory rate (Eigenberg *et al* 2000), blood parameters (Ingram *et al* 1997) and ear pinna temperature (Ingram *et al* 2002; Beausoleil *et al* 2004; Lowe *et al* 2005). Core body temperature has been measured using surgically implanted radio-transmitters (Lefcourt & Adams 1998) and intravaginal loggers (Bluett *et al* 2000). Systems have also been developed to measure behaviour remotely, such as foraging behaviour (Rutter *et al* 1997) and lying, standing and walking (Champion *et al* 1997). However, most of these remote systems still require handling the animal and in some cases minor surgery, catheterisation and other manipulations such as insertion of a logger into the ear canal or vagina. Some

systems require animals to carry bulky equipment, which itself could cause changes in physiology or behaviour. It may not always be possible to release animals carrying such devices into their normal herd, which may disrupt normal behaviour patterns (eg social behaviour). Therefore, remote systems may be useful in fundamental studies investigating acute stress responses to management practices such as transport or de-horning, but they may be less practical for assessing animal welfare on-farm. In commercial farming situations, animals are often exposed to multiple stressors or chronic stress, and while it is important to have accurate, non-invasive tools to measure acute stress, it is also vital to have tools capable of measuring chronic stress and recovery over longer periods of time on-farm.

Indicators of HPA activity can be measured in faeces (Palme *et al* 2000; Morrow *et al* 2002), urine (Pol *et al* 2002), milk (Verkerk *et al* 1996) and saliva (Cook *et al* 1996; Negrao *et al* 2003) thus avoiding blood sampling procedures. These indicators require minimal contact with the animal, the ideal being collection of faeces in a paddock or housing system where no restraint or sampling is required and there is no interference with the stress levels of subsequent samples. Morrow *et al* (2002) found a significant increase in faecal glucocorticoid excretion in dairy cows following administration of adrenocorticotropic hormone (ACTH), exposure to a novel environment or transport, suggesting that glucocorticoid metabolites measured in faeces can reliably indicate acute adrenal activity in dairy cattle. Delay times of faecal excretion need to be taken into account in the interpretation of stress responses that occurred at a certain time before the sampling period. This delay (approx 10-12 hours in cattle) is related to the digesta transit time between the bile duct and the rectum and is affected by season, feed intake and pasture digestibility (Morrow *et al* 2002). Salivary “free” cortisol may be a better indicator of stress than “ total” cortisol measured in blood because cortisol levels in saliva represent an ultrafiltrate of the free steroid fraction in blood and are biologically active (Cook *et al* 1996). Cook *et al* (1996) found that handling and transport stimulated significant increases in salivary cortisol in pigs and serum and salivary cortisol were significantly correlated. In addition, urinary cortisol concentrations have been shown to increase significantly in response to an ACTH challenge in pigs (Pol *et al* 2002). These authors found that increases in stereotypic behaviour were related to higher urinary cortisol levels and suggested that this may be a useful tool for measuring chronic stress. For dairy cows, collection of milk samples for

analysis of cortisol is a convenient sampling method that in modern facilities requires minimal or no animal handling, and is a procedure that cows become well habituated to. Verkerk *et al* (1996) found that milk and plasma cortisol concentrations were highly correlated in dairy cows. However, short duration increases in plasma cortisol were not well reflected in milk cortisol concentrations at the following milking, so samples need to be obtained during the period of elevated cortisol (Verkerk *et al* 1996). In summary, current non-invasive measures of stress have limitations and there is still a demand for more non-invasive ways to measure stress and study animal welfare.

Infrared thermography: application for measuring stress and assessing animal welfare

Infrared thermography (IRT) is the measurement of radiated electromagnetic energy. Electromagnetic radiation can be described as a stream of photons, which are particles that have no mass, each travelling in a wave-like pattern and moving at the speed of light. The photons with the highest energy correspond to the shortest wavelengths. In the electromagnetic spectrum, broad range infrared radiation wavelengths (3-12 micrometers) are longer than visible light and in animals, 40-60% of heat loss is within this range (Kleiber 1975). Small changes in temperature may result in substantial amounts of emitted photons (or radiated energy) that can be detected very sensitively using IRT.

IRT has been used for many years in human and veterinary medicine. Yang & Yang (1992) reviewed the applications of IRT in various fields of medicine, including pharmacy and dentistry. Purohit & McCoy (1980) also report on the use of IRT in equine medicine to detect leg or hoof problems in race horses.

When an animal becomes stressed, the HPA axis is activated and heat production, as a result of increases in catecholamines and cortisol levels as well as blood flow responses, will produce changes in heat production and loss from the animal (Schaefer *et al* 2002). This can be detected using a specialised infrared camera to collect real-time pictorial images at a distance from the subject, usually with no need for contact or restraint. Minimal restraint may be necessary in some cases to simplify image collection, depending on the animal's flight distance and handling experience, the area of the body that is of interest and how close the operator needs to be to the animal.

There are some limitations and factors that need to be taken into account when using IRT. Images must be collected out of direct sunlight and wind drafts, and hair coats should be free of dirt, moisture or foreign material. Dirt on the animal alters the emissivity and conductivity, and excess moisture increases local heat loss to the environment or dryer areas of the coat (Palmer 1981). The effects of weather conditions, circadian and ultradian rhythms, time following feeding, milking, lying and ruminating are also factors that need to be considered and require further investigation as part of validating IRT as a stress measurement tool.

IRT has been used successfully to detect cattle that are pre-disposed to producing Dark-Firm-Dry beef (Tong *et al* 1995), Pale-Soft-Exudative pork in swine (Schaefer *et al* 1989), and to monitor transport stress in cattle (Schaefer et al. 1988). Other studies have used IRT to assess inflammation due to hot iron branding in cattle (Schwartzkopf & Stookey 1997) and scrotal surface temperature as a measure of fertility in bulls (Kastelic *et al* 1996).

There are a number of body sites, alone or in combination that can be monitored with IRT to indicate the impact of a wide range of potential adverse events. For example, Cook & Schaefer (2002) found a significant increase in radiated heat from the dorsal surface of Wapiti in response to removal of velvet antler. Other studies have shown increases in eye temperature in response to velvet antler removal in elk and reindeer (Cook *et al* 2005) and de-horning procedures in calves (Schaefer, unpublished). Eye temperature, measured using IRT, was more effective at detecting bovine viral diarrhoea (BVD) as changes occurred as early as one day, compared to 5-6 days for other areas such as the nose, ear, body and hooves (Schaefer *et al* 2003).

Infrared thermography in the dairy industry

Uses of IRT in the dairy industry thus far include early detection of estrus (Hurnik *et al* 1985), mastitis (Scott *et al* 2000; Berry *et al* 2003) and lameness (Nikkhah *et al* 2005). Recent studies have focused on the use of infrared thermography to detect mastitis much earlier than previously possible (Scott *et al* 2000; Berry *et al* 2003). This is of considerable value since mastitis is a major welfare and economic concern for the dairy industry (Gill *et al* 1990). The use of somatic cell counts (SCC) is currently the industry

standard practice for detecting mastitis in milking cows. Changes in SCC are often found late into the time course of an udder infection and do not identify all classes of infection, subclinical infections, or those which take some time to display clinical signs. Furthermore, general clinical signs such as changes in core or rectal temperature are also late to develop during the course of an infection. However, a reliable sign of an inflammatory response is often the increase in temperature of the infected area *per se* (Marieb 1989). Thus, an alternative method for the early identification of mastitis would be to measure the radiated infrared temperature of the mammary gland, since it is the actual site of infection.

Using an endotoxin-induced mastitis model, Scott *et al* (2000) found that inflammation could be detected from temperature differences with IRT earlier than with SCC or bovine serum albumin (BSA). BSA concentration peaked at 6 hours post-induction, whereas IRT temperature increases were evident within one hour post-induction. In Figure 1 below, the background and legs of the cow are cooler and darker, while the udder is warmer and lighter. The skin temperature is affected by the flow of heat from the body core to the surface of the body, and by the flow of heat from the skin surface to the atmosphere. Such images can reveal very early signs of udder infections in lactating cows.

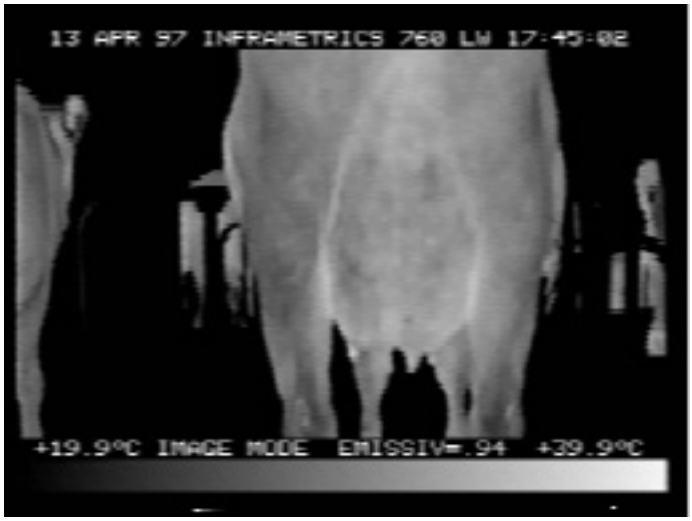

Figure 1: Infrared thermographic image (grey scale) of the udder and hind quarters of a dairy cow.

Berry *et al* (2003) used IRT to study the effects of environmental factors on the daily variation in udder temperature. They found a distinct circadian rhythm in udder temperature and a significant increase in udder temperature caused by exercise. The daily variation in udder temperature however, was smaller than the rise in temperature resulting from an induced mastitis response. They therefore concluded that IRT has promise as an early detection tool for mastitis if it is combined with detailed monitoring of environmental temperature.

Infrared thermography in the beef industry

Most beef calves globally are exposed to several transport and handling experiences within their lifetimes, typically involving co-mingling and some form of auction. These stressors often predispose calves to an increased incidence of diseases such as bovine respiratory disease (BRD) (Cusack *et al* 2003) and BVD (Houe 1999). These diseases have significant animal welfare and economic consequences for the beef industry, particularly in North America. The industry standard practice to identify calves with disease is the observation of clinical signs by an experienced cattle handler, at which point intervention measures are taken. Unfortunately, the appearance of clinical signs is usually several days or more into the course of the disease and by then the animal often requires considerable medical attention, including the use of antibiotics, in order to recover. In a BVD virus induction model, Schaefer *et al* (2003) used IRT as a non-invasive, early detection method for identifying calves with BVD. They found that increases in eye temperatures measured using IRT, were more consistent than other anatomical areas as mentioned earlier. There were also significant changes in eye temperatures several days to one week before other clinical signs of infection.

In such situations, the use of IRT for the non-invasive detection of early stages of disease in livestock would be possible. One such strategy which we have tested would be to incorporate an infrared scanning station into a water trough, as the animals visit this site on a regular basis (Figure 2). The scanning station is coupled to an electronic ID system, to automatically identify which animal is in attendance at the water station and to collect an infrared image of the eye region of that animal. From prediction indexes, such a system could then advise a feedlot operator or farm manager if an animal shows early signs of disease and thus requires medical attention.

Current research investigating infrared thermography as a tool to measure stress

The ability of IRT to detect and measure animal responses to stress has been a recent focus of research activity. Relationships between IRT and HPA activity were initially investigated by Cook *et al* (2001) who used IRT and cortisol to measure adrenocortical and metabolic activity in horses. Matched blood and saliva samples and IRT eye images were collected at set intervals before and after an ACTH challenge. The results showed a significant correlation between maximum eye temperature and both salivary and plasma cortisol suggesting that changes in eye temperature may be associated with activation of the HPA axis.

The study by Cook *et al* (2001) led to a need for further investigation into the relationship between the different components of HPA axis activity, IRT responses and changes in heat production and loss. In order to do this, we conducted a study to validate IRT eye temperature as a measure of stress, using dairy cattle as a model (Stewart *et al* 2005). Cows were given an ACTH challenge and subjected to psychological stress (social isolation). Increases in both cortisol and ACTH concentrations confirmed that the stress axis had been stimulated. IRT eye temperatures increased following both ACTH and saline (control) and also increased prior to the treatments, possibly due to effects of prior activity or handling stress. An interesting finding was that following social isolation, IRT eye temperature tended to fall rapidly and then increase again, which may reflect an acute sympathetic response due to the psychological stress. We also measured cortisol concentration and IRT eye temperature in these cows following two catheterisation procedures during this trial. IRT eye temperatures and cortisol concentrations did not increase significantly following the first procedure, however, both were significantly higher ($P < 0.001$ for IRT; $P < 0.05$ for cortisol) post catheterisation one week later, when the procedure was repeated. The increased response the second time the animals were catheterised suggests that the cows may have anticipated the procedure and that this caused the higher stress response. This supports the suggestion that some type of psychological component may be involved in the IRT eye response. IRT may therefore be capable of detecting acute sympathetic responses as well as HPA activity, however, research is currently being undertaken to investigate this possibility further.

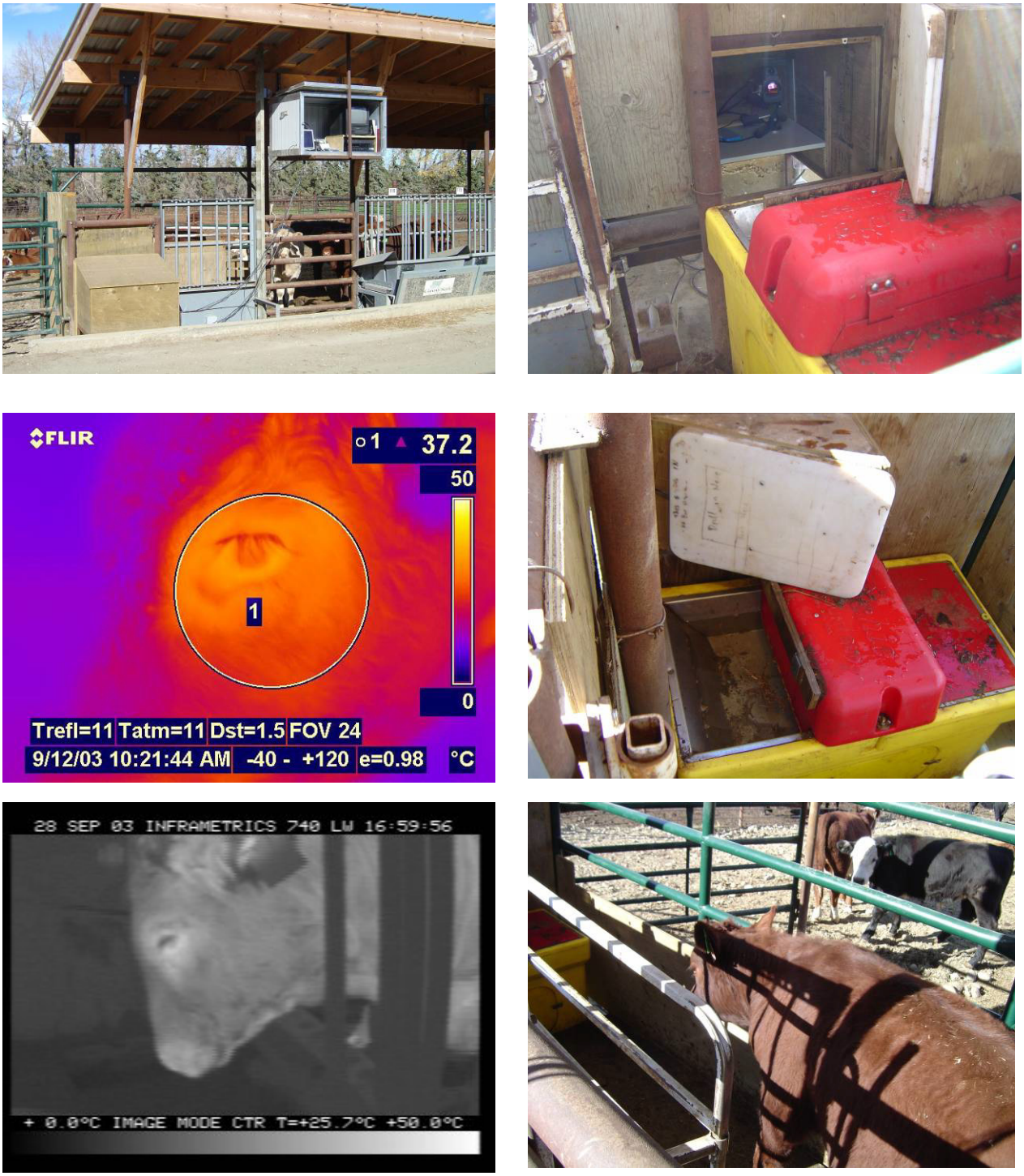

Figure 2. Automated infrared image collection for detection of early infection in cattle. The photos show (clockwise from top left) the collection site, infrared camera aimed at trough, electronic identification (EID) detection plate and water trough, animal entering for a drink, grey scale infrared image of an animal drinking and a close-up colour infrared image of the eye region.

Conclusions and animal welfare implications

Studies of animal welfare have commonly measured HPA axis activity, activation of the sympathetic system and behavioural responses to stress. However, a major problem for animal welfare researchers is that many of the methods used to measure these responses involve restraint or handling procedures, which may alter the stress response itself. Recently, there has been development of non-invasive or minimally invasive systems for measuring stress, but these have limitations and no single measure of stress is perfect. Reliable, non-invasive tools that can be used to measure acute and chronic stress during commercial practices and pre-slaughter are required. IRT fits this criteria and has great potential as a way to assess animal welfare. Uses in the dairy and beef industries have been developed and automated systems to monitor infection are currently being trialled. IRT equipment is portable, simple to use and animal restraint is minimal or unnecessary. There are some variables, such as weather conditions and circadian rhythms that need further investigation before IRT can be used reliably in an applied sense during commercial practices on-farm. However, IRT may have certain advantages over other non-invasive methods by offering insight into the metabolic consequences of stress and enabling measurement of short-term acute and long-term chronic responses to stress.

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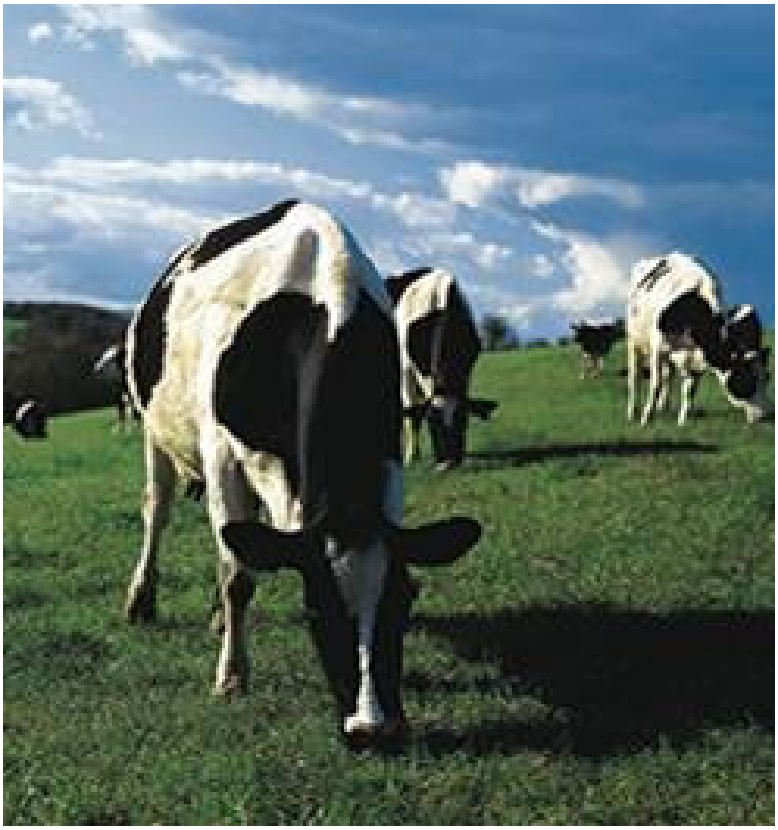
Chapter Three

Non-invasive measurement of stress in dairy cows using infrared thermography



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Abstract

The possibility that changes in eye temperature, measured using infrared thermography (IRT), can detect stress in dairy cattle was examined by six different stimulations of the stress axis. Six cows were given six treatments in a random Latin-square design: 1) Control (saline) 2) ACTH (0.05 mg Synacthen) 3) bCRH (20 µg) 4) bCRH (40 µg) 5) epinephrine (1.4 µg /kg liveweight) and 6) social isolation. Treatments were administered at time 0 and blood samples were taken at -30, -15, 0, 5, 10, 15, 20, 30, 40, 50, 60, 75, 90, 120, 180 and 240 min except for epinephrine which was sampled at -30, -15, -10, -5, 0, 2, 5, 10, 15, 20, 30, 45, 60, 90 and 120 min. Core body temperature was recorded every 10 min and eye images collected every 2 min. Eye temperature and cortisol increased following catheterization ($P<0.05$). ACTH increased following bCRH, cortisol increased following ACTH and bCRH ($P<0.001$) and NEFA increased following epinephrine ($P<0.001$). Core body temperature was unaffected by treatments. Eye temperature was unaffected by bCRH and epinephrine but was higher 30 and 60 min following control and ACTH ($P<0.001$). Our results provide evidence that exogenous HPA stimulation does not increase eye temperature. The increases in eye temperature following catheterization however raise the possibility that a cognitive component may be required for an eye temperature response to occur.

Keywords: Dairy cattle; Infrared thermography; Stress; Hypothalamic-pituitary adrenal axis; Eye temperature

1. Introduction

Activation of the hypothalamic-pituitary-adrenal (HPA) axis is a primary physiological response to stress in mammals. Measurement of HPA activity from plasma corticosteroids is commonly used to evaluate acute stress responses to management procedures in dairy cattle [1-3]. Unfortunately, many of the methods used to measure stress require restraint, handling and blood sampling of the animal that can cause a stress response and lead to confounding results. For example, collection of a single blood sample via jugular venipuncture or from a jugular catheter can cause increased plasma cortisol concentrations in dairy cows for up to 60 and 130 min, respectively [4]. Although HPA activity is a useful measure of stress, it does not comprise the entire

stress response (e.g., sympathetic and cognitive or emotional responses) and caution should be taken when interpreting HPA activity. The roles of cognitive and emotional responses to stress are being recognised as important considerations when interpreting stress responses [5]. Therefore, reliable, non-invasive methods capable of measuring the entire spectrum of stress responses would greatly improve our ability to assess animal welfare.

The close relationship between stress and the metabolic system is well documented [6]. When the sympathetic nervous system is activated due to stress, heart rate increases, adrenaline (epinephrine) is released, activation of β- receptors increases the availability of metabolic fuels, such as glucose and fatty acids, and blood flow is redistributed to the skeletal and heart muscles to prepare the animal for the 'fight or flight' response. The temperature of the extremities and skin is largely dependent on the amount of blood flowing through peripheral vessels [7], therefore, blood flow changes, as a result of stress, will alter the amount of radiated heat that is lost from thermoregulatory sites on the animal. For example, Blessing [8] found an alerting environmental stimuli caused a reduction in blood flow to the ear in rabbits and Vianna & Carrive [9] found the tail and paw temperatures of rats, measured using infrared thermography (IRT), decreased following a conditioned fear response. This initial acute response to stress presumably acts to increase perfusion pressure, redirecting blood flow to organs and the skeletal musculature, which has more urgent metabolic requirements and may also be a protective mechanism to reduce blood loss in the case of injury [8, 9].

IRT can detect changes in peripheral blood flow from the resulting changes in heat loss and therefore may be a useful tool for measuring stress in animals [10]. Recent studies, using IRT, have shown that the temperature of the eye may be a good indicator of stress [11-13]. Specifically, the temperature of small areas around the posterior border of the eyelid and the caruncula lacrimalis, which have rich capillary beds innervated by the sympathetic system, respond to changes in blood flow. IRT can detect subtle temperature changes in this region in response to stress caused by lying in humans [13]. Levine et al. [14] found that eye temperature increased in response to a sudden loud noise in humans, suggesting that startling is associated with increases in blood flow to the eye [14]. Eye temperature also increased in response to velvet antler removal in Wapiti [11]. Cook et al. [12] investigated the underlying causes for this increase in eye

temperature using IRT and plasma and salivary cortisol as measures of adrenocortical and metabolic activity in horses. Matching blood and saliva samples and eye images were collected at set intervals before and after an injection of adrenocorticotrophic hormone (ACTH). There was a significant correlation between maximum eye temperature and both salivary and plasma cortisol, suggesting that changes in eye temperature may be driven by activation of the HPA axis. However, the specific mechanisms involved in the observed relationship between the HPA axis and IRT responses are not known. It would be necessary to determine which components of the HPA axis are responsible and the time frame of the response in more detail to improve IRT as a technique for measuring stress.

The aim of the present study was to investigate the potential of using IRT to measure eye temperature as a means to detect stress in dairy cattle. This was achieved by measuring the eye temperature response to stimulation of different components of the stress system, including HPA, sympathetic and psychological. Furthermore, to assess which level of the HPA axis is involved, eye temperature was measured following administration of ACTH and bovine corticotrophin-releasing hormone (bCRH) which are recognised methods for evaluating HPA axis function in dairy cattle [2, 3, 15]. An epinephrine challenge was used to determine if the sympathetic nervous system affected eye temperatures. Epinephrine is a catecholamine secreted principally by the medulla of the adrenal gland and in response to a stressor, results in increased heart rate, hydrolysis of glycogen to glucose and the breakdown of lipids in fat cells. It also acts to dilate the pupils and constrict blood flow in the skin and gut. Finally, to investigate the eye temperature response to a psychological stressor, we used social isolation (i.e., cow isolated from the rest of their group). Our hypotheses were that eye temperature would increase when cows were subjected to ACTH, bCRH and isolation, and decrease in response to epinephrine. Catheterization for the above challenges provided an opportunity to evaluate the eye temperature response to this stressor.

2. Methods

The protocol and conduct of this study were approved by the Ruakura Animal Ethics Committee.

2.1 Animals and treatments

Six, non-lactating, non-pregnant, 3 to 5 year old Holstein Friesian cows with a mean body weight of 495 kg (range: 444 to 528 kg) were used in this study. All the cows were halter trained and brought into an enclosed barn, where the sampling took place three days per week, for three weeks prior to the start of the trial to acclimatise them to the facility, handling and the IRT procedures. Eight days prior to the start of the trial, all cows were treated with progesterone-releasing intravaginal devices (CIDR^{TM}, Inter-Ag Pty. Ltd., Hamilton, NZ) and 2 mg of oestradiol benzoate (CIDIROL, Bomac Laboratories, Auckland, NZ) so that each animal was in the luteal phase of its oestrous cycle during treatments. After eight days, CIDRs were removed and cows were treated with 2 ml of Estroplan (Parnell Laboratories Auckland, NZ) and 1ml of CIDIROL. Core body temperature was recorded every ten minutes using a Vemco TX minilog data logger (Vemco Ltd., Shad Bay, Nova Scotia, Canada) attached to a modified CIDR^{TM} and placed into the vaginal cavity of each cow at the same time that jugular catheters were inserted (18 hours before the start of the trial). Two hours prior to sampling, cows were brought into the barn, tethered and each fed approximately 30 kg silage. Cows had access to water *ad lib* and between sampling they were managed outdoors on pasture.

Six treatments were assigned randomly in a six by six Latin square design and were as follows: T1) physiological saline (2ml) T2) synthetic adrenocorticotrophic hormone ACTH $_{(1\text{-}24)}$ (0.05 mg Synacthen; Novartis Pharma AG, Basle, Switzerland) T3) standard dose synthetic bCRH (Cat. C-2671, Sigma, St Louis, MO, USA), 20 µg dissolved in 2 ml of physiological saline T4) high dose bCRH, 40 µg dissolved in 2 ml of physiological saline T5) Epinephrine (1.4 µg /kg liveweight) and T6) social isolation (one cow remained behind after the other cows left the barn at the end of the other treatments). Each exogenous treatment was administered once to each cow via jugular catheter. On each sampling day, treatments T1-T4 were administered at 0930 h, T5 was administered at 1130 h and T6 occurred at 1330 h when the other treatments were completed. There were three sampling days per week over two weeks, allowing a recovery of at least 48 hrs between treatments. Catheters were removed at the end of the first week (Day 6) and cows were given two days recovery before catheters were reinserted into the contralateral jugular for the second and final week of the trial.

2.2 Blood sampling

Blood sampling times for treatments T1-T4 were at -30, -15, 0, 5, 10, 15, 20, 30, 40, 50, 60, 75, 90 and 120 min in relation to treatments that were administered at time 0. Blood samples (25ml) were taken via jugular catheter into a syringe. This protocol has been shown to reliably evaluate plasma cortisol and ACTH responses to HPA challenges for 4-6 hrs [15]. For treatment T5, sampling intervals were -30, -15, -5, 0, 2, 5 10, 15, 20, 30, 45, 60, 90 and 120, as described by Kolver et al. [16]. Sampling intervals for T6 were the same as that for T1-T4, except that baseline (pre-treatment) samples were taken every 20 min. Blood samples were also collected from all cows before the catheterization procedure, via caudal vein venipuncture, and after catheterization, via jugular catheter, on week one and two.

Blood samples, collected via catheter, were immediately placed into two different vacutainer tubes. For NEFA and cortisol, blood was placed into tubes containing lithium heparin anticoagulant, placed on ice, centrifuged within one hour of collection and stored at -20^{o}C until assayed. For ACTH, blood was transferred into K_3-EDTA tubes, centrifuged immediately and stored at -80^{o}C until assayed. Plasma was assayed for concentrations of cortisol, ACTH and non-esterified fatty acids (NEFA).

2.3 Infrared thermography

Two identical cameras (ThermaCam S60, FLIR Systems AB, Danderyd, Sweden) were used to collect eye images so that frequent, simultaneous sampling from six animals could be achieved. Image collection started immediately before the first baseline blood sample and finished at the time of the last blood sample. Each infrared camera operator moved repeatedly between three cows, in the same order, taking one image of each at a time, resulting in an average of 2.37 min between images for each cow over the entire sampling period. All cows were scanned from the same side (left), angle (90^{o}) and distance (0.5-1.0 m). Eye images were also collected for each cow before and after each catheterization procedure.

Ambient temperature and relative humidity inside the barn were recorded every 30 min, and these values were used during image analysis to allow for atmospheric changes.

Images were recorded as JPEG files and stored on a compact flash card within the camera. Image analysis software (ThermaCam Researcher 2.7, FLIR Systems AB, Danderyd, Sweden) was used to determine the maximum temperature within an oval area traced around the eye, including the eyeball and approximately 1 cm surrounding the outside of the eyelids.

2.4 Hormone assays

Cortisol was measured using a double-antibody radioimmunoassay as described previously [2]. The minimum detectable level was 0.58 ng/ml. The intra-assay coefficient of variation (CV) for plasma pools measuring 4.4, 45.5 and 86.2 ng/ml were 0.0, 6.7 and 4.4% respectively and inter-assay CV's (n=2) for the same pools were 14.6, 12.7 and 9.4%. The concentration of ACTH in plasma was determined using a two-site immunoradiometric assay (Euro-Diagnostica B.V., Arnhem, The Netherlands) as described by Fisher et al. [2]. The sensitivity of the assay was 2.2 pmol/l. The intra-assay CV (n=2) for samples containing 5.6 and 50.7 pmol/l were 0.0 and 3.2% respectively, and inter-assay CV's (n=2) were 12.6 and 2.4%, respectively.

2.5 Non-esterified fatty acids

Plasma samples were assayed for concentrations of non-esterified fatty acids (NEFA) (colourimetric method: Wako, Japan) using a Hitachi 717 analyser (Roche) at 30^{o}C by Alpha Scientific Ltd (Hamilton, New Zealand).

2.6 Statistical analysis

The change in eye temperature during the 30 min pre-treatment period (excluding the first sample) was calculated by linear regression for each animal each day. These slopes were then analysed using a two way ANOVA to calculate the mean change for each treatment. The change in eye temperature in response to each treatment was calculated for each animal by subtracting the average temperature during the 30 min pre-treatment period from the average temperature during 30 or 60 minutes post-treatment. Changes in eye temperature were analysed using ANOVA to determine the treatment effects. The

same method was used for core body temperatures except that a REML (restricted maximum likelihood) analysis was used because not all animals had core body temperature measurements on all days. For the social isolation treatment, changes in eye temperature from pre-treatment to the first sample post-treatment and to 15 and 30 minutes post-treatment were calculated for each animal. The significance of these differences was tested using a Student's t-test. The peak height, the time to peak and the adjusted area under the curve (treatment response area minus control response area, AUC) for ACTH and cortisol concentrations were calculated for each animal each day, and analysed using ANOVA. For ACTH, both the AUC and the peak height were log transformed due to a non-normal distribution of the data. The average eye temperature and cortisol concentration immediately before and after the two catheterization procedures were calculated and a two-tailed Student's t-test used to analyse differences. A paired Student's t-test was also used to compare the plasma NEFA concentrations at the time that epinephrine was administered with the peak concentration at 5 min post-treatment.

3. Results

3.1 Cortisol, ACTH and NEFA concentrations

There were pronounced peaks in cortisol and ACTH concentrations following treatment with ACTH and bCRH but only minor increases following epinephrine or social isolation (Table 1). Cortisol peaked between 40 and 42 min following the ACTH and bCRH treaments. The AUC for ACTH was greater than for control following the high dose of bCRH ($P<0.001$), and the AUC for cortisol was greater following both ACTH and bCRH than for control ($P<0.001$). There was no effect of either epinephrine or social isolation on the AUC for ACTH or cortisol. NEFA concentrations increased significantly following epinephrine and peaked at 5 min post-treatment ($P<0.001$) (Figure 1).

3.2 Eye temperature

Eye temperature responses were variable across treatments (Figure 2). During the pre-treatment baselines, eye temperature increased ($P<0.01$) in both control and the ACTH

groups (Table 2). Average eye temperatures were higher 30 and 60 min after treatment for cows given ACTH and saline (control) compared to 30 min pre-treatment baselines ($P<0.001$) (Table 3). The first eye temperature measurement taken following social isolation was lower than baseline ($P<0.01$) and the level at 30 min ($P<0.001$).

Table 1. Pre-treatment mean, mean peak height and time to peak for plasma ACTH and cortisol concentrations 1 hour post- treatment and the adjusted (above control) AUC.

	ACTH				Cortisol			
Treatment	Pre-treatment mean (pg/ml)	Peak height (pg/ml)	Time to peak (min:sec)	AUC (pg/ml /min)	Pre-treatment mean (ng/ml)	Peak height (ng/ml)	Time to peak (min:sec)	AUC (ng/ml /min)
Control	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
ACTH	35.5	94.0	05:01	-180.1	2.0	45.1	41:40	4005.6*
CRH low	25.8	83.0	17:30	1086.4	2.4	37.7	40:00	2096.6*
CRH high	25.9	101.9	22:29	2865.1*	1.6	36.8	39:59	2589.2*
Epinephrine	31.5	38.0	15:20	-521.9	4.7	10.5	15:00	105.5
Isolation	32.8	39.6	20:00	-609.7	4.7	14.1	20:50	566.0
se	12.8	12.9	09:51	603.3	2.2	3.0	04:47	341.5

* significant ($P<0.001$)

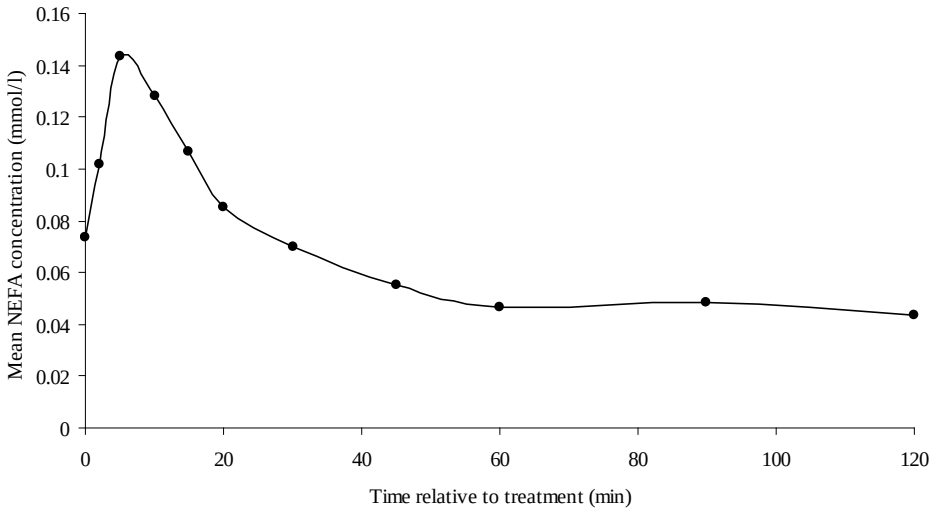

Fig. 1. Mean plasma concentration of NEFA following the epinephrine treatment.

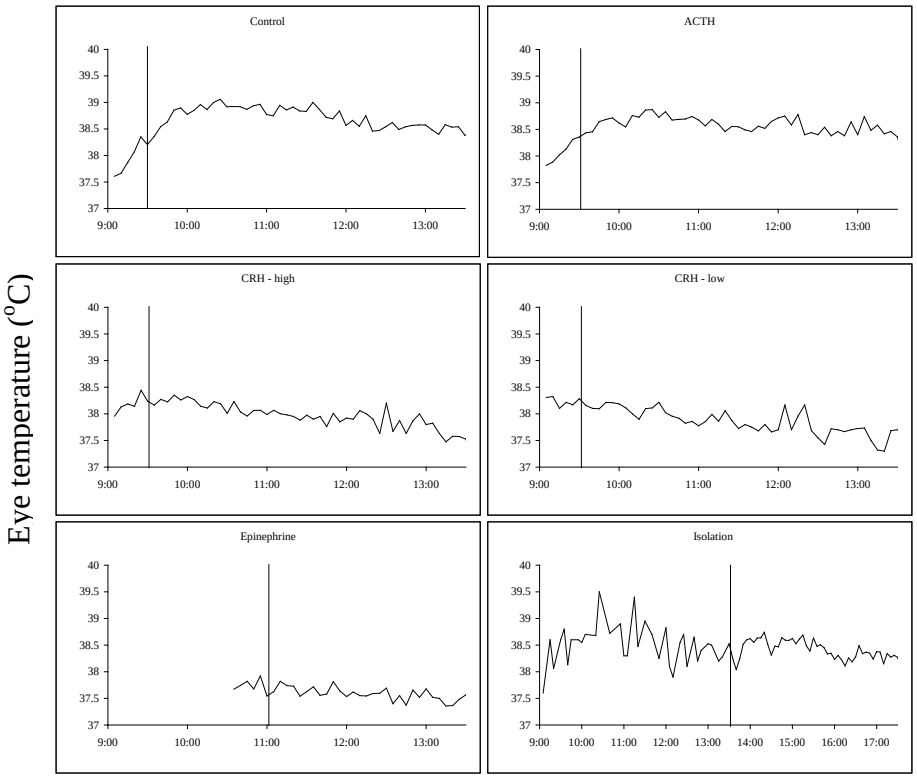

Fig. 2. Average eye temperature in response to the six treatments. Vertical lines indicate the time the treatments were administered.

Table 2. Rate of increase (degrees/min) in eye temperature during the 30 min pre-treatment period for each treatment.

Treatment	Slope (degrees/min)
Control	0.030
ACTH	0.023
CRH low	-0.003
CRH high	0.013
Epinephrine	-0.001
Isolation	-0.003
sed	0.009

Table 3. Average eye temperature 30 min before and 30 and 60 min post-treatment.

Treatment	30 min before	30 min after	60 min after	Difference 30min	Difference 60 min
Control	37.9	38.6	38.8	0.7 *	0.9 *
ACTH	37.8	38.4	38.5	0.5 *	0.6 *
CRH low	37.0	37.0	36.9	0.0	-0.1
CRH high	37.2	37.2	37.2	0.1	0.1
Epinephrine	36.6	36.5	36.5	-0.1	-0.1
Isolation	38.4	38.4	38.5	-0.1	0.0
sed				0.12	0.12

*significant differences pre and post-treatment (P<0.001)

3.3 Core body temperature

There was a tendency for core body temperature to increase after treatment for all animals, but there were no significant differences between the average core body temperature during baseline and 30 and 60 min post-treatment following any of the treatments (Table 4).

3.4 Responses to catheterization

Eye temperatures and cortisol concentrations did not increase significantly after catheterization in week one (P > 0.05), but they were higher (P=0.002 and P=0.026 respectively) after catheterization when the procedure was repeated one week later (Figure 3 and 4).

Table 4. Average core body temperature 30 min before and 30 and 60 min post-treatment.

Treatment	30 min before	30 min after	60 min after	REML estimates 30 min	REML estimates 60 min
Control	38.1	38.4	38.4	0.2	0.3
ACTH	38.5	38.5	38.5	0.1	0.2
CRH low	38.4	38.5	38.5	0.1	0.1
CRH high	38.3	38.5	38.4	0.2	0.2
Epinephrine	38.4	38.5	38.5	0.0	0.0
Isolation	38.3	38.4	38.4	0.1	0.1
sed				0.08	0.08

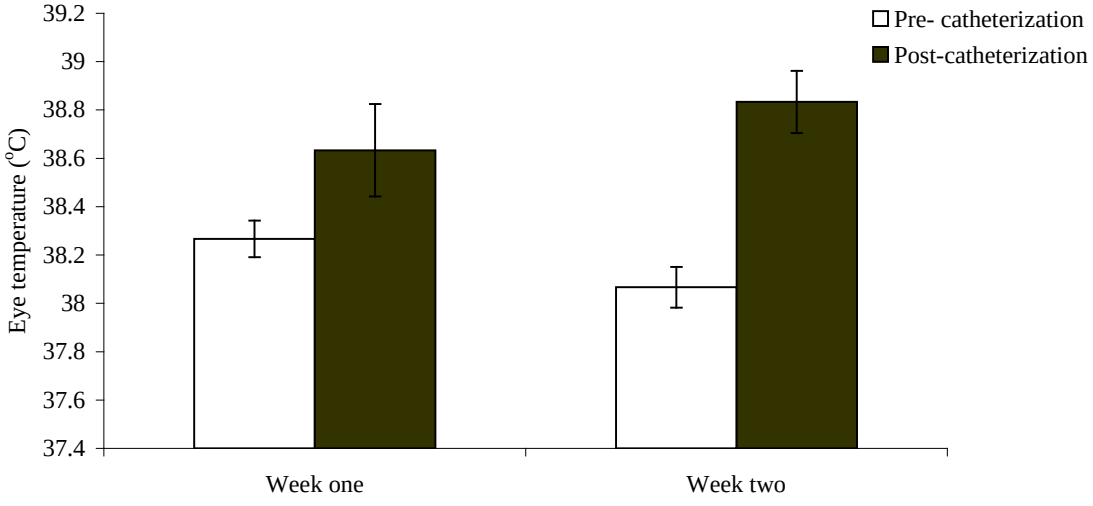

Fig. 3. Average eye temperature immediately before and after the first (Week one) and second (Week two) catheterization procedures.

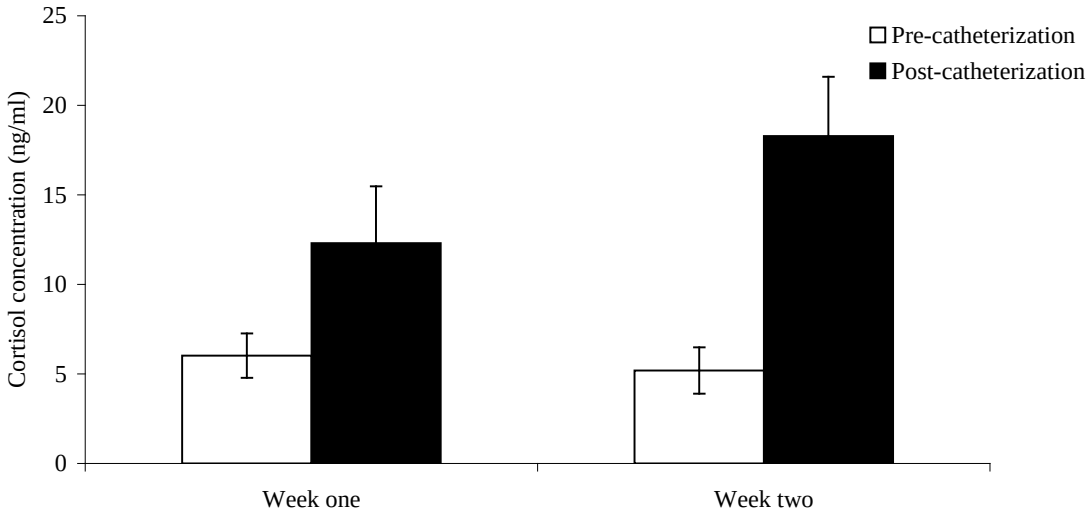

Fig. 4. Average plasma cortisol concentrations immediately before and after the first (Week one) and second (Week two) catheterization procedures.

4. Discussion and conclusions

We found no evidence to support our hypotheses that eye temperature, measured using IRT, increases in response to exogenous stimulation of HPA activity, or decreases in response to an epinephrine injection. The plasma cortisol and ACTH responses to the ACTH and bCRH treatments were consistent with previous studies [15] confirming that the HPA axis was stimulated. Our results are therefore inconsistent with those of Cook et al. [12] who found a correlation between eye temperature and cortisol levels following an ACTH injection in horses and suggested that the HPA axis may be driving the IRT response. Possible reasons for this discrepancy between studies are experimental design, as no control treatment such as saline was given in the study by Cook et al. [12], IRT and blood samples were taken less frequently (every 20 min) than in the present study, or a species difference between horses and dairy cows. It must also be noted that our ability to detect an increase in eye temperature after treatment may have been reduced by the increases in eye temperature prior to treatment. Although these dairy cows appeared highly habituated to human contact and were habituated to both the facilities and procedures, they may still perceive handling as stressful, causing temperature increases before treatment.

We predicted that a drop in eye temperature would be observed in response to epinephrine due to a sympathetically-mediated reduction in blood flow and the resulting decrease in heat loss from the orbital capillary beds. Following the epinephrine treatment, plasma NEFA concentrations increased, which indicates the mobilisation of fatty acids, and eye temperature tended to decrease gradually but not significantly. It is possible that the dose of epinephrine was not sufficient to cause a reduction in peripheral blood flow, or that the sampling interval was not frequent enough to see a drop in eye temperature if it was only short-term. For example, infrared nasal temperatures of monkeys, exposed to a potentially threatening person, decreased within 10-30 s [17]. Our experimental model was not designed to detect a rapid drop in eye temperature following treatment; however, the lower eye temperature of the first sample taken following social isolation suggests that a drop may have occurred. This result is insufficient to resolve this issue because sampling was not frequent enough to capture a rapid drop in eye temperature and baselines were too variable. Another explanation for a lack of a response could be that activation of the sympathetic system by exogenous epinephrine may not be representative of the body's entire repertoire (e.g., behavioral, neuroendocrine and autonomic) of responses to stress that would occur during the "fight or flight" response to a physical or psychological stressor. McMillan [5] reported several studies which demonstrate that sympathetic activation itself does not produce a stress response, but when stress or fear is present, sympathetic activation enhances the response. This may also explain why there were no treatment effects on core body temperature as the exogenous treatments given may not have stimulated a full and sufficient stress response required to cause a change in core body temperature.

Other studies have shown that an animal may not perceive and display signs of stress, even when physiologically challenged, unless there is a cognitive awareness that stress is occurring [5]. This importance of cognitive awareness may explain the lack of eye temperature responses following exogenous treatments. Stimulation of the stress axis using exogenous ACTH, CRH and epinephrine did not have a cognitive component, whereas social isolation and catheterization did. Following catheterization, the increase in both cortisol and eye temperature was larger following the second catheterization than the first, which may have been due to anticipation of the procedure, i.e., there was a perceived stressor as well as a physiological one. The possibility that there needs to be a cognitive component may also explain why other studies have shown increases in eye

temperature when there is a psychological component, such as in response to velvet antler removal [11] and lying in humans [13].

In conclusion, this study found evidence that exogenous stimulation of the HPA axis alone does not cause increases in eye temperature. However, an increase in eye temperature following catheterization raises the question of involvement of cognitive processing in this response and needs to be resolved.

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Chapter Four

Infrared thermography as a non-invasive method for detecting fear-related responses of cattle to handling procedures

Authors note: Chapter four is presented in the style of the journal Animal Welfare where it has been accepted for publication as:

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Abstract

Two experiments were conducted to determine whether maximum eye temperature, measured using infrared thermography (IRT), could non-invasively detect responses of cattle to handling procedures. Experiment one used six crossbred heifers randomly assigned to two groups in a crossover design and subjected to 1) being hit with a plastic tube on the rump 2) being startled by the sudden waving of a plastic bag. Experiment two used 32 crossbred bulls randomly assigned to three treatments: 1) control; restraint only, 2) electric prod; two brief applications of an electric prod or 3) startled; as in experiment one, accompanied by shouting. Exit speed (m/s) was recorded on release from the restraint. Maximum eye temperature was recorded continuously pre and post-treatment. In experiment one, eye temperature dropped rapidly between 20 and 40 seconds following both treatments (hitting; -0.23 ^{o}C, $P<0.05$ and startling; -0.32^{o}C, $P<0.01$) and returned to baseline between 60 and 80 seconds following hitting and between 100 and 120 seconds following startling. In experiment two, eye temperature dropped between 0 and 20 seconds following treatments (electric prod; -0.42 ^{o}C, $P<0.01$ and startling plus shouting; -0.57^{o}C, $P<0.001$) and returned to baseline by 180 seconds following startling plus shouting, but did not return to baseline for 5 minutes following electric prod. Exit speed tended to be faster following the electric prod. In conclusion, IRT detected responses that were possibly due to fear and/or pain associated with the procedures. IRT may therefore be a useful non-invasive method for assessing aversiveness of handling practices to cattle.

Keywords: animal welfare, cattle, eye temperature, fear, handling, infrared thermography

Introduction

Understanding and identifying animal handling techniques that can cause fear and pain in animals on commercial farms is of importance from the perspective of animal welfare and livestock industry economics. The increasing size of modern commercial farms, time constraints, and labour-saving technologies, such as robotic milking systems, reduce the contact animals have with humans and increasingly most of the contact that they do have is negative (Rushen *et al* 1999b) (eg restraint, transport, veterinary procedures). Negative attitudes of stockpeople towards animals on commercial farms are largely responsible for high levels of fear of humans that impact on animal welfare

and productivity (Hemsworth & Coleman 1998; Hemsworth 2007). Aversive handling of cattle by stockpeople (eg shouting, quick unpredictable movements) and the type of handling aids used (eg flags, sticks and prods) can produce a fear of humans that has a major impact on animal welfare, loss in animal production and increased risk of injuries to both animal and handler (Hemsworth & Coleman 1998; Rushen *et al* 1999b; Hemsworth 2003). Hitting and aversive handling of dairy cows has been shown to reduce milk yield (Breuer *et al* 1997; Rushen *et al* 1999a) and increase heart rate (Rushen *et al* 1999a), weight loss (Breuer *et al* 1997) and lameness (Chesterton *et al* 1989; Breuer *et al* 1997). In addition, excessive use of handling aids may in fact hinder rather than facilitate movement of cattle (Rickenbacker 1959). All of these factors have major economic cost to the farm and to livestock industries as a whole.

There is a lack of available tools to measure fear and pain responses of cattle, therefore, few studies have examined responses of cattle to different handling techniques and the use of specific handling aids. Researchers have used a combination of behavioural and physiological responses to measure fear. Some behavioural responses that have been used to measure fear in cattle include flight distance (Fisher *et al* 2000; Breuer *et al* 2003; Kilgour *et al* 2006), time to approach a handler (de Passillé *et al* 1996; Rushen *et al* 1998), vigilance (Welp *et al* 2004), vocalisations (Grandin 2001) and open-field tests (Kilgour 1975). Caution is required when interpreting behavioural responses, for example, in an open-field test, increased activity as a measure of fearfulness may be influenced by other factors such as novelty, social motivation, familiarity with the environment and handlers, curiosity and general activity or exploration (Rushen 2000). In addition, behavioural responses may not necessarily indicate the severity of a noxious experience as well as physiological indicators can, such as hypothalamus-pituitary-adrenal (HPA) axis or sympathetic activity (Mellor *et al* 2000).

Few studies have examined the physiological responses, such as HPA axis or sympathetic activity, which may be related to the fear of cattle to human handling. The acute physiological response to fear has two main components. Firstly, the rapid-onset, short-lived sympathetically-mediated catecholamine response, which activates the ‘fight or flight’ reaction, and secondly, the slower-onset, longer duration cortisol response, mediated by the HPA system (Mellor *et al* 2002). Changes in plasma cortisol concentrations in response to stress during painful husbandry procedures in cattle have

been well established (Stafford & Mellor 2005). However, fewer tools are available to measure the acute sympathetic response to fear. During the 'fight or flight' reaction, heart rate increases and blood flow is redirected away from the extremities to organs and musculature (vasoconstriction). A rapid drop in eye temperature, measured using infrared thermography (IRT), observed following disbudding of calves without local anesthetic, may be a sympathetically-mediated response via vasoconstriction (Stewart *et al* 2008). Nakayama *et al* (2005) found a drop in nasal temperature, measured using IRT, of rhesus monkeys after exposure to a threatening person. Eye temperature, measured using IRT, has been used as a non-invasive tool for measuring stress in other species (Stewart *et al* 2005) and increased in response to velvet antler removal in Elk (Cook *et al* 2005), jugular catheterisation of dairy cows (Stewart *et al* 2007) and a fright in humans (Levine *et al* 2001).

The objective of the present study was to determine if eye temperature, measured by IRT, could non-invasively detect responses of cattle to various handling procedures. The handling procedures studied were a sudden unpredictable movement (startling using a plastic bag) with or without human shouting and different handling aids commonly used on commercial farms to move cattle (hit with a plastic tube or a shock with an electric prod).

Materials and Methods

The study consisted of two experiments. The protocol and conduct of both experiments were approved by the Ruakura Animal Ethics Committee, Hamilton, New Zealand and the Lacombe Animal Care Committee, Alberta, Canada.

Experiment one

The first experiment took place at AgResearch Ltd, New Zealand. Six, non-pregnant Hereford x Friesian heifers (16 months old), weighing approximately 400kg were used. Two months prior to the start of the trial, all animals were halter-trained and brought into covered yards where the trial took place for 2 hrs per day (4 days per week) to habituate them to being handled and loosely tethered in the facility. Animals were randomly allocated into two groups and received two treatments in a crossover design. One treatment, hitting, consisted of three brief slaps on the rump with a 1m length of plastic tubing. The other handling treatment, startling, consisted of two brief sudden

shakes of a plastic bag in front of the animal's head. Two animals were given the startling treatment each day for three days, then given the hitting treatment over a following three days. All treatments were carried out by the same operator. Sampling occurred at the same time each day, between 9:00 and 12:00h, to reduce any circadian effects. Each animal was randomly selected for treatment and was brought into the yard and tethered loosely by a rope halter along with two companion animals. Infrared images of the eye region were collected at a consistent distance (approx. 0.5m) and angle (90^{o}) from the left side of the animal using an infrared camera (ThermaCam S60, FLIR Systems AB, Danderyd, Sweden). The camera was set to calculate and display the value and position of the maximum temperature within a circular area of analysis on each frame. The area of analysis was restricted to the medial posterior palpebral border of the lower eyelid and the lacrimal caruncle (Stewart *et al* 2008). From 10 minutes before until 10 minutes after treatment, the infrared camera was connected to a digital handycam (Sony DCR-TRV355E) to enable recording of each video frame. The maximum temperature imprinted on each frame was retrieved by converting the video into digital files (.mpeg) and examining each file on a frame by frame basis (25 frames per second) using The Observer, version 5.0 software (Noldus Information Technology, Wageningen, The Netherlands) over the period from 2 minutes prior to treatment to 3 minutes after treatment. The maximum temperature was averaged for each second and used for analysis. Ambient temperature and relative humidity in the yard were recorded and entered into the infrared camera to calibrate it for atmospheric conditions.

Experiment two

The second experiment was carried out at Lacombe Research Centre, Canada. This study used thirty two crossbred bulls, averaging 350 kg, randomly assigned to three treatment groups: 1) control; (n =13) restraint only, 2) electric prod; (n = 10) two brief 1 second applications of an electric cattle prod (9000 V, Hot Shot HS2000, Hot-Shot Products Co. Inc., Minnesota, USA) applied to the rump area or 3) startling plus shouting; (n = 9) two brief sudden shakes of a plastic bag in front of the animals head accompanied by a loud shout. Treatments were randomised and balanced across three test days and were carried out by the same operator. Sampling occurred at the same time each day, between 8:00 and 12:00h, to reduce any circadian effects. Each animal was brought into a restraining chute situated inside a barn and allowed a 5-minute rest period post-capture, followed by a 40-minute sampling period before being released.

Infrared images of the eye region were recorded continuously using a video cassette recorder (JVC HR-S9400U, Wayne, New Jersey, USA) connected to an infrared camera (ThermaCam S60, FLIR Systems AB, Danderyd, Sweden) situated 2 m at a right angle from the left side of the animal for 20 minutes pre and 20 minutes post-treatment. As each animal exited the restraining chute it interrupted an infrared beam and sensor unit set up 1 m from, and perpendicular to, the head gate. This event started a timing system that was stopped as the animal passed a second infrared beam and sensor unit 2 m from the first. The time time taken to travel between the two sensors and the distance travelled (2 m) were used to calculate a chute exit speed (m/s). Image analysis software (ThermaCam Researcher 2.7, FLIR Systems AB, Danderyd, Sweden) was used to determine the maximum temperature (^{o}C) within the area of the medial posterior palpebral border of the lower eyelid and the lacrimal caruncle every 1-3 seconds during a 5 minute pre-treatment and 5 minute post-treatment period. Ambient temperature and relative humidity inside the barn were recorded and entered into the infrared camera to calibrate it for atmospheric conditions.

Statistics

Average eye temperature (±SEM) was expressed as the difference from baseline (ie average over 20 seconds pre-treatment) at consecutive 20-second blocks post-treatment. A one-way ANOVA was then used to compare differences between treatments and a Student's t-test was used to compare differences at various periods post-treatment from baseline. A one-way ANOVA was also used to test for differences in exit speed between treatments.

Results

Experiment one

Eye temperature dropped rapidly between 20 to 40 seconds by 0.23 ^{o}C ±0.08 ($P<0.05$) and by 0.32^{o}C ±0.05 ($P<0.01$) after hitting and startling respectively. Eye temperature remained lower than baseline from 40 to 60 seconds following startling only (-0.22 ^{o}C ±0.07, $P<0.05$). Eye temperature returned to baseline levels between 60 and 80 seconds following hitting and between 100 and 120 seconds following startling (Figure 1 and 2).

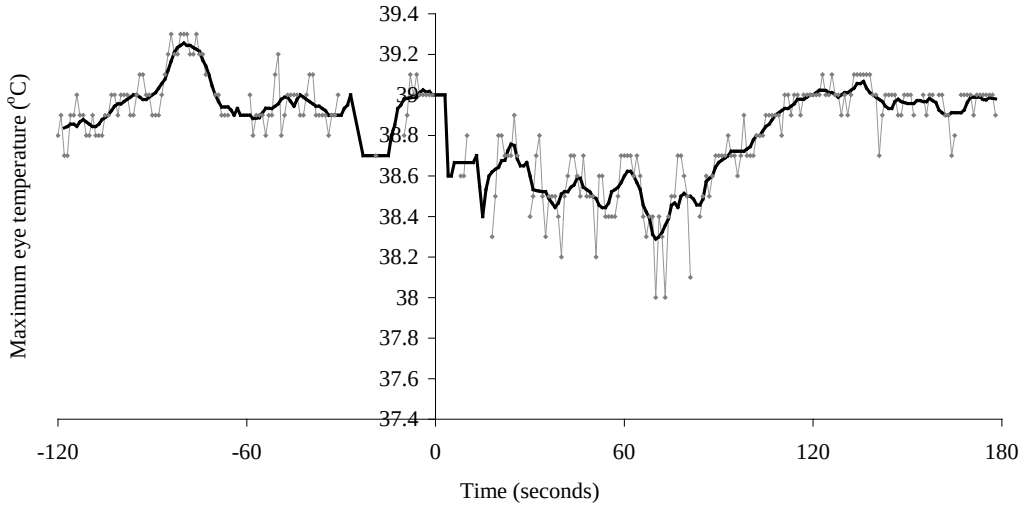

Figure 1. Maximum eye temperature (°C) for one calf before and after startling in experiment one. 0 seconds indicates the time of treatment. The solid black line represents a 19 second moving average and the grey line indicates the raw data for this individual.

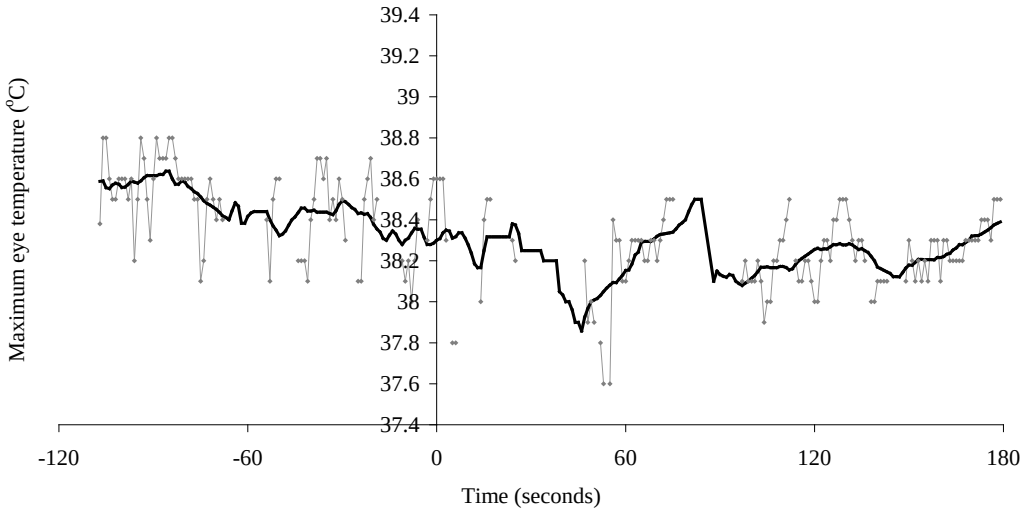

Figure 2. Maximum eye temperature (°C) for one calf before and after the hitting treatment in experiment one. 0 seconds indicates the time of treatment. The solid black line represents a 19 second moving average and the grey line indicates the raw data for this individual.

Experiment two

Eye temperature dropped rapidly from 0 to 20 seconds following both treatments (electric prod; -0.42^{o}C±0.12, P<0.01 and startling plus shouting; -0.57^{o}C±0.12, P<0.001) and was still lower than baseline in both treatments from 20 to 40 seconds post-treatment (electric prod; -0.32 ^{o}C±0.11, P<0.01 and startling plus shouting; -0.43^{o}C ±0.12, P<0.01). At 80 seconds post-treatment, eye temperature was lower than baseline (P<0.05) in the electric prod treatment only. Following startling plus shouting, eye temperature had returned to baseline levels by 180 seconds, however, following the electric prod, eye temperature did not reach baseline levels again during the entire 5-minute post-treatment period (Figure 3). Eye temperature did not change

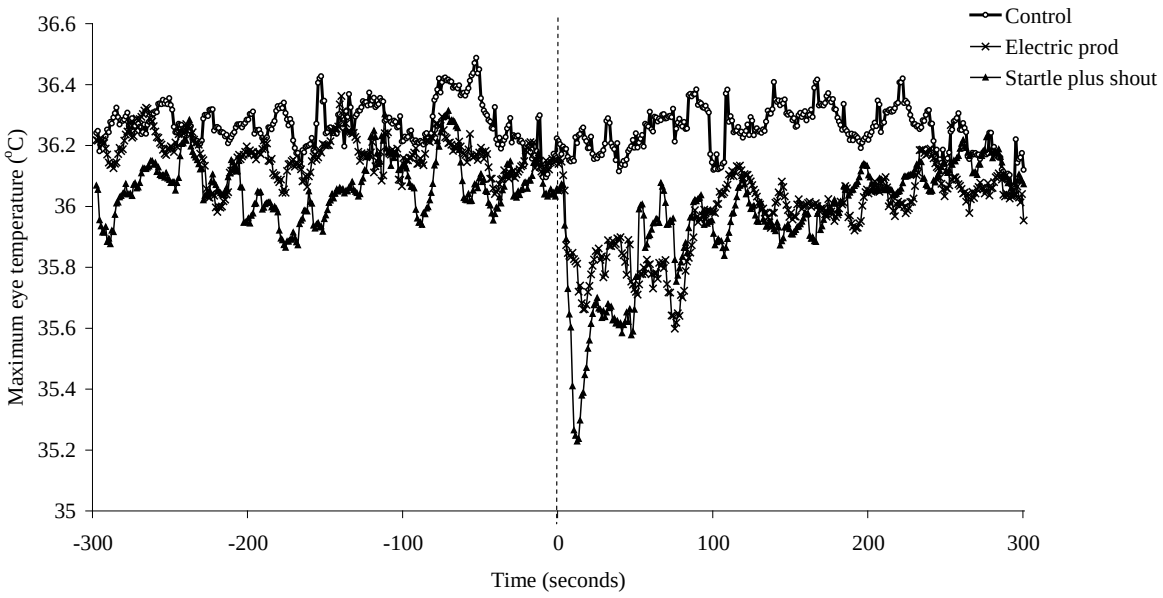

Figure 3. Maximum eye temperature (^{o}C) 5 min pre and 5 min post-treatment, using a 9 second moving average, following control (○, n =13), electric prod (x, n=10) and startle plus shout (▲, n=9) in experiment two. The dashed line (0 seconds) indicates the time of treatment.

following the control treatment. Compared to controls, eye temperature was lower for both the electric prod and startling plus shouting treatments from 0 to 20 seconds (P<0.05). From 20 to 40 seconds, only the startling plus shouting treatment had a lower eye temperature (P<0.05) than controls. There were no significant treatment differences between the electric prod and startling plus shouting. Exit speed from the chute tended

to be faster following the electric prod (mean 2.2±0.3 m/s) compared to the control (mean 1.7±0.3 m/s, P=0.188) or the startling plus shouting (mean 1.9±0.3 m/s, P=0.434) treatment.

Discussion

This study has shown that eye temperature, measured using IRT, can detect acute responses that may be due to the fear and/or pain associated with handling of cattle. Eye temperature dropped rapidly following all aversive treatments. The magnitude and duration of the drop in eye temperature was consistent with previous studies (Nakayama *et al* 2005; Stewart *et al* 2008). For example, following disbudding of calves without local anaesthetic, eye temperature dropped rapidly and was lower (-0.27˚C) within the first 5 min post-treatment than baseline (Stewart *et al* 2008). Similarly, the nasal temperature of monkeys dropped by 0.2˚C within 10-30 seconds (mean duration of the decrease 220-280 seconds) following exposure to a threatening person (Nakayama *et al* 2005).

The drop in eye temperature may be a sympathetically-mediated response. Following disbudding of calves without local anaesthetic, the drop in eye temperature was accompanied by a decrease in heart rate variability parameters that reflect a change in sympatho-vagal balance and may indicate an increase in sympathetic activity (eg the ratio of the low- (LF) to high-frequency (HF) power) (Stewart *et al* 2008). Blessing (2003) reported that fear and anxiety resulting from perception of a threat or possible dangerous event, with or without actually experiencing the actual physical attack or pain can cause sympathetically-mediated cutaneous vasoconstriction. When vasoconstriction occurs, blood flow to the peripheral capillary vessels is reduced and consequently skin temperature decreases (Vianna & Carrive 2005). Vianna and Carrive (2005) used IRT to measure stress responses of rats that were fear-conditioned by exposure to footshocks, and found a decrease in tail and paw temperature (-5.3 and -7.5 ^{o}C respectively) due to cutaneous vasoconstriction. This cooling of the extremities was associated with an increase in freezing immobility, a behavioural response to fear in rats. They suggested that the blood supply to the tail was very sensitive to the level of fear and that the stronger the fear the stronger and the longer the duration of the vasoconstriction.

The nature of the stimulus or the level of fear and/or pain that the animals experience may affect the duration of the drop in eye temperature. In experiment one, eye temperature took longer to return to baseline following startling compared to hitting. In experiment two, the initial drop in eye temperature was not significantly different following startling plus shouting compared to the electric prod, however, there was a longer lasting response and a tendency for exit speed from the chute to be faster following the electric prod. This could be interpreted as a difference in the degree of aversiveness between the two treatments. However, the aversiveness of an experience may also be due to its novelty or suddenness. Desire *et al* (2006) found that suddenness rather than unfamiliarity was responsible for a greater increase in heart rate of sheep exposed to a rapid compared to a slow appearance of a scarf, and suggested that the startle response is dependent on the suddenness of the event. The significance and interpretation of the responses found in the present study requires further investigation to determine the potential of IRT to measure the relative aversiveness of different handling procedures.

Shouting has been shown to be aversive to cattle (Waynert *et al* 1999; Pajor *et al* 2000). Pajor *et al* (2000) found that cows took longer and required more force to be moved down a race following repeated treatments with an electric prod or being shouted at compared to being hit with an open hand or having their tail twisted. However, Pajor *et al* (2003) found that cows in a Y-maze, showed no preference to being shouted at or being hit. Shouting has also been shown to increase the heart rate and movement of beef cattle in a restraining chute (Waynert *et al* 1999). The use of electric prods has been associated with vocalisations of cattle at commercial slaughter plants indicating that the devices are aversive to cattle (Grandin 2001). Lefcourt *et al* (1986) gave cows a range of electric shocks from 2.5 to 12.5mA and found that as mA increased, heart rate increased, cows became more agitated and some responded violently. It is difficult to compare the present results to that of other studies comparing the aversiveness of handling aids, because of possible inconsistencies in the type of negative handling and the force and way in which they are applied.

While there was no increase in eye temperature in the present study, other studies have shown that eye temperature can increase in response to fear or pain (Levine *et al* 2001; Cook *et al* 2005; Stewart *et al* 2007). In addition, Stewart *et al* (2008) found that after

an initial drop, eye temperature increased following disbudding with or without local anaesthetic. It is possible that the treatments in the present study produced insufficient stimulation to cause an increase in eye temperature. The mechanism for this increase is as yet unknown, however, there is evidence that it is not driven by changes due to heat, physical activity, increased HPA activity or local inflammatory processes (Stewart *et al* 2008). Due to the short time frame of the drop in eye temperature, studies that have only reported increases in eye temperature may have failed to detect an initial drop in eye temperature because sampling was too infrequent.

Several factors may influence the eye temperature response such as breed, temperament or experience with human contact. The present study was not designed to compare the effects of these on eye temperature, however, they warrant further investigation. To minimise the potential for confounding autonomic stimulation, animals should be habituated to the specific sampling conditions wherever possible. Other factors such as the angle and distance of the camera from the animal are also important to take into account when using IRT. However, it is still possible to achieve consistent measures of eye temperature in an outdoor, unrestricted situation. For example, an infrared camera located at a water trough was used to collect images automatically when animals visited the trough to drink (Stewart *et al* 2005; Schaefer *et al* 2007). In the present study, angle and distance were kept consistent and it is unlikely that these factors had any influence on the results. In addition, the drop in eye temperature was not due to evaporative heat loss caused by moisture in the eye. Evaporative heat loss depends on the surface area, and even a high rate of 4 g/m² trans-epidural water loss corresponds to only 150 W /m² (Mitchell 1977). The small surface area of the eye (1/10,000 of a square meter) translates into less than 0.02 W, which would produce an undetectable (substantially less than 0.1^{o}C) change in eye temperature. See reviews by (Stewart *et al* 2005) and (McCafferty 2007) for further discussion regarding limitations and recent advances in IRT applications.

It is important to note that IRT has shown promise as a non-invasive measure of sympathetic activity and while to date it has been validated during pain and fear responses its use may be extended to other situations where activity of the autonomic nervous system is changed, such as during pleasure or positive responses (eg provision of resources such as social contact, space or comfortable resting areas). Boissy *et al*

(2007) described the potential for heart rate variability parameters, combined with behavioural responses, to non-invasively monitor autonomic activity associated with positive emotions in animals. Similarly, IRT responses to positive situations warrant further investigation and may be complementary to heart rate variability responses for assessing emotional states in animals.

In summary, this study has shown that eye temperature, measured using IRT, can be used to detect responses to handling procedures in cattle. It is possible that the eye temperature response is due to the fear and/or the pain associated with the handling procedures, and is consistent with pain responses to disbudding in calves. The duration of the drop in eye temperature may relate to the level of fear and/or pain an animal is experiencing and may be used to compare the aversiveness of different handling methods. Eye temperature may therefore be a useful addition to behavioural and physiological methods for assessing fear and pain responses to handling of cattle.

Animal welfare implications

IRT has potential for non-invasively evaluating fear and/or pain responses in cattle during routine handling practices on commercial farms. It is clear from the results in the present study that the use of electric prods, hitting and shouting are all aversive to cattle during handling and moving and their use on-farm should be monitored and minimised to prevent reduced animal welfare and consequent economic costs to the livestock industries.

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Chapter Five

Eye temperature and heart rate variability of calves disbudded with or without local anaesthetic

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Abstract

The possibility that pain can be detected from changes in eye temperature and heart rate variability (HRV) during disbudding was examined in thirty calves, randomly assigned to four treatments: 1) sham handling (control), 2) Local anaesthetic (LA, cornual nerve injection) and sham disbudded, 3) sham LA and disbudded, 4) LA and disbudded. During a 40 min sampling period, maximum eye temperature, behavior and HRV parameters were recorded continuously. One week later, twelve disbudded calves were injected with adrenocorticotrophic hormone (ACTH) or saline and maximum eye temperature was recorded. There was a rapid drop in eye temperature during the 5 min following disbudding without LA ($P<0.05$). Eye temperature then increased and was higher than baseline over the remaining sampling period following both disbudding procedures ($P<0.001$), a response which could not be explained by increased physical activity. LA increased eye temperature prior to disbudding ($P<0.001$). Heart rate increased ($P<0.001$) during the 5 min following disbudding with and without LA, however, LF/HF ratio only increased during this time ($P<0.01$) following disbudding without LA. Eye temperature did not change following ACTH, suggesting that hypothalamus-pituitary-adrenal axis (HPA) activity is not responsible for the changes in eye temperature following disbudding. The increase in LF/HF ratio following disbudding without LA suggests an acute sympathetic response to pain, which could be responsible for the drop in eye temperature via vasoconstriction. HRV and eye temperature together may be a useful non-invasive and more immediate index of pain than HPA activity alone.

Keywords: Infrared thermography; Eye temperature; Heart rate variability; Behavior; ACTH

1. Introduction

Husbandry practices, which cause tissue damage (e.g., disbudding, castration), have the potential to cause pain. An animal's typical response to pain includes a cascade of autonomic, hormonal and behavioral activity, which comprise a stress response [1]. Therefore, to detect pain in animals a combination of physiological and behavioral measures are often used. Behavioral responses can provide a good indication of the duration and different phases of a painful experience, but may not indicate the maximal

intensity of a noxious experience as well as physiological indicators [1]. Plasma cortisol concentrations, which reflect hypothalamus-pituitary-adrenal axis (HPA) activity, have been widely used to evaluate painful procedures such as disbudding [2]. Caution is required when interpreting cortisol levels as they are not a direct measure of pain, but rather indicate the overall noxiousness of the experience [1]. In addition, the slow response of cortisol levels make them an insensitive measure of acute pain during the first few minutes of a noxious stimuli [3].

An immediate response to a stressful or painful (nociceptive) stimulus is for blood to be diverted from cutaneous capillary beds via sympathetically-mediated vasoconstriction, which consequently decreases skin temperature [4]. The effect of this vasoconstriction can be detected by infrared thermography (IRT) as a temperature change. Previous studies have shown that eye temperature responses, measured by IRT, are a useful non-invasive tool for measuring stress in animals [5]. More specifically, in cattle, the temperature of small areas around the medial posterior palpebral border of the lower eyelid and the lacrimal caruncle, which have rich capillary beds innervated by the sympathetic system, respond to changes in blood flow. Eye temperature is easily measured without the interference of fur or hair and has been shown to be a more consistent measure of temperature changes than other anatomical areas such as the nose, ear, body and hooves in response to stress and early detection of disease in cattle [6]. Eye temperature increased in response to velvet antler removal in Elk [7], bovine viral diarrhoea in calves [6] and jugular catheterization of dairy cows [8]. Velvet antler removal and jugular catheterization also resulted in increased plasma cortisol concentrations [7, 8]. It was suggested that increases in eye temperature as a result of stress were due to the corresponding increase in HPA activity [9]. However, a more recent study failed to find an increase in eye temperature in response to an adrenocorticotrophic hormone (ACTH) challenge in dairy cows [8] suggesting that the response is not due to HPA activity alone.

A recent study, using more intensive sampling techniques (i.e., every second rather than every 2-3 min), found that eye temperature initially drops in response to a fright or an electric prod in beef calves [10]. The time frame of this drop (approx. 1 min) in eye temperature suggests that it may be a sympathetically-mediated response reflecting the redirection of blood flow to organs and the skeletal musculature during the classical ‘fight or flight’ response. There is evidence to support this suggestion from other species; a reduction in blood flow to the ear in rabbits occurred in response to an

alerting environmental stimuli [4], the tail and paw temperatures of rats decreased following a conditioned fear response [11], and a drop in the nasal temperature of rhesus monkeys occurred during exposure to a threatening person [12].

Heart rate variability (HRV) can provide a more detailed measure of a stress response than simple time domain measures of cardiac activity, such as heart rate [13]. Using the cardiac interbeat interval (R-R interval) and calculating parameters in time, frequency and non-linear domains, it is possible to measure the balance between sympathetic and parasympathetic activity and provide a more detailed interpretation of autonomic activity [13]. Stressful or painful procedures can cause depressed parasympathetic/vagal tone and increased sympathetic activity and reduced HRV [13]. For example, insect harassment and diarrhoea in calves [14], transportation of horses [15], changes in milking systems in dairy cows [16], and gestation in pigs [17] all cause a decrease in HRV. Debate continues over interpretation of HRV and whether certain frequency parameters of HRV can directly measure sympathetic tone. For example, an increase in the LF (low frequency) /HF (high frequency) ratio of the power spectrum is regarded by some researchers as a reliable indicator of increased sympathetic nervous system activity [17, 18]. In contrast, studies that have used autonomic pharmacological blockades and stimulants suggest that sympathetic activity can not be measured by HRV parameters [19, 20]. For a recent review of HRV for assessing stress in farm animals, see von Borell et al. [21]. In the present study, because painful stimuli are known to cause an increase in sympathetic activity [1], we considered that cautery disbudding would be a useful model to measure changes in HRV in responses to pain.

Disbudding is a routine procedure carried out on young dairy calves to prevent horn growth, typically between the ages of 2-6 weeks, and there are clear indications from the literature that this procedure is painful (for a review see Stafford and Mellor [2]). The aim of this study was to investigate whether eye temperature and HRV respond to pain associated with disbudding of dairy calves. Increased physical activity raises body temperature [22], which could potentially affect eye temperature, therefore, the general level of physical activity during and after the procedure was measured by recording behavior. A secondary aim was to confirm that HPA axis activity is not driving the eye temperature response, by using an ACTH challenge in calves.

2. Method

The protocol and conduct of this study were approved by the Ruakura Animal Ethics Committee, Hamilton, New Zealand.

2.1 Animals

A total of thirty Holstein Friesian heifer calves, average age six weeks old (range 5-7 weeks), mean weight of 62.8kgs and scheduled for disbudding were used in this study. Calves were housed together indoors and managed under normal farm practice.

2.2. Treatments

Calves were randomly assigned to one of the following four treatments: 1) Control (n=8): Horn bud area handled to simulate handling for injection followed by simulated disbudding (cold iron held against each horn bud) 10 min later 2) Local anaesthetic control (n=8): Local anaesthetic (6 ml of 2% lignocaine hydrochloride; Lopaine, Ethical Agents Ltd, Auckland, New Zealand) administered by injection around each cornual nerve [23] and horn bud handled to simulate disbudding 10 min after injection 3) Disbudding (n=6): Horn bud area handled to simulate handling for injection and horn buds removed with a standard gas powered cautery iron (ABER LPG debudder, Shoof International Ltd, Cambridge, New Zealand) 10 min later 4) Local anaesthetic plus disbudding (n=8): Local anaesthetic administered as above and horn buds removed with a cautery iron 10 min later. The recommended minimum amount of time between injecting local anaesthetic and disbudding is 3 min [24], therefore, 10 min was considered to be enough time for the local anaesthetic to be effective.

On the days prior to treatment days, each calf was accustomed to wearing a heart rate monitor for approximately 1 hr, during which time they were restrained in a calf head bail (Cattlemaster, Te Pari Products, Oamaru, New Zealand) for 40 min, to familiarise them with the equipment, procedure and treatment area. One hour is considered to be a sufficient time period to accustom calves to wearing heart rate equipment [21]. Four calves (one from each treatment) were treated per day, one at a time. Treatments were balanced between test days and sampling occurred at the same

time each day, between 9:00 and 14:00h, to reduce any effects of circadian rhythms. All treatments were carried out by the same operator.

On treatment days, each calf was restrained for sampling in the calf head bail for a total of 40 min. This period included 15 min for baseline data collection, followed by injection of local anaesthetic or a sham injection, 10 min for the local anaesthetic to take effect before the disbudding or sham procedure (average 1:56 min:sec), and a further 15 min period to monitor post-treatment responses. Over this 40 min period, cardiac activity, eye temperature and behavior were recorded continously.

2.3 Infrared thermography

An infrared camera (ThermaCam S60, FLIR Systems AB, Danderyd, Sweden) was used to collect eye images. The maximum temperature (^{o}C) within the area of the medial posterior palpebral border of the lower eyelid and the lacrimal caruncle (Figure 1) was recorded on average every 38 sec immediately into an excel spreadsheet throughout the entire 40 min sampling period. All calves were scanned from the same side (left), angle (90^{o}) and distance (approx 0.5 m). It was not possible to collect images during the 2 min of the actual disbudding procedure because the view of the eye area was blocked by the operator of the cautery iron. Ambient temperature and relative humidity were recorded every 30 min inside the barn, and these values were entered into the camera to allow for atmospheric changes during the sampling period.

2.4 Heart rate variability

Heart rate and HRV were recorded continuously using Polar heart rate monitors (S810i™, Polar Electro Oy, Helsinki, Finland). Monitors were fitted 5 min before calves were placed into the head bail and removed at the end of the 40 min sampling period. To optimise conductivity, ultrasound transmission gel was applied liberally to the calf's coat at each electrode contact point. The electrodes and monitor were held in place by elastic bandaging (Vet-ban, VetSource Ltd, Ashburton, New Zealand), which was strapped firmly around the calf's thorax, immediately behind the forelimbs. At the end of each sampling period the stored heart rate was downloaded onto a computer for analysis. We examined equal time periods of 5 min to fulfill recommendations for analysis of HRV using Fast Fourier transformation (FFT) [21, 25]. Continuous

recordings of R-R (interbeat) interval data are prone to measurement errors [26] therefore, prior to analysis, a correction function within the Polar software (Polar Precision Performance Software; Version 4.03), set on default parameters, was used to correct for any artifacts (e.g., to eliminate ectopic heartbeats). An average error rate of 6% was accepted and included in the analysis. In addition, a visual inspection of the corrected data was performed to edit out any artifacts still existing. Time domain parameters included heart rate, the R-R interval and the root mean square of successive R-R interval differences (RMSSD). Frequency domain parameters included the high frequency power (HF, 0.30-0.80 Hz), the low frequency power (LF, 0.04-0.30 Hz) and the LF/HF ratio, which were calculated with FFT using advanced HRV software [27]. The frequency band widths were selected to take into consideration the calculated respiratory frequency of calves and the HF and LF power were presented as normalised units (nu) to account for inter-individual differences [21, 25]. Normalised units represent the relative value of each power component in proportion to the total power minus the very low frequency component (frequencies lower than the lower limit of the LF band). Respiration rate (breaths/min) was measured by counts of flank movements recorded using a video camera (DCR-TRV355E, Sony, Japan) positioned at the rear of the calf. The frequency band width of 0.30 to 0.80 Hz used for the HF power corresponds to range of 18 to 49 breaths per min. This frequency band width for the HF power was also used by Mohr et al. [14] for HRV analysis in calves.

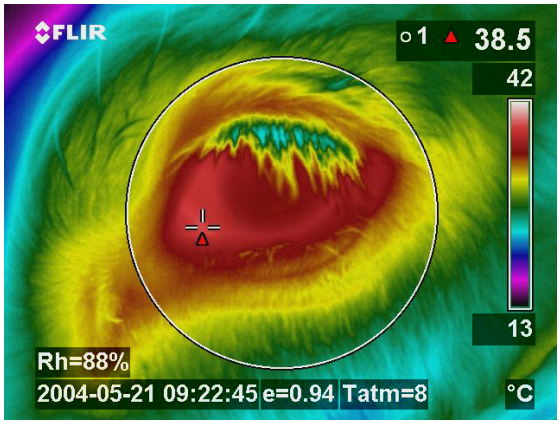

Figure 1. Infrared image of the eye region. The cross indicates the position of the maximum temperature within the area of the eye used for analysis, the medial posterior palpebral border of the lower eyelid and the lacrimal caruncle.

2.5 Behavior sampling

Two video cameras (DCR-TRV355E, Sony, Japan), one at the rear and the other at the front of the calf, were used to record physical activity and vocalizations on treatment days. All occurrences of the behaviors described in Table 1 were recorded continuously over the entire 40 min sampling period for each animal by video analysis.

Table 1. Description of each behavior recorded continuously during the 40 min sampling period.

Behavior	*Description*
Rear	One or both front legs are raised off the ground in a forward pawing action
Leg lift	Any foot raised off the ground and then replaced, often in a rapid movement (within 2 seconds)
Lunge	Both back legs leap forward or backwards together and land simultaneously
Crouch	Rump lowers to the ground, in a crouching motion, without the calf falling to the ground (recorded when the top of the tail reaches the point of the escutcheon or lower)
Fall	The calf collapses to the ground onto both knees and/or hocks
Slip	Hind leg is extended backwards or stretched forwards as it slides along the floor
Vocalize	Any audible noise made by the calf

2.6 ACTH challenge

In order to measure the eye temperature response to an ACTH challenge, one week after the last group of calves had been disbudded, twelve calves from either of the disbudded groups were randomly assigned to one of two treatments and given either 2 ml of physiological saline or 0.01 mg/100 kg live weight of synthetic ACTH $_{(1\text{-}24)}$ (0.05 mg Synacthen; Novartis Pharma AG, Basle, Switzerland) via intra-jugular injection. One calf was monitored at a time (six calves per day) over two consecutive days,

between 9:00 and 14:00h to reduce any effects of circadian rhythms. Treatment administration and blood sampling procedures were carried out by the same operator. Calves were restrained in the head bail for 35 min, which included 15 min of baseline data collection, followed by administration of the treatment (0 min) and then a further 20 min to monitor post treatment responses. During the 35 min sampling period, maximum eye temperature (^{o}C) was recorded, as described previously, on average every 35 sec. Blood samples were collected at 0, 20 and 40 min via jugular venipuncture into vacutainer tubes containing lithium heparin as an anticoagulant, placed on ice and centrifuged (2500 rpm for 15 min). Plasma was aliquoted off and stored at -20^{o}C until assayed for concentrations of cortisol.

2.7 Cortisol assay

Cortisol was measured using a double-antibody radioimmunoassay as described previously [28]. The minimum detectable level was 0.42 ng/ml. The intra-assay coefficient of variation (CV) for plasma pools measuring 7.3, 26.7 and 48.4 ng/ml were 1.7, 3.2 and 12.1% respectively and inter-assay CV's (n=2) for the same pools were 9.5, 10.7 and 9.6%.

2.8 Statistics

Counts of behavioral events were normalized to a frequency per minute. The incidences of vocalizing were too low to enable statistical comparisons to be made. All occurrences of each of the behaviors recorded, except vocalizing, were combined together as a single measure of physical activity (per minute) and a log transformation was used prior to analysis. Both behavior and maximum eye temperature were expressed as the difference from baseline (i.e., the first 15 min of sampling, before administration of local anesthetic) during various periods post-treatment, then an ANOVA was used to compare differences between treatments and a Student's t-test was used to compare differences at various periods post-treatment from baseline. The periods compared to baseline for behaviour were the first 2 min (during the disbudding or sham procedure) and the remaining post-treatment period (the last 13 min). For maximum eye temperature, recording started after the procedure, therefore, the periods compared to baseline were between 2-5 min and the last 10 min post-treatment. The

maximum eye temperature was averaged across each treatment and for presentation (Figure 2), a small amount of smoothing was applied using a loess smoother separately for each animal pre and post-treatment to allow interpolation and achieve averages at common time points. The effect of local anaesthetic on eye temperature was determined on the pooled data from both treatments given local anaesthetic by analyzing the difference from baseline during the 5 min before disbudding. Due to equipment failure HR and HRV data for one calf that was disbudded without local anaesthetic was missed from the analysis. A log transformation was used prior to analysis of the frequency domain parameters (HF, HF and LF/HF ratio) of HRV, then an ANOVA was used to compare differences between treatments and a Student's t-test was used to compare differences from baseline (i.e., 5 min prior to administration of local anesthetic) to 5 min post-treatment. The maximum eye temperature was averaged during the baseline period (first 15 min) and between 0 and 10 min, and 10 and 20 min after the ACTH challenge and a Student's t-test was used to compare differences between treatments. Cortisol concentrations were log transformed prior to analysis and a Student's t-test was used to compare differences between treatments at each sampling period (0, 20 and 40 min).

3. Results

3.1 Eye Temperature

There was a rapid decrease in eye temperature immediately following disbudding without local anaesthetic (Figure 2). Eye temperature had dropped from baseline by the time recording started, between 2-5 min post-treatment (-0.27˚C, $P<0.05$) and was lower than all other treatments ($P<0.05$, Figure 3). In contrast, following disbudding with local anaesthetic, there was only a small non significant decrease in eye temperature back to baseline levels (Figure 2). From 5 min post-treatment, eye temperature increased and was higher ($P<0.001$) for both disbudded groups compared to the two control groups during the last 10 min (Figure 4). Compared to baseline, eye temperature was 0.60˚C and 0.66˚C higher ($P<0.001$) during the last 10 min of the sampling period following disbudding without and with local anaesthetic respectively. Local anaesthetic administration caused an increase in eye temperature 5

min prior to disbudding (+0.19˚C, P<0.001). There were no significant differences in eye temperature following either the control or the local anaesthetic control treatment.

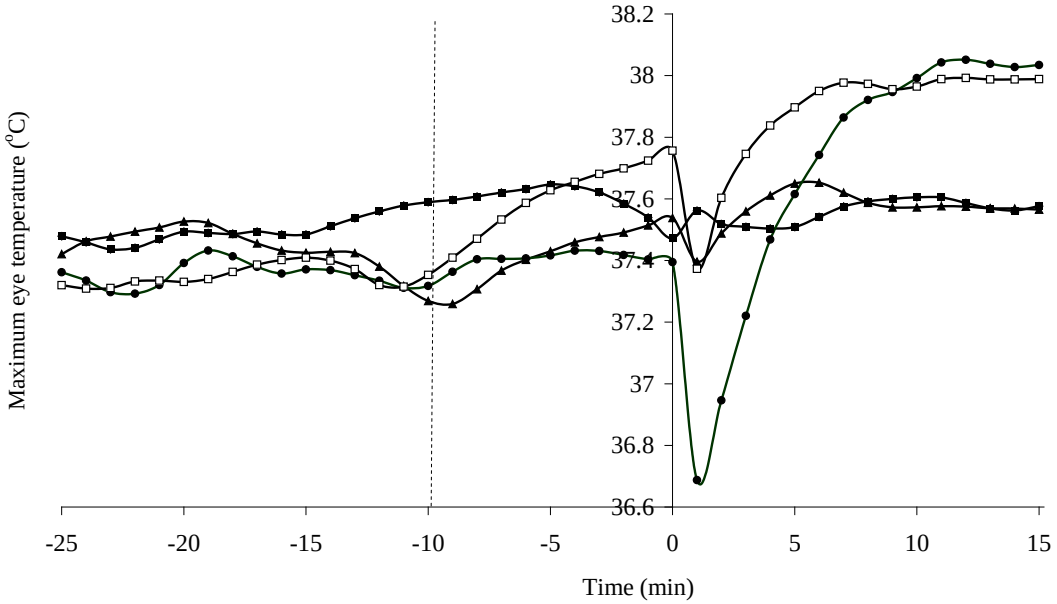

Figure 2. Maximum eye temperature (^{o}C) during the 40 min sampling period for control (■, n=8), local anaesthetic control (▲, n=8), disbudded with local anaesthetic (□, n= 8) and disbudded without local anaesthetic (●, n=6). Lines were smoothed using a loess smoother separately for each animal pre and post disbudding. The dashed vertical line indicates the time that local anaesthetic or the sham procedure was administered and 0 min indicates the time of treatment.

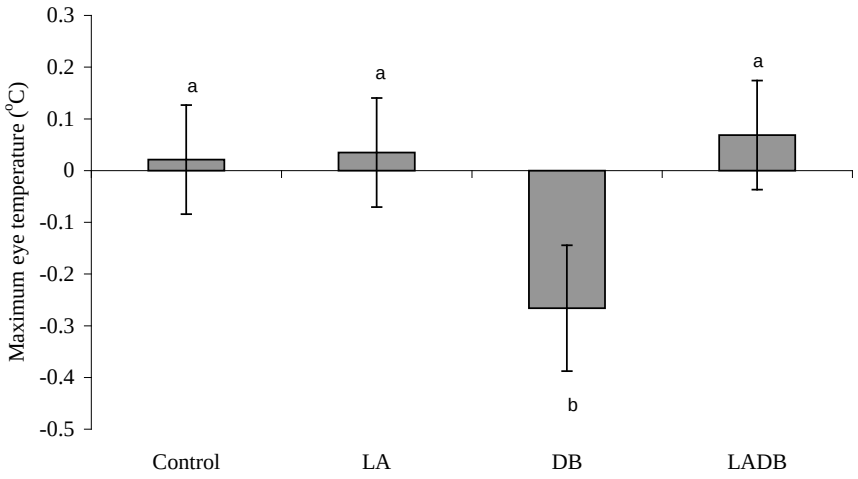

Figure 3. Change in maximum eye temperature (°C) ± S.E.M. from baseline (first 15 min) between 2-5 min post-treatment for control (n=8), local anaesthetic control (LA, n=8), disbudded without local anaesthetic (DB, n = 6) and disbudded with local anaesthetic (LADB, n=8). Significant differences (P<0.05) between treatments are marked by different letters (a and b).

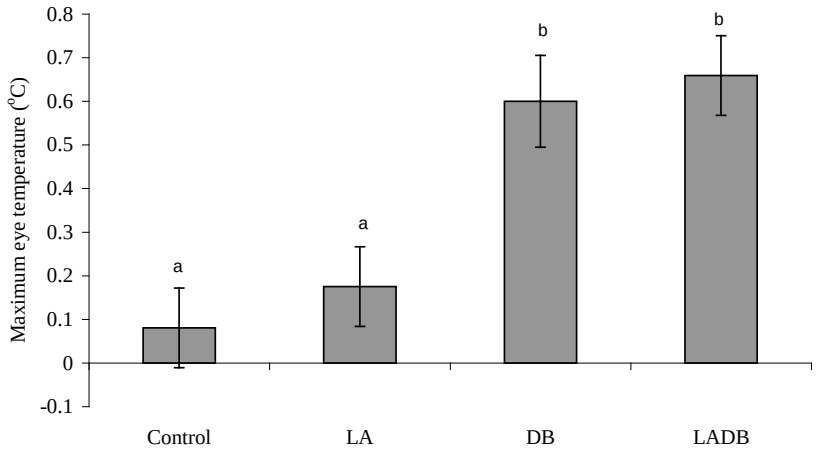

Figure 4. Change in maximum eye temperature (°C) ± S.E.M. from baseline (first 15 min) during the last 10 min of the sampling period for control (n=8), local anaesthetic control (LA, n=8), disbudded without local anaesthetic (DB, n=6) and disbudded with local anaesthetic (LADB, n=8). Significant differences (P<0.001) between treatments are marked by different letters (a and b).

3.2 Behavior

During the disbudding procedure (the first 2 min), both treatments, with or without local anaesthetic, had higher levels of physical activity than both of the control treatment groups ($P<0.01$). Physical activity was elevated 12.2±4.3 ($P<0.001$) times baseline levels during disbudding without local anaesthetic, 9.5±2.9 ($P<0.001$) times following disbudding with local anaesthetic and 2.0±0.6 ($P<0.05$) times for controls. During the remaining post-treatment period, controls had a higher level of physical activity than all other treatments ($P<0.01$), which was 4.4±1.8 ($P<0.01$) times baseline levels. There were no differences in physical activity from baseline during this last 10 min for any of the other treatments. There was no significant change in behavior at any time for local anaesthetic controls. Only four animals vocalized, two during the disbudding procedure, one with local anaesthetic and one without. The two other vocalizations were during the baseline period. All of the activity in the control animals was due to a high frequency of leg lifts (Table 2).

3.4 Heart rate and heart rate variability

Heart rate increased ($P<0.001$) during the 5 min following disbudding with and without local anaesthetic (Table 3), and then stayed elevated above baseline for the remainder of the sampling period following disbudding without local anaesthetic only (Figure 5). There were no significant changes in RMSSD for any treatment group. During the 5 min following disbudding without local anaesthetic, the parameter HFnu decreased and the LFnu and the LF/HF ratio increased from baseline. There were no significant changes in heart rate and HRV parameters following either the control or the local anaesthetic control treatment.

Table 2. Mean frequency of each behavior ± S.E.M. during the baseline period (first 15 min of sampling, prior to administration of local anaesthetic), the disbudding or sham procedure (2 min), and the remaining post-treatment period (last 13 min post-treatment) for control (n=8), local anaesthetic control (LA, n=8), disbudded without local anaesthetic (DB, n=6) and disbudded with local anaesthetic (LADB, n=8).

		Rear	Leg lift	Lunge	Crouch	Fall	Slip
Treatment	Interval						
Control							
	baseline	NA	2.7 ±0.1	NA	NA	NA	NA
	disbudding	NA	3.7 ±1.2	NA	NA	NA	NA
	post-disbudding	NA	7.8 ±2.3	NA	NA	NA	NA
LA							
	baseline	NA	2.6 ±0.5	NA	NA	NA	NA
	disbudding	NA	4.3 ±1.0	NA	NA	NA	0.1 ±0.1
	post-disbudding	NA	2.2 ±0.7	NA	NA	NA	NA
DB							
	baseline	NA	3.6 ±0.1	NA	NA	NA	NA
	disbudding	2.2 ±0.9	27.4 ±4.9	0.3 ±0.2	1.6 ±0.7	0.2 ±0.2	2.3 ±0.6
	post-disbudding	0.1 ±0.1	3.4 ±1.6	NA	NA	NA	0.1 ±0.0
LADB							
	baseline	NA	2.7 ±0.6	NA	NA	NA	0.1 ±0.0
	disbudding	1.4 ±0.6	22.3 ±3.2	0.4 ±0.2	1.1 ±0.5	0.1 ±0.1	2.3 ±0.7
	post-disbudding	NA	1.5 ±0.4	NA	NA	NA	NA

based on treatment means of non-transformed data

NA = no occurrences of behavior (0.0 ±0.0)

Table 3. Heart rate and heart rate variability parameters in time domain, the root mean square of successive R-R interval differences (RMSSD), and frequency domain, high frequency (HFnu) and low frequency (LFnu) power and the LF/HF ratio, during baseline (the 5 min prior to local anaesthetic administration) and the first 5 min post-treatment for control (n=8), local anaesthetic control (LA, n=8), disbudded without local anaesthetic (DB, n=5) and disbudded with local anaesthetic (LADB, n=8).

HRV parameter	Interval	Control	LA	DB	LADB	*ANOVA*
Heart rate (bpm)	5 min pre	83.5 (±5.4)	72.0 (±5.4)	94.0 (±6.8)	90.1 (±5.4)	ns
	5 min post	86.1 (±6.5)	71.1 (±6.5)	128.8 (±8.2)	110.9 (±6.5)	<0.001
	pre vs. post	ns	ns	<0.001	<0.001	<0.001
RMSSD (ms)	5 min pre	69.4 (±12.8)	72.3 (±12.8)	67.0 (±16.3)	63.1 (±12.8)	ns
	5 min post	72.3 (±12.8)	85.8 (±12.8)	41.2 (±16.1)	79.2 (±12.8)	ns
	pre vs. post	ns	ns	ns	ns	ns
HFnu	5 min pre	32.7 (±5.6)	39.5 (±5.6)	35.5 (±7.1)	34.8 (±5.6)	ns
	5 min post	28.1 (±4.5)	44.6 (±4.5)	15.9 (±5.6)	37.3 (±4.5)	<0.01
	pre vs. post	ns	ns	<0.01	ns	<0.01
LFnu	5 min pre	67.3 (±5.6)	60.5 (±5.6)	64.5 (±7.1)	65.2 (±5.6)	ns
	5 min post	71.9 (±4.5)	55.4 (±4.5)	84.1 (±5.6)	62.7 (±4.5)	<0.01
	pre vs. post	ns	ns	<0.01	ns	<0.05
LF/HF ratio	5 min pre	4.2 (±1.7)	2.5 (±1.7)	2.6 (±2.2)	3.7 (±1.7)	ns
	5 min post	4.0 (±1.7)	1.4 (±1.7)	6.2 (±2.1)	4.1 (±1.7)	ns
	pre vs. post	ns	ns	<0.01	ns	<0.05

Descriptive statistics are based on treatment means (±S.E.M) of non-transformed data.
Statistical significances for frequency domain parameters (LF, HF and the LF/HF ratio) are based on log transformed data. P values pre vs. post are based on results from the Student's t-test. ns = non-significant (>0.05).

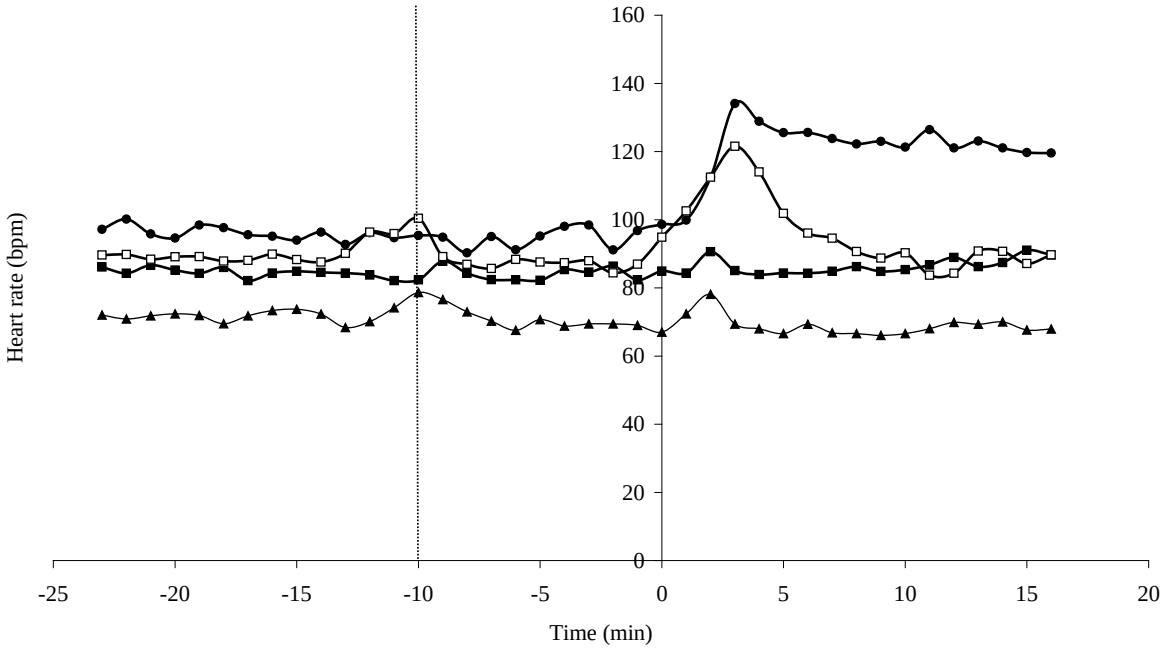

Figure 5. Mean heart rate (bpm) during the 40 min sampling period for control (■, n=8), local anaesthetic control (▲, n=8), disbudded with local anaesthetic (□, n=8) and disbudded without local anaesthetic (●, n=5). The dashed line indicates the time that local anaesthetic or the sham procedure was administered and 0 min indicates the time of treatment.

3.5 ACTH challenge

There were no significant differences in eye temperature before or after (between 0 and 10 min, and 10 and 20 min post-treatment) administration of either ACTH or saline (Figure 6). Cortisol concentrations were higher ($P<0.001$) following administration of ACTH compared to saline by the 20 min and 40 min sample (Table 4).

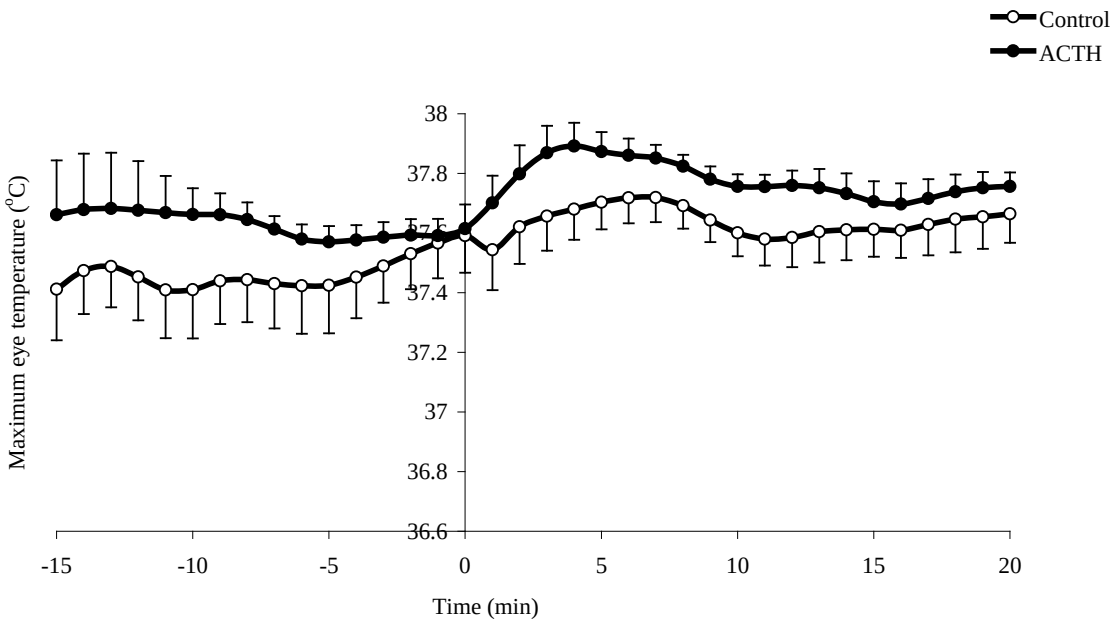

Figure 6. Maximum eye temperature (°C) ± S.E.M. following administration (0 min) of ACTH (●, n=6) or saline-control (○, n=6). Lines were smoothed using a loess smoother separately for each animal pre- and post-injection.

Table 4. Mean cortisol concentration (ng/ml) ± S.E.M. at 0, 20 and 40 min following administration of ACTH (n=6) or saline-control (n=6).

Post-treatment time (min)	Cortisol concentration (ng/ml)		
	Control	ACTH	
			P Value
0	3.5 (±1.1)	7.8 (±2.7)	0.168
20	6.4 (±1.6)	21.1 (±2.7)	0.001
40	3.9 (±0.8)	34.5 (±3.9)	<0.001

4. Discussion

The rapid drop in eye temperature following disbudding without local anaesthetic may have been caused by the redirection of blood from the capillary beds via sympathetically-mediated vasoconstriction. HRV parameters measured following disbudding without local anaesthetic indicated an acute decrease in HRV and a change in the sympatho-vagal balance. The short-term drop in eye temperature was followed by a prolonged elevation. While the mechanisms for this increase are unknown, the lack of any eye temperature changes following the ACTH challenge in the same animals that responded to disbudding, confirms that eye temperature responses are not a direct result of changes in HPA activity. Eye temperature and HRV may have benefits over measuring HPA activity (e.g., cortisol) when investigating acute responses to pain as they constitute a more immediate and detailed index of pain.

It was expected that local anaesthetic would reduce or eliminate the responses to pain measured in this study. When lignocaine is used as a cornual nerve blockade prior to disbudding, stress responses, such as cortisol concentrations following the procedure are virtually eliminated [2]. This is consistent with the present study in which local anaesthetic virtually eliminated the acute drop in eye temperature following disbudding. Complete elimination of all pain associated with disbudding may be practically difficult to achieve using a cornual nerve block. Incomplete or partial effectiveness of analgesia can result from insufficient dose or incorrect placement of local anaesthetic due to operator variability and individual differences in the neural topography of the horn bud area. Administration of local anaesthetic did abolish the changes in frequency domain HRV parameters, and prevented the prolonged elevation in heart rate following disbudding. The increase in eye temperature following administration of the local anaesthetic may have been due to a vasodilatory effect of lignocaine [29, 30].

The magnitude and duration of the drop in eye temperature following disbudding without local anaesthetic is consistent with that found following a fright in cattle [10] and also with the drop in nasal temperature of rhesus monkeys during exposure to a threatening person [12]. The results are also consistent with the sympathetic vasoconstriction in the paws and tails of rats [11] and the ears of rabbits [4] following exposure to stressful stimuli. The specific regions where blood flow changes have been recorded as a result of a fright or stress are species specific. Vianna and Carrive [11] concluded that skin vasoconstriction in response to a stressful stimulus is a

regional specific response, not a generalised response, as suggested previously by Nalivaiko and Blessing [31]. Nakayama et al. [12] found that nasal temperature changes of rhesus monkeys in response to stress were more consistent than eye temperature, whereas, Schaefer et al. [6] found that increases in eye temperature of calves in response to an induced viral infection were more consistent than temperature changes in other anatomical areas (i.e., nose, ear, body and hooves).

Changes in HRV occurred only following disbudding without local anaesthetic indicating higher levels of autonomic stimulation in these calves, possibly indicative of acute pain. Reduced vagal tone is reflected by a decrease in the time domain HRV parameter RMSSD and in the frequency domain parameter, the HF power [32]. Although no significant differences were found in the RMSSD, following disbudding without local anaesthetic there was a decrease in HF power, which indicated a reduction in vagal tone for these animals. The increase in LF power in this group of animals reflects a combined change in both vagal and sympathetic tone [18]. The increase in the LF/HF ratio following disbudding without local anaesthetic indicates a change in the sympatho-vagal balance and may suggest a possible shift towards sympathetic dominance. Similar increases in LF/HF ratio have been found in response to different milking systems [16] and insect harassment and diarrhoea in calves [14]. Relatively few studies have used HRV as a measure of stress in dairy cattle and no studies have measured HRV during painful husbandry procedures. Debate continues over the ability to directly measure sympathetic activity from HRV parameters [13, 21]. The increase in the LF/HF ratio is regarded by some researchers as a reliable indicator of increased sympathetic nervous system activity [17, 18]. However, Despres et al. [19] found that parasympathetic blockade with atropine had effects on HRV, but sympathetic blockade with atenolol did not. They concluded that the net vago-sympathetic effect (the ratio of the mean R-R in a defined situation to the mean R-R during double blockade), HR, the R-R interval and the RMSSD were valid indicators of parasympathetic tone of calves because of large variations in these parameters, due to atropine administration, and that no measure reflected sympathetic activity. However, measurements were collected while the calves were at rest, a state in which the authors suggested that sympathetic tone may be very low. Species-specific studies using such pharmacological approaches during stressful or painful procedures, when sympathetic activity is increased, may help better understand whether certain HRV parameters may indicate sympathetic activity.

There was an increase in eye temperature after disbudding, both with and without local anaesthetic. The mechanism and involvement of pain in this response is unknown. However, there is evidence that this is not driven by changes due to the heat of the hot cautery iron, increased physical activity or increased HPA activity. In addition, the time frame of the increase in eye temperature is not consistent with an effect of local inflammatory processes. This process is a complex cascade of events, involving cytokines and selective leukocytes, which take some time to develop [33] and anatomically any inflammatory response would occur at the wound site, around the horn bud, not around the eye. Following the initial drop in the tail temperature of rats, Vianna and Carrive [11] also found an increase in temperature above baseline, which corresponded with a decline in internal body temperature, suggesting a thermoregulatory response of the tail to dissipate the heat accumulated during stress by changing from a vasoconstricted to a vasodilated state. A direct effect of heat conduction from the cautery iron is also unlikely as similar increases in eye temperature have been reported in response to velvet antler removal in Elk [7], jugular catheterization in dairy cows [8] and a fright in humans [34], none of which involve application of heat. Furthermore, it is possible that studies that have only reported increases in eye temperature in response to stress, may have failed to detect an initial drop in eye temperature because sampling was too infrequent.

In the present study, physical activity increased during disbudding with or without local anaesthetic, which could have increased body temperature and induced vasodilation [22] and an increase in eye temperature. However, control animals also had high levels of activity during sham disbudding and had higher levels of activity than all other treatments during the post-treatment period and this caused no change in eye temperature. This suggests that the increase in eye temperature in the disbudded animals was not due to increased physical activity. The high level of activity observed in the control animals was due to a high number of leg lifts that may possibly be associated with restlessness in the restraint. Most of the behaviors observed during the procedure may have been attempts to escape and could be useful indicators of aversiveness even in a restrained situation, however, it is not possible to conclude from these results the relative contributions from the restraint itself or pain. Escape behaviors that only occur during the procedure but are not seen afterwards may reflect a specific acute pain [1] or may simply indicate a desire to escape [35]. It was not the aim of the present study to measure specific pain-related behaviors (such as head shaking and ear flicking) that

have been observed in other studies investigating pain responses following disbudding or dehorning [36-38], and restraint in the head bail prevented these behaviors from being observed.

In conclusion, the results from this study suggest that the drop in eye temperature following disbudding without local anaesthetic may be caused by sympathetic vasoconstriction and as such may be used to monitor events where sympathetic activity changes rapidly, such as with acute pain. A combination of eye temperature and HRV measures may provide more sensitive, detailed and immediate measures of acute pain than HPA changes. In addition, both IRT and HRV have the advantage of being relatively non-invasive.

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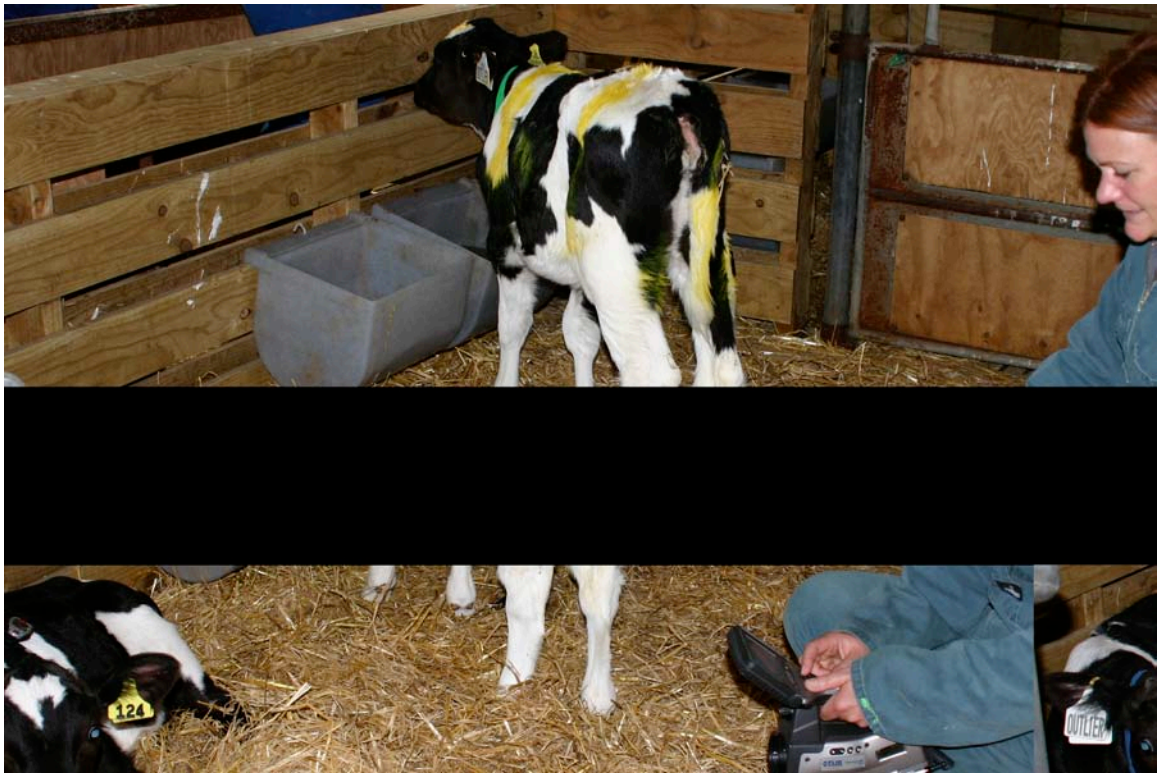
Chapter Six

Effects of local anaesthetic and a non-steroidal anti-inflammatory analgesic on behavioural and physiological stress responses of calves to cautery disbudding



Authors note: Chapter six is presented in the style of Journal of Dairy Science where it has been submitted. Results were also presented at the annual International Society for Applied Ethology conference and published as:

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ABSTRACT

This study examined the effects of local anaesthetic (LA) and a non-steroidal anti-inflammatory agent (NSAID), on physiological and behavioral responses of calves following cautery disbudding. Forty-six calves (average 4.5 weeks of age) were randomly assigned to six treatments: 1) sham handling (control), 2) sham LA and disbudded, 3) LA and disbudded, 4) LA and sham disbudded, 5) NSAID and LA, 6) NSAID and LA and disbudded. Maximum eye temperature (measured using infrared thermography) was recorded continuously for 30 min before and 15 min after disbudding; maximum eye temperature and behavior (standing, lying and ruminating) were recorded every 5 min for a further 3 h after treatments. Heart rate (HR) and heart rate variability (HRV) were recorded continuously for 30 min before and 3 h after disbudding, and short segments of 512 interbeat intervals (IBI) were examined. Between 2-4 min after disbudding without LA or NSAID, there was a rapid drop in eye temperature from baseline and HR was higher than in all other groups. HR remained elevated above baseline for 3 h after disbudding without LA or NSAID. The time domain HRV parameter, specifically the root mean square of successive differences (RMSSD), tended to decrease immediately following disbudding without LA, suggesting a decrease in vagal tone. Between 2-3 h following disbudding with LA, the high frequency (HF) power decreased and the low frequency (LF) power and LF/HF ratio increased. The RMSSD was lower following disbudding with LA compared to disbudding with LA and NSAID. These changes in HRV indicate a change in sympatho-vagal balance between 2-3 h. During the same time period, following disbudding with LA, there was a decrease in eye temperature, an increase in HR and decrease in lying behavior. There were no changes in HR, eye temperature or behavior for calves disbudded with LA and NSAID between 2-3 h. Time spent ruminating was lower over the entire 3 h following disbudding without LA compared to all other treatments, except disbudding with LA. Changes in eye temperature, HR, HRV and behavior 2-3 h following disbudding with LA indicate the onset of pain coinciding with the time that the LA effects wear off. The combination of LA and NSAID mitigated the stress responses associated with both the immediate and longer term (3 h) pain of disbudding and confirms this combination is more effective at alleviating pain than LA alone.

INTRODUCTION

Cautery disbudding is a routine husbandry practice performed on calves, typically between 2-6 weeks of age, which prevents horns developing by removing the horn buds using heat cauterisation. This procedure should be done at an early age because once the horn bud develops and becomes fixed to the skull, amputation dehorning is necessary (Weaver et al., 2005) and this is considered to be more painful than cautery disbudding (Stafford and Mellor, 2005). The purpose of both practices is to minimise the risk of injuries to stockpersons and other animals, the latter contributing to carcass downgrading through bruising and hide damage. Despite evidence (Stafford and Mellor, 2005) that demonstrates the welfare benefits of using analgesics, it is still common practice and permitted to disbud calves without local anaesthetic in most countries.

Use of local anaesthetic can reduce behaviors indicative of acute pain during disbudding (e.g., tail flicking, rearing, escape behavior) and other behaviors indicative of post-disbudding pain, such as head shaking and ear flicking (Stafford and Mellor, 2005). There is disagreement about the time course of pain associated with cautery disbudding and the controversy likely hinges on the different measures chosen to assess pain. Petrie et al. (1996) found that cortisol concentrations peaked at 30 min following disbudding and returned to baseline levels approximately 1 h after disbudding with or without local anaesthetic. They suggested that administration of local anaesthetic may be of little benefit in alleviating the overall pain response to disbudding and the associated pain may be reduced after 1 h. However, other studies using different methods of assessment have shown that the pain associated with disbudding lasts longer than 3 h. For example, following disbudding without local anaesthetic, pain-related behaviors (e.g., head shaking) lasted for 2 h (Graf and Senn, 1999) and heart rate (HR) remained high for up to 3.5 h (Grondahl-Nielsen et al., 1999).

Studies investigating pain associated with amputation dehorning have shown that a combination of local anaesthetic and a non-steroidal anti-inflammatory drug (NSAID) is required to alleviate both the acute pain and the pain experienced after dehorning, which is likely to be associated with inflammation (Stafford and Mellor, 2005). There are several reasons that the use of local anaesthetic alone may be unsatisfactory for pain alleviation. Lignocaine, the most commonly used local anaesthetic, is effective for approximately 2 h after administration, after which pain-

related behaviors, cortisol concentrations and inflammation-related pain increase (Stafford and Mellor, 2005). It can also be difficult to achieve a complete cornual nerve block in every animal. Incomplete or partial effectiveness of analgesia can result from insufficient dose or incorrect placement of local anaesthetic and individual differences in the neural topography of the horn bud area. Although many studies have investigated the benefits of using a combination of local anaesthetic and NSAID following dehorning (Stafford and Mellor, 2005), less information is available regarding the benefits of using a combination of local anaesthetic and NSAID during cautery disbudding.

Researchers have used various measures to detect pain and investigate the efficacy of various analgesics, however, each measure has advantages and limitations (Mellor et al., 2000). To date, most assessments of pain induced by husbandry practices have employed changes in plasma cortisol concentrations and/or pain-related behaviors. Pain-related behaviors can provide good indices of the duration and the different phases of a painful experience (Mellor et al., 2000). Caution is required when interpreting cortisol levels as they are not a direct measure of pain, but rather indicate the overall noxiousness of the experience (Mellor et al., 2000). Therefore, further research is required to expand the range of physiological parameters used for the assessment of pain in farm animals. Mellor et al. (2002) described plasma catecholamine concentrations as an additional index of pain to cortisol during castration and tailing of lambs and scoop dehorning of calves. They found a peak of adrenaline within 5 min following dehorning without local anaesthetic, indicating sympathetic stimulation of the adrenal medulla.

Other indices for assessing pain during husbandry procedures that have been described more recently include electroencephalographic responses (Johnson et al., 2005), heart rate variability (HRV) and infrared thermography (IRT, Stewart et al., 2008). Eye temperature, measured using IRT, dropped rapidly immediately following disbudding without local anaesthetic (Stewart et al., 2008) and in response to a fright or an electric prod (Stewart et al., In Press). It was concluded that this response may have been caused by the redirection of blood from the capillary beds in the eye via sympathetically-mediated vasoconstriction, reflecting the redirection of blood flow to organs and the skeletal musculature during the classical ‘fight or flight’ response. Therefore, IRT may be able to detect sympathetic activity. HRV is the variation between consecutive heartbeats and provides a more detailed interpretation of cardiac

activity in terms of autonomic nervous system (ANS) activity than HR alone (Malik and Camm, 1995). HRV is measured by determining the constantly changing interval between successive R waves of the electrocardiograph (interbeat interval, IBI), from which time and frequency [(e.g., high frequency (HF), and low frequency (LF) power)] domain parameters of HRV can be calculated. Time and frequency domain analysis of HRV indicate the balance between sympathetic and parasympathetic activity and so may reflect the level or nature of the stressor. A decrease in HRV reflects a shift of the autonomic balance towards a more sympathetic dominance (Malik and Camm, 1995). HRV has been widely used to assess ANS regulation of cardio-vascular function in human and veterinary medicine and although there is an increasing number of studies that have used HRV to indicate stress in farm animals (von Borell et al., 2007), little is known about HRV responses to painful husbandry procedures. Stewart et al. (2008) found a decrease in the HF power and an increase in the LF/HF ratio 5 min following disbudding without local anaesthetic, indicating a change in sympatho-vagal balance. For a recent review of HRV for assessing stress in farm animals, see von Borell et al. (2007).

The aim of this study was to use a combination of physiological (eye temperature, HR and HRV) and behavioral responses (lying and ruminating) to evaluate the effects of using a NSAID in combination with local anaesthetic to alleviate pain following cautery disbudding of calves. The hypothesis was that administration of local anaesthetic and NSAID prior to disbudding would eliminate physiological and behavioral responses.

MATERIALS AND METHODS

The protocol and conduct of this study were approved by the Ruakura Animal Ethics Committee, Hamilton, New Zealand, and steps were taken to statistically minimise the number of animals exposed to treatments without pain relief.

Animals

Forty-six Holstein Friesian mixed sex calves (23 heifers and 23 bulls), average age 4.5 weeks (range 4 to 5.5 weeks), mean weight of 53 kg (range 42-65 kg),

scheduled for disbudding, were used in this study. Calves were housed together in an indoor calf rearing facility and managed under normal farm practice.

Treatments and Procedure

Calves were randomly assigned to one of the following six treatments:1) Control (n=8, C): Saline injected (5 ml) around each cornual nerve and horn bud area handled to simulate disbudding (cold cautery iron held against each horn bud) 10 min later 2) Disbudding (n=6, DB): Saline injected (5 ml) around each cornual nerve and horn buds removed with a standard gas powered cautery iron (ABER LPG debudder, Shoof International Ltd, Cambridge, New Zealand) 10 min after saline injection 3) Local anaesthetic (LA) and disbudding (n=8, LADB): LA (5 ml of 2% lignocaine hydrochloride, Lopaine, Ethical Agents Ltd, Auckland, New Zealand) administered by injection around each cornual nerve then 3-4 ml infiltrated subcutaneously around the base of each horn bud (Weaver et al., 2005) and buds removed with a cautery iron 10 min later after last LA injection 4) Local anaesthetic control (n=8, LAC): LA administered as above 10 min before horn bud handled to simulate disbudding 5) NSAID and local anaesthetic control (n=8, NSAID+LA): Meloxicam (Metacam®, Boehringer Ingelheim Ltd, Auckland, New Zealand; ½ life of approximately 26 h in cattle), a preferential COX-2 inhibiting NSAID, injected intra-venously (0.5 mg/kg liveweight into jugular vein) 30 min before the start of sampling (55 min before disbudding), LA administered as above (to both caudal nerve and horn buds) 10 min before horn bud handled to simulate disbudding 6) NSAID and local anaesthetic and disbudding (n=8, NSAID+LADB): Metacam administered and LA administered as above and buds removed 10 min later with cautery iron. Treatments were balanced for sex, randomised and balanced across test days and were carried out by the same operator.

Four calves were treated each day. The day before treatment, each calf acted as a companion animal for the calf undergoing treatment that day to habituate them to the treatment area. On this training day, each calf also had its coat clipped down the right side of the body to improve contact for the HR electrodes, and calves were given time to habituate to wearing the HR equipment (1 h, S810i™, Polar Electro Oy, Helsinki, Finland) and restraint in a calf head bail (20 min, Cattlemaster, Te Pari Products, Oamaru, New Zealand). To facilitate jugular vein injections, the coat on the necks were

also clipped. On treatment days, each of the four calves and their companion calf were confined as pairs within four holding pens (2.3 x 2.5m), on straw bedding, with access to food and water. Thirty min before the start of sampling, each calf was fitted with a HR monitor and injected intravenously with either NSAID or saline. After 30 min, the calf was moved from its holding pen and restrained in the head bail for a 45-min sampling period. This period included 15 min for baseline data collection, followed by a cornual nerve injection of local anaesthetic or saline (average injection time of 53 sec), then an average of 04:37 min:sec for the local anaesthetic to take effect. This was followed by a local infiltrate around each horn bud or a sham infiltrate (no needle) for saline-treated calves (04:48 min:sec), then an average of 04:46 min:sec before the disbudding or sham procedure was administered (average 02:11 min:sec). A further 15-min was used to monitor responses after treatments. During this 45-min sampling period, cardiac activity and maximum eye temperature were recorded continuously. Calves were then released back into the holding pen with their companion for a further 3 h where maximum eye temperature and behavior were recorded at 5 min intervals.

Infrared Thermography

An infrared thermography camera (ThermaCam S60, FLIR Systems AB, Danderyd, Sweden) was used to collect images of the eye. The maximum temperature (^{o}C) within the area of the medial posterior palpebral border of the lower eyelid and the lacrimal caruncle (Stewart et al., 2008) was recorded immediately into an Excel™ spreadsheet on average every 20 sec throughout the baseline and 15 min after treatment, and then every 5 min for the following 3 h sampling period in the holding pens. Continuous recordings of eye images (29 frames/sec) were also achieved by connecting the infrared camera to a laptop and using image analysis software (ThermaCam Researcher 2.7, FLIR Systems AB, Danderyd, Sweden). These continuous recordings were used to determine acute eye temperature responses to disbudding during the 5 min after treatment. All images were taken from the same side (left), angle (90^{o}) and distance (approx 0.5 m). It was not possible to collect images during the actual disbudding procedure because the view of the eye area was blocked by the operator of the cautery iron. Ambient temperature (^{o}C) and relative humidity (%) inside the sampling area were recorded and entered into the infrared camera to calibrate it for atmospheric conditions.

Heart Rate and Heart Rate Variability

Continuous interbeat intervals (IBIs) were recorded using Polar HR monitors (S810i^{TM}, Polar Electro Oy, Helsinki, Finland). Monitors were fitted to each calf in their holding pen 30 min before being moved into the calf head bail at the start of sampling and removed after the 3 h. To improve conductivity, ultrasound transmission gel was applied liberally to the clipped site at each electrode contact point. The electrodes and transmitter were built into an elastic strap, provided with the Polar HR monitor, which was strapped firmly around the calf's chest, immediately behind the forelimbs, with the HR monitor attached. At the end of each sampling period the stored IBI data were downloaded on to a computer for analysis. Time domain parameters included mean HR (beats/min) and root mean square of successive differences (RMSSD), and frequency domain parameters included the HF (0.30-0.80 Hz) and the LF (0.04-0.30 Hz) powers and the LF/HF ratio which were calculated with Fast Fourier Transformation (FFT) using HRV software (Niskanen et al., 2004). To fulfill recommendations for analysis of HRV using FFT made by the Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology (1996) and von Borell et al. (2007), short segments of data containing 512 beats were examined. In addition, frequency band widths were selected to take into consideration the respiratory frequency of calves and the HF and LF power were presented as normalised units (nu) to account for inter-individual differences. Normalised units represent the relative value of each power component in proportion to the total power minus the very low frequency component (frequencies lower than the lower limit of the LF band). Continuous recordings of IBI data are prone to measurement errors (Marchant-Forde et al., 2004) therefore, before analysis, a correction function within the Polar software (Polar Precision Performance Software; Version 4.03), set on default parameters, was used to correct for any artifacts (e.g., to eliminate ectopic heartbeats). Only data sets with an error rate of less than 5% were included in the analysis.

Behavior in holding pens

Standing (defined as upright with all four hooves on the ground) and lying (flank in contact with ground) were recorded using instantaneous scan sampling every 5 min for 3 h at the same time as each eye temperature recording. At the same sampling

intervals, we also recorded whether or not the calf was ruminating (regurgitation, re-mastication and re-swallowing of food characterised by repetitive jaw movements).

Statistical Analysis

Acute Eye Temperature, Heart Rate and Heart Rate Variability Responses. A REML (restricted maximum likelihood, Genstat, version 10) analysis showed there were no effects of sex on maximum eye temperature or HR. Therefore, a one-way ANOVA was used to compare treatment differences in maximum eye temperature and HR and a Student's t-test was used to compare differences from baseline at various periods following treatments (the first 15 min before administration of local anaesthetic). For maximum eye temperature, recording started after treatment, therefore, the period between 2-4 min was compared to baseline for acute responses to treatments. Due to equipment failure, eye temperature profiles between 2-4 min were only available for a total of 16 calves: Control (n=3), DB (n=3), LADB (n=3), LAC (n=2), NSAID+LA (n=2) and NSAID+LADB (n=3).

Eye Temperature, Heart Rate and Heart Rate Variability Responses to Local Anaesthetic Wearing Off. Changes in maximum eye temperature and HR between 2-3 h (in response to local anaesthetic wearing off) were calculated from the average 30 min before and 30 min after 2.5 h post disbudding. A one-way ANOVA was used to compare treatment effects and a Student's t-test was used to compare differences before and after 2.5 h. To analyse the effects of local anaesthetic wearing off on HRV parameters (between 2-3 h) a one-way ANOVA was used to compare differences between treatments and a Student's t-test was used to compare differences in the group disbudded with local anaesthetic to other treatments. Time and frequency domain analysis of 512 IBI pools were compared before (IBI pool -6 to -1) and after (IBI pool 1 to 6) 2.5 h after treatments (six 512 IBI pools covered approx 30 min). Over the 2-3 h, eye temperature recordings for one calf in the NSAID+LADB group and HR recordings for one calf in the DB group were lost due to equipment failure.

Behavior after Disbudding or Sham Disbudding. A generalised linear mixed model (GLMM; Genstat, version 10) analysis was used to compare treatment differences in behavior, with random effects to account for repeated observations on the same animals for calf and calf by time interactions and fixed effects for treatment, time and sex. A Student's t-test was also used to compare behavior following disbudding

without anaesthetic against all other treatments over the 3 h sampling period. Comparisons between 1 and 2, and 3 h were made using relevant means and standard errors from the GLMM.

RESULTS

Eye temperature

There was a 0.5°C decrease ($P<0.05$) in eye temperature immediately following treatment for the DB group, compared to baseline levels (Table 1). A 0.8°C increase in eye temperature also occurred following treatment for the NSAID+LADB group ($P<0.01$). There were differences between treatments ($P<0.05$) in the change in eye temperature between 2-3 h; eye temperature decreased 0.6°C during this time ($P<0.001$) for the LADB group. There were no significant differences in eye temperature in the 2-4 min or the 2-3 h after disbudding for any of the control treatments.

Heart Rate and Heart Rate Variability

HR increased ($P<0.001$) during the 5 min following treatment for the DB group and then stayed elevated above baseline for the remainder of the 3 h sampling period (Figure 1). There were no significant changes in HR from baseline during the first 5 min for any other treatment. RMSSD tended to decrease ($P=0.07$) immediately following treatment for the DB group, however, there were no other significant changes in HRV during this period for any other treatments. Between the interval of 2-3 h after treatment, HR increased for the LADB group ($P<0.05$). There were no other significant changes in HR for any other treatment group between 2-3 h. During this interval for the LADB group, the HF power decreased and the LF/HF ratio increased (Figure 2) and both of these frequency domain parameters were significantly different ($P<0.05$) for this group of calves compared at all other treatments by the second IBI pool following 2.5 h. Following LADB, the LF power was higher ($P<0.05$) by the first IBI pool following 2.5 h compared to DB or NSAID+LADB only. The RMSSD was significantly lower following LADB ($P<0.05$) compared to NSAID+LADB only by the first IBI pool following 2.5 h.

Table 1. Maximum eye temperatures (^{o}C) ± S.E.M. and number of successful recordings for the baseline period (15 min before administration of LA/saline), the acute response (2-4 min after treatment) and for the 2-3 h period after treatment for control, disbudded without local anaesthetic (DB), disbudded with local anaesthetic (LADB), local anaesthetic control (LAC), NSAID and local anaesthetic control (NSAID+LA) and disbudded with NSAID and local anaesthetic (NSAID+LADB).

		Control	DB	LADB	LAC	NSAID +LA	NSAID +LADB	*P Value*
		n=3	n=3	n=3	n=2	n=2	n=3	
2-4 min	15 min before disbudding	37.7 (±0.3)	37.8 (±0.3)	37.7 (±0.3)	37.6 (±0.4)	37.2 (±0.4)	37.7 (±0.3)	0.882
	2-4 min after disbudding	37.7 (±0.4)	37.3 (±0.4)	38.1 (±0.4)	37.6 (±0.5)	37.4 (±0.5)	38.5 (±0.4)	0.310
	Difference	0.0 (±0.2)	-0.5 (±0.2) *	0.3 (±0.2)	0.0 (±0.3)	0.2 (±0.3)	0.8 (± 0.2) **	0.023
		n=8	n=6	n=8	n=8	n=8	n=7	
2-3 h	2 to 2.5 h after disbudding	37.8 (±0.1)	37.9 (±0.1)	38.1 (±0.1)	37.9 (±0.1)	37.9 (±0.1)	37.8 (±0.1)	0.378
	2.5 to 3 h after disbudding	37.6 (±0.1)	38.0 (±0.1)	37.5 (±0.1)	37.9 (±0.1)	37.8 (±0.1)	37.8 (±0.1)	0.108
	Difference	-0.2 (±0.1)	0.0 (±0.1)	-0.6 (±0.1) ***	0.0 (±0.1)	-0.1 (±0.1)	-0.0 (±0.1)	0.011

Statistical significances, *P<0.05, ** P<0.01 and ***P<0.001 in the difference from baseline are based on results from the Student's t-test.

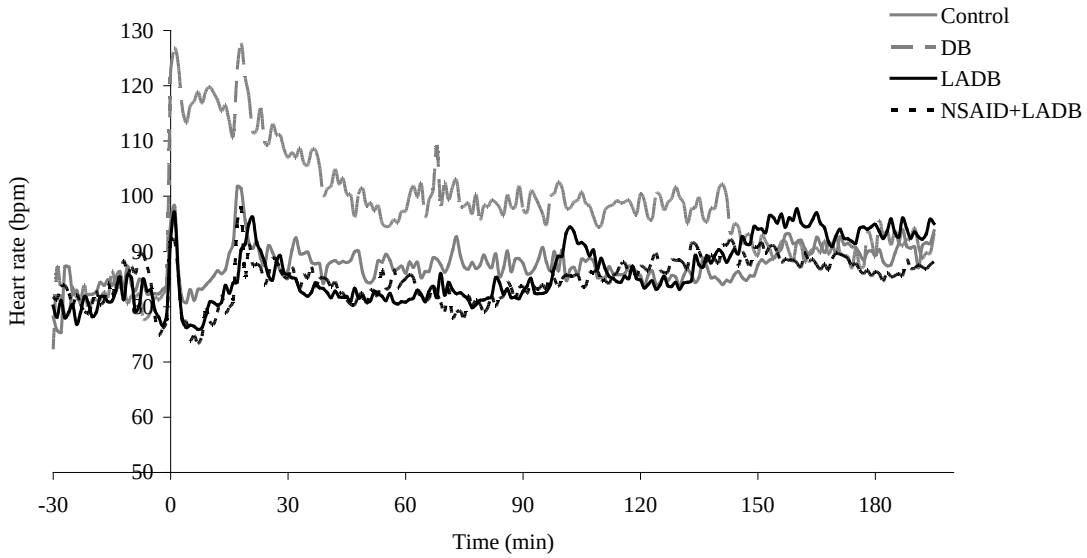

Figure 1. Average HR for control (n=8), disbudded without local anaesthetic (DB, n=5), disbudded with local anaesthetic (LADB, n=8) and disbudded with NSAID and local anaesthetic (NSAID+LADB, n=8).

Behavior

Calves in the LADB group showed a decrease in time spent lying ($P<0.001$) between 2 and 3 h after disbudding (Figure 3). There were no significant differences in lying behavior for any other treatment. Calves in the DB group spent less time ruminating during the 3 h after disbudding compared to all other treatment groups ($P<0.05$) except LADB (Figure 4).

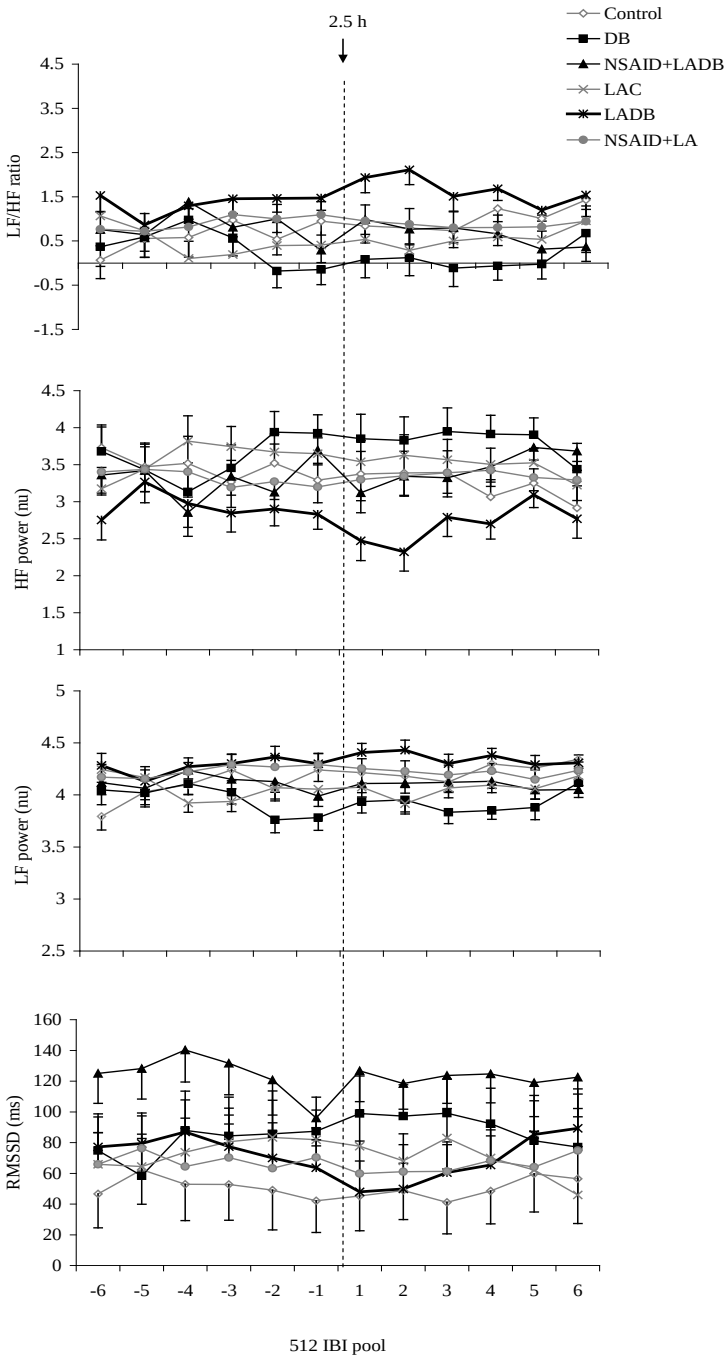

Figure 2. Time domain (root mean square of successive R-R interval differences, RMSSD) and frequency domain (high frequency (HFnu) and low frequency (LFnu) power and LF/HF ratio) analysis of 512 IBIs 30 min before (sample -6 to -1) and 30 min after (sample 1 to 6) 2.5 h following treatments. Control (○, n=8), disbudded without local anaesthetic (◀, DB, n=5), disbudded with local anaesthetic (**x**, LADB, n=8), local anaesthetic control (x, LAC, n=8), NSAID and local anaesthetic control (●, NSAID+LA, n=8) and disbudded with NSAID and local anaesthetic (▲, NSAID+LADB, n=8).

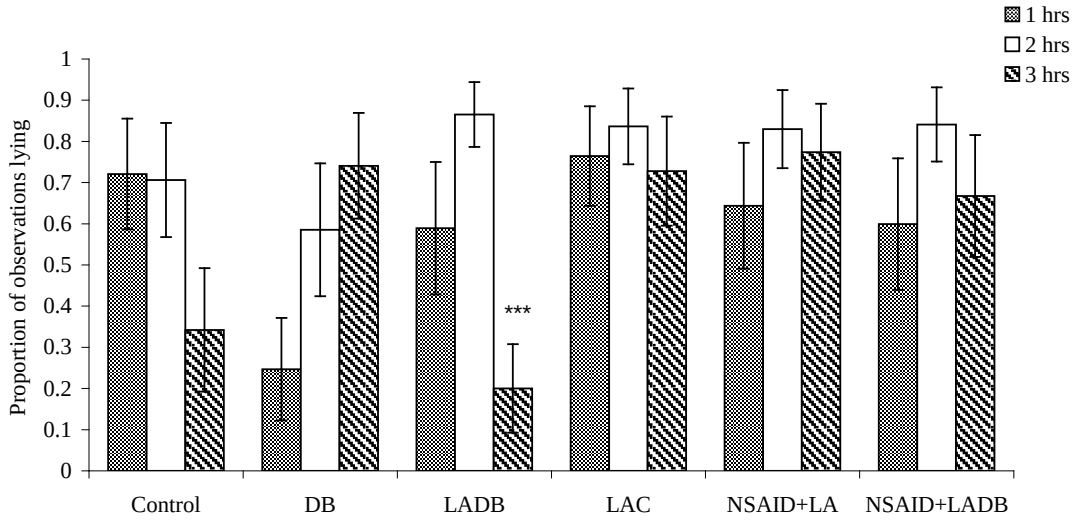

Figure 3. Proportion of observations/h (± S.E.M.) lying for control (n=8), disbudded without local anaesthetic (DB, n=6), disbudded with local anaesthetic (LADB, n=8), local anaesthetic control (LAC, n=8), NSAID and local anaesthetic control (NSAID+LA, n=8) and disbudded with NSAID and local anaesthetic (NSAID+LADB, n=8) during 1 (▨), 2 (□) and 3 hours (▩) after treatments (***P<0.001, statistically different between 2-3 h).

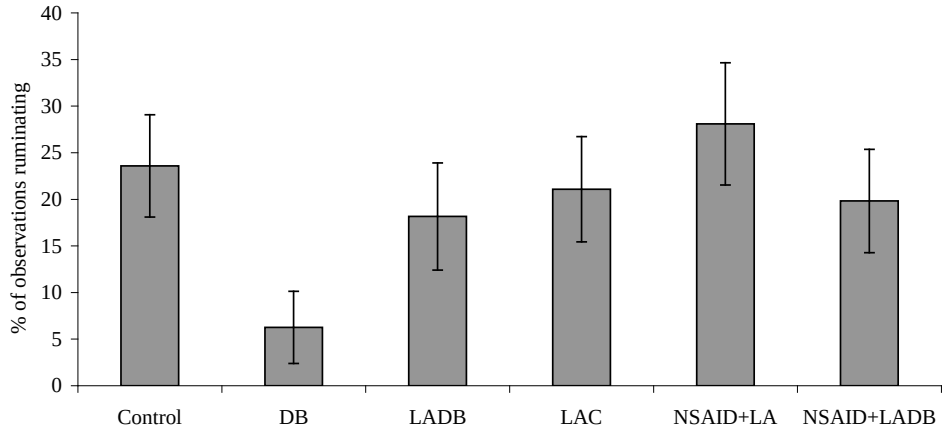

Figure 4. Percentage of total observations (± S.E.M.) ruminating during 3 h after treatments for control (n=8), disbudded without local anaesthetic (DB, n=6), disbudded with local anaesthetic (LADB, n=8), local anaesthetic control (LAC, n=8), NSAID and local anaesthetic control (NSAID+LA, n=8) and disbudded with NSAID and local anaesthetic (NSAID+LADB, n=8).

DISCUSSION

The results of this study support the hypothesis that administration of local anaesthetic and NSAID abolishes the immediate pain caused by cautery disbudding as determined by changes in the eye temperature, HR and behavioral responses following the procedure. Despite the low number of recordings completed during the immediate period due to equipment failure, the rapid drop in eye temperature immediately following disbudding without local anaesthetic was consistent with the findings reported by Stewart et al. (2008). The subsequent drop in eye temperature by 2-3 h following disbudding in the animals that had been administered with local anaesthetic is of considerable interest. These animals also had an increase in HR and a decrease in lying behaviour, except when they had received NSAID. This suggests that there was an onset of pain about this time, possibly due to the effects of the local anaesthetic wearing off and that the NSAID abolished these responses.

These results are consistent with studies investigating the effect of administering NSAID on cortisol secretion during amputation dehorning (Stafford and Mellor, 2005) and also behavioral responses to cautery disbudding (Faulkner and Weary, 2000). Faulkner and Weary (2000) found that the NSAID ketoprofen, administered with the milk ration on the day of disbudding, reduced head shaking and ear flicking and increased weight gain over the 24 h following cautery disbudding. The present study has used alternative physiological responses rather than cortisol levels and supports the view that a combination of local anaesthetic and NSAID is more effective at alleviating pain following cautery disbudding than local anaesthetic alone.

The present study further extends knowledge as it has demonstrated that eye temperature also drops after 2-3 h, a time that is likely associated with the clearance of the local anaesthetic. Other studies have also shown changes in behavior at this time. For example, Graf and Senn (1999) found increased head shaking in the 3rd hour after disbudding in calves that had received local anaesthetic. Neither the control group nor the group treated with both local anaesthetic and NSAID had such a drop in eye temperature, suggesting that this response was associated with the onset of pain. The drop in eye temperature is thought to be due to sympathetically-mediated vasoconstriction in response to the pain induced by disbudding, as previously reported (Stewart et al., 2008). It is unclear why eye temperature increased immediately after disbudding in calves treated with both local anaesthetic and NSAID. It is possible that

there was an interaction between the NSAID and the response to disbudding. A NSAID/DB treatment would have helped tease out this possible interaction, but this treatment group was not included in order to minimise the number of calves disbudded without full pain relief.

The reduction in time spent ruminating in the 3 h after disbudding without local anaesthetic was consistent with other studies following amputation dehorning (McMeekan et al., 1999; Sylvester et al., 2004) and cautery disbudding (Grondahl-Nielsen et al., 1999) and suggests that these calves experienced a higher level of discomfort. The calves disbudded with both local anaesthetic and NSAID spent a similar amount of time ruminating compared to the control groups. Less time spent lying in the 3rd hour after disbudding with local anaesthetic alone is inconsistent with the study by McMeekan et al. (1999) who reported the opposite effect. They found an increase in lying behavior 2 h following dehorning with or without local anaesthetic compared to those given local anaesthetic and NSAID. However, both standing still and lying will reduce general activity, and could serve to minimise pain and assist in healing (Mellor et al., 2000).

In the present study, the immediate increase in HR following disbudding without local anaesthetic was similar to levels reported in other studies (Schwartzkopf-Genswein et al., 2005; Stewart et al., 2008). The prolonged response in HR over 3 h after disbudding was consistent with the study by Grondahl-Nielsen et al. (1999) who found that HR was elevated for 3.5 h after disbudding without local anaesthetic, whereas cortisol concentrations had declined after 1 h. This suggests that the initial cortisol response may be a consequence of the immediate noxious stimulus of the cautery rather than representing the ongoing pain experienced. These differences between measures highlight the importance of measuring multiple physiological parameters over an extended period in order to assess pain.

Cardiac responses to disbudding were analysed in detail using HRV parameters. The RMSSD tended to decrease immediately after disbudding without local anaesthetic change indicating a small reduction in vagal tone. The lack of significant change in the frequency domain parameters associated with the procedure is not consistent with previous findings where the HF power decreased and the LF power and the LF/HF ratio increased from baseline, indicating an acute change in the sympatho-vagal balance of the ANS (Stewart et al., 2008). Despite the modest changes in HRV immediately after disbudding, it did change 2-3 h post disbudding. The calves that were disbudded with

local anaesthetic had a lower HF power and RMSSD than other treatment groups indicating a reduction in vagal tone, with a higher LF power and LF/HF ratio, reflecting a change in the sympatho-vagal balance (von Borell et al., 2007). This change in ANS activity may indicate of the onset of pain as the effects of the local anaesthetic start to wear off. Increases in the LF/HF ratio have been found in response to different milking systems (Hagen et al., 2005), insect harassment and diarrhoea in calves (Mohr et al., 2002), but its use as an index of pain in cattle has only been reported previously by Stewart et al. (2008). Further research is required to improve our understanding of the underlying mechanisms of HRV and how it may reflect the experiences of pain and stress in farm animals (von Borell et al., 2007).

In conclusion, the combination of local anaesthetic and NSAID successfully mitigated behavioral and physiological responses to disbudding up to 3 h after the procedure. These results support other findings that have shown this combination is more effective at alleviating pain than local anaesthetic alone during amputation dehorning procedures. Furthermore, these results show that NSAIDs are also beneficial for alleviation of post-operative pain associated with cautery disbudding. Non-invasive measures of eye temperature, HR and HRV may provide additional information to other indices for assessing responses to painful husbandry procedures.

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Chapter Seven

Eye temperature, heart rate variability and catecholamine responses of calves castrated with or without local anaesthetic

Authors note: Chapter seven is in the process of being revised for submission to an international journal. An abstract has been submitted for presentation at the 4th International Workshop on the Assessment of Animal Welfare at Farm and Group Level, September 2008, Ghent, Belgium.

Abstract

The role of the autonomic nervous system (ANS) in mediating eye temperature responses during painful procedures was examined in 30, four-month-old calves randomly assigned to four treatments: 1) sham handling control (C), 2) surgical castration (SC), 3) local anaesthesia and sham handling (LAC), 4) local anaesthesia and SC (LASC). Two weeks later, 16 control calves were infused with adrenaline or saline. Maximum eye temperature (^{o}C) measured using infrared thermography (IRT), heart rate HR and heart rate variability (HRV) were recorded continuously for 25 min before and 20 min after castration and for 15 min before and after adrenaline infusion. HRV was analysed by examining short segments of 512 interbeat intervals (IBI) before and after treatments. Blood samples, collected via jugular catheter, were used to determine plasma concentrations of noradrenaline and adrenaline following SC and C, cortisol concentrations after all treatments and packed cell volume (PCV) following adrenaline infusion. During the 20 min after castration, eye temperature increased ($P<0.001$) following LASC (+0.28 ^{o}C) and SC (+0.47 ^{o}C), and there was a small increase (+0.10^{o}C) following C that was less than both castration treatments ($P<0.001$). During the 2 min following SC, there was a small drop in eye temperature that coincided with a short-lived increase in HR (+15.3bpm, $P<0.001$), adrenaline ($P<0.05$) and noradrenaline ($P<0.001$). Following SC, there was an increase in the root mean square of successive differences (RMSSD, $P<0.001$) and the high frequency (HF) power ($P<0.05$), and a decrease in the low frequency (LF)/HF ratio ($P<0.05$). Following LASC, there were increases in HR (+6.3bpm, $P<0.001$) and the RMSSD ($P<0.01$) and a decrease ($P<0.05$) in the LF power. Cortisol increased above baseline by 15 min following both castrated groups ($P<0.001$), but was greater for SC ($P<0.05$) than LASC. There were no HR, HRV, catecholamine or cortisol responses to C or LAC. Adrenaline infusion caused a decrease in eye temperature (-1.4 ^{o}C, $P<0.01$) and HR (-9.3bpm, $P<0.05$) and an increase in RMSSD ($P<0.001$), PCV ($P<0.001$) and cortisol ($P<0.001$). There were no responses to saline. Local anaesthetic did not eliminate responses to surgical castration. The synchronised drop in eye temperature and increased catecholamine and HR responses following SC suggested a short-lived sympathetic response. The increase in RMSSD, HF and decrease in LF and LF/HF ratio suggests that this was followed by an increase in parasympathetic tone, which may have been responsible for the increase in eye temperature. The drop in eye temperature after adrenaline confirms a

sympathetically-mediated response. HRV and IRT may complement each other as a non-invasive alternative to measuring catecholamines as an index of autonomic activity for assessment of acute pain in cattle.

Keywords: Calves; Infrared thermography; Eye temperature; Heart rate variability; Catecholamines; Cortisol; Castration

Introduction

Acute responses to noxious stimuli cause activation of the autonomic nervous system (ANS) and hypothalamic-pituitary-adrenal (HPA) axis. These are the two main systems that mediate the stress response and are primarily involved in metabolic homeostasis. Activation of the sympathetic division of the ANS and the resultant release of catecholamines (e.g., adrenaline and noradrenaline) from the adrenal medulla occur extremely rapidly and are the primary component of the 'fight or flight' reaction. In contrast, the cortisol response that is mediated by the HPA axis is slower, more persistent and more easily measured. Increases in HPA activity (i.e., cortisol levels) in response to painful husbandry procedures in calves have been well documented (Stafford and Mellor, 2005a; b), however, less attention has been given to measuring ANS responses to pain in cattle, possibly because of the lower cost and ease of measuring cortisol. Methods that have been used to measure sympathetic nervous system (SNS) activity include heart rate variability (HRV), pupillary diameter, skin resistance, peripheral blood flow and plasma catecholamine concentrations. Relatively few studies have measured catecholamines in farm animals, which may be because of the high cost of assays and practical difficulties in collection and measurement of catecholamines, due to their low concentrations and short half life (1-2 min) in plasma (Hjemdahl, 1993). Catecholamines are involved in mobilising energy, blood vessel dilation and increasing muscle contractility, cardiac output, respiration and other responses required for the rapid 'fight or flight' response. Increased catecholamine concentrations have been found in response to branding (Lay et al., 1992a; Lay et al., 1992b) isolation (Lefcourt and Elsasser, 1995), simulated transport (Locatelli et al., 1989) and dehorning (Mellor et al., 2002) in cattle; and ring castration and tailing (Mellor et al., 2002) and transport (Parrott et al., 1994) of sheep.

Recently, it has been suggested that eye temperature responses, measured by infrared thermography (IRT), may be a useful, non-invasive measure of SNS activity in cattle (Stewart et al., 2008). The drop in eye temperature found following disbudding of calves without local anaesthetic may be caused by the redirection of blood from the capillary beds via sympathetically-mediated vasoconstriction (Stewart et al., 2008). This explanation for the drop in eye temperature was supported by synchronised changes in HRV that indicated an increase in SNS activity in response to the pain of disbudding. However, further investigation of the role of the ANS in regulating the eye temperature response is required to fully understand the underlying mechanisms. In the present study, surgical castration of calves was used to measure changes in ANS activity in response to pain and to further validate the eye temperature responses using a different model of pain than disbudding in cattle. Castration is a common husbandry practice used by farmers to modify temperament, prevent unwanted breeding and to alter carcass quality (see Stafford and Mellor, 2005b for a recent review on pain alleviation and different methods of castration). Surgical castration causes a greater acute pain response, but heals faster and causes less chronic pain than other methods of castration (Molony et al., 1995; Stafford and Mellor, 2005b), which makes it a suitable model to assess acute ANS responses to pain in this study. In cattle, the effects of surgical castration on growth and feed intake (Fisher et al., 1996), heart rate (HR) (Schwartzkopf-Genswein et al., 2005), cortisol concentrations (Stafford and Mellor, 2005b) and behaviour (Molony et al., 1995; Schwartzkopf-Genswein et al., 2005) have been investigated. The aim of the present study was to examine the role of the ANS in mediating the eye temperature response during painful procedures by measuring eye temperature, HR, HRV and catecholamine responses following surgical castration of calves. A secondary aim of this study was to confirm that the previously observed drop in eye temperature in response to pain or a fright is due to sympathetic activity by measuring eye temperature responses to an adrenaline challenge.

Method

The protocol and conduct of this study were approved by the Ruakura Animal Ethics Committee.

Animals and treatments

A total of 30 four-month-old Friesian cross calves with a mean weight of 106±3kg (range 101 to 111 kg), kept together outdoors at pasture and managed under normal farm practice were used in this study. Calves were halter trained and brought into the yards where the trial took place 3-5 days per week for three weeks prior to the start of the trial to acclimatise them to the facility, handling and procedures. During this time calves were also acclimatised to restraint in a head bail and to wearing the HR monitors. The trial took place over eight days and 3-5 calves were sampled per day, one at a time. During the day prior to treatment, calves were clipped down the right side of the body to allow maximum contact for the HR monitors and necks were clipped to facilitate jugular catheters. Indwelling jugular catheters were inserted the day prior to treatment to allow the calves to settle overnight and were removed at the end of sampling the following day. The same veterinarian carried out all drug administrations and castration procedures. Calves were restrained in a calf crush and head bail and randomly assigned to four treatments:

1. Control (n=8; C): Scrotum and testes handled to simulate administration of local anaesthetic for the time normally required for administration (approximately 1.5 min) and again 10 min later for the time normally required to complete the castration procedure (approximately 1.5 min).
2. Surgical castration (n=6; SC): Scrotum and testes handled to simulate administration of local anaesthetic as above. 10 min later the testes and cords were exteriorised by an incision across the base of the scrotum and each testes pulled out separately by gentle traction until the spermatic cords broke.
3. Local anaesthesia control (n=8; LAC): 5 ml of 2% lignocaine hydrochloride (Lopaine, Ethical Agents Ltd, Auckland, New Zealand) administered by injection into each testicle through the distal pole and into the distal end of the scrotum followed by a subcutaneous infiltration (7 ml) at the neck of the scrotum (Weaver et al., 2005). 10 min later the scrotum and testes were handled to simulate the castration procedure as described above.
4. Local anaesthesia plus surgical castration (n=8; LASC): Lopaine administered as described above, then a prick test (4 areas 5 mm from the base of the scrotum

pricked with a needle) 10 min later to ensure loss of sensation before proceeding with surgical castration.

On treatment days, each of the calves scheduled for treatment that day were brought into a holding pen (3.1 x 2.8 m) with access to straw bedding, hay and water and allowed approximately 1 hr to settle before sampling started. At approximately 15 min prior to sampling, each calf was fitted with a HR monitor and then moved from the holding pen into the calf crush and restrained in a head bail where they remained during a 45 min sampling period. This period included 10 min for baseline data collection, followed by injection of the local anaesthetic (including infiltration) or a sham injection (average 01:37±00:26 min:sec), 10 min for the local anaesthetic to take effect before the castration or sham procedure (average 01:43±00:25 min:sec) and a further 20 min period to monitor post-treatment responses. Over this 45 min period, cardiac activity and eye temperature were recorded and blood samples were collected.

To prevent post-operative pain and infection, all castrated calves were treated with 0.5 mg/kg of Meloxicam (Metacam®, Boehringer Ingelheim Ltd, Auckland, New Zealand; ½ life of approximately 26 hrs in cattle), a preferential COX-2 inhibiting non-steroidal anti-inflammatory agent (NSAID), injected via jugular catheter before the jugular catheters were removed. Catheters were then removed and calves were released at the end of the 45 min sampling period.

Adrenaline challenge

Following a 2-week recovery period after sham castration treatments, a total of 16 calves (n=8 from the control treatment and n=8 from LAC), with an average weight of 124±5kg (range 108 to 130 kg), were used for the adrenaline challenge. Treatments took place over four days and four calves were sampled per day, one at a time. During the day prior to treatment, calves were re-clipped and two indwelling jugular catheters were inserted, one each side. Two catheters were required, one for the infusion and one for blood sampling, in order to prevent contamination of the blood samples. Catheters were removed at the end of sampling the following day. Calves were randomly assigned to one of two treatments and given either Epinephrine HCL (4 µg /kg/min, Cat. E4642, Sigma Aldrich, St Louis, MO, USA) or the equivalent volume of physiological saline

administered via jugular infusion using a Baxter Flo-Gard 6201 Volumetric Infusion Pump (Baxter Healthcare Corporation, Deerfield, USA).

On treatment days, each of the four calves scheduled for treatment were brought into the same holding pen used for castration and the procedure was the same as that during castration, except that it took place over a 30 min sampling period. This period included 15 min for baseline data collection, followed by infusion of the saline or adrenaline (average 05:02 ± 00:04 min:sec), and a further 10 min period to monitor post-treatment responses. Over this 30 min period, cardiac activity and eye temperature were recorded and blood samples were collected.

Infrared thermography

An infrared thermography camera (ThermaCam S60, FLIR Systems AB, Danderyd, Sweden) was used to collect images of the eye. Images were collected approximately every 20 sec. All images were collected from the left side of the calf (approximate distance 0.5 m) at a right angle. Maximum temperature (^{o}C) of the medial posterior palpebral border of the lower eyelid and the lacrimal caruncle (Stewart et al., 2008) was recorded from each image immediately into an excel spreadsheet over the 45 min sampling period. In addition, a firewire cable was used to connect the infrared camera to a laptop computer to achieve continuous recordings of eye temperature during the sampling period using image analysis software (ThermaCam Researcher 2.7, FLIR Systems AB, Danderyd, Sweden). When manual recording was not possible for short periods due to calves moving their head around, recordings of eye temperature were achieved by analysing images from the software. Both methods of recording and analysis (i.e., immediately entering values into excel on the day and software analyses) were shown to be compatible (see Appendix 3). Ambient temperature (^{o}C) and relative humidity (%) inside the sampling area were recorded every 30 min and entered into the infrared camera to calibrate it for atmospheric conditions.

Heart rate and heart rate variability

Continuous interbeat intervals (IBIs) were recorded using Polar heart rate monitors (S810i^{TM}, Polar Electro Oy, Helsinki, Finland). Monitors were fitted to each calf in their

holding pen approximately 15 min before being moved into the calf crush for the start of sampling and removed at the end of the sampling period before being released. To increase conductivity, ultrasound transmission gel was applied liberally to the clipped site at each electrode contact point. The electrodes and transmitter were built into an elastic strap, provided with the Polar HR monitors, which was strapped firmly around the calf's thorax, immediately behind the forelimbs, with the HR monitor attached. At the end of each sampling period the stored HR was downloaded via a serial interface to a computer for analysis. Time domain parameters included mean HR, R-R interval and root mean square of successive differences (RMSSD), and frequency domain parameters included the high frequency power (HF, 0.30-0.80 Hz), the low frequency power (LF, 0.04-0.30 Hz) and the LF/HF ratio, which were calculated with Fast Fourier Transformation (FFT) using HRV software (Niskanen et al., 2004). In order to fulfill recommendations for analysis of HRV using FFT made by the Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology (1996) and von Borell et al. (2007), we examined short segments of data containing 512 beats, frequency band widths were selected to take into consideration the respiratory frequency of calves and the HF and LF power were presented as normalised units (nu) to account for inter-individual differences. Normalised units represent the relative value of each power component in proportion to the total power minus the very low frequency component (frequencies lower than the lower limit of the LF band). Continuous recordings of IBI data are prone to measurement errors (Marchant-Forde et al., 2004) therefore, prior to analysis, a correction function within the Polar software (Polar Precision Performance Software; Version 4.03), set on default parameters, was used to correct for any artifacts (e.g., to eliminate ectopic heartbeats). Only data sets with an error rate of less than 5% were included in the analysis.

Blood sampling and assays

To enable blood sampling every 30 sec for acute changes in catecholamine concentrations, blood samples (6 ml) were collected via jugular catheter attached by silicone tubing to a peristaltic pump into pre-chilled EDTA tubes (placed on ice) from -2 until 10 min relative to the time of castration (time 0). For cortisol assays, blood samples (7 ml) were taken via jugular catheter into a syringe at -20, -10, 15 and 20 min

in relation to the time of castration and at -10, -5 and 15 min in relation to the start of the adrenaline infusion, and immediately placed into tubes containing lithium heparin anticoagulant. All blood samples were placed on ice and centrifuged immediately (or at least within 10 min of collection) for 10 minutes at > 2500 rpm. Plasma was stored at -80^{o}C until assayed. Plasma was assayed for concentrations of catecholamines (adrenaline and noradrenaline) and cortisol. During the adrenaline challenge, blood samples were also taken for measurement of packed cell volume (PCV). These were taken at -5 and 5 mins in relation to the start of infusion. Cortisol was measured using a double-antibody radioimmunoassay as described previously (Fisher et al., 2002). The minimum detectable level was 1.0 ng/ml. The inter-assay coefficient of variation for plasma pools measuring 8.3, 24.4 and 63.4 ng/ml were 1.8, 10.0 and 5.8% respectively. For the catecholamine assay, 20 ul of 600 nmol/l N-methyl dopamine internal standard was added to 1 ml of plasma and extracted on alumina. The alumina was washed with sodium bicarbonate solution, then water and eluted with 0.2M acetic acid. The extracted catecholamines were separated and measured by reverse phase high performance liquid chromatography (HPLC) with electrochemical detection as described previously (Goldstein et al., 1981; Eisenhofer et al., 1986). The extraction efficiency for N-methyl dopamine was 99.3%.

Statistics

A one-way ANOVA was used to compare treatment differences in maximum eye temperature, cortisol and catecholamine concentrations, HR and HRV and a Student's t-test was used to compare differences at various periods after castration from baseline. Maximum eye temperature in the 20 min post-treatment period was compared to baseline (the first 10 min before administration of local anaesthetic). For HRV, time and frequency domain analysis of the 512 IBIs (approximately 5 min) post-treatment were compared to baseline (the 512 IBIs prior to administration of local anaesthetic). Due to high error rates, HRV data from seven calves were not included in the analysis and HR profiles for one calf were not included. HR during the injection of local anaesthetic and the 2 min post-treatment were compared to baseline. A one-way ANOVA was also used to compare treatment differences in maximum eye temperature, HR and HRV following the adrenaline challenge. Catecholamine concentrations were measured in 12 calves following control (n=6) or castrated without local anaesthetic (n=6).

Results

Eye temperature following castration

During the first 2 min following castration without local anaesthetic, there was a small, non-significant drop in eye temperature, which was followed by a rapid increase (Figure 1). There were no treatment differences during the baseline period. Eye temperature increased above baseline within the first 5 min ($P<0.001$) following both castration treatments and stayed elevated for the entire 20 min post-treatment period. Eye temperature ±S.E.M. was 0.28±0.05^{o}C and 0.47±0.05^{o}C higher ($P<0.001$) than baseline during the 20 min post-treatment period following castration with and without local anaesthetic respectively. This increase was greater in the calves castrated without local anaesthetic compared to those castrated with local anaesthetic ($P<0.05$). Eye temperature following the control treatment also increased during the 20 min post-treatment (+0.10±0.05^{o}C, $P<0.05$), but the increase was less than that following both castration treatments ($P<0.001$).

Heart rate and heart rate variability following castration

HR (±S.E.M.) increased during the first 2 min following castration with local anaesthetic (+6.3±2.4 bmp, $P<0.05$) and without local anaesthetic (+15.3±2.8 bmp, $P<0.001$) then dropped rapidly back to baseline levels (Figure 2). This increase in HR was greater following castration without local anaesthetic compared to castration with local anaesthetic ($P<0.05$). During the 2 min following the injection of local anaesthetic, HR increased ($P<0.001$) by 6.4±1.4 bpm (LASC) and 7.1±1.3 bpm (LAC), but no significant changes occurred during sham local anaesthetic. During the 512 IBIs post-treatment, RMSSD was higher than baseline with ($P<0.01$) or without local anaesthetic ($P<0.001$, Table 1). Following castration without local anaesthetic, HF also increased ($P<0.05$) and LF/HF decreased ($P<0.05$) from baseline. Following castration with local anaesthetic, LF decreased ($P<0.05$) from baseline. There were no changes in HR or any HRV parameter following both sham castration treatments (Control and LAC).

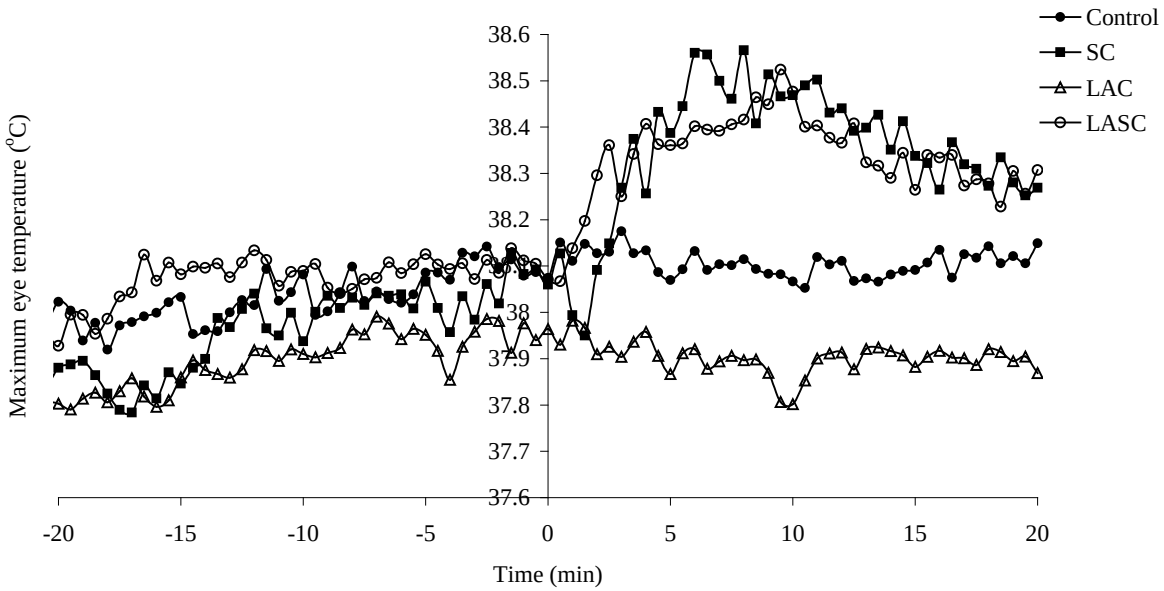

Figure 1. Maximum eye temperature (^{o}C) for control (●, n=8), castrated without local anaesthetic (■, n=6), local anaesthetic control (Δ, n=8) and castrated with local anaesthetic (o, n=8). 0 min indicates the time of treatment.

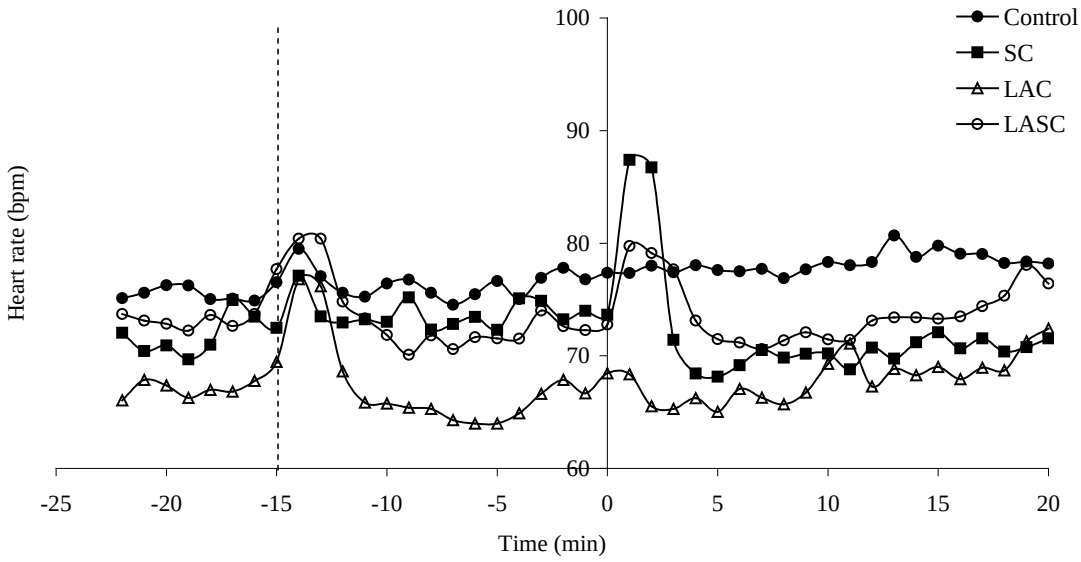

Figure 2. Mean heart rate (bpm) for control (●, n= 8), castrated without local anaesthetic (■, n=6), local anaesthetic control (Δ, n=8) and castrated with local anaesthetic (o, n=7). The dashed line indicates the time that local anaesthetic or the sham procedure was administered and 0 min indicates the time of treatment.

Table 1. HR variability parameters in time domain, the root mean square of successive R-R interval differences (RMSSD), and frequency domain, high frequency (HFnu) and low frequency (LFnu) power and the LF/HF ratio, during baseline (the last 512 IBIs prior to local anaesthetic administration) and the response (the first 512 IBIs post-treatment) for control (n=8), local anaesthetic control (LA, n=7), castrated without local anaesthetic (SC, n=3) and castrated with local anaesthetic (LASC, n=5).

HRV parameter	Interval	Control	SC	LAC	LASC	*ANOVA*
RMSSD (ms)	Baseline	35.2 (±8.0)	31.3 (±13.1)	37.1 (±8.6)	47.1 (±10.1)	ns
	Response	32.9 (±7.6)	57.1 (±12.3)	33.3 (±8.1)	65.2 (±9.6)	0.043
	P value	ns	0.001	ns	0.002	0.001
HFnu	Baseline	36.9 (±4.9)	20.9 (±8.0)	35.8 (±5.3)	43.2 (±6.2)	ns
	Response	34.4 (±4.6)	31.8 (±7.5)	33.3 (±4.9)	53.3 (±5.8)	ns
	P value	ns	0.020	ns	ns	ns
LFnu	Baseline	63.1	79.1	64.2	56.8	ns
	Response	65.6	68.2	66.7	46.7	0.030
	P value	ns	ns	ns	0.050	ns
LF/HF ratio	Baseline	2.0 (±0.5)	4.9 (±0.8)	2.3 (±0.6)	1.5 (±0.7)	ns
	Response	2.3 (±0.5)	2.8 (±0.8)	2.5 (±0.5)	1.0 (±0.6)	ns
	P value	ns	0.050	ns	ns	ns

Descriptive statistics are based on treatment means (±S.E.M) of non-transformed data.
Statistical significances for frequency domain parameters (LF, HF and the LF/HF ratio) are based on log transformed data. P values are based on results from the Student's t-test. ns = non-significant (P>0.05).

Cortisol and catecholamine concentrations following castration

Cortisol concentration had increased at 15 and 20 min following both castrated treatment groups (P<0.001), but was higher following castration without local anaesthetic at 15 min (P<0.01) and 20 min (P<0.05) compared to castration with local anaesthetic (Figure 3). There were no changes in cortisol concentrations following the control or local anaesthetic control treatments. Following castration without local

anaesthetic, noradrenaline concentrations increased (P<0.001) and reached peak levels at 2 min (Figure 4). Noradrenaline concentrations after castration without local anaesthetic were higher (P<0.01) than controls for the post-treatment period up until 5.5 min, then concentrations returned to baseline. Adrenaline concentrations increased from baseline (P<0.05) following castration without local anaesthetic and reached peak levels at 2 min, then returned to baseline levels by 3 min, however, concentrations were not significantly different to controls (Figure 5). There were no changes in noradrenaline or adrenaline in the control treatment.

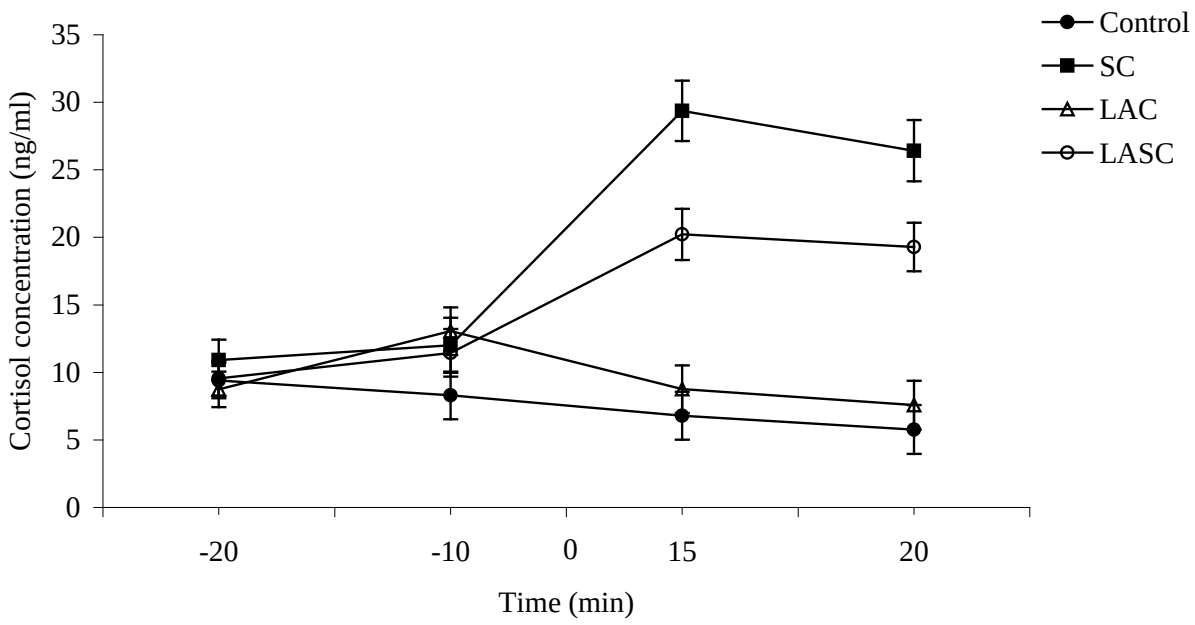

Figure 3. Mean cortisol concentration (ng/ml) for control (● n= 8), castrated without local anaesthetic (■, n=6), local anaesthetic control (Δ, n=8) and castrated with local anaesthetic (o, n=8). Treatments were administered at time 0 min.

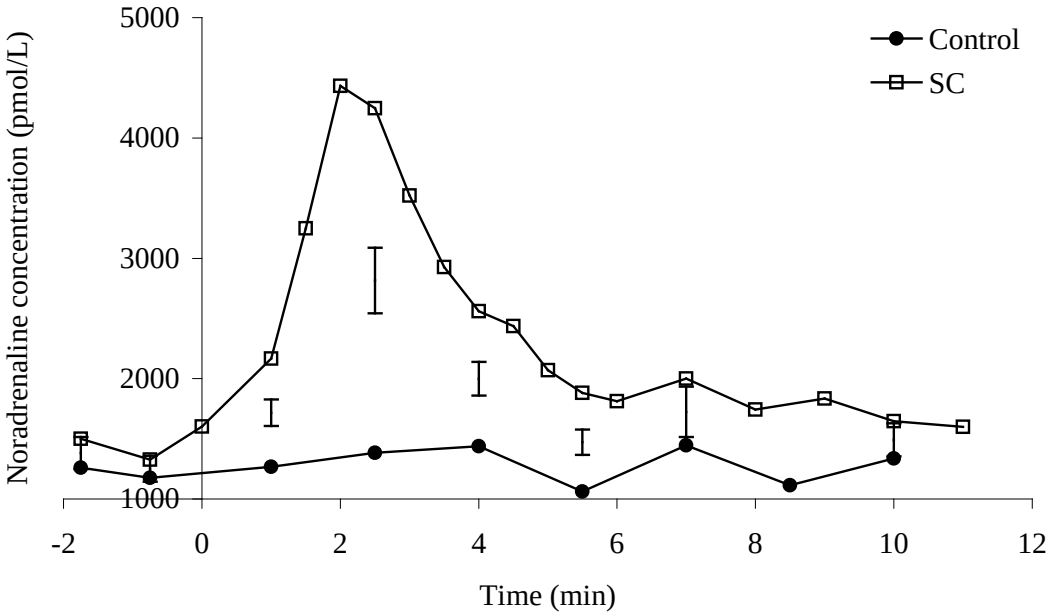

Figure 4. Noradrenaline concentrations following control (●, n=6) or castrated without local anaesthetic (□, n=6). Error bars represent standard errors of the differences (sed).

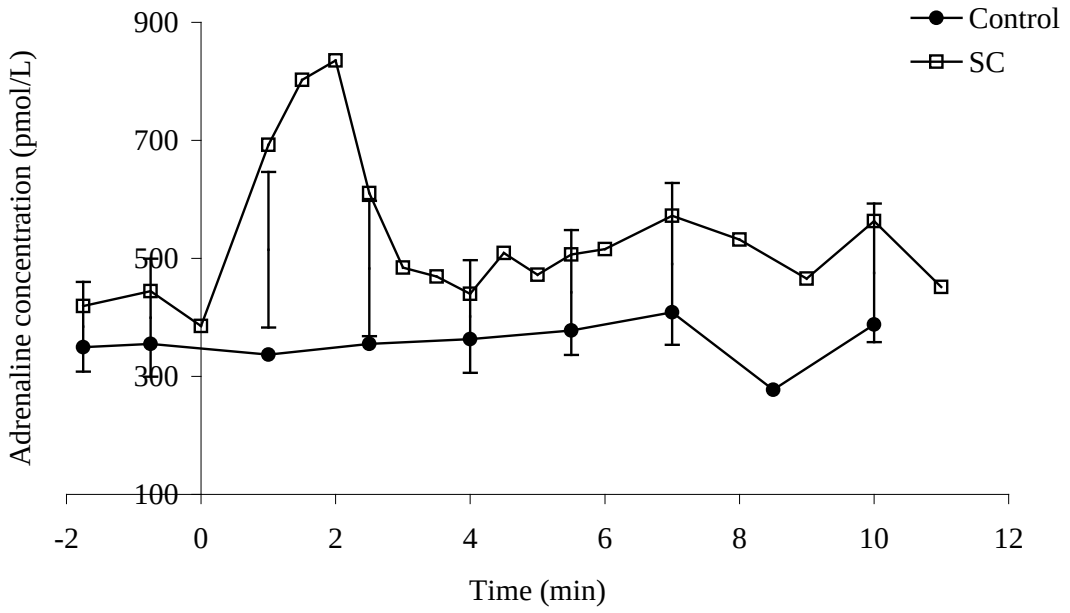

Figure 5. Adrenaline concentrations following control (●, n=6) or castrated without local anaesthetic (□, n=6). Error bars represent standard error of the differences (sed).

Adrenaline challenge

Eye temperature dropped rapidly in the first 5 min during the adrenaline infusion (average change -0.14±0.05 ^{o}C, P<0.01) and was lower compared to saline (P<0.001, Figure 6). During the same time period, HR decreased in response to adrenaline (-9.3±3.3 bpm, P<0.05), but was not significantly different to control (Figure 7). There was high individual variability in the HR response to adrenaline (HR decreased in 5 out of 8 calves). At the end of the adrenaline infusion, HR increased and was higher (P<0.01) than control in the last 10 min post-treatment. There were no significant changes in the HF, LF or the LF/HF ratio following adrenaline or saline. RMSSD increased (P<0.01) in response to adrenaline from 34.2±9.6 ms (baseline) to 124.3±22 ms. Cortisol concentrations were higher (P<0.001) at 15 min following adrenaline compared to saline (Figure 8). PCV was higher (P<0.001) after the adrenaline infusion. There were no changes in eye temperature, HR, HRV, cortisol or PCV after saline.

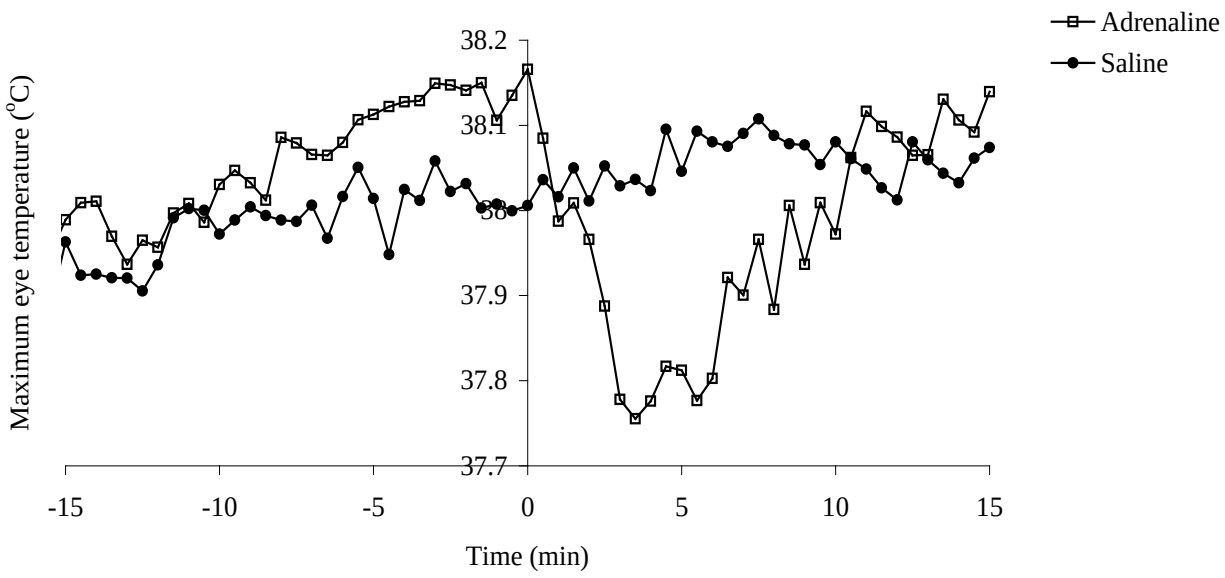

Figure 6. Maximum eye temperature (^{o}C) following infusion (0 to 5 min) of adrenaline (□, n=8) or saline (●, n=8).

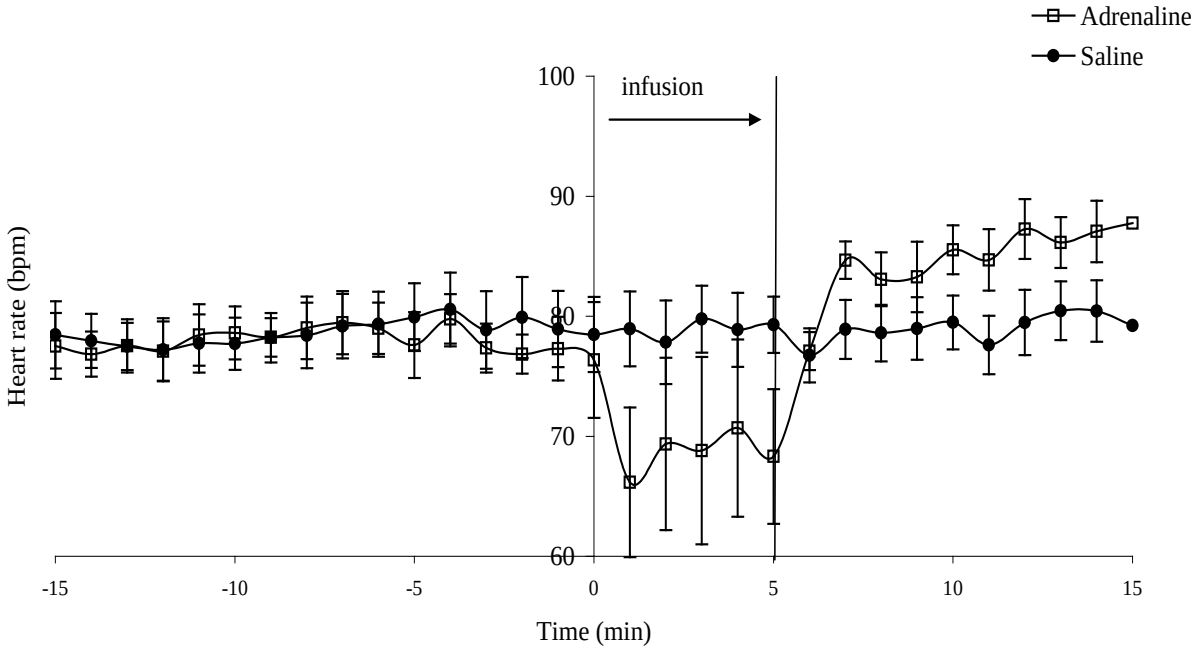

Figure 7. HR (bpm) following infusion (0 to 5 min) of adrenaline (□, n=8) or saline (●, n=8).

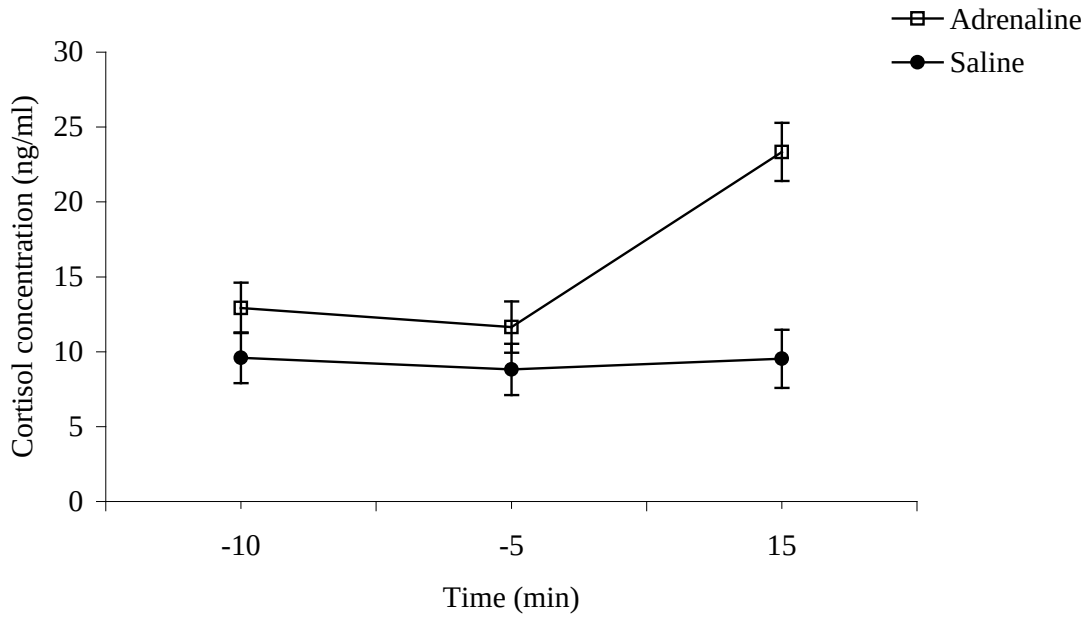

Figure 8. Mean cortisol concentration (ng/ml) following infusion of adrenaline (□, n=8) or saline (●, n=8).

Discussion

The changes in eye temperature and time and frequency domain HRV parameters, and increase in cortisol and catecholamine concentrations indicated acute pain in response to surgical castration. Local anaesthetic was not effective enough to completely eliminate pain-related responses such as changes in eye temperature, HR, HRV and cortisol concentrations. Stafford et al. (2002) found that administration of local anaesthetic did not reduce the plasma cortisol response to surgical castration and that a combination of local anaesthetic and NSAID was required to eliminate the cortisol response. Local anaesthetic alone is not sufficient enough to eliminate pain-related responses during surgical castration because the sites of injection are distal to the areas in the body cavity where stretching and tearing of the spermatic cords would generate most nociceptior impulses (Stafford and Mellor, 2005b). In addition, the injection itself caused an increase in HR to levels similar to those found after castration, which suggests that the injection itself may be stressful for calves, without providing optimal pain relief. In future studies, it would be useful to measure HR, HRV and eye temperature responses following local anaesthetic and NSAID.

The increase in eye temperature following castration was consistent with responses to velvet antler removal in Elk (Cook et al., 2005), jugular catheterisation of dairy cows (Stewart et al., 2007) and a fright in humans (Levine et al., 2001). Prior to the increase in eye temperature following castration without local anaesthetic, there was a non significant, short-lived drop in eye temperature. Although this drop was not as pronounced, the response is consistent with an acute drop in eye temperature followed by an increase after disbudding without local anaesthetic (Stewart et al., 2008). The drop in eye temperature was explained as a sympathetically-mediated response due to vasoconstriction; however, the mechanism for the increase is still unknown. However, it was suggested previously that the increase in eye temperature following disbudding was not driven by changes due to heat (from the cautery iron), physical activity, increased HPA activity or local inflammatory processes (Stewart et al., 2008). The present results provide further evidence that the increase in eye temperature is not due to heat or physical activity as no heat was applied and although behaviour was not recorded, calves were never observed to struggle and remained still during and after castration. The adoption of an immobile stance following castration may function to avoid or

reduce stimulation of hyperalgesic tissues therefore reducing pain (Molony and Kent, 1997). It is possible that the increase in eye temperature may be caused by vasodilation due to increased parasympathetic activity or the release of vasodilators such as nitric oxide (endothelium-derived relaxing factor), adrenaline, prostaglandins, bradykinins and histamine during pain.

The decrease in the LF/HF ratio and increase in RMSSD and HF following castration without local anaesthetic indicates increased parasympathetic activity (Kleiger et al., 1995). The parasympathetic nervous system (PNS) branch of the ANS acts to lower HR and carry noxious impulses from the pelvic viscera, including the testes (King, 1987). Therefore, the cardiac responses to castration may be due to direct stimulation of the PNS. The peak in HR at 2 min following castration without local anaesthetic coincided with peak plasma catecholamine concentrations and a small (non significant) drop in eye temperature indicating a short-lived increase in sympathetic activity. Lay et al. (1992a) also found that adrenaline and HR responses coincided following hot iron branding. In the present study, HR returned to baseline rapidly following the initial increase. This rapid decrease in HR may be due to an adaptive response of the PNS to lower the HR. Few studies have measured HR during castration of cattle, however, decreases in HR have been reported following surgical castration of calves (Schwartzkopf-Genswein et al., 2005) and ring castration of sheep (Johnson et al., 2005).

Plasma catechoalmines have a short half-life of 1-2 min (Hjemdahl, 1993), however, few studies have measured catecholamines in cattle within seconds of a painful husbandry procedure. In the present study, the acute increase in catecholamine concentrations indicates stimulation of the SNS in response to the pain associated with castration. Castration caused a more marked affect on plasma noradrenaline levels compared to plasma adrenaline levels, which suggests that the increased sympathetic activity may have been predominantly due to peripheral sympathetic neural stimulation (Ahmed et al., 1994). In addition, noradrenaline may have been responsible for the synchronised drop in eye temperature as it stimulates α-adrenergic receptors, which causes peripheral vasoconstriction. It is difficult to compare concentration levels across different experiments because of factors such as age, temperament, sex, and the different level of stress associated with the particular procedure. However, baseline

plasma catecholamine concentrations were similar to those found previously in cattle (Locatelli et al., 1989; Lefcourt and Elsasser, 1995), but were lower than values reported by Lay et al. (1992a; b). Consistent with the rapid response time in the present study, adrenaline concentrations peaked at 30 seconds following hot iron branding (Lay et al., 1992a) and adrenaline and noradrenaline concentrations reached peak levels by 30 seconds and returned to baseline within 5 min following electric stunning of sheep (Lowe et al., 2000). Mellor et al. (2002) found that noradrenaline concentrations peaked at 30 min and adrenaline concentrations peaked at 5 min following dehorning of calves. However, given the short-lived nature of catecholamines, the acute responses to dehorning may have been overlooked as the first blood sample was not taken until 5 min post-treatment.

The drop in eye temperature in response to the adrenaline challenge was similar to that during disbudding (Stewart et al., 2008), which confirms that this is a sympathetically-mediated response. In a previous study no change in eye temperature was detected following an adrenaline challenge in dairy cows (Stewart et al., 2007), however, this was probably because the dose that was used (1.4 µg /kg) was not sufficient to cause a reduction in peripheral blood flow. In the present study, the increased PCV following adrenaline confirmed sympathetically-mediated contraction of the spleen, which functions to release stored red blood cells that carry the extra oxygen required during the 'fight or flight' response. Increased cortisol concentrations confirmed an increase in hypothalamic-pituitary-adrenal activity in response to the adrenaline challenge. The decrease in HR and increase in RMSSD during the infusion of adrenaline was unexpected and suggests a small increase in parasympathetic activity. However, there was a high level of individual variability in cardiac responses to adrenaline. These results were inconsistent with increased HR and decreased RMSSD following an adrenaline challenge in humans (Ahmed et al., 1994). However, they were consistent with the decrease in HR and increase in HF power following an adrenaline infusion in children during anaesthesia (Wodey et al., 2003). Exogenous adrenaline administration results in peripheral vasoconstriction (as indicated by a drop in eye temperature in the present study) and increased systolic blood pressure. This leads the ANS to increase parasympathetic activity, which in the present study was indicated by increased RMSSD, in order to decrease successively the HR, the cardiac output and finally the blood pressure (Wodey et al., 2003). In addition, the differences in the cardiac response

to an adrenaline challenge between studies may be dependent on the dose (Wodey et al., 2003) and the duration of administration (Ahmed et al., 1994).

In conclusion, there was evidence that local anaesthetic may have alleviated some of the pain caused by castration, however, it did not eliminate eye temperature, HR, HRV or cortisol responses. The synchronised drop in eye temperature and increases in the catecholamine and HR responses within the first 2 min following castration without local anaesthetic suggested a short-lived sympathetic response. The increased eye temperature and changes in HRV suggest a subsequent increase in parasympathetic tone. The increase in eye temperature in response to castration may be due to parasympathetically-mediated vasodilation in response to the pain of surgical castration. The drop in eye temperature in response to an adrenaline challenge confirms this is a sympathetically-mediated response. IRT and HRV may complement each other as a non-invasive alternative to measuring catecholamines as an index of ANS activity for assessment of acute pain and its alleviation in cattle.

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Chapter Eight

General Discussion

This chapter summarises the main findings and overall conclusions from this thesis. A discussion of the limitations and future areas of research worthy of further investigation that have arisen from this research are also outlined. This thesis is the most recent and more comprehensive attempt to validate eye temperature, measured by infrared thermography (IRT), as a non-invasive measure of stress and pain in cattle. The main approach was to examine autonomic nervous system (ANS) and hypothalamic-pituitary-adrenal (HPA) axis activity in response to various routine handling practices and painful husbandry procedures practiced on-farm. A number of exogenous challenges were used to provide some insight into the underlying physiological mechanisms driving the eye temperature responses.

1.1. Validation of eye temperature as a measure of stress and pain

1.1.1 Main findings

The first hypothesis tested was that eye temperature would increase in response to exogenous stimulation of HPA activity. This hypothesis was based on the study by Cook et al. (2001) who found a correlation between eye temperature and cortisol levels following an adrenocorticotrophic hormone (ACTH) injection in horses, and suggested that the HPA axis may be driving the increase in eye temperature. As well as attempting to repeat these findings in cattle, the first study (Chapter 3) took this one step further to assess which level of the HPA axis might be involved. Cattle were given either ACTH, bovine corticotrophin-releasing hormone (bCRH) or exposed to a psychological stressor (social isolation). In addition, an epinephrine challenge was given to determine if the sympathetic nervous system (SNS) had a role in the eye temperature response. This first study provided no evidence to support the hypothesis as no changes in eye temperature occurred following any of the exogenous challenges. It was concluded that it may be possible that stimulation of the stress axis using exogenous challenges alone was not sufficient to cause a change in eye temperature. Stimulus in this manner is not representative of the body's entire repertoire of responses to stress that would occur during the 'fight or flight' response to a physical or psychological stressor and therefore, a cognitive component may need to be involved. This idea was supported by the finding that eye temperature increased in response to catheterisation in the same study, which had a cognitive component. Therefore, the next experiment (Chapter 4)

investigated eye temperature responses to various aversive handling procedures in cattle. This was the first time that eye temperature images were collected at very frequent intervals and a rapid drop in eye temperature was detected immediately following an electric prod, shouting and startling. It was suggested that this response may be due to sympathetically-mediated vasoconstriction due to the rapidity of the response.

A small study was also undertaken that used a pharmacological approach to investigate fear responses of sheep exposed to a barking dog (Appendix 1). Sheep were given either saline or diazepam before being exposed to a dog. No treatment differences were found in HR or behaviour, suggesting that diazepam did not modify the fear response of sheep to a dog. It was suggested that diazepam may have paradoxically increased anxiety in some sheep. There appeared to be interpretive difficulties associated with dose rates and the appropriate fear model in the use of diazepam to study anxiety in sheep.

Pain is a major stimulus for the SNS, therefore, eye temperature and changes in heart rate variability (HRV) were measured in response to disbudding in calves, to investigate the role of the SNS (Chapter 5 & 6). In addition, calves were given an ACTH challenge two weeks after disbudding and as in the first study, there was no response in eye temperature, which confirmed that eye temperature responses were not due to changes in HPA activity (Chapter 5). Responses to pain following disbudding without local anaesthetic included a rapid drop in eye temperature, a prolonged heart rate (HR) increase (up to 3 hrs) and an acute HRV response [reduced high frequency (HF) power and increases in the low frequency (LF) power and the LF/HF ratio] that indicated an acute change in the sympatho-vagal balance with a possible shift towards increased sympathetic activity. The rapid drop was followed by an increase in eye temperature. The mechanism for this increase has not yet been determined. However, there was evidence that the increase was not driven by changes due to heat (i.e., heat of the cautery iron), physical activity, increased HPA activity or local inflammatory processes following disbudding. The synchronised changes in HRV provided further evidence that the drop in eye temperature may be driven by SNS activity. However, there is some debate over the ability to directly measure SNS activity from HRV parameters. The increase in the LF/HF ratio is regarded by some researchers as a reliable indicator of increased SNS activity but not by others, therefore, some caution is required when

interpreting HRV responses (von Borell et al., 2007). In addition, there were inconsistencies in the acute HRV response between the two disbudding studies that are difficult to explain. In the study in Chapter 6, the only acute change in HRV following disbudding without local anaesthetic was a tendency for the root mean square of successive differences (RMSSD) to decrease, thereby indicating a small reduction in vagal tone. These discrepancies between studies are not entirely surprising as analysis of HRV and assessment of autonomic responses are not straight forward, which may explain why they have not been used a great deal to assess welfare of cattle previously. However, this research provides the first published data that has measured HRV responses to painful husbandry procedures in farm animals. One unpublished study (Marchant-Forde, unpublished) measured HRV responses to disbudding in calves, however, calves were sedated with Xylazine, which induces bradycardia and subsequently increases parasympathetic tone, therefore, the results were not comparable to the present findings in conscious animals. Despite the lack of acute changes in HRV in Chapter 6, there were changes in both eye temperature and HRV at a later stage when the local anaesthetic wore off. Lignocaine, the local anaesthetic that was used, is only effective for approximately 2 hrs after administration, and when the effects wear off, pain-related behaviours, cortisol concentrations and inflammation-related pain all increase (Stafford and Mellor, 2005a). A drop in eye temperature was detected by IRT in the animals disbudded with local anaesthetic due to the onset of pain at this time. This drop in eye temperature was accompanied by a decrease in lying behaviour and parasympathetic tone (decreased HF), and an increase in HR and SNS activity (increased LF and LF/HF ratio). The administration of local anaesthetic and a non-steroidal anti-inflammatory agent (NSAID) abolished the eye temperature, HR, HRV and behavioural responses associated with the pain caused by disbudding.

In Chapter 7, to confirm the role of the ANS in mediating the eye temperature response, plasma catecholamine concentrations were measured in addition to HRV. Surgical castration of calves was studied as it causes a greater acute pain response than other methods of castration, which made it a suitable model to assess acute ANS responses to pain. In addition, surgical castration provided other important differences to disbudding, involving a different area of the body and a different type of noxious stimuli, without the use of heat.

Following castration without local anaesthetic, the small, non-significant drop in eye temperature, which coincided with an increase in catecholamines and HR, indicated a short-lived sympathetic response that may have been associated with the initial phase of the procedure (the incision into the scrotum). This was followed by an increase in eye temperature and an increase in parasympathetic tone (increase in RMSSD and HF and decrease in LF and LF/HF ratio). The responses to castration with local anaesthetic differed in that there was no drop in eye temperature and the eye temperature, HR and HRV responses were not as marked, indicating that local anaesthetic had some effect but did not completely eliminate pain-related responses to castration. Surgical castration was performed as it would be during normal farm practice and there was no attempt to separate out the two components of the procedure. However, the lack of drop after castration with local anaesthetic may indicate that the pain of the initial incision was blocked by local anaesthetic, but not the traction of the testes. This would be predicted because the sites of the injection are distal to the areas of the body cavity where stretching and tearing of the spermatic cords generate most nociceptor impulses (Stafford and Mellor, 2005b).

The finding that eye temperature and HRV responded differently to castration compared to disbudding was interesting and may have been due to the nature of the pain. Castration caused a more marked increase in eye temperature than disbudding and increased parasympathetic tone, whereas, disbudding caused a more marked sympathetic response. In addition, the increase in HR was much more marked and prolonged (3 hrs) following disbudding compared to castration (2 min). There are many factors that could influence these different physiological responses, such as the location of the pain and the type of tissue involved, different intensities of pain and the level of fear associated with the particular procedure. The pain stimuli during castration, is made up of two components; a somatic stimulus from the scrotum and a deeper, visceral stimulus from tearing and stretching of the testicular structures (Johnson et al., 2005). On the other hand, the pain during disbudding is of a somatic nature involving third or fourth degree burns to the skin tissue localised around the periphery of the horn bud during removal (Weaver et al., 2005). In addition, disbudding requires a significant amount of head restraint by the operator, which could result in a different background type of fear response associated with human handling during the procedure. A similar

drop in eye temperature that occurred following aversive handling procedures may also be due to the associated level of fear.

As mentioned earlier, the mechanism for the increase in eye temperature is still to be determined. However, the suggestion that the increase was not due to heat, physical activity or local anti-inflammatory processes was supported by the finding that eye temperature also increased after castration, which did not involve heat or any observed physical activity and would have caused local inflammatory responses well away from the head area and the eye. It is possible that the increase in eye temperature may be due to parasympathetically-mediated vasodilation. The marked increase in parasympathetic tone after castration may be associated with deep visceral pain due to the stretching and tearing of the spermatic cords as the parasympathetic nervous system (PNS) branch of the ANS carry noxious impulses from the pelvic viscera, including the testes (King, 1987). The PNS acts to lower cardiac output and blood pressure, resulting in vasodilation, which could have caused an increase in eye temperature. The release of vasodilators in response to pain may also be partly responsible. For example, the endothelium of blood vessels use nitric oxide (otherwise known as endothelium-derived relaxing factor) to signal the surrounding smooth muscle to relax, thus resulting in vasodilation and increased blood flow. It is possible that the release of other vasodilators could also be involved such as adrenaline, prostaglandins, bradykinins and histamine. These neurohumoral substances have been shown to have profiles of release associated with pain (Mellor et al., 2000).

Up until this point, the role of the SNS in the drop in eye temperature had been investigated by measuring HRV and catecholamines, however, the finding that eye temperature did not drop in response to an epinephrine challenge in the first study (Chapter 3) was unresolved. It was possible that in the first study the dose of epinephrine was not sufficient to cause a reduction in peripheral blood flow and that the sampling interval may not have been frequent enough to detect a rapid drop in eye temperature. Therefore, the epinephrine challenge was repeated two weeks after castration (Chapter 7) using an infusion of epinephrine at a higher dose rate and more frequent sampling and detailed analysis of eye temperature. This time, eye temperature dropped rapidly in response to the epinephrine challenge and was similar to the responses found after disbudding. This finding confirmed that the drop in eye

temperature is likely to be caused by the redirection of blood from the capillary beds via sympathetically-mediated vasoconstriction. In addition, the finding that an exogenous challenge caused an eye temperature response also provided evidence that a cognitive component is not necessary to cause a change in eye temperature. The decrease in HR and increase in the RMSSD during the infusion of epinephrine suggested a small increase in parasympathetic activity. Although this result was unexpected, it may be due to central effects of epinephrine on the heart. Administration of epinephrine results in peripheral vasoconstriction (as indicated by a drop in eye temperature) and increased systolic blood pressure. This leads the ANS to increase parasympathetic activity in order to decrease successively the HR, the cardiac output and finally the blood pressure.

1.1.2 Practical considerations and some limitations

One limitation in the present experiments was that due to ethical issues, steps were taken to statistically limit the number of animals exposed to treatments without any pain relief in order to meet the requirements of the ethics committee and gain ethics approval. As with any physiological measurement there was a certain level of individual variability in the parameters measured, therefore, greater animal numbers would have improved the data profiles obtained and assisted with interpretation of the results.

Other factors such as the angle and distance of the camera from the animal are also important to take into account when using IRT. It is unlikely that these factors had any influence on the present results as distance and angle were kept consistent by lightly restraining animals in a head bail or rope halter. Angle was sometimes affected if animals moved their heads around or struggled during procedures. The advantage of using the two different methods of image analysis (outlined in Appendix 3) concurrently was that it made it possible to go back to software analysis and capture images on a frame by frame basis when this occurred. In addition, this enabled very frequent sampling intervals of eye image analysis when necessary to capture rapid changes that occurred over a matter of seconds or minutes. Although angle and distance should be kept consistent wherever possible, recent research has shown that it is also possible to achieve consistent measures of eye temperature in an outdoor, unrestricted situation. An automated system for remotely collecting eye images has been used by placing an infrared camera at a water trough and collecting images when animals visit

the trough to drink (Stewart et al., 2005; Schaefer et al., 2007). Further development of this system will allow non-invasive, early detection of disease in calves on farms.

Radiative surface temperature may also be influenced by wetting or evaporation and some concerns have been raised that the drop in eye temperature could be due to evaporative heat loss caused by moisture (or tears) in the eye. Indeed, trans-dermal water loss can represent a meaningful evaporative heat loss factor. Some reports suggest that the evaporative loss can account for several percent of total body heat loss, for example, in a typical daily heat budget for a homoeothermic animal such as a human (Kleiber, 1975). However, the evaporative heat loss will depend greatly on the surface area involved and even at a high rate of trans-epidural water loss of 4 g/m² will still only correspond to 150 W /m² (Mitchell, 1977). Given the comparatively small surface area of the eye (1/10,000 of a square meter) this translates into less than 0.02 W, which would produce an undetectable (substantially less than 0.1^{o}C) change in eye temperature. Furthermore, as a minor point, the emissivity of water is estimated at approximately 0.96 which is very close to the emissivity of skin, therefore, in itself any moisture on the eye surface would have little interference with the measurement of radiated heat from the eye.

Several other factors may influence the eye temperature response such as breed, temperament or experience with human contact. To minimise the potential for confounding autonomic stimulation, animals were habituated to the specific sampling conditions. Animals were always habituated to handling, restraint or haltering to facilitate collection of eye temperature and the same animals were studied in basal conditions before being exposed to treatments. The calves that were used were ideal models for validation purposes as they were familiar with humans and had been well handled and easily tamed prior to experiments. However, there may be difficulties related to the use of IRT in on-farm studies. For example, individual variability of starting points could make it difficult on-farm where the history of the animal is unknown and baselines are unable to be achieved. Large numbers of animals would therefore be necessary to enable accurate interpretation of the data. As with HRV, a within-individual change in eye temperature recorded before and after treatments is more meaningful than between group comparisons. Another limitation would be that radiative surface temperature is influenced by solar radiation, therefore, data collection

is limited to indoor areas out of direct sunlight. Even when it is possible to collect images indoors it is important to take into account any radiative surfaces or lighting that may influence the results or steps should be taken to eliminate them. All of the present experiments were undertaken indoors and any direct light was eliminated, however, this may limit the use of IRT in some on-farm situations. Semi-outdoor facilities or situations where animals are exposed to low solar radiation can be used as long as in the case of comparative studies, conditions are equivalent between groups.

1.1.3 Future research

Acute autonomic responses to aversive procedures in cattle have only been touched upon here and much more research is required to fully understand the relationship between autonomic activity and pain in farm animals. This research investigated acute responses to stress and pain, but there was also evidence to show that eye temperature and HRV can detect the slower onset of pain associated with local anaesthetic wearing off. However, to determine the full potential of IRT and HRV, investigation into their use for quantifying chronic responses to aversive situations also warrants further research. Furthermore, research is required to determine if different autonomic responses can detect different types and intensities of stress or pain for non-invasive assessment of animal welfare. This would involve studies designed to expose animals to different degrees of pain and would help to determine whether the magnitude and duration of the eye temperature response reflects the intensity of the stress or degree of pain. Eye temperature responses also need to be validated in different breeds of cattle and other species. The full capability of IRT and HRV for detection of disease and the effects of individual traits (coping styles, temperament) and previous experience also deserve attention. The detection of changes in ANS activity using a combination of IRT and HRV lends itself to studying different emotional states in animals; negative as well as positive. In recent years, assessment of ANS activity measured by changes in HRV has received considerable attention as a marker of positive emotions in humans but very few studies have used HRV to study animal emotions (Boissy et al., 2007). For example, an increase in parasympathetic tone, indicated by changes in HRV, was linked to relaxation of horses while immersed in warm spring water (Kato et al., 2003). Research combining eye temperature, HRV and behaviour may be a suitable approach to assess positive emotional states in animals.

Further research to fully understand the underlying mechanisms of the eye temperature response is also necessary. Several suggestions have been made in this thesis regarding the mechanism that may be driving the increase in eye temperature, but the studies presented here were not designed to investigate this response. Also, it may be necessary to further confirm the role of the SNS in the drop in eye temperature. Experimental studies involving the use of pharmacological inhibition or stimulation of the ANS activity, such as atropine sulphate (a parasympathetic tone blockade), atenolol (a sympathetic tone blockade) and isoproterenol (a sympathetic stimulant) would be required. These studies would also be required to further understand regulatory mechanisms contributing to HRV responses in farm animals. In addition, to improve ease of HRV analysis, software development for automatic elimination of artefacts from the data would be useful. Finally, the cost of IRT equipment may have limited its use in animal welfare research in the past; however these costs are reducing, which will hopefully provide more opportunities for future research.

1.2 Animal welfare implications

The results from this thesis have implications for pain management on-farm and confirm that a combination of local anaesthetic and a NSAID are required to alleviate both the acute pain and pain in the hours after the procedure, which is likely to be associated with inflammation (Stafford and Mellor, 2005a). Although this has been studied extensively by measuring HPA and behavioural responses after dehorning procedures (Stafford and Mellor, 2005a), the present results show for the first time using measures of physiological responses other than cortisol levels, that this combination is more effective at alleviating the pain induced by cautery disbudding as well.

Local anaesthetic was not sufficient enough to eliminate pain responses to surgical castration. These results are consistent with the finding that local anaesthetic did not reduce the plasma cortisol response to surgical castration (Stafford et al., 2002). As explained earlier, this is because the effects of the local anaesthetic do not reach the areas of the body cavity where the deeper visceral pain associated with traction of the spermatic cords occurs. A combination of local anaesthetic and NSAID is required to

eliminate the cortisol response to castration (Stafford et al., 2002). Future studies should investigate the efficacy of local anaesthetic and NSAID during different methods of castration using eye temperature and HRV as non-invasive measures of ANS activity in response to pain.

The lack of analgesic use during painful husbandry procedures on-farm is mainly due to practical and economical factors; difficulty in administering the drugs, low cost of the animal versus the cost of the treatment and therefore increased cost of production. These are ongoing issues for farmers and veterinarians that are difficult to resolve. In New Zealand, the Code of Welfare for Painful Husbandry Procedures (NAWAC, 2005) currently only require local anaesthetic to be used in cattle over the age of nine months for dehorning and six months for castration, and these science based codes are slow to change. The recent increase in public awareness of animal welfare issues and consumer expectations is increasing the pressure on industries and retailers to provide 'welfare friendly' products. Perhaps the best chance of change will hinge on these types of pressures that are leading retailers to develop their own animal welfare standards and changes will occur sooner than is possible by relying on regulatory and policy bodies. Indeed, supermarkets in the UK and USA are already developing animal welfare standards and welfare labelling systems to meet this demand.

1.3 Final conclusions

Some practical hurdles with IRT need to be overcome before it can be applied for more widespread use in other farm animal species or in on-farm situations. However, eye temperature and HRV, offer advantages over other indicators of stress and pain due to the ability to non-invasively collect data with little interference, therefore minimising the confounding factors associated with other measures. A combination of eye temperature and HRV measures may be a complementary index to other indicators currently used to measure pain and stress, and could replace invasive procedures, such as measurement of plasma catechloamines, to measure ANS responses for assessing animal welfare. This combination may provide more sensitive, detailed and immediate measures of acute pain than HPA changes and could have wider applications to test the efficacy of analgesics and measure animal emotions.

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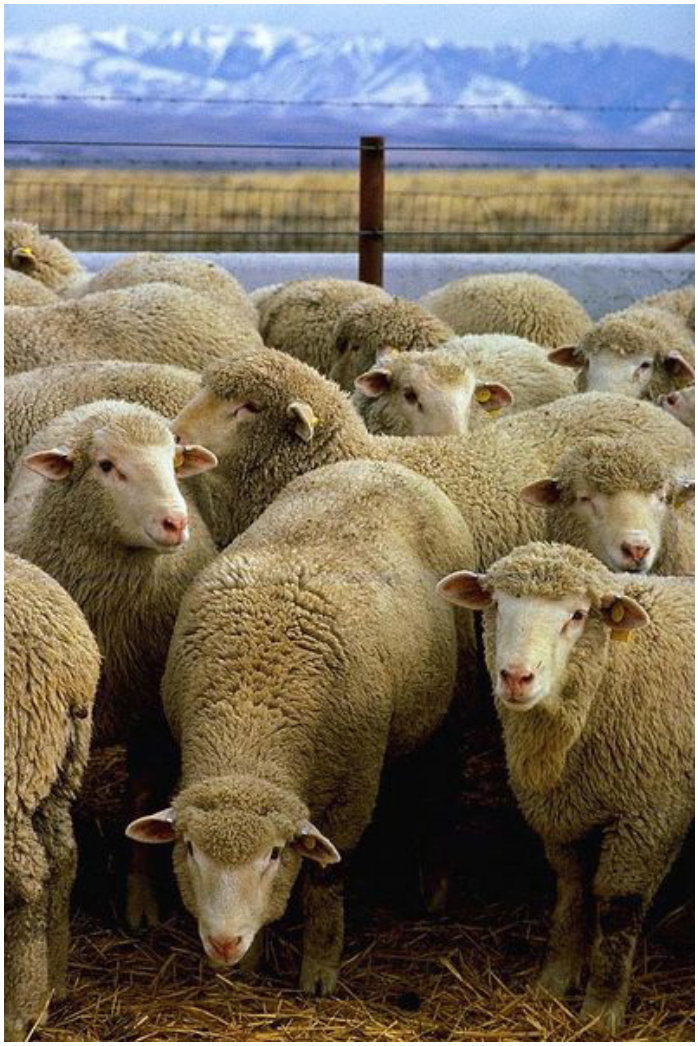
Appendix One

The use of diazepam as a pharmacological method for evaluating anxiety in sheep



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The use of diazepam as a pharmacological method for evaluating anxiety in sheep

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ABSTRACT

The aim of this study was to examine the effects of diazepam, an anti-anxiety drug, on the fear responses of sheep and its usefulness as a pharmacological method to study welfare. Fourteen, eight month old Romney ewes were randomly assigned to two treatments and injected via jugular venepuncture with diazepam (0.37 mg/kg) or saline (controls) 20 min before exposure to a barking dog. Heart rate (bpm) and escape attempts were recorded throughout a 10 min period before injection (baseline), 20 min post injection and 15 min following the dog. Average heart rate was lower in diazepam-treated sheep during the first 5 min following injection ($P<0.01$) and was significantly higher ($P<0.01$) for both diazepam and saline-treated sheep during the 5 min following exposure to the dog compared to baseline (+23.1 and +21.5 bpm for diazepam and saline respectively). Heart rate then decreased back to baseline levels during the last 10 min. Diazepam-treated sheep tended to have a higher number of escape attempts during the 5 min following exposure to the dog ($P=0.08$) compared to controls, suggesting that diazepam may have paradoxically increased anxiety in some sheep. The lack of differences between treatments suggests that diazepam did not modify the fear response of sheep to a dog. There appear to be interpretive difficulties associated with dose rates and the appropriate fear model in the use of diazepam to study anxiety in sheep.

Keywords: sheep; fear; diazepam; heart rate; behaviour.

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INTRODUCTION

Fear and anxiety are emotional states that occur as coping mechanisms in situations that are perceived as being threatening or dangerous. Fear is considered to have a direct cause whereas anxiety is an overall, non-specific level of arousal (Boissy, 1995). The presence of these emotions are important in the assessment of animal welfare. Sheep are exposed to a range of potentially fearful experiences during routine farm management such as transport, exposure to unfamiliar conspecifics and mustering by humans and dogs. During lambing, human disturbance is one factor that may cause increased anxiety in ewes and can result in delayed parturition, desertion of lambs or premature movement from the lambing site, all of which can compromise ewe-lamb bonding and ultimately impact on lamb survival (Fisher & Mellor, 2002).

It is common for studies of animal welfare to use behavioural and physiological techniques to measure fear and stress. Pharmacological approaches are less commonly used, but manipulation of an animal's emotions using drugs may be a useful way of studying fear-related responses to aversive experiences. Benzodiazepines, such as diazepam, are anti-anxiety drugs that facilitate the inhibitory activity of γ-aminobutyric acid (GABA) receptors, which balance the excitatory effects of glutamate and regulate general neural excitability (Roy-Byrne, 2005). Diazepam, administered intravenously, has a rapid distribution rate to and from the central nervous system resulting in a quick onset of action (approx 1-5 min) (Horn & Nesbit, 2004; Mandelli *et al.*, 1978). Benzodiazepines are frequently used to study anxiety and fear in laboratory animals however very few studies have used these drugs to study anxiety in farm animals.

Recently, diazepam has been used to study anxiety in cattle and pigs. Sandem *et al.* (2006) used diazepam as a tool to validate the degree to which the eyes are opened (i.e., the percentage of white in the eye area) as an indicator of 'frustration' in dairy cows. They induced 'frustration' by preventing cows from accessing visible food and found that the cows treated with diazepam showed a reduced percentage of white in the eye compared to untreated cows. A standard test for fear and anxiety in rodents is an elevated plus-maze in which anxious animals chose to avoid arms of the maze, which have no sides, and an increased risk of falling, Andersen *et al.* (2000) used this test and found that diazepam-treated pigs spent more time on open arms of an elevated plus-maze than pigs administered saline, which was consistent with results from rodent studies (Cole & Rodgers, 1995). Ferreira *et al.* (1992) found that treatment of parturient

ewes with diazepam facilitated maternal behaviour and enhanced acceptance of alien lambs. They suggested that diazepam may attenuate the ewe's negative emotional response to the odour of the alien lambs.

The aim of this study was to examine the effects of diazepam on coping responses of sheep to the presence of a dog. There is evidence to show that exposure to a dog, is particularly stressful and causes increases in fear-related behaviour (Beausoleil *et al.*, 2005; Hansen *et al.*, 2001) and heart rate (Baldock & Sibly, 1990; MacArthur *et al.*, 1979). A barking dog was therefore chosen as a fear stressor for sheep in this study. Our hypothesis was that diazepam administration would reduce fear responses, measured by heart rate and escape behaviour, of sheep to a dog compared to saline-treated controls.

MATERIALS AND METHODS

Fourteen, eight month old Romney ewes (average weight 34.6 kg) were used in this study. Ten additional ewes of the same breed and age were used as companions. All the sheep were grazed on pasture under routine farm management. One month prior to treatment, the sheep were moved into an indoor barn each day (for approx. 3 hours) to allow acclimatisation to the indoor pens and handling and to reduce fear of human contact. The sheep were also acclimatised to restraint in a head bail and to wearing a heart rate monitor strap. The day prior to treatment, sheep were clipped around the girth and neck area to provide maximum contact for heart rate monitors and to facilitate jugular vein injection.

On treatment days, the sheep were randomly assigned to two treatments (7 sheep per treatment) and given a jugular intravenous injection of either diazepam (Pamlin, Parnell laboratories; 0.37 mg/kg) from commercially available vials (5 mg/ml) or an equivalent volume of saline (control treatment). Injections were administered while each sheep was manually restrained against the side of the pen by one other person.

One sheep was treated at a time and either 2 or 3 sheep were treated per day. During treatment, each sheep was held individually in the monitoring pen (1.0 x 1.4 m) and restrained in a head bail, in order to limit movement, facilitate a stronger fear response, due to the inability to escape from the dog, and maintain a consistent position and distance from the dog. Two unrestrained companion sheep were held in identical

neighbouring pens either side of the monitoring pen. To prevent the companion sheep habituating to the dog, two different companion sheep were used each treatment day.

Polar S810i heart rate monitors (Polar Electro Oy, Finland) were fitted immediately after the sheep were moved into the monitoring pen prior to treatment. To optimise conductivity, ultrasound transmission gel was applied to the skin at each electrode contact point. The electrodes and transmitter were built into an elastic strap, designed for human use, which was strapped firmly around the sheep's girth area, immediately behind the forelimbs, and a heart rate monitor attached. Once heart rate monitors were fitted, the sheep were given 5 min to settle before being restrained in the head bail. Continuous recordings of heart rate (bpm) and behaviour (via video recording) were then collected over a 45 min sampling period. The sampling period included a 10 min baseline period, followed by injection of diazepam or saline, a 20 min period before exposure to a barking dog (0 min) and a final 15 min recovery period. The dog, a 6 yr old female huntaway farm dog, was led into the sampling area on a short leash and positioned approx 1m in front of the sheep (average time, 15 s), commanded to bark 2 to 3 times, and then led away. Sheep were familiar with the handler but had no prior experience with the dog.

Behaviour was analysed from videos and the number of occurrences of attempts to escape (i.e., the number of times all four feet lifted off the ground) per min was recorded during the 5 min before and 5 min after exposure to the dog.

Statistical analyses

Heart rate and behaviour were expressed as the difference from baseline (i.e., the difference was calculated between the mean baseline pre-injection (the first 10 min) and various periods following exposure to the dog). A Student's t test was used to compare differences between treatments and differences at various periods following exposure to the dog from baseline.

RESULTS

Baseline heart rate was similar for both groups (96.7±2.7 and 102.9±2.5 bpm for diazepam and saline respectively). For both treatments, average heart rate was significantly higher during the 5 min following exposure to the dog compared to

baseline (P<0.01) and then decreased back to baseline levels during the last 10 min (Figure 1). Average heart rate increased during the first 5 min post injection but was lower for diazepam-treated sheep during this period (P<0.01) (Figure 1). Figure 2 shows the average number of occurrences of attempts to escape per min for the 5 min before and 5 min after exposure to the dog. All sheep stood calmly prior to the dog entering. Sheep administered with diazepam tended to have a higher number of attempts to escape during the 5 min following exposure to the dog (P=0.08) compared to saline-treated sheep. There were no significant differences between treatments in heart rate or behaviour of sheep in response to the dog.

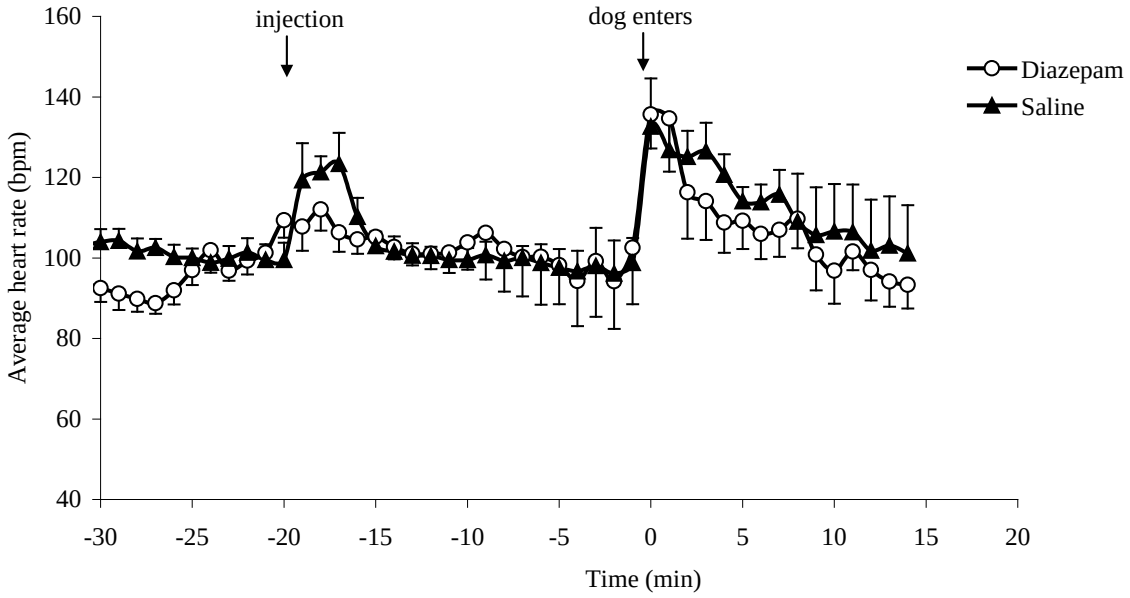

FIGURE 1: Average heart rate (bpm) ± S.E.M. for both diazepam (○) and saline (▲) treated sheep over the 45 min sampling period. 0 min indicates the time that the dog entered and the treatment was administered via jugular injection at -20 min.

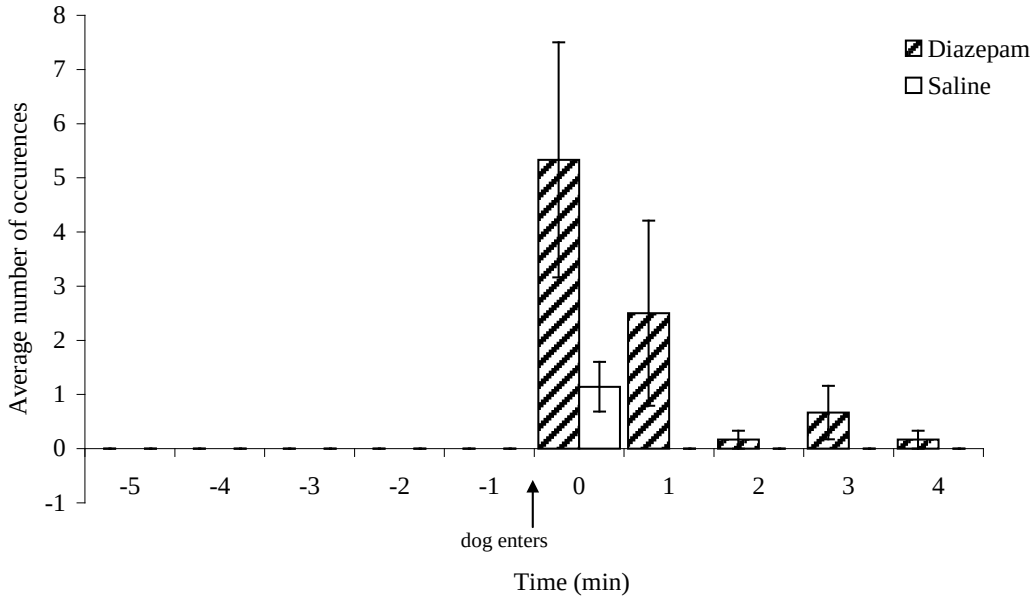

FIGURE 2: Average number of occurrences of attempts to escape per min ± S.E.M for diazepam (▨) and saline (□) treated sheep during the 5 min before and 5 min after exposure to the dog. 0 min indicates the time that the dog entered.

DISCUSSION

Exposure to a barking dog produced a stress response that was consistent with others studies that have measured heart rate responses of sheep to a dog and human handler (Baldock & Sibly, 1990; MacArthur *et al.*, 1979). The lack of any differences in heart rate and behavioural responses to the dog between diazepam and saline-treated sheep suggests that diazepam did not modify the fear response of sheep to a dog. These results are inconsistent with studies in other farm animal species such as dairy cattle (Sandem *et al.*, 2006) and pigs (Andersen *et al.*, 2000). The results do not support our hypothesis that diazepam would reduce the fear response of sheep to a fearful experience and suggest that caution should be used when assuming that human drugs will have similar effect on other animals.

The lower heart rate of diazepam-treated sheep immediately after administration of the jugular injection compared to saline-treated sheep suggests that diazepam may have reduced anxiety and the fear response to handling required for the injection. There is little data on the effects of diazepam on heart rate, however, a reduction in heart rate

following administration in humans has been reported (D'Amelio *et al.*, 1973). The effects of the diazepam should have lasted long past the administration period as other studies have shown longer lasting effects of diazepam in sheep (Ferreira *et al.*, 1992).

The tendency for diazepam-treated sheep to show more escape behaviours in response to the dog than saline-treated sheep suggests that diazepam may have actually increased anxiety in some animals. It was noted that some of the diazepam-treated sheep also appeared to be more anxious (e.g., more excited) after being released from the pens at the end of sampling. This stimulatory effect may not be totally unexpected as benzodiazepines can produce paradoxical stimulant effects that cause increased aggressive and hyperactive behaviour (Speth *et al.*, 1980). It would have been useful to observe other fear-related behaviours, however, restraint in the head bail prevented other anti-predator behaviours, such as foot stamping and avoidance behaviours, which have been observed in other studies investigating behavioural responses to a dog (Baldock & Sibly, 1990; Beausoleil *et al.*, 2005).

Our inability to distinguish any differences between treatments may be due to the dose rate or individual variability of responses to the dog. The dose rate used in this study was slightly lower than Ferreira *et al*. (1992) used to investigate the effects of diazepam on acceptance of ewes to alien lambs (20mg/4ml) and lower than doses used to induce sedation in sheep (0.5-1.5 mg/kg) (Bolte & Stupariu, 1978; cited in Ferreira *et al*., 1992). Ferreira *et al.* (1992) showed that the diazepam dose used in their study did not affect the level of activity of sheep in an open field test. However, it is evident that the optimal dose rates for the use of diazepam as a pharmacological method for measuring anxiety in sheep are unknown. Another possible explanation for the lack of effect of diazepam in this study may be the type of fear stressor that was used. For example, Sandem *et al.* (2006) prevented cows from accessing visible feed to induce 'frustration', which is a different type of stressor to the acute type of fear or fright response used in this study. It is possible that pre-treatment of diazepam to sheep exposed to a more chronic stressor that is more representative of anxiety, such as weaning or social isolation, may cause a different response.

In conclusion, the results from this study suggest that there are interpretive difficulties in the use of diazepam to study fear in sheep. The paradoxical and variable effects indicate that the responses to diazepam administration in sheep may differ depending on the dose or model of anxiety used.

ACKNOWLEDGEMENTS

The authors would like to thank the Animal Genomics group at AgResearch for the use of their sheep and the Ruakura farm staff for their assistance during the trial.

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Appendix Two

Conference abstracts

Authors note: Appendix two is a collection of abstracts published in proceedings of the various international conferences where results from this thesis were presented.

Infrared thermography as a non-invasive measure of stress in dairy cows.

Stewart, M., Webster, J.R., Verkerk, G.A., Colyn, J.J., Schaefer, A.L.

Journal of Animal Science 2005, 83, 374

Presented at *American Society of Animal Science, American Dairy Science Association Joint meeting*, Cincinnati, Ohio, July, 2005

Infrared thermography of the eye (ET), to detect heat produced by stress, may be a useful non-invasive way to measure the welfare impact of husbandry practices on domestic livestock. This study examined the ET of dairy cows during stimulation of the stress axis by intravenous hormonal administration or social isolation. Six cows, acclimated to handling, were each given six treatments in a random Latin-square design: 1) 5ml saline 2) ACTH (0.05 mg Synacthen) 3) bCRH (20 μg) 4) bCRH (40 μg) 5) epinephrine (1.4 μg /kg liveweight) and 6) isolation (I). Treatments were administered at time 0 and blood was sampled via jugular catheter while standing beside each cow at -30, -15, 0, 5, 10, 15, 20, 30, 40, 50, 60, 75, 90, 120, 180 and 240 min except for epinephrine which was sampled at -30, -15, -10, -5, 0, 2, 5, 10, 15, 20, 30, 45, 60, 90 and 120 min. Body temperature was recorded every 10 min and ET was recorded approximately every 2 min from 30 min pre-treatment (ThermaCam S60). Plasma samples were assayed for ACTH, cortisol and non-esterified fatty acids (NEFA). ACTH increased after bCRH, and cortisol increased after ACTH and bCRH ($P<0.001$). Neither cortisol nor ACTH changed after epinephrine or I. NEFA increased after epinephrine ($P<0.01$). ET increased prior to treatment in many cases. Compared to pre-treatment, ET was higher 30 and 60 min after saline and ACTH ($P<0.001$), but not after other treatments. ET tended to drop rapidly by the first sample after I ($P=0.057$) and then increase again (by 30 min, $P<0.001$). Body temperature was not affected by any treatment. Increases in cortisol, ACTH and NEFA confirmed stress axis activation. Pre-treatment increases in ET, possibly due to prior activity or handling stress, confounded post-treatment effects. The changes in ET found after I are novel, suggestive of an acute sympathetic response and may reflect psychological stress which was unique to social isolation.

Objective measurement of pain and fear in cattle using infrared thermography.

Schaefer, A.L., Stewart, M., Webster, J.R., Cook, N.J., Colyn, J.J., Lepage, P., Church, J.S., Haley, D.B.

Proceedings of the International Society of Applied Ethology North American Regional Meeting, Vancouver, Canada, June 7-8, 2006, pp 55.

The aim of the present study was to investigate the ability to non-invasively and objectively detect pain and fear in a bovine model using infrared thermography of the orbital (eye) region. Twenty one crossbred beef calves averaging 350 kg were used in the study. Animals were randomly assigned to three treatment groups designated as control (C, no interference), pain (P, two brief 1 second applications of a conventional electric cattle prod) or fear (F, the sudden shaking of a plastic bag in front of the animals head and the accompanying shout of a fearful word). Each animal was brought into a conventional cattle restraining chute and allowed a five minute rest period. Continuous infrared images were then collected from a distance of 2 meters using a FLIR S60 broadband infrared camera for a further five minutes before and after treatment. A sudden drop in orbital temperature occurred in both F (- 0.36±0.11 C) and P (-0.47±0.10 C) over the first 50 seconds after treatment ($P<0.05$). There was a delayed return to baseline in P compared to F and orbital temperature was still lower than baseline in P at 50-100 and 150-200 seconds after treatment ($P<0.05$). The data suggest that it is possible to non-invasively detect both acute pain and fear in cattle and potentially differentiate between these two stressors.

Changes in eye temperature, measured using infra-red thermography, can detect pain due to disbudding in calves.

Stewart, M., Webster, J.R, Stafford, K.J., Dowling, S.K.

Proceedings of the 40th International Congress of the International Society for Applied Ethology, Bristol, England, August, 2006, p97.

The possibility that changes in eye temperature can detect stress and pain in livestock was examined in calves using disbudding as a painful procedure. The effects of local anaesthetic (LA) on the eye temperature response of thirty calves to disbudding with a cautery iron was studied using a 2x2 factorial design. All animals were handled the same and in place of treatments, sham procedures were carried out. Maximum temperature of the eyelid and caruncula lacrimalis, measured using infrared thermography (IRT), heart rate variability (HRV), interbeat interval (IBI), and activity (e.g. kicking, slipping, falling and rearing) were recorded continuously for 40 min for each animal, including 15 min baseline, LA administration (10min pre-treatment) and 15 min post-treatment. Differences between treatments were detected by ANOVA. There was no change in eye temperature following sham procedures. Eye temperature dropped rapidly following disbudding without LA and was lower 2 min post-treatment than baseline (-0.3˚C, P<0.05). Eye temperature then increased and was higher for the last 10 min post-treatment than baseline (+0.6˚C P<0.0001). A rise in eye temperature also occurred following disbudding with LA (+0.7˚C, P<0.0001). Activity was higher than baseline during the first 2 min post-treatment following disbudding with and without LA (P<0.0001). The IBI was lower than baseline for 5 min post-treatment following disbudding without LA (-156ms, P<0.0001) and with LA (-97ms, P<0.001) and HRV was lower during this period following disbudding without LA only (P<0.01). The synchronised drops in eye temperature, IBI and HRV suggest that the fall in eye temperature may be caused by an acute sympathetically mediated change in blood flow to the eye. Furthermore, IRT is a useful non-invasive method to detect acute sympathetic responses arising from pain and stress that may occur during management of livestock.

Effects of local anaesthetic and a non-steroidal anti-inflammatory analgesic on stress responses of calves to disbudding.

Stewart M., Stookey, J.M., Stafford, K.J., Tucker, C.B., Rogers, A.R., Dowling, S.K., Verkerk, G.A., Webster, J.R.

Proceedings of the 40th International Congress of the International Society for Applied Ethology, Merida, Mexico, July, 2007, p235.

We examined the effects of local anaesthetic (LA) and a non-steroidal anti-inflammatory agent (NSAID) on physiological and behavioural responses of calves following disbudding (DB). Forty six mixed sex calves (average 4.5 weeks) were randomly assigned to 6 treatments: control, LA, NSAID, DB, DB+LA and DB+LA+NSAID. Eye temperature (measured using infrared thermography) was recorded continuously for 30min before and 15min after cautery disbudding; eye temperature and behaviour (lying, ruminating) were recorded every 5min for a further 3hr post disbudding. Heart rate (HR) and heart rate variability (HRV) were continuously recorded. ANOVA was used to detect treatment differences. During the first 2min after disbudding without LA or NSAID, there was a rapid drop in eye temperature (-0.5^{o}C, $P<0.05$) and HRV ($P<0.001$) from baseline and HR was higher ($P<0.001$) than all other groups. HR remained elevated 3hr post-disbudding for the DB group. The simultaneous drops in HRV and eye temperature immediately after disbudding without analgesia suggest an acute sympathetically-mediated reduction in blood flow to the eye in response to pain. Ruminating was lower than other treatments for DB ($P<0.05$), except LADB. Between 2-3hr following disbudding with LA, there was a rapid decrease in eye temperature (-0.6^{o}C, $P<0.001$), an increase in HR ($P<0.05$) and decrease in lying behaviour ($P<0.01$). There were no changes in HR, eye temperature or behaviour for calves disbudded with LA and NSAID during this time. Changes found between 2-3hr following disbudding with LA suggests the onset of pain at this time, which coincides with the time that LA effects wear off. The combination of LA+NSAID mitigate the stress response associated with the immediate pain of disbudding and confirms this combination is more effective at alleviating pain than LA alone. Eye temperature measurements may be an additional and useful way to assess the duration and efficacy of pain mitigation by analgesics.

Detection and alleviation of pain due to castration in calves

Stewart, M., Verkerk, G.A., Schaefer, A.L., Stafford, K.J.,
Worth, G.M., Clark, K., Webster, J.R.

Submitted for presentation at the *4th International Workshop on the Assessment of Animal Welfare at Farm and Group Level,* September 2008, Ghent, Belgium.

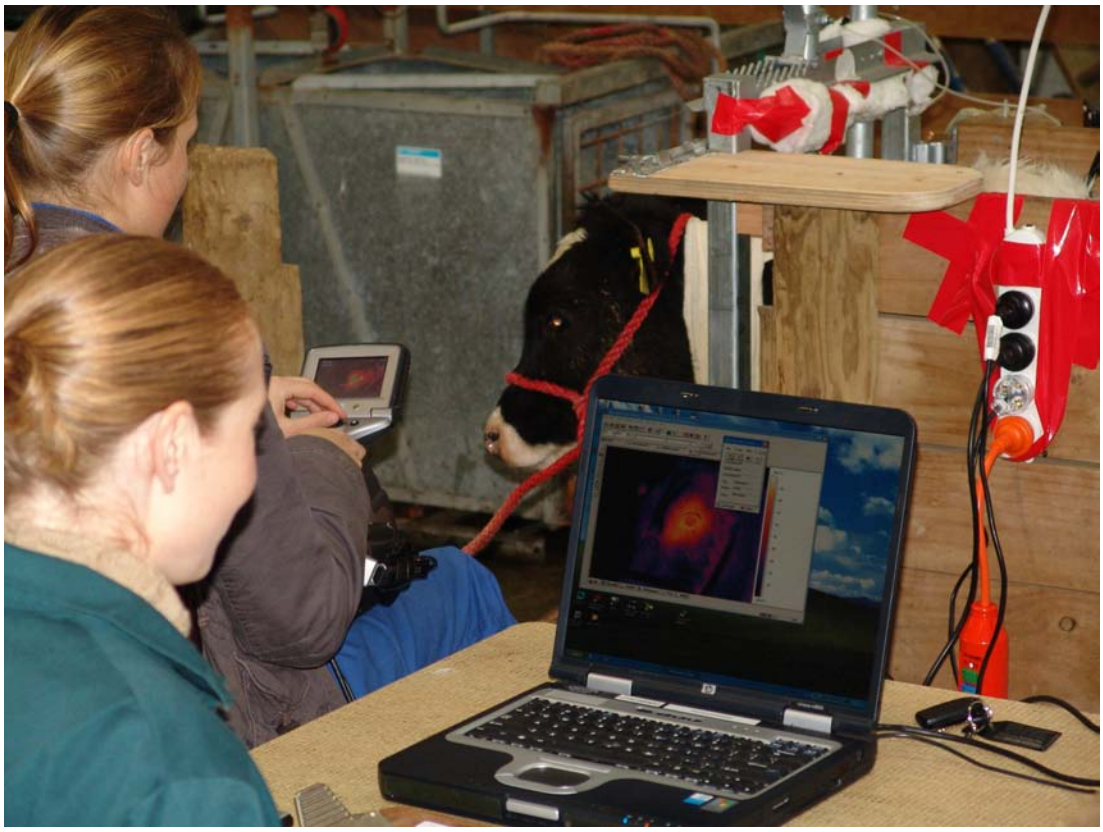
Autonomic nervous system (ANS) activity increases in responses to pain, however, few studies have used ANS activity to assess pain in cattle or its alleviation using local anaesthetic (LA). This study used a novel combination of eye temperature, heart rate and variability (HR and HRV) and catecholamines to detect pain-related changes in ANS activity in response to castration. Thirty, four-month-old calves were randomly assigned to treatments: 1) sham castration (handling only, H), 2) surgical castration by scrotal incision and traction of the testes (SC), 3) LA+H and 4) LA+SC. Maximum eye temperature (^{o}C), measured using infrared thermography, and HR and HRV (measured over 512 interbeat intervals (IBI), using Polar S810i^{TM}) were recorded before and after castration, and analysed using ANOVA. Catecholamine concentrations (noradrenaline and adrenaline) were determined in plasma collected via jugular catheter, before and after SC and H.

During the 20 min after SC and LA+SC, eye temperature increased (0.47 and 0.28 ^{o}C respectively, $P<0.001$). Eye temperature also increased after H (0.10^{o}C) but less than castration treatments ($P<0.001$). During the first 2 min, HR increased following SC and LA+SC (+15.3bpm and +6.3bpm respectively, $P<0.001$) and catecholamines increased after SC ($P<0.05$) but not after H. Both eye temperature and HR responses were greater in SC than LA+SC ($P<0.05$). Changes in HRV indicative of increased parasympathetic tone (e.g., the root mean square of successive differences) occurred in the first IBI after both castration treatments. HR and HRV were unaffected by H or LA+H.

LA reduced but failed to eliminate pain-related responses due to surgical castration. Catecholamine and HR increases indicate a short-lived sympathetic response to SC. The increased eye temperature and HRV changes suggests a subsequent increase in parasympathetic tone. The combination of HRV and eye temperature is a useful non-invasive way to detect acute pain and its alleviation in cattle.

Appendix Three

Compatibility of two methods for analysing IRT images



In Chapter 7, two different methods of IRT analysis of maximum eye temperature (^{o}C) were used concurrently. The advantage of using both of the methods concurrently is that it makes it possible go back to the software analysis and capture images on a frame by frame basis when an animal is moving excessively (e.g., struggling during a painful procedure) or moving their head, and it enables collection of images at very frequent sampling intervals. In addition, it provides a useful system to back up data in the case of any unexpected circumstances that lead to data being lost (e.g., equipment failure). Using the first method, the camera was set to calculate and display the position and value of the maximum temperature within a circular area of analysis on each frame. The displayed values from each image were immediately entered into an excel spreadsheet at the time of data collection. Using the second method, a firewire cable was used to connect the infrared camera to a laptop (Compaq nc8000, Hewlett-Packard Company, China) to achieve continuous recordings (29 frames per second) of eye temperature during the sampling period using image analysis software (ThermaCam Researcher 2.7, FLIR Systems AB, Danderyd, Sweden). Sequence files (.SEQ) of continuous recordings for each calf were saved and maximum eye temperature was analysed frame by frame at a later date, using the ThermaCam Researcher software.

In Chapter 7, the software analysis was used when it was not possible to collect images accurately using the first method due to calves moving their head. Figure 1 shows that the eye temperature results using the two different methods were very similar. The results from a Student's t-test used to test for differences between the two methods are shown for each calf in Table 1. There were no significant differences between the methods for Calf 1 and 2, but a small difference of 0.014 ^{o}C ($P<0.05$) was found between the two methods for Calf 3. The results show that the two methods are compatible, therefore, it is possible to use either method or a combination of both.

Table 1. Difference between two methods of IRT analysis calculated as the average bias (^{o}C, ± standard deviation) for each calf.

	average bias	P Value
Calf 1	-0.002 (±0.099)	0.713
Calf 2	-0.009 (±0.118)	0.283
Calf 3	0.014 (±0.103)	0.033

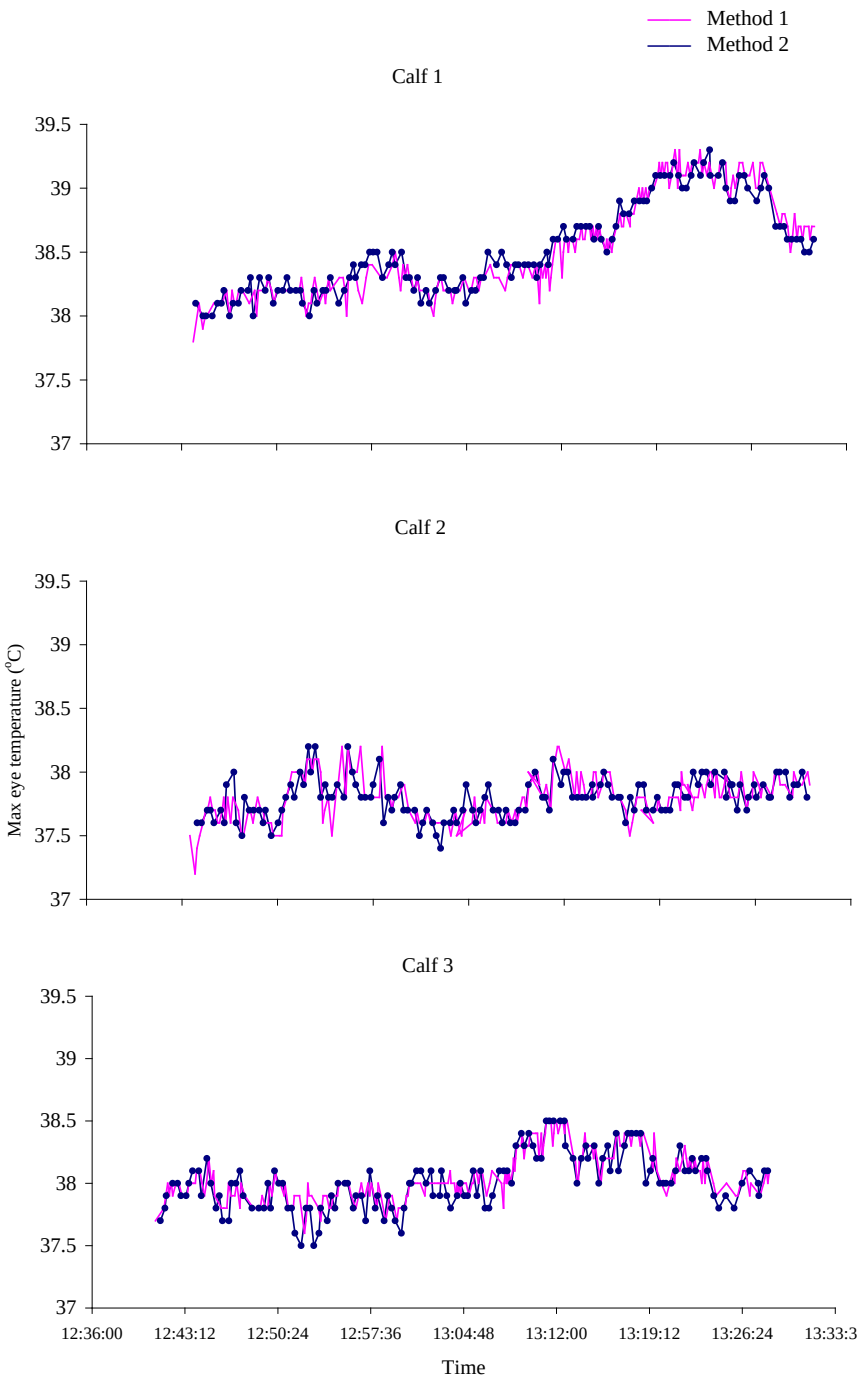


Figure 1. Maximum eye temperature (^{o}C) recorded using two different methods of IRT analysis. The pink line represents the data collected on the day (Method 1) and the blue line represents the data collected using ThermaCam Researcher software (Method 2).