AN INVESTIGATION OF HAIR CORTISOL AS A BIOMARKER OF LONG-TERM STRESS IN BEEF CATTLE

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ABSTRACT

Long-term stress studies can be difficult in beef cattle due to a lack of objective measures of long-term stress and because beef cattle are often raised extensively, making repeated sampling difficult. Hair cortisol (HC) has been proposed as a measure of systemic cortisol over an extended period of time, accumulated over a period of days to weeks, however its usefulness in beef cattle has not yet been investigated. Therefore, the objective of this thesis was to investigate the use of HC concentration as a physiological measure of long-term stress in beef cattle. Beef calves were studied over two stressful procedures that occur in modern production: i) castration, and ii) weaning in order to study HC concentration as an objective measure of long-term stress, and explore various factors that influence HC concentration such as sex, temperament, dam parity and calf age. For study 1, bull calves located on two farm sites, site 1: Hereford cross (n=73), at 47 + 9.6 d of age (mean ± S.D.), site 2: Black Angus (n=85), 48 ± 11.3 d of age, were equally divided across three treatments: surgical castration (CS, n=52), surgical castration with meloxicam (CM, n=54), and sham castration (S, n=52). All treatments were balanced for calf age. Hair samples were collected from an area on the left hip prior to castration on d 0, with hair regrowth collected on d 14 from the d 0 sample location. Calf standing time was recorded by accelerometers from d 0-7 as a previously validated measure of pain on a sub-sample of 129 calves (CS=47; CM=42; S=40, numbers per treatment were balanced across farm sites). Treatment effects on HC were analyzed via the MIXED procedure (STATA® 12). Standing time was analyzed using repeated measures MMLR. On d 14 post castration, HC concentration was 13.5% higher in CS than S calves ($P = 0.025$) and tended to be higher than CM ($P = 0.06$). Standing time across treatments showed, CM tended to stand more than S calves on d 0-4 following castration ($P = 0.052$), with no other differences determined. For study 2,

calves were weaned at 5-7 months of age (186 ± 15.5 days, mean ± S.D.) via two-stage (TS, n = 80) or abrupt weaning (AW, n = 81), and balanced for sex across treatments. Nose-flaps were inserted on d 0 in all TS calves and removed on d 7; all cow-calf pairs were physically separated from their dam on d 7. Hair samples were collected from the right hip of all calves on d 0, 7 and 14; d 0 and 7 hair samples included the full hair shaft while hair collected at d 14 included regrowth from the d 0 sample location. Standing time was recorded as a behavioural measure of stress, as a surrogate for number of steps, on a sub-sample of calves (n = 49/treatment). Treatment effects of HC were analyzed using the MIXED procedure (STATA® 12); standing time was analyzed using two-sample t-tests per day. On d 14, TS calves had significantly higher HC concentration (pg mg^{-1}) than AW calves (1.39 ± 0.15 vs. 1.16 ± 0.15, respectively, LSM + S.E.; $P = 0.02$). AW calves stood 6% more than TS calves on the day of weaning (P = 0.007) and 12% more the following day after weaning (P = 0.0003). Treatment differences were detectable in HC concentrations in both studies, suggesting that HC responds to HPA axis activity from applied stressors. Following castration, HC concentration was highest in the most aversive treatment and lowest in the least stressful treatment on d 14. However, total standing time post-castration used as a measure of pain did not reflect HC concentration. The HC concentration in TS was higher than AW calves one week following physical weaning, suggesting TS calves experienced more HPA axis activity over the two week period. Conversely, AW calves stood more than TS calves in the first two days following physical weaning, indicating AW was a more severe stressor compared to TS weaning. This signifies that HC may be more sensitive to long-term than acute stressors. Using the combined results from the two applied studies, HC appears to be a promising medium for measuring long-term stress in beef cattle.

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LIST OF ABBREVIATIONS

kg	Kilogram
mg	Milligram
min	Minute
d	Day
h	Hour
ADG	Average daily gain
HC	Hair cortisol
HPA	Hypothalamic-pituitary-adrenal
CRH	Corticotropin releasing hormone
ACTH	Adrenocorticotropic hormone
NSAID	Nonsteroidal anti-inflammatory drug
CS	Surgical castration with saline
CM	Surgical castration with meloxicam
S	Sham castration
MMLR	Mixed model linear regression
TS	Two-stage wean
AW	Abrupt wean

1 INTRODUCTION

Understanding the degree of stress experienced by animals due to various management practices is the first step in improving animal welfare. Thus, it is crucial that our means of quantifying stress accurately measure the physiological response to the stressor in question. Plasma cortisol has been used extensively as a physiological measure of acute stress in farm animals, although its reliability to accurately measure stress has been questioned (Rushen, 1991). Cortisol has been measured in mediums other than plasma, including saliva, urine and feces; both plasma and salivary cortisol have been utilized to quantify the acute stress response. However, cortisol is quickly metabolized in plasma and saliva, and therefore, to study long-term stress requires repeated sampling which is stressful and often invasive. Circulating cortisol can be accumulated in urine for up to a period of 24 hours or in feces up to a period of a few days, however, levels are highly variable due to digesta and time spent in the gastrointestinal tract. Cortisol concentration in hair is able to document the accumulation of cortisol in the hair shaft over a period of days to months and is more sensitive to prolonged than acute stressors. Hair collection is potentially a less stressful means of sampling because it reduces the number of times sampling is required. Additionally, hair collection via clipping is non-invasive, compared to venipuncture or catheterization which are both painful and stressful for the animal. Handling is a perceived stressor for beef cattle, as they are not regularly handling, which can cause an immediate rise in cortisol at the time of collection. However, hair cortisol is unaffected by an immediate rise in cortisol at the time of collection because cortisol will not be deposited in the hair shaft until cells in the follicle become keratinized and grow above the skin surface (Pragst and Balikova, 2006), making hair cortisol a more stable measure which is not impacted by handling.

Castration is a painful procedure routinely performed on bull calves to decrease aggression (Tarrant, 1981), prevent unwanted breeding (Stafford and Mellor, 2005), and improve meat quality due to increased marbling and tenderness (AVMA, 2009). The 2014 Code of Practice for the Care and Handling of Beef Cattle will require pain control to be administered at the time of castration in bulls over 9 months of age as of January, 1 2016 and bull calves over 6 months of age as of January 1, 2018 to help mitigate post-castration pain. The pain control administered to bull calves at castration could be an analgesic, anesthetic or combination of both. However, the impact of stress following castration and effectiveness of pain control on mitigating post-castration pain are not well documented in young calves, in part due to a lack of suitable mediums by which to measure long-term stress in beef cattle. Castrating bulls is most appropriate when they are young, due to a decrease pain response compared to older calves, yet there is a small amount of research documenting the effects of pain medication during castration in this age group of animals.

Abrupt weaning is routinely performed in beef cattle and causes significant stress in calves following separation. Comparatively, two-stage weaning has been documented to decrease the behavioural stress response following weaning (Haley et al. 2005). However, there has been little physiological measurement of long-term stress following weaning to investigate the level of stress experienced by beef calves. Castration and weaning are stressful events that frequently occur at a critical period before transport to the feedlot. A combination of stressors such as castration, weaning, transport and mixing increase risk of contracting disease at the feedlot, likely due to immunosuppression caused by chronic stress. The effect of concurrent stressors is often synergistic, thereby increasing the magnitude of stress experienced by an

individual. Decreasing stress prior to transportation to the feedlot has the potential to decrease illness upon arrival which is of importance for both welfare and production economics.

Should HC prove to be a useful measure for long-term stress, it can be applied to future studies investigating stress in beef cattle. Reliable measures of physiological stress, in combination with behavioural measures, are necessary to evaluate the impact of routine husbandry procedures on beef cattle. Through the measurement of HC concentration, the effects of long-term stress in beef cattle and how these problems impact welfare may be more clearly understood.

1.1 Thesis Objectives

Acute stress in beef cattle has been studied extensively but changes in cortisol due to long-term stress have not been well investigated due to a lack of biomarkers that can document cortisol over a period of days to months. A number of other species including non-human primates (Davenport et al. 2006), grizzly bears (Macbeth et al. 2010), and dairy cattle (Comin et al. 2013; Burnett et al. 2015) have established the validity of HC detection and used it to measure long-term stress. Chapter 2 of this thesis entails a review of important literature related to the deposition of cortisol into the hair shaft and factors that influence HC concentration. The review also looks at previous physiological, behavioural and production measures that have been used to study castration and weaning stress in beef cattle. The principal objective of this research was to determine if HC can be used as an objective measure of long-term stress in beef cattle. In order investigate HC concentration in relation to long-term stress, two studies were performed that are outlined in Chapters 3 and 4.

Stress is a physiological and acute event needed for the fight or flight response (Cannon, 1929), which lasts for a few seconds or minutes. Anything beyond the initial stress response is

considered long-term or chronic stress. Surgical castration is a stressful event that can be used as a model of stress (Earley and Crowe, 2002) and was therefore chosen to document changes in HC concentration that occur after a stressor. The objective of this study was to investigate if HC in young bull calves (2-4 months of age) varied in a predictable manner following different methods of castration. Calves were surgically castrated, with and without meloxicam, and sham castrated to determine if HC concentration could identify differences in stress response to the different treatments. An additional objective was to determine if meloxicam, a nonsteroidal anti-inflammatory drug (NSAID), administered at the time of castration would decrease post-operative pain, and influence HC concentrations and the behaviour of calves following castration.

The study outlined in Chapter 4 was performed to investigate the usefulness of HC as a measure of long-term stress in beef cattle following artificial weaning, a routine management procedure and a severe stressor experienced by calves. Two weaning protocols, two-stage and abrupt weaning, were chosen because they were considered to cause contrasting levels of stress, as determined in behavioural observations from previous studies (Haley et al. 2005). The objective of this study was to investigate if HC is a useful physiological measure of stress following a social stressor. The HC concentrations of the different treatments were compared to determine if differences were detectable in HC concentration. Over the course of the studies outlined in Chapter 3 and 4, data should help to determine if HC is a useful measure of stress in beef cattle.

2 LITERATURE REVIEW

2.1 Stress Response

2.1.1 Activation of the HPA Axis

Stress is a general term describing both external and internal forces exerted on an individual causing adaptation mechanisms to adjust and return body processes to homeostasis (Cannon, 1932). Upon a stressful event, or perception of a threat (Gaab et al. 2005), the hypothalamic-pituitary-adrenal (HPA) axis and sympathetic nervous system (SNS) are triggered in response, while the parasympathetic nervous system is suppressed. In addition to the HPA axis and SNS being triggered in response to a stressor, an immunological cascade begins when trauma occurs (Von Borell, 2001). These neuroendocrine systems are involved in the regulation of energy fluxes, particularly, the ability to mobilize energy from energy storage tissues in the event of a stressor, which is then used by defense mechanisms in order to cope with the stressor at hand (Turner et al. 2012). Activation of the SNS initiates a cascade of reactions including increased heart rate and respiratory rate regulated by the release of norepinephrine (Jansen et al. 1995). Acute stress triggers the initiation of an immunological cascade in response to coping with challenges in the environment, however, increased glucocorticoids due to chronic stress have been documented to supress immune function (Salak-Johnson and McGlone, 2007).

The physiological response of animals to various stressors has been termed the ‘fight or flight’ response (Cannon, 1929) or ‘general adaption syndrome’ (Selye, 1936). Upon the perception of a threat, the HPA axis responds to the stressors following the SNS by the synthesis and secretion of neuropeptides corticotropin releasing hormone (CRH) and arginine vasopressin (AVP) from the hypothalamus. This initiates the pituitary to release adrenocorticotropic hormone

(ACTH), which acts on the adrenal cortex to release glucocorticoids (Manteuffel et al. 2002). A stressor of longer duration triggers a sustained release of CRH, leading to a longer release of total cortisol. However, increased cortisol exerts negative feedback on the anterior pituitary gland and the subsequent catecholamines secreted by the adrenal medulla exert negative feedback on the hypothalamus (Axelrod and Reisine, 1984), these processes return glucocorticoids to homeostasis following a stressor. Adaptive functions in response to acute stressors that quickly return the system to homeostasis are beneficial to the animals' survivability because chronically elevated glucocorticoids from long-term stressors become detrimental to basic functioning.

The HPA axis evolved to help cope with stressors occurring in nature, but in food animal production, livestock are subjected to stressors they wouldn't normally encounter (Cook, 2012), such as castration, artificially imposed weaning, transportation and restrictive housing. Long-term stressors, such as restrictive housing, are more likely to have detrimental effects on biological functioning due to hyperactivation of the HPA axis. Increased glucocorticoid levels over an extended period of time cause immunosuppression (Salak-Johnson and McGlone, 2007) via the suppression of cytokine synthesis and release, as well as having detrimental effects on reproduction (Wingfield and Sapolsky, 2003).

2.1.2 Cortisol as a Measure of Stress

Measuring HPA activity is a common approach to the study of stress and welfare in farm animals (Mormède et al. 2007) and cortisol is the primary glucocorticoid used to measure HPA axis activity in mammals (Minton, 1994). Therefore, cortisol has long been a standard physiological measurement taken when evaluating stress. Previously, cortisol concentrations have been measured via serum (Veissier and Le Neindre, 1988), saliva (Fell and Shutt, 1986),

urine (Higashiyama et al. 2009) and feces (Morrow et al. 2002). While these measures have contributed substantially to the understanding of the acute stress responses following aversive management practices, the usefulness of plasma cortisol comes into question (Rushen, 1991).

Cortisol release is pulsatile and episodic following a diurnal pattern in cattle, with a periodicity of approximately 90 minutes (Thun et al. 1981). Due to the rapid metabolism of systemic cortisol, demonstrating HPA axis activation from chronic stress is difficult without repeated sampling or catheterization. Handling and restraint of animals which is required for sample collection and venipuncture for collection of plasma are in and of themselves major stressors, influencing glucocorticoid release. Following the onset of a stressor, glucocorticoids are released into the bloodstream within minutes (Sapolsky et al. 2000). Handling can confound cortisol levels from applied stressors by increasing glucocorticoids at the time of sampling. Non-invasive collection procedures are favourable because they may decrease handling stress at the time of collection, therefore mitigating a rise in cortisol due to sample collection. Additionally, imposing additional stress on animals during research is not desirable and by utilizing non-invasive collection techniques, stress studies can be refined. Examples of non-invasive mediums include salivary, urinary, fecal and HC, as they do not require venipuncture for sample collection.

Salivary cortisol increases within two minutes of plasma cortisol increase (Cook, 2012) making it a useful measure of acute stress. However, plasma and salivary cortisol are point samples (Davenport et al. 2006), providing information only about HPA activity at the time it is collected. Additional mediums, which are less invasive than plasma collection, have been developed to measure cortisol over extended periods of time. Cortisol is accumulated in urine for up to 24 hours and fecal cortisol can be accumulated in the gastrointestinal tract over a period of

days. Like HC, fecal cortisol does not fluctuate with immediate stressors and therefore, is not confounded by handling stress during collection. However, cortisol concentration in feces is highly variable due to biological functions such as passage time through the gastrointestinal tract, bacteria present, type and mass of food material (Cook, 2012). While non-invasive measures are preferable, salivary, urinary and fecal cortisol are less favourable to effectively document long–term stressors because each medium is more sensitive to acute stressors than HC and would require repeated sampling over an extending period of time. Depending on the collection method used, this would cause disruption to the animals. In addition, repeated sampling is a less favourable technique due to the increased labour to collect samples and cost of analysis on a larger number of samples

Unlike any of the previously mentioned cortisol measures, HC is sensitive to prolonged stressors (Davenport et al. 2006) and is not influenced by short term stress (Ashley et al. 2011). One of the major advantages of HC as a measure of stress is that upon collection, samples are not confounded by the rapid increase of cortisol during handling. Furthermore, mediums which integrate long periods of time are less sensitive to acute stressors but are more sensitive to long-term changes, making HC a more precise measure of long-term stress. Changes in HPA activity are documented over a period of weeks to months in hair (Davenport et al. 2006; Macbeth et al. 2010) and can be used to detect long-term stress in wild and domestic species.

When using HPA axis activity as a measure of stress, it is important to keep in mind that the HPA axis is highly variable. Corticosteroids follow diurnal and seasonal patterns, are influenced by feed intake, environmental factors such as temperature and humidity, age and physiological state (Gratacós-Cubarsí et al. 2006). Additionally, large differences in glucocorticoid levels occur across species, breeds and individuals (Mormède et al. 2007) making

it difficult to use cortisol as a measure of stress without accounting for the additional variables which impact it.

2.1.3 Welfare Implications

One of the more commonly accepted definitions of animal welfare refers to 'the state of an animal with regards to its ability to cope with its environment' (Broom, 1986). Animal welfare is most commonly assessed using a multi-faceted approach including considerations for: health and functioning, affective states, and natural living. In an attempt to quantify the magnitude of stressors on an animals' ability to cope, multiple mediums are used to capture physiological responses following applied treatment. Using HC represents an additional medium to measure HPA activity following applied stressors and may improve the understanding of physiological stress responses in beef cattle.

Stress research in beef cattle can be difficult due to extensive management systems and a lack of long-term stress measures. Husbandry practices that cause physical and emotional distress, such as branding or restrictive housing, can cause long-term stress leading to a continued production of glucocorticoids which negatively impact productivity, health and welfare (Moya et al. 2013). As a tool to measure stress experienced by the animal over time, HC would be a useful tool in determining long-term stress as experienced by the animal, without potentially confounding or masking results, due to its low invasiveness to the animal during hair collection. Up to this point in time, HC has been used in a number of studies including wildlife (Macbeth et al. 2010), humans (Russell et al. 2012), and intensively raised animals (Comin et al. 2012). To our knowledge, the applied stress studies outlined in chapters 3 and 4 of this thesis are the first to study HC as a long-term measure of stress in beef cattle.

Present day consumers and veterinarians are highly focused on the welfare of food animals. It is important to address welfare concerns within the beef industry to keep up with changing consumer demands and because it is vitally important for the care for our animals. By understanding what production practices are detrimental to beef cattle welfare, it creates an opportunity to improve them. Additionally, improving stressful practices which if severe enough may cause immunosuppression, in combination with low-stress management has the potential to decrease incidence of disease at the feedlot. Thus, improving animal welfare and mitigating economic loss.

2.2 Hair Cortisol

2.2.1 Mechanism of Incorporation

There are multiple suggested mechanisms for the incorporation of cortisol into the hair shaft. These include passive diffusion, incorporation from sweat or sebum (Henderson, 1993; Cone, 1996) and incorporation from an independent HPA axis located in the skin and hair follicle (Ito et al. 2005; Sharpley et al. 2009). However, of the proposed mechanisms, passive diffusion is the most widely accepted model of cortisol incorporation into hair.

Passive diffusion proposes that as cortisol increases in the bloodstream, it is passively diffused from capillaries into the growing cells at the base of the hair follicle and subsequently becomes trapped in the keratin matrix of the hair shaft (Henderson, 1993). Hair originates from the follicle 3-5 mm below the skin surface, which is surrounded by a rich capillary network providing the hair with necessary metabolites for growth (Pragst and Balikova, 2006). As cortisol systemically increases, it is passively diffused from the surrounding capillary system into

the hair follicle, which will later grow above the skin surface into the hair shaft. Eventually, after keratinization, the hair shaft grows above the skin surface where it can be collected for analysis.

Another route of cortisol incorporation into the hair shaft includes penetration of cortisol via surrounding sweat and sebum which contains cortisol (Pragst and Balikova, 2006), thereby increasing total HC concentration. If hair is soaked too long in sweat, it is likely the cuticle will swell, causing sweat to be incorporated into the hair shaft, artificially increasing HC concentration (Russell et al. 2014).

Additionally, some research suggests the presence of a self-regulating HPA system is present within the skin and hair, thus the hair follicle may be capable of producing its own cortisol (Ito et al. 2005; Sharpley et al. 2009). With the presence of an independent HPA system located in the skin and hair follicle, an injured site may have the potential to secrete its own cortisol, as well as creating an independent negative feedback loop in the hair follicle to reach homeostasis.

Of all the proposed mechanisms of cortisol incorporation into the hair shaft, passive diffusion is the most generally accepted and widely assumed pathway. However, the precise mechanism of cortisol incorporation into the hair shaft is unclear and requires further research.

2.2.2 Measurement of Hair Cortisol Across Species

Male rock hyrax were the first species to be studied used HC by Koren et al. (2002) when studying long-term stress in relation to social rank. Since then, researchers across a multitude of species, ranging from wild to domestic, have utilized HC as a measure of long-term stress (see Table 2.1). Repeated sampling is not required to investigate long-term stress because HC forms a stable matrix within the hair shaft (Pragst and Balikova, 2006), which can be analysed long after

a stressor has occurred making it ideal for studying stress in wild species (Ashley et al. 2011; Macbeth et al. 2012). The validity of HC as a measure of stress has been verified in dairy cows (González de la Vara et al. 2011; Burnett et al. 2014; Tallo-Parra et al. 2015) and the ability to detect cortisol in the hair shaft of beef cattle has been shown (Moya et al. 2013). However, HC has not yet been used to detect different treatment stressors in beef cattle.

Table 2.1. Animal studies using hair cortisol as a long-term indicator of stress

Year	Reference	Objective	Species
2002	A novel method using hair for determining hormonal levels in wildlife L. Koren et al.	Investigated the relationship between hair cortisol concentration and male social rank	Rock hyrax (*Procavia capensis*)
2006	Analysis of endogenous cortisol concentrations in the hair of rhesus macaques M.D. Davenport et al.	Developed and validated a reliable method for measuring hair cortisol concentration in rhesus monkeys. Investigated whether HC was impacted by relocation stress and if HC varied along the hair shaft	Rhesus macaque (*Macaca mulatta*)
2010	Hair cortisol concentration as a non-invasive measure of long-term stress in free-ranging grizzly bears (Ursus arctos): considerations with implications for other wildlife B.J. Macbeth et al.	Developed an accurate and reliable technique to detect HC concentration in grizzly bears. Investigated additional variables which influenced HC	Grizzly bear (*Ursus arctos*)
2011	Glucocorticosteroid concentrations in feces and hair of captive caribou and reindeer following adrenocorticotropic hormone challenge N.T. Ashley et al.	Validated hair cortisol concentration of caribou and reindeer following ACTH challenge	Alaskan Caribou (*Rangifer tarandus granti*) and Reindeer (*R. t. tarandus*)
2012	Hair cortisol levels to monitor hypothalamic-pituitary-adrenal axis activity in healthy dairy cows A. Comin et al.	Evaluated hair cortisol concentration in healthy dairy cows	Friesian dairy cows (*Bos taurus*)
2013	Hair cortisol as a marker of hypothalamic-pituitary-adrenal axis activation in Friesian dairy cows clinically or physiologically compromised	Investigated hair cortisol concentration as a measure of HPA axis activation in clinically or physiologically compromised cows	Friesian dairy cow (*Bos taurus*)

	A. Comin et al.		
2015	Relationship of concentrations of cortisol in hair with health, biomarkers in blood, and reproductive status in dairy cows T. A. Burnett et al.	Determined the relationships between hair cortisol and clinical disorders, reproductive status. Evaluated the association between hair cortisol concentrations metabolic status and acute inflammation	Holstein dairy cow (*Bos taurus*)

2.2.3 Factors Influencing HC Concentration

2.2.3.1 Influence of Hair Colour on HC

Multiple studies, in a variety of species, have investigated the impact of hair colour on HC. In dogs, light hair has been found to have higher cortisol levels than dark hair, within the same individual and as a group (Bennett and Hayssen, 2010). This has also been observed in dairy cows, Holsteins specifically, with greater HC concentration in white than black hair (González de la Vara et al. 2011; Burnett et al. 2014) within the same animal and across animals. The mechanisms creating differences in HC due to hair colour are not well understood but may be related to melanocyte development (Slominski et al. 2005), melanocyte differentiation (Roulin et al. 2008) or the amount of physical space within the hair shaft. It is proposed that the amount of cortisol absorption is related to the amount of space left in the hair shaft from the melanocytes pigmenting dark hair which leave less space for cortisol to be contained within the hair shaft (Burnett et al. 2014).

However, Tallo-Parra et al. (2015) found higher HC concentrations in black hair than white hair in dairy cows. The discrepancy in results from this study may be due to a difference in sampling location. White hair was collected from the frontal region of the head and black hair was a mixture of hair from the frontal region of the head as well as the occipital crest, while hair in the other studies was collected from the top line. Hair from the head and neck contain more cortisol than hair from the hip and tail (Moya et al. 2013), which may have attributed to the observed differences in HC from different coloured hair as well. When hair was graded from light to dark in grizzly bears, hair colour had no impact on cortisol concentration (Macbeth et al. 2010).

2.2.3.2 The Influence of Animal's Age on HC

Cortisol is crucial to fetal organ development as well as initiating parturition (Mastorakos and Illias, 2003). Placental CRH stimulates fetal ACTH release, leading to a surge of fetal cortisol initiating parturition (Mastorakos and Illias, 2003). The fetal HPA axis is essential to development of the fetus, as well are preparing for survival of the neonate following parturition (Mastorakos and Illias, 2003) and assists in adapting from the intrauterine to extrauterine environment (Flint et al. 1979). Therefore, younger animals who are closer to birth have higher HC concentrations than older animals. As animals age and hair growth continues, HC decreases due to dilution from hair growth without cortisol increasing in the hair shaft. In a study by González da la Vara et al. (2011), 15 day old heifers had HC concentrations approximately 10 fold higher than 2 year old cows. This is likely due to their proximity to their birth and dilution of cortisol as hair growth occurred.

2.2.3.3 Influence of Multiparous Cows and Primiparous Heifers on HC

In previous studies, multiparous cows have been documented to have higher HC than heifers (Burnett et al. 2014; Burnett et al. 2015). While this mechanism is not well understood, it is likely due to the amount cortisol needed to initiate parturition. Primiparous heifers may need a lesser surge of cortisol to initiate parturition than multiparous cows, leading to increased HC concentration in multiparous cows compared to primiparous heifers. Multiparous cows tend to have higher HC than primiparous cows at parturition (Burnett et al. 2014), contrary to beliefs that primiparous cows suffer more than multiparous cows during the transition period around parturition. In an additional study by Burnett et al. (2015), multiparous cows had consistently higher HC than primiparous cows through 126 days in milk. Although differences in HC between multiparous and primiparous cows are significant and consistent, any explanation as to

why this variation occurs at this point is merely speculative. However, it is a variable that needs to be considered when performing HC analysis in dams and their offspring.

2.2.3.4 Hair Growth: Anagen, Catagen and Telogen Phase

Hair growth is cyclical and undergoes periods of growth, transition and rest. The anagen phase is a long period of active growth followed by the catagen phase which is a short transitional stage where cell division stops and the hair shaft becomes fully keratinized, followed by the telogen phase, which is a period of no growth (Harkey, 1993). Factors affecting hair growth rate include body region, age, gender, seasonal and climatic factors and hormone levels (Gratacós-Cubarsí et al. 2006). Season may affect HC concentration (Comin et al. 2012) by changing the rate of hair growth and the hair growth cycle (Courtois et al. 1996). Blood-borne substances, including cortisol, are able to diffuse from capillaries into the cells of the hair follicle and become deposited in the hair shaft (Cone, 1996) during periods of active growth (Davenport et al. 2006; Pragst and Balikova 2006). In order for cortisol to be incorporated into the hair shaft, the hair must be actively growing in the anagen phase (Harkey, 1993; Wenning, 2000). The cortisol concentration in a full intact hair shaft represents systemic HPA activity during the active growth phase of the hair being evaluated (Macbeth et al. 2010). The most reliable HC results occur when hair is shaved close to the skin stimulating new hair growth, creating optimum conditions for steroid incorporation (Davenport et al. 2006). Retention and stability of drugs within the hair shaft over time is considered good (Pragst and Balikova, 2006). Therefore, if hair is collected long after a stressor occurs, cortisol will still be present in the hair shaft.

In beef cattle, hair grows at a consistent rate of approximately 6-7 mm per month (Gaillard et al. 1997; Durant et al. 2002) during the anagen phase, which is affected by a number of previously discussed factors. Theoretically, stressors could be retrospectively documented

based the cortisol concentration in the hair shaft and its respective position from segmental analysis.

2.2.3.5 Variability of HC Concentration due to Body Region

The cortisol concentration in hair varies depending on the body location from which it is collected, likely due to the different growth rates of hair. Areas with increased hair turnover have the most recent cortisol deposition because hair in that area spends more time in the anagen phase (Macbeth et al. 2010). Grizzly bears have higher HC in hair samples collected from the neck compared to the shoulder and rump, likely because of the variation of moult patterns (Macbeth et al. 2010). Dairy cows have higher HC in the tail switch compared to the hip and top line which have higher cortisol than the shoulder (Burnett et al. 2014). A study by Moya et al. (2013) investigated variability of HC due to body region in Angus cross bulls, and found the tail switch had higher HC when compared to the neck and shoulder, while neck and hip hair had higher cortisol than the shoulder. Cattle undergo a full moult approximately every 3 months (Schwertl et al. 2003) and areas with increased turnover are more likely to contain higher levels of cortisol because hair spends more time in the anagen phase, extending the period in which cortisol can be incorporated into the hair shaft. The body has shorter growth rest-cycles than the tail which spends more time in the anagen phase, so it is expected the tail hair will contain more cortisol than the body hair due to a greater accumulation of cortisol during the elongated anagen phase (Fisher et al. 1985). This may explain why, upon resampling, the tail had greater HC compared to the head and shoulder.

2.2.4 Applications and Future Uses of HC

One of the beneficial aspects of HC is that it is more sensitive to long-term stressors than acute stressors. Clinical disease and lameness can be classified as chronic stressors, increasing the HPA axis activity repeatedly over a period of weeks to months. Prior studies using plasma cortisol to detect relationships in HPA activity between healthy and physiologically compromised animals found no differences between healthy animals and those with laminitis (Walker et al. 2010) or mastitis (Forslund et al. 2010; Lavon et al. 2010). However, physiologically or clinically compromised dairy cows have been found to have greater HC concentrations than their healthy cohorts (Comin et al. 2013; Burnett et al. 2015), successfully documenting HPA activity present during chronic stress. Keeping this in mind, there are a number of variables which influence HC including dam parity, age, hair colour and body location, as previously discussed which need to be considered when analyzing HC for differences between groups. Should measurement of HC be able to systematically document HPA activation over a period of weeks to months, it would prove a powerful tool for use in applied studies aimed to determine the stress response of animals to different management practices.

2.3 Techniques for Measuring Castration Stress

In order to determine pain induced stress caused by castration, studies have used different tools to measure the physiological and behavioural responses of bulls at varying ages both during castration and post-procedurally. Pain associated with castration results in behavioural changes, some of which are likely performed to alleviate or minimize painful stimuli (Molony and Kent, 1997). Physiological changes occur due to activation of the HPA axis and SNS caused by stress and trauma to the tissue. The physiological and behavioral changes occurring after castration can be used to detect the severity of treatments.

2.3.1 Behavioural Measures of Pain due to Castration

Behavioural changes as a result of pain, in mammals, can be described in five main categories: avoidance and defense behaviours, vocalization, behaviour directed toward the painful area, posture and behaviours to reduce stimulation of the painful area and general changes in activity (Prunier et al. 2012).

Defense or avoidance behaviours are performed during a painful procedure in order to avoid or escape from a painful stimulus. Avoidance and escape behaviours are basic instincts in avoiding trauma that will ultimately be harmful to the animal; exaggerated attempts of escape are believed to indicate more painful stimuli. For example, during painful procedures, calves receiving a more aversive treatment struggle and kick more (Fisher et al. 2001). Additionally, cattle have been documented to vocalize during painful procedures (Schwartzkopf-Genswein et al. 1997). Currah et al. (2009) used vocalization as a measure of stress during castration, however, only 14% of animals vocalized, providing non-significant results between castration treatments surgically castrated with and without pain medication. This indicates the role of individual differences in how an animal responds to pain, and as such, measuring a variety of behaviours indicative of pain is wise. Post-procedurally, acute pain can be documented by increased foot stamping and kicking (Molony et al. 1995).

Following castration, behaviour directed toward the painful area increases, most likely in an attempt to relieve pain (Prunier et al. 2012). Increased licking of the site, head turning, slow tail movement and alternate lifting of the hind legs all increase following castration (Fisher et al. 1997) signifying pain. In calves castrated by rubber rings, these changes can persist up to 48 d (Molony et al. 1995), suggestive of chronic stress.

Postural and behavioural changes occur in order to reduce stimulation of a painful area. Number of steps as well as stride length deceases following castration and in calves castrated without pain medication (Currah et al. 2009). Additionally, calves castrated with elastic bands spent reduced time lying and shortened stride length up to 28 days after banding (González et al. 2010). Standing time in calves increases following castration to decrease stimulation of the injured site and signifies pain (White et al. 2008; Brown et al. 2015). Behavioural changes such as abnormal postures (Molony et al. 1995; Ting et al. 2003a), crouching and statue standing (Webster et al. 2013) and increased tail wagging following castration (Robertson et al. 1994) appear to be more sensitive and specific measures of pain than total lying time. It is important to note that behaviours, like standing, are socially facilitated, and if multiple treatment groups are housed together in the same pen results can be confounded.

In a study by Petherick et al. (2014a) the amount of walking and the number of leg and tail movements were higher in banded bulls than surgically castrated weaner (7 – 10 months old) and mature bulls (22-25 months old) at 0 – 1.5 h but not thereafter. Additionally, at 24 h post castration, surgically castrated bulls walked less and stood more than band castrated calves. This study provides a good example that pain related behaviours differ with castration method. Active restlessness was observed in banded animals while surgically castrated bulls minimized movement and stimulation of the area.

2.3.2 Physiological Measures of Stress due to Castration

Measurement of HPA axis activity is the most common way to measure farm animals' physiological stress response. Among the HPA measures, corticosteroids are the most prevalent, even though their value to evaluate animal welfare has been questioned (Rushen, 1991). Changes in plasma cortisol have been measured before and after castration in a variety of studies to

determine biological significance as well as the comparison of cortisol levels between treatment groups (Fisher et al. 1996; Earley and Crowe 2002; Stafford et al. 2002; Ting et al. 2003b; Ting et al. 2004; Coetzee et al. 2007; Coetzee et al. 2010). However, cortisol release is pulsatile with a periodicity lasting approximately 90 minutes (Mormède et al. 2007) and long-term changes are difficult to monitor without repeated collections. A study by Petherick et al. (2014b), compared band and surgical castration by repeatedly collecting plasma over an extended period of time. They found greater plasma cortisol in banded animals 2 h following castration than surgically castrated animals, while at 24 h post castration, surgically castrated bulls had higher cortisol than banded bulls. Two to four weeks post castration, banded bulls had higher plasma cortisol than surgically castrated bulls. Up to four weeks following castration, plasma cortisol continued to be elevated in banded bulls, indicating chronic stress. However, in a study by González et al. (2010), calves castrated by banding had salivary cortisol levels that returned to sham castration salivary cortisol levels two hours following the application of bands and remained non-significant for the remainder of the study (tested at 0, 0.5, 1, 2, 4, 24, 48, 168 and 336 h following band application). Behavioural results from this study also showed that band castrated calves had shortened meal durations and shorter stride length than sham castrated calves up to six weeks following the application of bands. The discrepancy between salivary cortisol and behaviours suggests that salivary cortisol is not a useful measure of chronic stress, or the pain caused by banding castration was not severe enough to cause a stress response captured in salivary cortisol.

Eye temperature and heart rate (Stewart et al. 2010) have been used to measure sympathetic nervous system (SNS) activation following castration. Both heart rate and eye temperature increase immediately following castration, and eye temperature has been found to

remain elevated for up to 20 minutes following castration (Stewart et al. 2010), indicating activation of the SNS. In addition, substance P is a neuropeptide present in the areas of the neuroaxis involved in integration of pain and stress anxiety (Coetzee, 2013). In a recent study measuring the influence of castration on levels of substance P, castrated calves had significantly higher substance P levels than their sham castrated cohorts over the course of four hours, even though differences in plasma cortisol levels were not detectable (Coetzee et al. 2008).

While measuring inflammation and immune responses can indicate the amount of trauma to a site, inflammation does not necessarily indicate pain. However, inflammation is indicative of infection or trauma which are often cause pain. Haptoglobin (Hp) is an acute phase protein synthesized by hepatocytes in response to inflammatory processes normally occurring with the immunological cascade due to infection, stress or injury (Chrousos, 1995). Plasma fibrinogen, is another acute phase protein used as a measure of active inflammation, was used in 5.5 month old dairy calves in response to banding or burdizzo castration. Castrated calves had higher plasma fibrinogen levels following castration compared to controls but not between castration treatments, indicating castrates experienced greater inflammation following castration (Pang et al. 2006). Brown et al. (2015), investigated the acute phase response following surgical castration, measuring Hp, and found that bulls castrated at weaning had Hp levels significantly higher than non-castrated bulls up to four days following castration. Interestingly, administration of oral meloxicam briefly reduced Hp levels in castrated bulls. This presents Hp as an effective measure of injury following castration and oral meloxicam decreases the inflammation response following castration. A combination of acute phase proteins and cortisol as a measure of stress following castration, would provide a robust understanding of the amount of trauma and stress occurring to the animal.

There are multiple tools used for detecting HPA axis and SNS activity occurring both during and post-castration. Additionally, inflammation can be used to determine the magnitude of trauma. While these mediums have been highly beneficial in understanding the acute stress response of castration, long-term effects are difficult to document without repeated sampling. The advantage of HC is its ability to measure HPA axis activity over an extended period of time with the added benefit of offering a non-invasive, sampling technique.

2.3.3 Production Measures Following Castration

Production parameters such as average daily gain (ADG) and dry matter intake (DMI) are often measured to evaluate economic value of specific production practices. However, they are fairly crude measurements that rarely yield differences following a painful procedure over an extended period of time (Stafford and Mellor, 2005). Following castration in bull calves, finishing phase ADG and carcass quality did not differ between castration treatments (Brown et al. 2015), or from d 0-42 (González et al. 2010). Coetzee et al. (2012) found differences in the ADG of castration treatments in the first few weeks following castration (d 0-14), but not thereafter (d 15-28). However, weaned calves treated with oral meloxicam to control post procedural pain had lower bovine respiratory morbidity rates (Coetzee et al. 2012). Treating weaned calves with meloxicam at the time of castration may reduce long-term stress which causes immunosuppression or it may aide in overcoming infection. Production measures are generally too crude to measure treatment differences in young animals because they are rapidly growing and growth rate is affected by many variables such as genetics and feed type. Additionally, growth rate is highly variable between animals which weakens it as a measure of stress when measured at a group level. However, if production differences are detectable, it provides economic incentives to adopt improved practices promoting increased value.

2.4 Techniques for Measuring Weaning Stress

Natural weaning is a gradual process, occurring over time as the milk supply diminishes, resulting in fewer nursing bouts of shorter duration until complete cessation of nursing (Weary et al. 2008). In nature, calves are weaned by the dam before the birth of the next calf or when the milk supply is no longer the calf's main source of nutrients. Even following weaning, the cow-calf pair stay in close proximity for an extended period of time (Reinhardt and Reinhardt, 1981). Traditionally, in North America, calves are artificially weaned through abrupt separation of the dam and calf, and cessation of nursing occurs as a result of the separation. This causes significant stress for the calf, and has been shown to increase susceptibility to disease (Lynch et al. 2010).

2.4.1 Behavioural Measures of Weaning Stress

Following separation from the dam, calves have been observed to vocalize more and spend more time walking (Veissier and Le Neidre, 1989; Veissier et al. 1989). Calling by offspring signals separation distress and is performed in an attempt to reunite with the dam, reuniting with the parent following separation increases survival potential of the young, making vocalization beneficial to the offspring's chance of survival (Fraser, 2008). In abruptly weaned calves, separation distress is evident with an increase in calls by the calf following separation from the dam (Newberry and Swanson, 2008; Enríquez et al. 2010). In response to the stress caused by abrupt weaning, alternatives such as two-stage and fenceline weaning have been studied. Fenceline weaning involves separating calves from their dams via a fence, physically preventing nursing, but calves can still have visual and auditory contact with the dam (Price et al. 2003). An alternative to both abrupt and fenceline weaning is two-stage weaning. During two-stage weaning, plastic nose-flaps are inserted in the nostrils of calves to prevent them from suckling but cows and calves remain together where they can maintain physical contact. After 5-

7 days of wearing nose-flaps, calves are physically separated from the dam. When weaned by two-stage (Haley et al. 2005; Loberg et al. 2008; Lambertz et al. 2015) or fenceline weaning (Price et al. 2003; Johnsen et al. 2015) calves vocalize less than abruptly weaned calves, signifying that calves weaned via two-stage or fenceline weaning were less distressed following separation from the dam. Furthermore, dairy calves separated by a fence allowing physical contact with a foster cow, performed less high pitched calls and called fewer times, compared to calves weaned and separated by a solid wall that only allowed auditory contact following weaning (Johnsen et al. 2015).

Calves are observed walking more following abrupt weaning, as compared to fenceline or two-stage weaning (Price et al. 2003; Haley et al. 2005; Loberg et al. 2008; Enríquez et al. 2010; Lambertz et al. 2015). Additional behaviours observed in calves following abrupt weaning include an increase in time spent walking, a decrease in lying and resting (Veisser and Le Neidre, 1989; Price et al. 2003; Haley et al. 2005) and an increase in vigilance behaviours, such as a raised head position with forward ears (Johnsen et al. 2015).

Rumination decreases in calves following weaning, indicating distress (Loberg et al. 2008; Enríquez et al. 2010). However, a reduction in rumination time may also be due to the increased amount of time spent vocalizing and walking after separation. Calves weaned abruptly have also been observed to spend less time eating than calves in contact with their dam or weaned through fenceline and two-stage weaning (Price et al. 2003; Haley et al. 2005; Enríquez et al. 2010).

The majority of behavioural responses to weaning are observed within the first two days of complete visual and auditory separation of the cow-calf pair (Price et al. 2003; Haley et al. 2005; Enríquez et al. 2010; Lambertz et al. 2015), and generally decrease by the third day.

Behavioural effects of increased CRH disappear after two days, even if CRH remains chronically elevated (Morimoto et al. 1993; Buwalda et al 1997; Salak-Johnson et al. 1997), suggesting that even if the physiological stress response continues, behavioural effects will no longer occur. An absence of behavioural changes indicates decreased stress, however, behavioural responses will diminish even with sustained physiological stress responses. Therefore, it is important to combine behavioural and physiological measures of stress in order to increase the strength of a study by providing a more robust understanding of the stressor effects.

2.4.2 Physiological Measures of Weaning Stress

In addition to behavioural changes following weaning, physiological stress responses occur to help cope with the stressor at hand including HPA axis and SNS activation, followed by an immunological response. The SNS is triggered following separation of the cow-calf pair and responds with increased catecholamine, norepinephrine and epinephrine release in combination with activation of the HPA axis. The highest levels of plasma cortisol have been found the day following separation, showing a significant decrease when the cow-calf pair is reunited (Lefcourt et al. 1995). Measured in saliva, cortisol also increases in dairy calves following weaning from a foster cow (Loberg et al. 2008) and heart rate has been found to be lower in calves weaned by two-stage weaning than abrupt weaning at the time of separation (Loberg et al. 2008), suggesting that calves weaned through two-stage weaning experience a lesser stress response than abruptly weaned calves upon separation from the dam. In addition to SNS and HPA axis activation, the immune system responds to stressors, including weaning. At weaning, neutrophils increase with impaired phagocytic function causing a greater transitory reduction in immune function at weaning (Lynch et al. 2010), which temporarily increases disease susceptibility.

Physiological measures of weaning in beef cattle are fairly limited and previous research on the physiological effects of weaning are minimal. Beef cattle are generally extensively managed and thus not habituated to handling because their contact with people is limited. Handling is therefore a perceived stressor for beef cattle which can increase glucocorticoid levels at the time of sampling, confounding results of the applied stressor. Weaning is a common management practice which until recently had few low-stress alternatives. There is now a growing shift within the beef industry to adopt low-stress weaning strategies such as fenceline and two-stage weaning, but there has been limited research investigating the physiological responses of calves to these weaning procedures. Furthermore, it is not uncommon for calves to be weaned immediately prior to transport to feedlot, exposing them simultaneously to multiple stressors and confounding the ability to measure a stress response directly related to only weaning.

2.4.3 Production Measures Following Weaning

Weaning occurs long before slaughter and production measures are generally crude indicators of stress when measured long after the stressor has occurred. When comparing ADG of calves weaning abruptly or with two-stage weaning, ADG was lower for calves weaned by two-stage weaning during stage 1 (cessation of milk but remaining with the dam), but higher during stage two (removal from the dam) than abruptly weaned calves (Haley et al. 2005), resulting in overall there being no treatment difference. However, calves that were completely separated from their dams never compensated for early weight losses, even after 10 weeks following weaning (Price et al. 2003), compared to fenceline weaned calves. Growth additionally depends on a number of variables including feed type, animal age and genetics. Measuring ADG over a long period of time are often not useful measures of stress when trying to determine the

comparative stress of treatments because compensatory growth of cattle following cessation of stressors makes it hard to detect production differences.

2.5 Conclusion

The use of HC as a measure of long-term has been investigated in multiple species with the exception of beef cattle. There are a number of variables impacting cortisol deposition into the hair shaft which need to be taken into consideration when performing HC analysis in order to see treatment effects from applied studies. Previously, there have been limited tools to investigate long-term stress effects of castration and weaning in beef calves which ultimately have the ability to impact feedlot health. If proven to be an effective tool, HC would be highly useful in future stress studies, creating the opportunity to better understand long-term stress in beef cattle.

3 AN INVESTIGATION OF HAIR CORTISOL AS A MEASURE OF LONG-TERM STRESS IN BEEF CATTLE: RESULTS FROM A CASTRATION STUDY

The chapter presents an experiment performed to investigate the use of hair cortisol as a measure of stress following surgical castration in beef calves performed with and without the use of an NSAID, and in comparison to sham castrated calves. Differences in hair cortisol concentration were found between the methods of castration used in this experiment.

Chapter 3 is in preparation for submission for publication. The copyright of this chapter will belong to the journal in which it is published.

This manuscript was originally drafted by Kate Creutzinger with suggested comments from Drs. Joseph Stookey, Yolande Seddon and Fernando Marquez. Experimental design, animal handling, and data collection was under taken by Kate Creutzinger and Travis Marfleet with advice from Drs. Joseph Stookey and John Campbell. Data analysis was performed by Kate Creutzinger under the guidance of Drs Cheryl Waldner, Yolande Seddon and John Campbell.

3.1 Introduction

Behavioural and physiological research on beef cattle can be challenging due to a lack of reliable, appropriate objective measures of pain and stress. This makes it difficult to monitor how routine husbandry practices, such as castration, affect cattle welfare. Measuring hair cortisol (HC) concentration is a non-invasive method for assessing hypothalamic-pituitary-adrenal (HPA) axis activity across species, making it an attractive research tool for assessing HPA axis activity in a variety of wild and domestic animal species (Koren et al. 2002; Davenport et al. 2006; Accorsi et al. 2008; Macbeth et al. 2010; Moya et al. 2013). Analysis involves extraction and quantification of unbound cortisol incorporated into the hair shaft from systemic circulation over time, while hair is in the active growth phase (Macbeth et al. 2010). The technique has potential application for identifying stress over several days to months (Meyer and Novak, 2012), compared to plasma and salivary cortisol which require multiple collections to measure glucocorticoids over time as cortisol is quickly metabolized in plasma and saliva. By comparison, cortisol is stable within the hair shaft for an extended period of time (Cone, 1996; Pragst and Balikova, 2006), facilitating retrospective analysis. Although the exact mechanism of cortisol incorporation into the hair shaft is unclear, diffusion of cortisol from blood capillaries surrounding the follicle to follicular cells is the leading theory for cortisol deposition into hair (Henderson, 1993). The ability to monitor HPA activity over a period of weeks to months makes HC especially beneficial in beef research because beef cattle are often extensively raised and therefore rarely handled. Compared to other methods of measuring cortisol, HC is not confounded by a rapid release of cortisol, which is frequently attributed to handling and venipuncture for sample collection.

Previous studies have validated the technique for detecting cortisol in hair of beef cattle

(Moya et al. 2013) and studies in dairy cows have shown HC can be used to distinguish healthy from physiologically compromised animals (Comin et al. 2013). However, HC analysis is a relatively new technique in bovine research and there are multiple variables that need to be taken into consideration when using it to measure physiological stress. Variation in HC concentration has been attributed to hair colour (González de la Vara et al. 2011; Burnett et al. 2014), body location (Moya et al. 2013; Burnett et al. 2014), age (González de la Vara et al. 2011) and number of parities (Burnett et al. 2014; Burnett et al. 2015). It is important to recognize these variables when using HC as an objective tool to measure physiological stress, in order to account for them and correctly interpret results.

Castration is a common husbandry procedure performed on beef calves that promotes a variety of benefits such as decreased aggression and mounting behaviour resulting in fewer injuries (Tarrant, 1981), improved meat quality associated with increased marbling and tenderness (AVMA, 2009), and preventing unwanted breeding (Stafford and Mellor, 2005). However, castration is a significant stressor due to clinical pain caused by the procedure of either surgical or banding castration which injures peripheral tissue (Stafford et al. 2002). Post-castration inflammation and pain can result in production losses if severe enough (González et al. 2010). Additionally, bull calves castrated post-weaning have increased susceptibility to bovine respiratory disease (Massey et al. 2011), and are at a greater risk of contracting disease when castrated without meloxicam (Coetzee et al. 2012).

The 2014 National Farm Animal Care Council, Code of Practice for the Care and Handling of Beef Cattle recommends administration of pain control at the time of castration for calves of all ages. Pain control, anesthetics, analgesics or a combination of both, will be required during castration for bulls over 9 months of age beginning January 1, 2016 and required in bulls

over 6 months of age starting January 1, 2018 (NFACC, 2013). However, little scientific evidence is available to show the benefit of nonsteroidal anti-inflammatory drugs (NSAID) at the time of castration, prompting the need for reliable, objective measures of pain and stress post-castration. To address this shortcoming, the objective of this research was to determine the usefulness of HC in demonstrating HPA axis activity in beef calves from one to six months of age following surgical castration. In particular, it was of interest to determine if HC changed in a predictable manner following surgical castration with saline, surgical castration with meloxicam and sham castration, and was able to distinguish differences in the stress response to different treatments. An additional objective was to determine if an NSAID (meloxicam) administered at the time of castration would decrease post-operative pain, and influence HC concentrations and the behaviour of calves following castration.

3.2 Materials and Methods

This work was approved by the University of Saskatchewan's Animal Research Ethics Board and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

3.2.1 Animals

Castration trials were carried out at two separate locations. At site 1 (Goodale Research Farm), Hereford-cross bull calves (n=73) weighing 94 ± 16.5 kg (mean ± S.D.) were 47 ± 9.6 d of age at d 0 of the trial (Table 3.1). At site 2 (Western Beef Development Center), Black Angus bull calves (n=85) weighing 88 ± 15.9 kg and 48 ± 11.3 d of age on d 0 of the trial were studied. Per farm site, calves were listed in order of age in days (ascending), the treatment for the youngest calf in each group of three calves was randomized then calves were assigned treatment by ascending order based on the treatment randomly selected for the youngest calf. Three treatments included: (1) surgical castration (CS, n =52), (2) surgical castration injected with meloxicam (CM, n = 54),

and (3) sham castrated (sham castrates were handled as if to be castrated but not) (S, n = 52).

3.2.2 Study Design and Data Collection

On d 0, all calves were weighed on a digital chute scale and either castrated or sham castrated on a tip table. Calves in the CS and CM groups were surgically castrated by longitudinally incising the scrotum with a Newberry knife. The testes and spermatic cord were expressed manually, emasculators were applied for approximately 30 s to crush and cut the spermatic cord. Surgical castration was performed by the same two trained persons in order to minimize procedural variation. Surgically castrated calves were given meloxicam (0.5 mg/kg BW; Metacam, Boehringer Ingelheim, Burlington, ON) or sterile saline immediately prior to castration and were injected subcutaneously anterior to the left shoulder at the time of castration. The veterinarian administering the saline and meloxicam kept other researchers blind to which drug was administered. Sham castration was performed in an identical manner to surgical castration. Calves were placed on a tip-table as if they were to be castrated, the testicles were handled gently, and calves were then released without surgical castration.

Electric trimmers (Oster Golden A5, Sunbeam Products, Inc.) with a detachable size 40 blade were used to shave a 10 cm x 10 cm patch of hair as close to the skin as possible on the left hip, between the coxal tuber and ischial tuber, of each calf. Hair was collected and stored in dry paper envelopes at room temperature in the dark prior to cortisol analysis. Following castration and hair sampling, the entire group of calves were reunited with their dams and moved to pasture for two weeks. Calves and their dams were maintained on grass-pasture and had constant access to water through automatic watering bowls. On d 14, calves were reweighed and hair samples were collected. The d 14 hair sample included two weeks of hair regrowth from the d 0 sample location and was collected using the previously described equipment and method. Calves were checked

and recorded for the presence or absence of scrotal infection at d 14; scrotal infections were defined as a swollen scrotum with discharge present. Additionally, researchers documented whether calves were born to multiparous cows or primiparous heifers, and the parity of their dams.

3.2.3 Calf Standing Time Post-Castration

A subset of animals (n=129) were randomly selected to wear accelerometers (HOBO pendant G accelerometers, Onset Computer Corporation, Pocasset, MA) through a random number generator (randomnumbergenerator.com) and balanced by treatment (CS, n = 47; CM, n = 42; SC, n = 40), calves wearing accelerometers were not balanced by age. Total standing and lying time (min d^{-1}) were used as a previously validated behavioural measure of pain following castration because increased standing time (White et al. 2008) and statue standing following castration are indicative of pain (Molony et al. 1995). Accelerometers were encased in foam padding fitted to the lateral aspect of the hind leg distal to the hock, as described by Ito et al. (2009), with Elastoplast wrap (Elastoplast Canada, St-Laurent, PQ). The accelerometers were positioned so the x-axis was perpendicular to the ground pointing in the distal direction and the y-axis was oriented parallel to the ground pointing in the cranial direction.

Data loggers are validated to accurately measure standing time when measured in intervals ≥ 30 s (Ledgerwood et al. 2010), therefore, accelerometers were programmed to record at 100 Hz every 30 s from d 0 to d 7. Loggers were set to automatically commence sampling roughly three minutes following calf chute exit.

Standing and lying time was assessed in min day^{-1} from the data recorded by accelerometers following the protocol described by Bonk et al. (2013). The degree of tilt on the logger Y axis was the measure of interest with <60° indicating standing and readings of ≥60° indicating the calf lying down. Walking and standing yield the same coordinates on accelerometers, therefore

discrimination between walking and standing was not possible, so any upright position was counted as standing in this study.

Throughout the course of the study, at total of nine accelerometers were either lost or failed to log data correctly (CS=5, CM=2, S=2). Analysis was performed on the data from the 120 remaining accelerometers.

3.2.4 Chute Exit Speed

Chute exit speed was recorded on all calves on d 0, following weight measurement and prior to castration, as a measure of temperament (Vetters et al. 2013). More reactive animals have been shown to have faster chute exit speeds correlated with higher serum cortisol concentration (Curley et al. 2006), thus it was predicted calves in this study calves with higher chute exit speeds would have higher HC concentrations. Exit speed from the handling chute for individual calves was determined using a Sony Cybershot video camera (Sony, New York, NY, USA). Video was reviewed by a single individual and exit speed (m s^{-1}) was calculated based on the time taken for a calf to traverse a fixed distance (3.05 m) immediately upon exiting from the head gate. Time began when calves crossed a set marker upon exiting the chute and ended when they crossed a second set marker, the nose was used as the point of reference for crossing the markers. Each animal was assigned a chute score on d 0, while in the squeeze chute during weight measurement, as a measure of temperament (Voisinet et al. 1997). Observations were taken by a single observer to reduce variability. Scores were assigned to calves on a 5 point scale from 1 (calm, no movement) to 5 (violently struggling).

3.2.5 Hair Sample Preparation

Hair samples were stored in paper envelopes at room temperature in the dark until processing. A subset of samples were used for cortisol analysis (CS, n = 40; CM, n = 33; SC, n =

33). The subset of samples used for cortisol analysis was balanced between farm locations and balanced for calf age upon random selection. Of the calves selected for HC analysis n = 94 (CS, n = 39; CM, n = 28; SC, n = 30) wore accelerometers. For cortisol analysis, 100 mg of calf hair was loosely separated and any significant debris removed. Samples were washed three times in 0.4 mL methanol per mg hair, and allowed to dry overnight in plastic dishes on the bench top at room temperature. Washed and dried hair samples were ground to a uniform fine powder with a Retsch MM 301 Mixer Mill (Retsch Inc, Newton, PA, USA) at 30 Hz for 0.03 min mg^{-1} of hair using 10mL stainless steel grinding jars with a 12 mm stainless steel grinding ball. Grinding hair into a fine powder is preferential to mincing for maximum cortisol extraction (Davenport et al. 2006). Ground samples were stored in individual 0.5 mL plastic vials in cryoboxes until extraction.

Table 3.1 Calf demographic at d 0 of the study. Age presented as mean ± S.D.

	Site 1			Site 2		
	CS	CM	S	CS	CM	S
n	23	25	25	29	29	27
Age	47.6 ± 9.1	46.5 ± 10.2	47.4 ± 9.6	48.1 ± 10.7	47.4 ± 11.4	48.3 ± 12.0
Hobos, *n*	22	20	20	20	20	20
Hair analysis, *n*	21	18	18	19	16	15
Breed	Hereford cross			Black Angus		

3.2.6 Steroid Extraction and Quantification

Cortisol extraction was performed by immersing 25 mg of ground hair in 0.5 mL of HPLC-grade methanol (EMD Chemicals, Gibbstown, NJ, USA), gently vortexing, and placing on an automatic slow rotator at 18 rotations per minute for 24 h. At 24 h, samples were centrifuged (15 min at 4,500 rpm) and supernatants transferred to 12 mm glass tubes (VWR, Radnor, PA, USA). Supernatants were dried under a steady stream of N_2 gas in a heating block set to 38°C. Powder hair samples were immersed in 0.5 mL methanol, gently vortexed (30 s), centrifuged (15 min at

4,500 rpm) and supernatant was added to the 12 mm glass tube where previous supernatant was collected. The wash step was repeated once more before proceeding with extraction for a total of three collections. After each collection, supernatants were dried under a stream of N_2 gas. Steroids were concentrated at the bottom of glass tubes using three consecutive methanol washes of decreasing volume (0.4, 0.2, 0.15 mL) followed by drying under N_2 at 38°C. Concentrates were reconstituted with an EIA sample buffer before cortisol analysis was performed with an Oxford EA-65 cortisol EIA kit (Oxford Biomedical, Lansing, MI, USA). Samples were reassayed if the coefficient of variation between duplicate samples varied by >15%. All assays included a cortisol standard curve to determine unknown cortisol concentrations with values expressed as pg of cortisol per mg of ground hair used for extraction.

3.2.7 Statistical Analysis

All statistical analyses were performed in using commercial software (Stata® 12, Software, StatCorp. LP, College Station, TX, USA). Individual variables were screened via bivariate associations with HC d 0 or d 14, any variable with $P \leq 0.20$ was included in the backwards stepwise linear regression. A backwards stepwise linear regression was used to determine significant variables (Appendix A) in relation to HC concentration d 0 and d 14, variables included treatment, calf age (days), primiparous heifer vs. multiparous cow, dam age, chute score, chute exit speed (m s^{-1}) and farm/breed. Only significant variables as determined by the backwards stepwise linear regression ($P < 0.05$) were included in the final mixed model linear regression (MMLR) to determine any effect of treatment on HC concentration d 0 and d 14. For analysis of HC concentration between treatments at d 14, HC at d 0 was included in the model as a covariate. Interactions were examined between treatment and other variables that remained in the final model and reported if $P \leq 0.05$. Variables included in the MMLR were checked for collinearity.

The correlation between chute exit speed and HC concentration was examined using Pearson correlations separately for HC d 0 and d 14. Chute exit speed was compared between farm locations using a two-sample t-test. Prior to application of the Person correlations at t-tests, continuous variables were checked for normality using the Shapiro-Wilks normality test.

Average daily gain (ADG) was calculated for d 0 to d 14. A backwards stepwise linear regression was performed to identify potential confounders or effect modifier variables. These included age, primiparous heifer vs. multiparous cow, dam age, chute score, chute exit speed, scrotal infection and farm/breed. Significant variables ($P \leq 0.05$) were included in a MMLR to explore differences between treatment groups.

Calf standing time was determined per day (12:00am-23:59pm) for the first five days of the study, d 0-4. A backwards stepwise linear regression was applied to the data using variables age, primiparous heifer vs. multiparous cow, dam age, chute score, chute exit speed, scrotal infection and farm/breed. Once significant variables were determined, a repeated measures MMLR with an autoregressive correlation structure was applied to standing time (min day^{-1}) from d 0-4, variables included treatment, day, age, farm, and treatment by day interaction. Standing time was further assessed for healthy calves vs. those with visible scrotal infection using two-sample t-tests each day following castration.

The residuals from all models were checked for normality using the Shapiro-Wilks test for normality. Standardized residuals were checked for extreme outliers. Data used the in two-sample t-tests was tested for normality by applying Shapiro-Wilks to the data set prior to statistical analysis. Statistical significance was set to a *p*-value of ≤ 0.05

3.3 Results

3.3.1 Hair Cortisol

The MMLR for d 0 HC included age. Variables included in the MMLR for HC concentration d 14 included HC d 0 as a covariate, treatment, farm/breed and primiparous heifer vs. multiparous cow. There was no significant difference in HC at d 0 between treatment groups (Table 3.2). However, age of calves significantly influenced HC at d 0 (Figure 3.1), with older calves having lower HC concentrations. On d 14 HC concentrations of the CS calves was 13.5% higher and significantly greater than S calves, calves in the CM treatment tended to have a lower concentration of HC than CS calves ($P < 0.1$), but were not different from S (Figure 3.2**)**. Calves born to multiparous cows had significantly greater HC concentration at d 14 than those born to primiparous heifers (1.88 ± 0.27 vs. 1.15 ± 0.13, LSM ± S.E., respectively; $P = 0.005$). The HC concentrations at d 14 were significantly greater from calves located at site 2 compared to calves at the site 1 (2.11 ± 0.13 vs. 1.51 ± 0.24, respectively; $P < 0.001$). No other variables were found to significantly influence HC at d 14.

3.3.2 Chute Exit Speed

Chute exit speed was not correlated with HC concentration at d 0 ($r = 0.03$, d.f. = 147, $P = 0.79$) or d 14 ($r = 0.11$, d.f. = 147, $P = 0.26$) HC concentration. There was a significant difference for chute exit speed between farms (t(145) = 5.77; $P < 0.001$), calves at site 1(2.25 ± 1.19 m s^{-1}, mean ± S.E.) had faster chute exit speeds than calves located at site 2 (3.75 ± 1.78 m s^{-1}).

3.3.3 Average Daily Gain

Results of the MMLR showed no significant difference in ADG between treatments (coef = 0.01, 95% CI = -0.06 - 0.08, $P = 0.73$). However, both scrotal infection and farm/breed significantly affected ADG. Calves with scrotal infection had significantly lower ADG than their cohorts which did not develop scrotal infection following castration (1.25 ± 0.27 kg day^{-1} vs.

1.40 ± 0.28 kg day^{-1}, mean ± S.E., respectively; P = 0.027). Additionally, there was a significant difference in ADG between farms. Calves at site 1 had significantly lower ADG than calves at site 2 (1.09 ± 0.05 kg day^{-1} vs. 1.64 ± 0.07 kg day^{-1}, respectively; P < 0.001).

Table 3.2 Final MMLR at d 0 and d 14 for HC concentration

	coefficient	95% CI	*P*
D0			
CS vs. CM	-0.70	-0.70 – 0.55	0.820
CS vs. S	0.16	-0.47- 0.78	0.620
CM vs. S	0.23	-0.42 – 0.88	0.489
Age	-0.09	-0.12 - -0.06	<0.001
D14			
CS vs. CM	-0.23	-0.47-0.01	0.059
CS vs. S	-0.27	-0.51- -0.04	0.024
CM vs. S	-0.04	-0.29- 0.20	0.725
Dam parity	-0.47	-0.80- -0.14	0.005
Farm/breed	-0.50	-0.71- -0.28	<0.001

Final MMLRs included treatment and significant variables as determined by backwards stepwise linear regression

3.3.4 Standing Time

Calves in the CM group tended to stand more than S calves (P = 0.052). There were no other differences between treatments. There was no significant interaction of treatment by day on standing time. Standing time across all treatment groups differed significantly between days 0-4, calf age significantly impacted standing time (older calves stood more than younger calves), and calves located at site 2 stood significantly more than calves located at site 1 (Table 3.3).

Calves that developed scrotal infection following castration stood significantly more on d 0, 2 and 3; and tended to stand more on d 1 and 4 (Figure 3.3).

Table 3.3 Standing time repeated measures linear regression. Values reported in min d^{-1}				
Variable	estimate	SE	coef	*P*
Day				
0 (reference)	372[a]	48		
1	547[b]	49	174.3	
2	613[cd]	44	240.8	< 0.001
3	604[d]	44	231.2	
4	661[e]	43	288.6	
Treatment				
CS	568	107	28.0	
CM	574	106	36.7	NS*
S (reference)	534	116		
Farm location				
Site 1 (reference)	523	102		< 0.001
Site 2	597	104	72.4	

Values with different superscripts [a, b, c, d, e] within the same column and within a variable and significantly different (P < 0.001). *Age (d) was also included in the repeated measures model as a continuous variable (coef = 2.0, 95% CI = 0.43 – 3.55, *P*-value = 0.012). CM vs. S P = 0.052; CS vs S = NS; CS vs. CM = NS. Overall significance of the model was *P* < 0.001.

3.4 Discussion

Castration is known to be a painful and acute stressor (Stafford et al. 2002; Brown et al. 2015), and has been used as an applicable model of stress to better understand the cortisol response (Earley and Crowe, 2002). On d 14, HC was significantly greater in CS than S calves and tended to be higher than CM calves, while the HC concentration from CM calves did not differ from S calves. Thus, the null hypothesis that HC concentration would not differ between treatment groups can be rejected.

Pain-induced stress caused by surgical castration can be reduced through the application of local anesthetic, NSAIDs, or a combination of both (Early and Crowe, 2002; Stafford et al. 2002; Ting

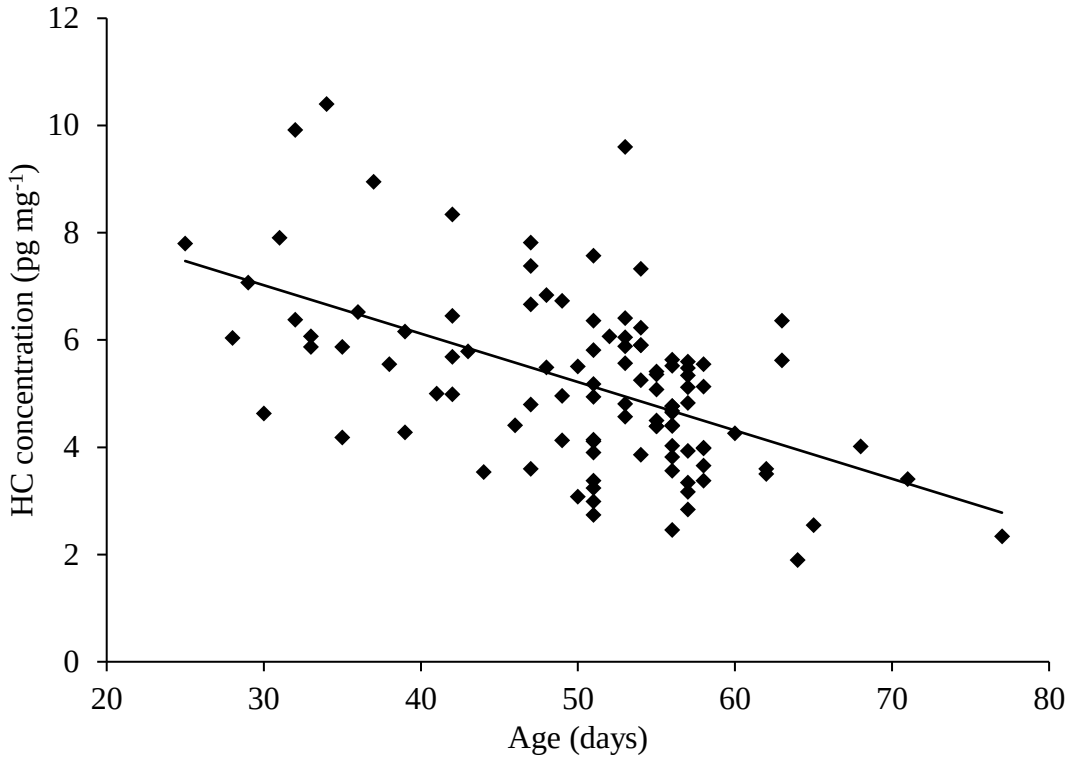

Figure 3.1 Effect of age on HC at day 0. As day in age increased there was a decrease in HC ($R^2 = 0.29$; coef = -0.09; $P < 0.001$).

et al. 2003). The current data suggest CS calves experienced greater stress than S, as documented by greater HC at d 14. It can also be concluded this is likely be due to the pain from castration because S calves were handled in an identical manner, but not castrated. Because HC tended to be lower in CM than CS calves, it suggests that meloxicam has a beneficial effect to help reduce stress following castration. Interestingly, HC in CM calves did not differ from S, suggesting that the cortisol response in CM calves was more similar to that of S than CS calves, further demonstrating a benefit of providing pain relief. Pain has been shown to be alleviated by NSAIDs which effectively reduce the area under the cortisol curve by 29% (Coetzee et al. 2013), likely due to analgesic and anti-inflammatory effects that continue post-castration (Coetzee et al. 2013; Roberts et al. 2015). Oral meloxicam has a mean half-life of 27.54 hours in cattle and when delivered intravenously meloxicam has a relatively long mean half-life of 20.74 hours

(Coetzee et al. 2009). From this, it can be estimated that when administered subcutaneously, meloxicam will have a half-life of approximately 20-27 hours, which is longer than most available NSAIDs. The differences observed between CS and CM calves, but not CM and S, are likely due to pain relief up to a full day following castration from subcutaneous meloxicam. Results from the applied model of castration, provide evidence to suggest that HC is a useful measure of long-term stress in beef cattle. Additionally, it indicates that meloxicam mitigates post-castration stress.

Accelerometers were used to determine lying and standing time as a measure of pain-induced stress (Brown et al. 2015). Physical and overt behavioural signs of pain are considered to be more reliable than some biochemical measures (Millman, 2013) because they are specific to painful stimuli that biochemical measures cannot always detect. Castration pain can be characterized by recently castrated steer calves standing in abnormal positions, including statue

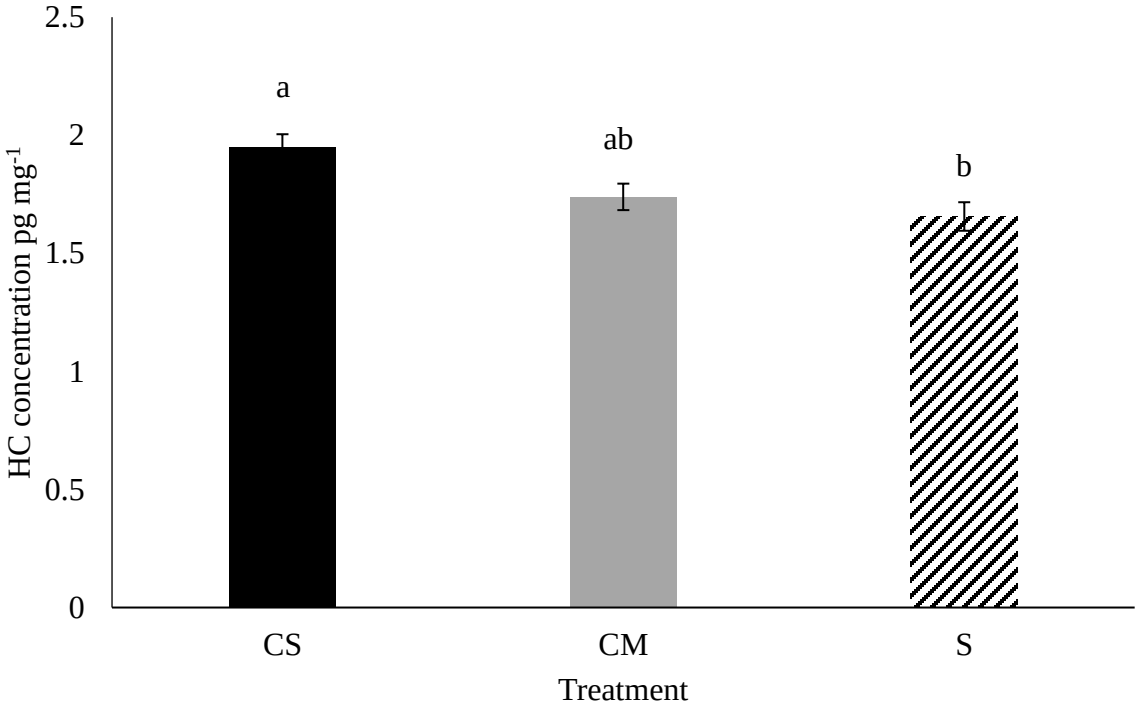

Figure 3.2. Mean HC (pg mg^{-1}) of calves of calves per treatment. Values are represented as LSM ± S.E. Significant statistical differences ($P \leq 0.05$) as distinguished by different letters, a and b

with increased standing time as an indicator of increased pain (Molony et al. 1995; White et al. 2008; Millman 2013).

Standing time tended to be higher in CM than S calves following castration, however, no additional differences were detectable. Increased standing time in CM calves compared to S is likely due to increased pain following castration, in an attempt to reduce stimulation of the injured area. It is also possible CM calves spent more time standing than CS calves because they spent more time grazing or performing more activities due to decreased pain and inflammation, from administration of meloxicam. Unfortunately, there is no additional data provided by the accelerometers, activities performed by calves or number of steps taken, therefore this is merely speculative. On the assumption that calves in pain stand more (Molony et al. 1995; White et al. 2008; Brown et al. 2015), it was predicted that CM calves would stand less than CS because meloxicam has a half-life greater than 24 hours (Coetzee, 2009) and provides calves with pain relief for approximately a day following castration. Beef cattle are prey animals and therefore tend not to display signs of pain in an attempt to not draw attention to themselves (Currah et al. 2009). This could be the cause of CS animals not standing significantly more than S, as compared to CM calves which tended to stand more. It is important to consider that standing time is a crude measure which does not provide a large amount of detail about performed behaviours. There is a large amount of individual variation between animals, and this needs to be considered when analyzing standing time (White et al. 2008).

Calves with scrotal infection stood for approximately 80 min more per day than animals which healed normally, statistical differences were observed on d 0, 2 and 3 of the study, and calves with scrotal infection showed a tendency to stand more on d 1 and 4 than their healthy cohorts. The amount of scrotal infection did not differ between CS and CM calves. Increased

standing time from calves with scrotal infection is likely due to increased discomfort (Molony et al. 1995). A greater duration of standing time in calves with scrotal infection compared to normally healed calves, provides more evidence to suggest that increased standing time indicates increased discomfort in beef calves.

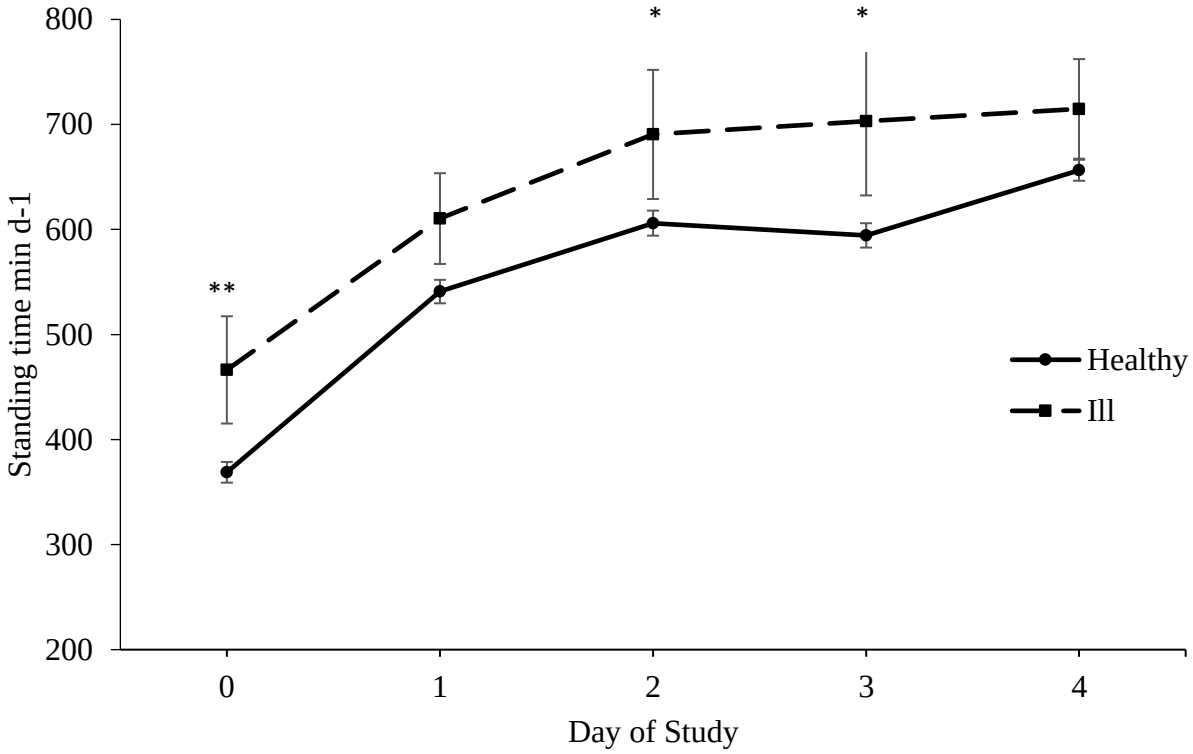

Figure 3.3 Standing time of healthy calves vs. those with scrotal infection. Total standing time, min day^{-1}, is represented as mean ± S.E. for calves with scrotal infection present (Ill) and calves without scrotal infection (Healthy) following castration d 0-d 4, castration occurred on d 0. Where asterisk appear * $P < 0.05$, ** $P < 0.01$.

Cortisol measured in the hair shaft is directly related to the systemic unbound cortisol while hair is in the active growth phase (Macbeth et al. 2010), providing evidence that d 14 HC measurement from this study is a measure of the long-term stress experienced by the calves in the period post-castration. In this study, HC appears to be a valid tool to measure long-term stress in beef calves following castration, however, a number of variables impact HC and need to be considered. Calves born to first time heifers had lower HC on d 14 than calves born to

multiparous cows. This finding agrees with work by Burnett and coworkers (2014), who found multiparous cows had higher HC than primiparous heifers, although the difference was not statistically significant.

Fetal cortisol is essential for fetal organ development and inducing parturition (Flint et al. 1979; Mastorakos and Ilias, 2003). In this study, calf age was found to influence the d 0 HC measurement, with older calves having a lower HC concentration. As day in age increased, HC at d 0 decreased, following a negative relationship. As calves age and are more removed from gestation and parturition, lower HC values may be due to dilution of cortisol in the hair shaft from hair growth without additional stressors (González de la Vara et al. 2011; Meyer and Novak, 2012). Cortisol observed within the hair shaft is cumulative and values are subject to dilution caused by hair growth. Variability of HC is greater in young animals, therefore, when using HC as a measure of stress it is important to keep age of the subjects in mind.

There was a significant difference in calf HC concentration between sampling collection sites. Each farm site also had varying breeds, Hereford cross (site 1) and Black Angus (site 2). Calves located at site 2 has significantly greater HC concentration than calves at site 1. The variation in HC concentration between farms is likely due to a difference in breed between the two locations. Unfortunately, because farm and breed are highly correlated including effects such as: environment, management and hair colour which are all variables impacting HC (Bennett and Hayssen, 2010; González de la Vara et al. 2011; Tallo-Para et al. 2015). Due to the multitude of differences between the two locations it is impossible to define what is affecting HC concentration differences. While there is a clear difference in HC between the farm locations, without further research, we are unable to determine which factors impact HC.

In the future to get a better idea of baseline HC, it may be beneficial to shave an area

several weeks before taking the initial baseline sample. This would remove the animals' HC history up to the point of the study, and allow for a true baseline measurement to determine if there was a biological difference. Stressors prior to a study are generally unknown and cannot be controlled for when determining baseline values. By shaving a patch earlier and collecting regrowth it would allow, regrowth to be compared to baseline samples and see if an increase in HC following treatment application is observable. While this has not been performed in other studies using HC, it may be useful in the future when studying applied stressors.

In this study, variables other than castration treatment, influenced the total amount of standing time by calves. Calves at site 2 stood more than calves at site 1 in the days following castration. As previously discussed, breed varied by location and breed temperament could have an impact on the amount of standing, additionally, it could be due to the environment itself. In addition, as the age of calves that were used in this study increased, standing time also increased following castration. This supports the idea that older animals experience more discomfort following castration than younger calves (Bretschneider et al. 2005).

There were no differences between treatments for ADG. Production parameters such as weight gain, may be too crude a measure to quantify pain experiences by animals (Stafford and Mellor, 2005), at a time of high growth a stressor would have to be very severe to depress weight gain. However, there were differences in ADG between farm location/breed and between calves with and without scrotal infection following castration. Calves located at site 2 had higher ADG than calves at site 1. The difference in ADG may be due to a variety of variables such as breed, management, environment and type of forage consumed. Not surprisingly, calves with scrotal infection had lower mean ADG than their healthy cohorts. Illness caused by scrotal infection is potentially a long or severe enough stressor that production is decreased and a difference in ADG

is observable as has been reported in other studies where sickness negatively affects weight gain (Coetzee et al. 2012)

Chute exit speed was taken as an additional measure to be compared to HC as a way to evaluate temperament. No differences were observed in chute exit speed due to treatment nor was any relationship determined between exit speed and HC. However, calves at site 1 had faster mean exit speeds than calves at site 2. Faster chute exit speeds at site are likely due to a breed difference, between Black Angus and Hereford calves. Calves with more excitable temperaments have been found to have faster chute exit speeds and a lower ADG (Petherick et al. 2002), as with the calves at site 1 compared to site 2. Unfortunately, no direct relationship was found between HC and chute exit speed.

3.5 Conclusion

The HC levels measured in calves following castration responded in a predictable manner, with higher HC values in CS than CM and S calves. Treatment differences were detectable using HC concentration in beef cattle, making HC a potential tool for measuring long-term stress. Increased total standing time in calves with scrotal infection compared to standing time further validates increased standing time as a measure of pain. However, standing time, used as a behavioural measure of pain, between treatments did not reflect HC concentration findings. Multiple variables including age, farm/breed, multiparous cow vs. primiparous heifer impacted HC concentrations in this study. Therefore, when performing HC analysis in the future, it is important to consider the potential confounding variables.

4 AN INVESTIGATION OF HAIR CORTISOL AS A MEASURE OF LONG-TERM STRESS IN BEEF CATTLE: RESULTS FROM A WEANING STUDY

Results from the investigation in Chapter 3 revealed treatment differences in hair cortisol concentration between various methods of castration, an applied physical stressor, indicating that hair cortisol may be a useful measure of applied stress in cattle. Chapter 4 explores an experiment investigating the use of hair cortisol as a measure of long-term stress following weaning, a psychosocial stressor, in beef calves. Weaning was performed as traditional abrupt weaning or two-stage weaning, an alternative method of weaning in the following experiment. The results of this experiment found that hair cortisol concentration varied between the two weaning methods, further indicating that hair cortisol may be a useful measure of long-term stress in beef cattle.

Chapter 4 is in preparation for submission for publication. The copyright of this chapter will belong to the journal in which it is published.

This manuscript was originally drafted by Kate Creutzinger with suggested comments from Drs. Joseph Stookey, Yolande Seddon and Fernando Marquez. Experimental design, animal handling, and data collection was under taken by Kate Creutzinger with advice from Drs. Joseph Stookey and John Campbell. Data analysis was performed by Kate Creutzinger under the guidance of Drs Cheryl Waldner, Yolande Seddon and John Campbell.

4.1 Introduction

North American beef producers generally wean beef calves by abrupt separation from the dam near 6-7 months of age. This is considerably different from natural weaning where the dam prevents the calf from suckling before the birth of its next calf, and even after weaning, the pair remain in close proximity for an extended period of time (Reinhardt and Reinhardt, 1981). Following abrupt separation of the cow-calf pair, time spent vocalizing and walking drastically increases for both cow and calf in an attempt to reunite (Newberry and Swanson, 2008), which in turn, decreases the time calves spend eating and lying (Veissier and Le Neindre, 1989). Abrupt weaning represents a significant social stressor and creates the need for low-stress weaning alternatives.

Alternatives to abrupt weaning such as fence-line (Price et al. 2003) and two-stage weaning (Haley et al. 2005) have been investigated in an attempt to decrease weaning stress. Two-stage weaning is performed by first preventing the calf from suckling by inserting an anti-suckling nose-flap, while leaving the calf in physical contact with its dam, followed by physical separation from the dam seven days later. In comparison to abrupt weaning, two-stage weaning more closely mimics natural weaning; the dam and calf remain together following the cessation of nursing and once separated, calves are no longer dependent on the dam for nutrients. Following separation from the dam, two-stage weaning has been found to result in calves performing less vocalization and locomotion (Haley et al. 2005) and increased time spent grazing (Enríquez et al. 2010), compared to abrupt weaning. These overt behavioural changes indicate that the two-stage weaning process decreases calf distress following separation.

The most common measure of physiological stress response in mammals is through the HPA axis activity (Mormède et al. 2007). Hair cortisol (HC) has been used as a measure of long-

term stress in multiple species, including cattle, in which validation studies have been performed in both dairy (Comin et al. 2013; Burnett et al. 2015) and beef cattle (Moya et al. 2013). Measurement of HC can detect HPA axis activity over a period of weeks to months (Davenport et al. 2006; Comin et al. 2013) via passive diffusion of glucocorticoids into the hair follicle during the active growth phase (Cone, 1996), making it an objective, biomarker of long-term stress (Manenschijn et al. 2011; Meyer and Novak, 2012). Cortisol is stable within the hair matrix for an extended period of time (Pragst and Balikova, 2006) and is not influenced by acute changes in the HPA axis activity (Ashley et al. 2011). Traditionally, measuring the physiological effects of long-term stress in beef cattle has been challenging because measurement of cortisol in the common mediums, serum and saliva, reflects an acute stress response and does not provide information on the influence of long-term stressors on the HPA axis activity over time. Consequently, repeated sampling is required which can reduce the validity of the results due to increased stress from handling and sample collection. A stable, long-term measure of stress, such as HC, is needed to help facilitate understanding of the physiological impacts of long-term stressors, such as weaning. This is particularly important for assessment of management practices which may impact calf welfare and performance.

The objective of this study was to investigate the use of HC as a tool for measuring long-term stress in beef cattle, as evaluated by two distinct methods of weaning, abrupt (AW) and two-stage (TS), which are believed to generate stress of different magnitude in calves. The hypothesis of this study was that HC concentrations measured post weaning, would differ between AW and TS weaned calves and the change in HC would reflect the behavioural changes associated with these two distinct weaning protocols.

4.2 Materials and Methods

4.2.1 Animals and Experimental Design

A total of 161 Hereford x Angus calves between 5-7 months in age (age: 186 ± 15.5 days; weight: 246 ± 40.5 kg, mean ± S.D.), were randomly assigned to two weaning protocols: AW or TS weaning. Treatments were balanced for age and sex (see Table 4.1). Prior to weaning, all calves were maintained in one group, on grass pasture with their dams with free access to water via automatic waterers.

At the start of the trial on d 0, all calves were temporarily separated from their dams and moved single file through a handling facility, restrained in a headgate, individually weighed on a digital scale and had hair samples collected. During initial handling, a subset of 49 calves per treatment group (balanced for sex) were randomly selected and fitted with accelerometers (HOBO Pendant G Data Loggers, Onset Computer Corporation, Bourne, MA). Calves in the TS treatment (n = 81) were fitted with QuietWean™ nose-flaps (JDA Livestock Innovations Ltd., Saskatoon, SK). The nose-flap, made of flexible plastic, served as an anti-suckling device and prevented calves from suckling but did not interfere with eating, drinking or grazing. The fitting of the QuietWean™ nose-flaps initiated the first stage of the weaning process, the cessation of milk intake. Calves in the AW treatment were handled in an identical manner, but were not fitted with nose-flaps and were allowed to nurse for an additional 7 d. All calves were reunited with their dams after handling on d 0 and the entire group was moved back to the pasture where they were previously kept.

On d 7, all calves were handled in a manner identical to d 0, for reweighing and the collection of hair samples. At that point, nose-flaps were removed from all TS calves. Thereafter, both groups were permanently separated from their dams and moved into pens where visual and tactile contact with their dam was not possible. Calves from both treatment groups were placed

in two pens, separated by gender. All calves had access to water via automatic waterers installed in pens and were provided a diet of ad libitum alfalfa brome grass hay mixture.

On d 14, calves were again handled in a manner similar to d 0 and 7. A final collection of hair samples was taken and calves were reweighed and accelerometers removed. At that point, AW calves had been weaned for 7 d, after simultaneous removal of dam and milk, while TS calves were prevented for suckling for 14 d and physically separated from their dam for 7 d.

4.2.2 Hair Collection

Hair samples from all animals were collected from within a small area of the right hip, to decrease any possible HC concentration variability due to body region. On d 0, a 10 x 10 cm square area from the right hip, at a middle point between the coxal tuber and ischial tuber, was clipped as close to the skin as possible using electric clippers (Oster Golden A5, Sunbeam Products, Inc., Boca Raton, FL) with a detachable blade (size 40).

Hair samples collected on d 7 were obtained by clipping a 10 x 10 cm square area immediately adjacent to the left of the d 0 collection site, making certain there was no overlap with the d 0 sample area. Hair samples on d 14 were collected from the d 0 sample area, representing 14 days of hair regrowth. Dry individual hair samples were stored in paper envelopes at room temperature (21 °C) until further processing. González de la Vara et al. (2011), previously determined that HC is stable within the hair shaft for at least 11 months when stored in this manner.

Of the 161 calves on trial, hair samples from a total of 70 calves were selected for HC analysis. Calves were randomly selected for HC analysis using a random number generator, with an equal number of calves selected per treatment group (35/treatment), and balanced for gender.

Each calf selected provided all three hair samples collected on day 0, 7 and 14. Of the calves selected for HC analysis n = 46 (AW, n = 24; TS, n = 22) wore accelerometers.

4.2.3 Analysis of HC Concentration

Analysis of HC was performed following the protocol developed by Macbeth et al. (2010). A 100 mg subset of hair was removed from each sample for processing and analysis. Hair samples were washed three times in 98% methanol to remove excess surface contaminants such as dirt, debris or endogenous secretions, such as sebum or sweat, which can also contain cortisol (Pragst and Balikova, 2006; Russell et al. 2014). After washing, hair samples were left to air dry overnight.

Clean, dry hair samples were ground into a homogenous, fine powder using a Retsch MM 301 Mixer Mill (Retsch Inc, Newton, PA) with 10 mL stainless steel grinding jars and 12 mm stainless steel grinding ball at 30 Hz for 0.03 min mg^{-1} of hair. Grinding hair into a fine powder is preferential to mincing for maximum cortisol extraction (Davenport et al. 2006). Ground samples were stored in individual 0.5 mL plastic vials in cryoboxes until cortisol extraction.

4.2.3.1 Steroid Extraction

For cortisol extraction, 25 mg of ground sample was immersed in 0.5 mL of 98% HPLC grade methanol (EMD Chemicals, Gibbstown, NJ) and set to spin on a slow rotator (18 revolutions per minutes) for 24 hours. After rotating for 24 hours, samples were centrifuged (15 min, at 4500 rpm, 20°C) to separate the supernatant containing cortisol and powdered hair. Collected supernatant was transferred to a 12 mm glass tube to be evaporated under a stream of N_2 gas. After collecting the supernatant, samples were immersed in 0.5 mL fresh 98% HPLC grade methanol and centrifuged again to ensure all steroids had been extracted. This was repeated for a total of three collections into a single sample.

Methanol was evaporated under a stream of N_2 gas at 38°C after each collection. After the three collections were completely dry, steroids were concentrated at the bottom of the tube with three consecutive methanol rinses of decreasing volumes (0.4, 0.2, and 0.15 mL). Following each rinse, methanol was evaporated under a stream of N_2 gas at 38°C as to concentrate the steroids to the bottom of the 12 mm glass tube as much as possible. Concentrated samples were reconstituted with 0.2 mL phosphate buffer (12 hours, 4°C) in the dark. After reconstitution, samples were gently vortexed, collected and transferred to plastic vials. The reconstituted samples were centrifuged (15 min at 4500 rpm, 20°C) to separate any remaining solids from the cortisol solution. Supernatant was collected and used for analysis.

Cortisol analysis was performed using an enzyme linked immunoassay kit (Oxford EA-65 Cortisol EIA Kit, Oxford Biomedical, Lansing, MI). Samples were re-assayed if the coefficient of variation between duplicate samples varied by >15%. Cortisol concentrations were reported in pg of cortisol per mg of hair.

4.2.4 Calf Standing Time

Accelerometers, fitted to a sub-sample of calves (n = 49/treatment) were used to measure lying and standing time from d 0 to d 14, as a behavioural measure of stress. Previous studies have shown that abruptly weaned calves spend more time standing following separation from the dam (Newberry and Swanson, 2008). Calves wearing accelerometers were chosen randomly via an online number generator and blocked by treatment and sex. Accelerometers were encased in foam padding and attached on d 0, to the lateral aspect of the right front metacarpal area using Tensoplast (BSN Medical Ltd, Pinetown, South Africa). The accelerometers were positioned so the x-axis was perpendicular to the ground pointing in the distal direction and the y-axis was oriented parallel to the ground pointing in the dorsal direction. HOBO Onset Pendant G Data

Loggers have been validated to accurately measure standing time when measured in intervals greater than 30 s (Ledgerwood et al. 2010), therefore accelerometers were set to log coordinates every 60 s, which allowed battery life and data collection to extend for the entire 14 d period. Accelerometers were removed from the calves on d 14.

Standing and lying time was assessed in min day^{-1} from the data recorded by accelerometers following the protocol described by Bonk et al. (2013), where an x coordinate ≤120° recorded by the accelerometer was considered lying. Discrimination between walking and standing was not possible, since both behaviours could yield the same coordinates on the accelerometers, so any upright position was tallied as standing in this study. Over the period of the study, four accelerometers were lost (TS n = 3; AW n = 1). Therefore, standing time could only be analysed for 94 of the 98 calves originally fitted with accelerometers.

4.2.5 Chute Exit Speed

Chute exit speed was recorded on all calves as a potential measure of temperament (Vetters et al. 2013). More reactive animals have been shown to have faster chute exit speeds correlated with higher cortisol concentration (Curley et al. 2006), thus it was predicted in this study that calves with higher chute exit speeds would have higher HC concentrations. Calves were recorded exiting the chute with a video camera (Sony Handycam DCR-SR47, Sony Corporation 2009, Tokyo, Japan) on d 0 and d 7 of the study. Chute exit speed (m s^{-1}) was defined by the time it took for calves to traverse a fixed distance (3.05 m) upon exiting the chute, using the calf's nose as marker across the defined points. All video was watched by a single reviewer to minimize observer variability. Data collected on d 0 of the study had to be discarded due to camera malfunction. Therefore, statistical analysis of chute exit speed was performed on data collected on d 7.

Table 4.1 Calf demographics at day 0 of study and experimental groupings. Age and weight are listed as mean ± S.D.

Item	AW	TS
n	80	81
Weight (kg)	242 ± 39.6	250 ± 41.2
Age (d)	187 ± 14.9	185 ±16.1
Heifers, *n*	38	40
Steers, *n*	42	41
Hobos, *n*	49	49
Hair analysis	35	35

4.2.6 Statistical Analysis

Data analysis was performed using commercial software (Stata® 12, Software, StatCorp. LP, College Station, TX, USA). A backwards stepwise linear regression was used to determine significant variables relating to HC d 0, 7 and 14. Individual variables were screened via bivariate associations with HC d 0 or d 14, any variable with $P \leq 0.20$ was included in the backwards stepwise linear regression. Variables tested within the backwards stepwise linear regression (Appendix A) included treatment, age, sex, chute exit speed and multiparous cow vs. primiparous heifer. Once significant variables were identified they were then included into separate mixed model linear regression (MMLR) for d 0, 7 and 14 (Table 4.2). To determine effects on HC at d 0the MMLR included variables treatment, sex, age and chute exit speed. Treatment effects were investigated at d 7 and d 14, with d 0 HC included in the model as a covariate. The MMLR for d 7 only explored the effect of treatment on HC, with no additional variables in the model. For d 14 HC, the model included the variables treatment and sex only. Variables in the MMLR were checked for collinearity. Assumptions of the models were assured

by testing the residuals of all models independently for skewness and kurtosis, as well as the Shapiro-Wilks test for normality.

Two-sample t-tests were used to determine difference between treatments for standing time, chute exit speed and average daily gain (ADG). Standing time was analyzed individually by day, d 0-14. The ADG was calculated for time periods d 0-7, d 7-14 and d 0-14, to explore differences in the periods of measurement. Prior to application of two-sample t-test, all data was checked for normality using the Shapiro-Wilks test. Statistical significant was set to a *p*-value of ≤ 0.05.

4.3 Results

4.3.1 Hair Cortisol Concentration

Results from individual MMLRs show there were no significant differences in HC between treatment groups on d 0 or d 7 (Figure 4.1). However, at d 14 HC was significantly greater in TS than AW calves. Factors that had a significant relationship with the concentration of HC at d 0 included, age (coef = -0.01, 95% CI = -0.01 - -0.001, $P = 0.022$), chute exit speed recorded on d 7 (Figure 4.2) and sex (coef = 0.75, 95% CI = 0.53 – 0.96, $P < 0.001$). Steers had significantly greater HC than heifers on d 0 and d 14 (Figure 4.3). No other variables impact d 7 HC.

4.3.2 Standing Time

There was no difference in total standing time between treatment groups on days 0-6 of the study. On d 7, the day of weaning, and d 8, the day following weaning, AW calves spent significantly more time standing than TS calves (Figure 4.4). By d 9, standing time returned to pre-separation standing time values with no significant difference between groups. However, TS

calves stood more than AW calves on d 10 and d 11. Standing time did not significantly differ between treatments on d 12 – 14.

Table 4.2 Final HC concentration MMLR at d 0, 7 and 14

	coefficient	95% CI	*P*
D 0			
Treatment	0.17	-0.05 – 0.40	0.134
Sex	0.75	0.53 – 0.96	<0.001
Age (d)	-0.01	-0.01 - -0.001	0.020
Chute exit speed (m s^{-1})	-0.09	-0.17 - -0.01	0.025
D 7			
Treatment	-0.09	-0.24 – 0.07	0.264
D 14			
Treatment	0.22	0.03 – 0.41	0.008
Sex	0.31	0.08 – 0.55	0.024

Final MMLRs included treatment and significant variables as determined by backwards stepwise linear regression. D 7 and d 14 included HC d 0 as a covariate

4.3.3 Chute Exit Speed

Chute exit speed, measured on d 7 following release of individual calves from the headgate, was significantly greater ($t(159)= -2.48$, $P = 0.014$) in TS (2.53 ± 0.15 m s^{-1}, mean ± S.E.) than AW calves (2.05 ± 0.13 m s^{-1}).

4.3.4 Average Daily Gain

There were no significant differences in ADG between treatment groups at any time period (Table 4.3).

Table 4.3. Average daily gain (ADG, kg d-^{1}). Values are presented as mean ± S.E.

	Treatment			
ADG	AW	TS	SE	*P*
D 0-7	0.79	0.77	0.10	0.92
D 7-14	1.4	1.45	0.10	0.79
D 0-14	1.09	1.11	0.07	0.79

4.4 Discussion

Results from this study revealed that calves weaned in the TS group had significantly higher HC than AW calves on d 14, as measured in hair regrowth. A higher HC concentration indicates TS calves experienced a greater stress response over the two week period of the study. Exploring the behavioural response, the increased standing time found in AW calves on d 7 and 8 (the days following separation of the calves from dams), suggests AW calves were more acutely stressed by separation from the dam, as determined by previous studies indicating AW calves experience a more severe stress response at this time (Haley et al. 2005; Loberg et al. 2008). Considering the current literature and the methodologies of the two weaning strategies tested, the HC results from this study may not be that surprising. It is recognised that HC is less sensitive to acute stressors than long-term stressors (Davenport et al. 2006; Ashley et al. 2011), therefore it is important to interpret HC data with these considerations. The HC at d 14 measured systemic HPA activation from d 0-14 of the study when hair was in the active growth phase (Macbeth et al. 2010). Although TS calves have greater overall HC, it does not necessarily indicate a more severe stress response. The TS calves wore nose-flaps from d 0 – 7, likely creating seven days of stress not experienced by AW calves. Therefore, by weaning calves in two stages, it potentially extended their stress response to weaning over the entire 14 day period, including seven days wearing the nose-flap and being deprived of milk prior to physical

separation from the dam. Comparatively, AW calves experienced weaning stress up to a total of seven days, following physical separation from the dam. The standing time results suggest that following separation from the dam, both groups had an increase in stress response, as seen by increased standing time. However, the AW calves showed a greater acute behavioural stress response, following separation from the dam, that reduced by day 9. Conversely, the standing

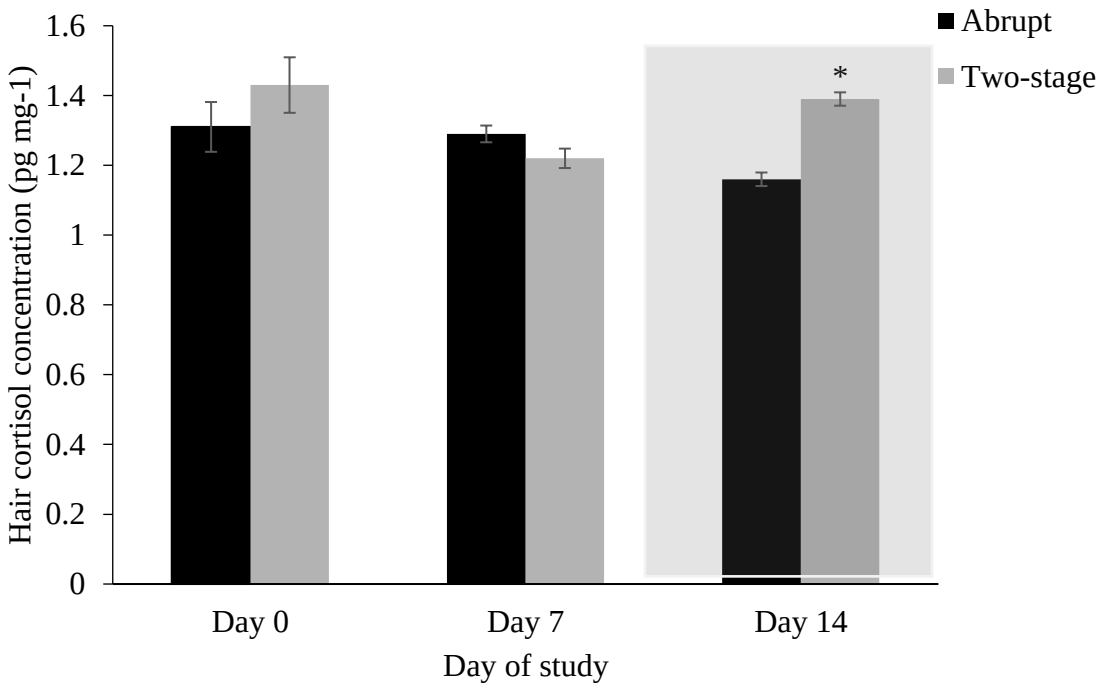

Figure 4.1 Mean HC concentration (pg mg^{-1}) of AW and TS calves, on each day of hair collection. Samples collected on d 0 and 7, included the full hair shaft while d 14 samples included 2 weeks of regrowth over study period. Values are presented as LSM ± S.E.. Where asterisk appear, * = $P < 0.05$

time results of TS calves, suggest that TS calves experience a less severe acute stress response than AW calves. However, TS may experience a stress response lasting for a longer duration created by the two stage process, and this may be responsible for higher HC levels observed in TS calves. If AW calves experienced a more acute stress response to weaning, as the behavioural

data suggests, it may not be possible to determine from the HC how strong an acute stress response the AW calves had if it was too short in duration to be captured in the hair growth.

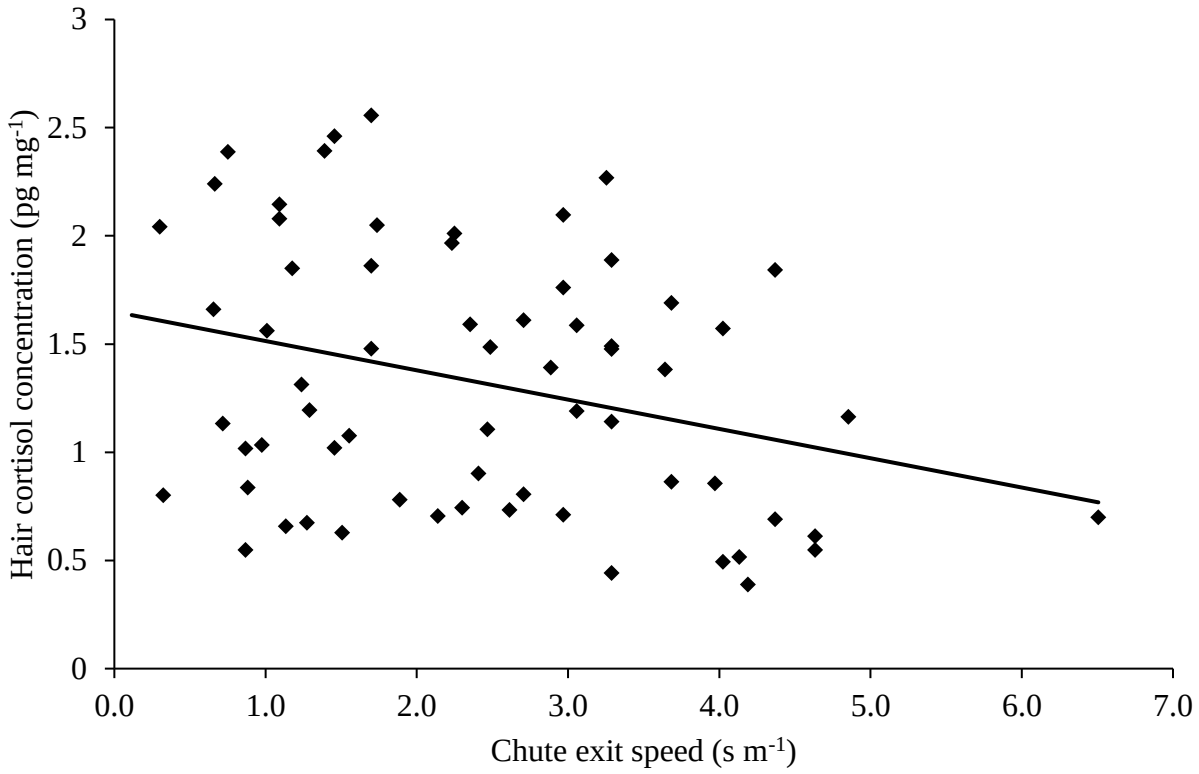

Figure 4.2. Chute exit speed and d 0 HC concentration relationship. Higher HC had faster chute exit speeds, as HC decreased chute exit speed decreased (coef = -0.09, S.E. = 0.04, 95% CI = -0.17 - -0.01, *P* = 0.025). Results are from the d 0 MMLR, raw values are presented.

Circulating, unbound cortisol is incorporated into the hair shaft while it is in the active (anagen) growth phase and HC is not necessarily related to the maximum cortisol secretion but rather the amount of time plasma cortisol is raised. Therefore, prolonged stressors will have a higher impact on HC concentrations than acute stressors (Ashley et al. 2011). Time spent standing was greater in AW than TS calves the first two days following physical weaning from the dam, indicating a greater stress response to physical weaning than TS calves. Increased standing time is used as an indicator of stress following weaning because it represents the time calves spend searching for their dam in attempts to reunite (Enríquez et al. 2010). On the third

and fourth day following weaning, AW calves stood for significantly less time than TS calves. This could likely be due to exhaustion from walking following weaning or due to the fact that behavioural responses to a stressor cease on the third day following the stressor, even if physiological changes continue (Buwalda et al 1997; Morimoto et al. 1993; Salak-Johnson et al. 1997).

Even though TS calves wore nose flaps d 0 – 7 of the study, there were no treatment differences in HC concentration on d 7. However, hair collected on d 7 included the full hair shaft, and thus contained cortisol levels from before the period of study. Hair samples collected on d 14 were two weeks of regrowth from the d 0 sample site. Hair growth is cyclical and undergoes periods of growth (anagen), transition (catagen) and rest (telogen) (Gratacós-Cubarsí et al. 2006). In order for cortisol to be incorporated into the hair shaft it must be actively growing in the anagen phase (Harkey, 1993; Wenning 2000). If hair in the d 7 collection sample was not actively growing at the time of the stressor, it is likely that cortisol was not incorporated into the hair shaft. Clipping hair on d 0 stimulated growth in the collection area, because hair was actively growing, circulating, unbound cortisol over the 14 d period of the study was incorporated into the hair shaft. Additionally, the inability to see a treatment difference at d 7 could be due to a dilution effect. A stress response occurring over a few days is likely not visible within the entire hair shaft (González de la Vara et al. 2011). If repeating this study, using hair regrowth to measure the d 7 sample would be desirable, to try and capture the stress response to insertion of nose flaps and cessation of milk, as tracked through cortisol stored in fresh hair growth. Additionally, collecting more behavioural measures, such as vigilance behaviours, vocalization and distance walked, following weaning could be directly compared to HC between all subjects and would strengthen the study.

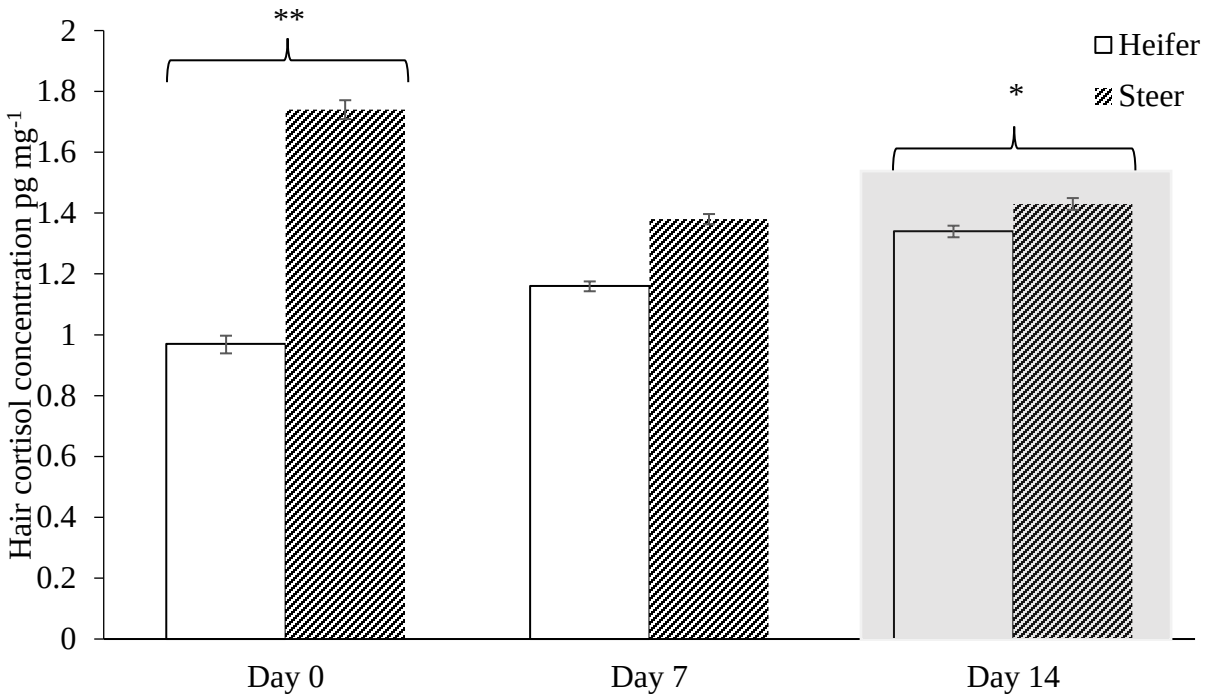

Figure 4.3 HC concentration (pg mg^{-1}) by sex on each day of hair collection. Hair samples collected on d 0 and d 7 included the full hair shaft while hair collected at d 14 included two weeks of regrowth. Values are presented as LSM ± S.E. Where asterisk appear, * = $P < 0.05$, ** = $P < 0.01$

Previous studies have documented that additional variables such as hair colour (Bennett and Haysson, 2010), body region (Moya et al. 2013) and multiparous cow vs. primiparous heifer (Burnett at al. 2014; Burnett et al. 2015) influence HC concentration. Steers had greater HC than heifers on d 0 and d 14, which is likely due to physiological differences between genders. No research has been conducted to explore the impact of gender on cortisol levels in beef calves, and future studies are warranted to establish its significance. Additionally, as seen in Chapter 3, there was a negative relationship between age and HC at d 0; younger calves had higher HC compared to older calves. In this study, d 0 hair samples included the entire hair shaft documenting the age effect. González de la Vara and coworkers (2011) showed that 15-day old heifers had HC concentrations approximately 10 fold higher than 2 year old cows, likely due to their proximity to birth. Cortisol is crucial to fetal organ development, and a surge of fetal cortisol initiates

parturition (Flint et al. 1979; Mastorakos and Illias, 2003). As the animal ages and hair growth occurs, HC decreases due to dilution from hair growth without cortisol increasing in the hair shaft.

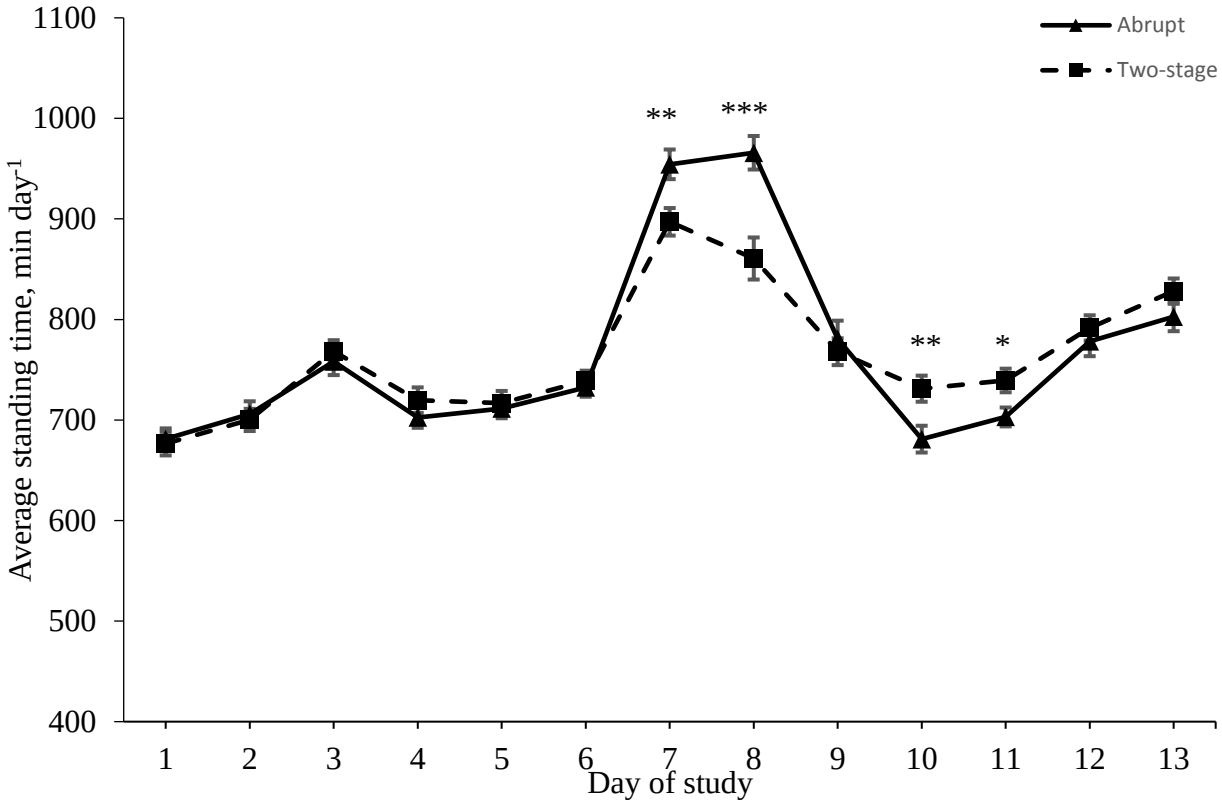

Figure 4.4 Total average standing time (min day^{-1}) per day of study. Values are presented as mean ± S.E. Where asterisk appear, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.

A significant relationship was observed between chute exit speed recorded on d 7 and d 0 HC, calves with higher HC at d 0 had faster chute exit speeds and calves with lower HC had slower chute exit speeds. This is most likely due to a difference in temperament with more reactive calves having faster chute exit speed and higher cortisol levels and calmer calves have slower chute exit speed and lower cortisol levels (Curley et al. 2006; Café et al. 2011). Additionally, treatment differences in chute exit speed were observed. TS calves had slower chute exit speeds on d 7 than AW calves. It is possible, TS calves had slower chute exit speeds

on d 7 than AW calves on d 7 because they were already weaned from their dam and separation at the time of hair collection was more stressful for AW than TS calves as they were not accustomed to being separated from their dam. Unfortunately, the loss of exit speed data on d 0, was not available to help explain the observed differences in treatment groups.

4.5 Conclusion

When measured in active hair regrowth, this study revealed greater HC concentration in TS than AW calves at d 14. This suggests that TS calves experienced stress for a prolonged duration over the 14 d period and this was able to be captured in the active hair growth phase. The lack of difference in HC between treatments at d 7 may have resulted because not all of the hair was actively growing therefore cortisol was not incorporated into the hair shaft. Furthermore, hair collected at d 7 included cortisol accumulated prior to the study, creating a dilution effect where cortisol changes from weaning methods would have been too small to observe in the full length hair shaft. The behavioural data was suggestive that AW responded with a stronger acute stress response following separation from the dam that TS calves. Considering the behavioural standing time results alongside the HC, total accumulated HC appears to be more sensitive in detecting long-term, rather than acute stress, when comparing AW and TS treatments because cortisol is present in the blood stream over a greater time period and is gradually accumulated into the hair shaft as growth occurs. Future studies are warranted to establish the significance of many variables affecting HC concentration, such as gender, age, hair colour, calf temperament and stage of the hair cycle.

5 GENERAL DISCUSSION AND CONCLUSIONS

The objective of this thesis was to investigate the use of HC as means to measure the long-term physiological stress response in beef cattle. An objective measure of long-term stress in beef cattle will be of use in future applied research to better understand how management practices affect beef cattle.

Castration is a routine procedure in beef cattle production. Various methods of castration exist, but all cause pain and therefore, are of concern for calf welfare. Castration is known to generate a stress response and as such can be used to model stress (Early and Crowe, 2002). In Chapter 3, castration was the applied stressor used to evaluate hair cortisol. Results found that the concentration of cortisol measured from a 14 day period of hair regrowth following castration was significantly higher in surgically castrated calves (no pain control), compared to sham castrated calves, and tended to be higher than calves castrated with meloxicam at the time of castration. In addition, there was no difference in hair cortisol concentration between sham castrated calves and those castrated with administration of meloxicam. Handling and castration are separate sources of stress on beef calves, however, it is expected that castration would cause a long-term stress response due continuous clinical pain, in part caused by inflammation. Sham castration is a short term stressor caused by handling which ends after release from the chute. Castration with meloxicam would likely have a rise in cortisol, as calves were surgically castrated, however, hair cortisol concentrations in calves castrated with meloxicam were not different from sham castrated calves suggesting meloxicam has positive effects on reducing the stress response, likely due to decreased post-operative pain and inflammation. This finding is of great importance to the beef industry, particularly so, as the Code of Practice for the care and

handling of beef cattle requires the administration of pain control when performing castration on bulls (NFACC, 2013).

In this study, meloxicam was administered at the time of castration, improving labour efficiency, while still effectively mitigating post-castration stress. Also noteworthy in the current study is the fact that the tendency for a reduction in hair cortisol in calves castrated with administration of meloxicam, demonstrates that the meloxicam must have reduced calf stress for an extended duration of time in order to reflect as a reduction in HC. Total standing time in calves starting on the day of castration through day four following castration, did not show any differences between treatments. Beef cattle are prey species and therefore try to minimize pain related behaviours that may decrease their likelihood of survival. It may be that castrated calves tried to minimize pain related behaviours following castration. However, other behavioural measures such as abnormal standing posture (Molony et al. 1995; Ting et al. 2003a) and tail movement (Robertson et al. 1994) may be more sensitive and specific measures of pain in beef calves compared to standing time. Going forward, these findings will add to the support of meloxicam in improving the welfare of bull calves at the time of castration.

Weaning was chosen as a different applied stressor to further evaluate hair cortisol in Chapter 4. Abrupt weaning is the method of weaning commonly employed in commercial practice and is known to be a highly stressful event for calves as they are abruptly and simultaneously removed from milk and the dam. Previous studies (Haley et al. 2005; Enriquez et al. 2010), showed that behaviourally, two-stage weaning appears to be a much less aversive method than abrupt weaning. However, no physiological measures were collected by Haley et al. (2005). Results from Chapter 4 showed that the cortisol concentration measured in 14 days of hair regrowth was greater in calves weaned via two-stage weaning compared to abruptly weaned

calves. At day 14, calves in both groups had been separated from the dam for seven days, with calves in the two-stage group having not suckled from the cow for an additional seven days prior to separation from the dam. Higher hair cortisol at day 14 in TS calves compared to AW calves is indicative of greater HPA axis activity, over the two week period prior to collection of hair regrowth, which could be a stress response of lower magnitude that continued for an extended period of time as indicated by behavioural data following weaning which found abruptly weaned calves spent increased time standing for the two days immediately following separation of the cow-calf pair. Increased standing time represents increased walking following separation (Haley et al. 2005), as the coordinates of the accelerometers could not distinguish between walking and standing. This suggests abruptly weaned calves suffered a more severe, acute stress response to physical separation from the dam. Acute stressors do not influence hair cortisol concentration in the same manner as long-term stressors because total hair cortisol is influenced by the amount of time cortisol is present in the bloodstream rather than total plasma cortisol concentration. It is likely that the greater hair cortisol concentration in two-stage weaned calves is due to a gradual accumulation of cortisol in the hair regrowth over the 14 day period of the study due to two-stage weaning being a lower magnitude stress that occurred over a longer period, including the seven days pre-weaning during the wearing of nose flaps and cessation of suckling and post-weaning following separation from the cow. Findings from this study further suggest that hair cortisol is more sensitive to long-term than acute stressors.

The findings detailed in Chapters 3 and 4, suggest that hair cortisol has great promise to be a useful measure of long-term HPA axis activity in beef calves. Explored under two applied stressors commonly experienced by calves in commercial beef production, treatment differences in hair cortisol concentration were detected. In Chapter 3, differences in hair cortisol occurred in

a logical manner with the highest concentration in the most severe treatment group. In Chapter 4, treatment differences in hair cortisol illuminated effects of two-stage weaning on calves which had not yet been determined. The rise in hair cortisol in both studies validates hair cortisol as a measure of HPA axis activity in beef calves. However, it is important to keep in mind the number of variables which influence hair cortisol concentration. Chapters 3 and 4 identified differences in HC due to calf age, sex, dam parity, temperament as tested by chute exit speed and location or breed. Having taken these variables into consideration, it was possible to observe treatment differences. However, it can be assumed this may not be the case if these variables are not considered. In the future, more research will be needed to further investigate the variables which influence hair cortisol.

The studies outlined in Chapters 3 and 4 could certainly be improved through the addition of more outcome measures to each study. For example, collection of additional behavioural measures such as distance walked, vocalization and eating bouts could have provided more information to understand the relationship between behavioural and physiological responses to stress. As was found in Chapters 3 and 4, collection of hair regrowth is the best measure of hair cortisol in period which hair was actively growing, otherwise, detecting a change in hair cortisol while collecting hair from a period before the study began is not possible. Thus, it is important when studying applied stressors to collect hair regrowth occurring over the period of interest in order to document HPA axis activity. In the future, it may also be beneficial to collect hair regrowth prior to stressor application in order to gain a better understanding of baseline hair cortisol and if changes following stressors are biologically significant. Following this research, future studies should investigate the impact of variables on hair cortisol concentrations as well as the effect of handling on immediate changes in hair cortisol. As a measure of long-term stress,

hair cortisol has a broad application to investigate the impact of management practices on beef cattle and how they may be improved. Overall, the results of this thesis research provide evidence to suggest great promise for the use of hair cortisol as a tool to evaluate long-term stress in beef cattle.

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APPENDIX A

Table A.1 Statistical significance of all variables included in d 0 and 14 backwards stepwise linear regression for HC concentration in response to castration

Variables	d 0	d 14
Treatment		
CM	NS	0.059
S	NS	0.025
Age	<0.001	NS
Heifer vs. cow	NS	0.005
Dam Age	NS	NS
Chute score	NS	NS
Chute exit speed	NS	NS
Farm/breed	NS	<0.001

Treatment CM and S used CS as reference. *P*-values are presented

Table A.2 Statistical significance of all variables included in d 0, 7 and 14 backwards stepwise linear regression for HC concentration in response to weaning

Variables	Cortisol d 0	Cortisol d 7	Cortisol d 14
Treatment	NS	NS	0.02
Age	<0.001	NS	NS
Heifer vs. cow	NS	NS	NS
Sex	<0.001	NS	0.008
Chute exit speed	0.025	NS	NS

P-values are presented