

THESIS

EVALUATION OF DIFFERENT HAIR CHARACTERISTICS AND THE IMPACT OF
LIVER ABSCESS PRESENCE ON STRESS RELATED PHYSICAL AND PHYSIOLOGICAL
PARAMETERS ASSOCIATED WITH WELL-BEING IN BEEF FEEDLOT STEERS

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In partial fulfillment of the requirements

For the Degree of Master of Science

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Fort Collins, Colorado

Summer 2019

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ABSTRACT

EVALUATION OF DIFFERENT HAIR CHARACTERISTICS AND THE IMPACT OF LIVER ABSCESS PRESENCE ON STRESS RELATED PHYSICAL AND PHYSIOLOGICAL PARAMETERS ASSOCIATED WITH WELL-BEING IN BEEF FEEDLOT STEERS

Physiological and behavioral parameters are commonly used to assess cattle welfare. The overall objective of these studies was to understand the impact of animal-based characteristics and the presence of a metabolic disease on the overall well-being of beef feedlot steers through the measurement of physical and physiological parameters.

The objective of Experiment 1 was to determine the impact of hair color and length, and animal age on hair cortisol concentration in beef feedlot steers. Nineteen beef crossbred steers were used for this study. Seven of the steers ($1,043 \pm 6.8$ kg; approx. 9 years of age) were fitted with ruminal fistulas and duodenal cannulas and classified as old steers. The other twelve steers (680 ± 4.5 kg; approx. 2.5 years of age) were fitted with only ruminal fistulas and classified as young steers. One steer was euthanized due to health problems within the first week of the study. Steers were categorized into one of three groups: old with black hair (OB, $n = 3$); old with white hair (OW, $n = 3$); young with black hair (YB, $n = 12$). Hair samples from the right rump region of each steer were collected throughout a period of six weeks from six different areas. Only samples collected during Week 6 were used for analyses. Older steers exhibited greater hair cortisol concentrations than younger steers ($P < 0.001$). The white hair of the old steers yielded higher concentrations of hair cortisol as compared to black hair from young steers (OW = 10.89

± 2.03 pg/mg and YB = 0.98 ± 0.11 pg/mg, respectively; $P < 0.001$). Hair cortisol concentration was not impacted by duration of growth ($P = 0.33$). However, cortisol concentrations exhibited a weak, positive correlation with hair length ($r = 0.33$, $P = 0.01$). Additionally, the average hair growth per week of beef steers in the winter months was calculated to be 0.90 mm. Further research should be performed to improve our understanding of the effect of hair characteristics on hair cortisol concentrations as related to the well-being of cattle.

The objective of Experiment 2 was to evaluate the relationship between liver abscess presence and stress-related parameters in beef feedlot cattle, utilizing both physiological (hair and serum cortisol, ocular temperature) and behavioral measurements (mobility scoring). The ultimate goal of the study was to establish an initial understanding of the welfare state of cattle with liver abscesses so that management practices can be maintained or changed to allow for the production of cattle to be continually practiced in an efficient and sustainable manner. Three hundred and sixty-three beef breed, *Bos taurus* feedlot cattle were categorized by the liver abscess score assigned during the slaughter process. The liver abscess scoring groups were: no liver abscess presence (NLA; $n = 316$); mild liver abscess presence (MLA; $n = 21$) and severe liver abscess presence (SLA; $n = 24$). Two animals were unable to be assigned liver abscess scores. No relationships were identified between the presence of liver abscesses and the measured parameters – ocular infrared thermography ($P = 0.55$), hair cortisol ($P = 0.96$) and serum cortisol ($P = 0.21$). Furthermore, hair color was not statistically significant when compared across liver abscess scores ($P = 0.70$). All animals exhibited normal mobility with no visual signs of lameness. The data indicate that under the conditions of this experiment, where adhesions to the body wall were not tabulated, liver abscesses did not impact measured stress-

related outcomes. Additional research is necessary to understand the impact of liver abscess presence on other stress-related parameters associated with well-being in cattle.

Further research should be performed to increase our understanding of feedlot cattle welfare, in relation to all associated factors – health and disease, environment, and management-related. Both studies also concluded that future research should be focused on establishing benchmark values of hair cortisol concentration for other applications and validations such as hair sampling techniques, metabolite analysis methodology, and potential health indicators.

ACKNOWLEDGEMENTS

My journey as a graduate student has been one of trials and tribulations. I wouldn't be where I am today without the people and experiences that I have had the opportunity to learn from along the way.

I would like to thank my advisor, Dr. Temple Grandin, for always providing intelligent insight and a visual big picture perspective for research, science and life. I enjoyed our graduate student lunches that were filled with important life lessons, recent news stories and animal science discussion.

I would like to thank my co-advisor, Dr. Lily Edwards-Callaway, for helping me throughout the past two years. I greatly appreciate your guidance, trust and willingness to answer any questions that I had.

I would like to thank Dr. Terry Engle for providing a voice of reason and a realistic perspective. Your passion for your work and true care for students is something that I will always remember.

I would like to thank Dr. Bernie Rollin for broadening my outlook and exposing me to new perspectives concerning agriculture and life.

Thank you to my entire committee for setting inspiring examples of the type of scientist, mentor and person I wish to become.

Throughout my research at Colorado State, I have had the honor of getting to know and enlisting the help of many students. Thank you to all who helped with my data collections in various environments, especially in the frigid temperatures of a Colorado winter – all for cattle

hair! Thank you to Morgan Schaeperkoetter for enduring graduate school alongside me, reminding me to enjoy life and teaching me something new each day. Thank you to Dennis Willson for asking thought provoking questions and keeping things simple. Most of all, thank you to Arquimides Reyes for providing a listening ear, constant support and understanding, and reminders of the big picture as needed.

Lastly, I would like to thank my family. Without you, none of this would have been possible. Your constant support, guidance and understanding (especially making the big move to Colorado) has been what kept me moving forward. Thank you to my father for instilling a deep appreciation for animals and agriculture, as this is what fuels my goals and passion to this day. Thank you to my sisters, Brook, Megan and Shae, for making me smile each day and offering endless support and love. Thank you to my mother for being one of the strongest, most hardworking and caring people I know – you set the example for the person that I strive to become.

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CHAPTER I

INTRODUCTION

Today's beef industry continues to strive for a balance between efficient and quality production, consumer transparency, positive animal welfare practices and sustainability for the future. Animal husbandry has been at the forefront of farmers' and ranchers' livelihood for generations throughout the evolution of the livestock production industries. The current, targeted focus on animal welfare related to the management of livestock species is being driven by the interest and curiosity of consumers insistent to be aware and informed of all events from farm to fork. This cumulation of forces, including both producer and industry integrity, as well as, consumer demand, has made the well-being of animals a topic for continuous research.

The understanding and quantification of animal well-being can be difficult due to the subjective nature of the field. The methodology used to measure animal welfare should be practiced in an as objective way as possible through the utilization of trained observers, clearly defined behavioral observations and physiological basal levels, and repeatable reliability. Given the subjective nature, a broad-spectrum approach is often utilized when measuring animal well-being to provide an extensive perspective as to the animal's state in relation to the environment.

Additionally, beef producers, specifically feedlots, have been dealing with several management-derived issues, known as production diseases. Liver abscesses have been deemed one of the production diseases. Abscessed feedlot cattle have incurred nutritional, production, and carcass quality losses. The exact etiology of this disease is unknown, along with the many long-standing and undiscovered impacts. The presence of disease affects many aspects of an

animal, including the overall well-being. The body of knowledge related to the well-being of feedlot cattle focused on the impact of production diseases and liver abscesses is rather small as compared to the resulting impact that liver abscesses have had on the beef industry.

Therefore, the overall objective of this body of research is to understand how physical characteristics of an animal and collected cattle hair, as well as, the presence of a production disease, specifically liver abscesses, impact the stress-related parameters associated with well-being in feedlot cattle. From a broad perspective, this research was designed to assess the overall welfare state of feedlot cattle, while advancing knowledge pertaining to a novel biomarker of chronic stress, known as hair cortisol.

CHAPTER II

LITERATURE REVIEW

2.1 Beef Cattle Welfare

Animal welfare is a critical aspect of the cattle industry. Animal welfare is defined as “the state of an individual in relation to its environment” (OIE, 2018; AVMA, 2019). An assessment of animal welfare is usually based on the examination of an animal’s mental state (Dawkins, 1990; Duncan and Petherick, 1991), physical state (Broom, 1996), the human-animal interaction (Tannenbaum, 1991; Fraser, 1995), natural living and behavior (Fraser et al., 1997), and/or physiological parameters (Wilson et al., 2002). Several frameworks such as the Five Freedoms (FAWC, 2009), Fraser’s Three Circles (Fraser et al., 1997) and Five Domains (Mellor et al., 2016) are often used to help assess animal welfare. The criteria for assessing animal welfare is commonly divided into factors that are associated with the animal’s environment (input-based measures) and factors that are associated with the state of the animal (outcome-based measures) (Rushen and de Passillé, 1992). Input-based measures related to beef cattle may include space allowance, group size, water and feed availability, diet type, and shelter access. Outcome-based measures related to beef cattle may include body condition score, disease incidence, lameness or mobility score, vocalization, and physiological parameters (metabolite concentrations, heart rate, respiratory rate, etc.). Essentially this approach allows for humans to “let the animal tell the story” (Vogel, personal communication), which is a critical piece to assessing, interpreting and understanding animal welfare.

2.1.1 Well-being of Feedlot Cattle

Raising beef cattle for meat production is a large sector of the animal production industry. Approximately 12 million head of cattle are housed in feedlots of 1,000 or more head, as reported by the USDA National Agricultural Statistics Service (NASS) (USDA-NASS, 2019). Beef Quality Assurance (BQA) is a U.S. based program that serves to increase consumer confidence by way of educating and upholding a commitment for quality throughout the entire beef industry (BQA, 2019). The BQA Feedyard Assessment is used as a general production and animal welfare benchmarking tool for feedlot managers and producers in the United States. Woiwode et al. (2016) found that there is a high compliance rate with the BQA Feedyard Assessment across the U.S. Furthermore, the implementation of routine assessment and monitoring could lead to even greater changes pertaining to feedlot cattle handling practices (Woiwode et al., 2016). According to Grandin (2014), two of the biggest welfare issues in feedlots include muddy pen conditions and heat stress. Other areas involving beef cattle and feedlot related welfare issues are handling practices, transport, stocking densities, and metabolic problems from high-grain diets (Rushen et al., 2009), as well as, protection from cold stress, shade provisions, and water requirements (Grandin, 2016).

With a large population of cattle being raised in a feedlot environment, a multitude of research has been performed concerning the well-being of feedlot cattle. Studies have focused on many welfare related topics, including cattle mobility (Thomson et al. 2015; Edwards-Callaway et al., 2017), handling and acclimation techniques (Noffsinger et al., 2015), hot weather and heat stress (Dikmen et al., 2012; Gaughan and Mader, 2014), preconditioning management (Hilton, 2015), lying behavior (Wagner, 2016), pen cleanliness and mud (Mader, 2011), space requirements (Keane et al, 2018), effect of shade (Sullivan et al., 2011; Hagenmaier et al., 2016),

effect of wind (Mader et al., 1999), transport (González et al., 2012), foot disease (Kulow et al., 2017), and other common nutrition and health-related diseases (Galyean and Rivera, 2003; Avra et al., 2017).

As previously mentioned, behavioral and physiological parameters are common methods used to assess welfare. Physiological measures are often used to diagnose or measure various welfare issues in feedlot cattle, such as heat stress and lameness. Wilson et al. (2002) highlighted specific physiological metrics that are applicable for assessing the welfare of beef cattle in feedlots, which included adrenal weight, adrenal index, and immunological variables (serum immunoglobulin A and WC1+). Another study found that several biomarkers, serum amyloid-A and haptoglobin, were greater in lame cattle than in non-lame feedlot, which shows that these parameters may be useful for lameness identification and mitigation (Marti et al., 2018). Marti et al. (2018) reported that biomarkers of stress, specifically hair cortisol, serum cortisol and rectal temperature, were unsuccessful when used to differentiate between lame and non-lame cattle. Body temperature can be used to diagnose heat stress in cattle (Davis et al., 2003; Mader, 2003). Panting scores are generally used to assess heat stress in cattle (Mader et al., 2006; Gaughan and Mader, 2014); however, this method is known to be quite subjective (Unruh et al., 2016). The objective heat load measurement in cattle has been successfully assessed using infrared (IR) thermography technology, though this technology has not been demonstrated as effective in predicting heat stress (Unruh et al., 2016). The plasma concentration of secreted heat shock protein 70 was found to be a reliable indicator of chronic stress in feedlot cattle (Gaughan et al., 2013). Moreover, Tucker et al. (2015) identified the topics of acidosis and liver abscesses as an area of future research related to beef cattle welfare.

2.1.2 Well-being of Research Fistulated Cattle

Fistulated cattle are used for many applications in research. Studies with fistulated or cannulated cattle are generally focused on nutrition and digestion in both beef (Beauchemin et al., 1994) and dairy (Dittmann et al., 2016) cattle. Other specific applications have involved protein metabolism (Jin et al., 2018), rumen bacterial communities (Hernandez-Sanabria, 2012) and their regulation abilities (Il'shat et al., 2017), along with mineral supplementation (Engle and Spears, 2000). Although this type of research involves an initial invasive surgical insertion technique to establish the fistula, the use of fistulated cattle for research has led to an improved understanding of rumen nutrition and microbiology. The impact of fistula presence on the welfare of cattle is a topic that has not been researched, with the exception of some studies briefly alluding to the welfare of cattle post-surgery. In one such study, over a period of three weeks after fistula surgery, Kristensen et al. (2010) evaluated dairy heifer calves using health and performance parameters to determine the effects of this surgical technique on calf rumen development. They found that the surgical technique did not have any effect on feed intake, body weight, and rumen characteristics.

2.2 Quantifying Stress

Stress is defined as a complex, multidimensional phenomenon promoted by several stressors or stimuli that causes behavioral and physiological responses with the intent to maintain body homeostasis (Moberg, 1985). This response can take place as two different forms of stress – eustress and distress. Eustress is generally perceived as a neutral or positive condition (Trevisi and Bertoni, 2009), whereas distress creates negative effects (Webster, 1983) and takes place when the animal does not adapt to the stressor resulting in a reduction in well-being (Broom, 2003). The type of stress that results is dependent upon the perception of the stressor by the

individual animal. For example, a stressor that an animal perceives as dangerous and having the potential to cause deleterious effects, such as failed reproduction (Moberg, 1987) and altered metabolism (Elsasser et al., 2000), is considered to be distress (Moberg, 2000). An animal may experience eustress during times of pleasure related to play and copulation reproduction behavior (Lay Jr., 2000). A stress response is known to activate two endocrine systems: the sympathetic nervous system (SNS) and the hypothalamic-pituitary-adrenal axis or system (HPA) (Moberg, 2000). Other pathways are often involved including the immune system and metabolic pathways (Trevisi and Bertoni, 2009). The type of stress – positive or negative – must be taken into consideration when quantifying stress.

2.2.1 Acute vs. Chronic

In addition, there are two types of stress related to the duration of the stressor's impact on the animal and its response. Acute stress is defined as a stress that persists for a duration of minutes to hours (short term), whereas chronic stress lasts for a duration of days to months (long term) (Dhabhar, 2000). Moreover, a short period of time involving a negative situation that permits a rapid, complete recovery of physiological balance constitutes acute stress, while a condition that does not allow full recovery resulting in a maladaptation is regarded as chronic stress (Trevisi and Bertoni, 2009).

The occurrence and physiological quantification of acute stress can be difficult since the resulting effect is a rapid biological response. Hormones are usually released after the stressor takes place further resulting in either rapid increased (catecholamines, vasopressin, glucagon, glucocorticoids, and adrenocorticotrophic hormone (ACTH) or decreased (serotonin) concentrations. (Trevisi and Bertoni, 2009). Other physiological and metabolic effects occur, such as increased heart rate, higher blood pressure, stimulation of immune function, and an

increased energy mobilization (Sapolsky et al., 2000). Alternately, chronic stress is related to a long-lasting state, which may be linked to long duration illness or poor health, that sustains a constant effect on the animal (Webster, 1994). The ability to measure stress over an extended period of time also proves to be difficult, especially related to establishing basal levels. Perhaps, the biggest problem with measuring stress, whether acute or chronic, is that of the individual animal variation of the stress response (Moberg, 2000). Many factors have been shown to influence an animal's perception of a stimulus, including early life experience (Boivin et al., 1994; Probst et al., 2012), age (Bretschneider, 2005), genetics (Grandin, 1992), social cohort relationships (Veissier and Le Neindre, 1992), and human-animal interaction (Lensink et al., 2000; Probst et al., 2013). The past experiences pertaining to the nature of stressor perception can often be more controlled in a laboratory environment that can shape most the animal's life; however, this becomes nearly impossible when concerning herds of livestock or non-domesticated, wild species (Moberg, 2000).

Common methods of quantifying stress include endocrine indicators, physiological indicators, performance and health indices, immune indices, and biochemical indicators (Trevisi and Bertoni, 2009). Thorough, consistent research has led to the consensus that specific biomarkers of glucocorticoids (cortisol) can be used to quantify acute stress in cattle (Hemsworth et al., 1989; McMeekan et al., 1998; Llonch et al., 2016). However, researchers and scientists are still working to find consistently reliable biomarkers for chronic stress in animals. A reliable biomarker of chronic stress “must lead to subtle and long-term changes in a physiological function even if individuals have seemingly accepted the condition of their lives” (Kelly et al., 1997). Recently, hair cortisol has been evaluated as a representation of chronic stress in cattle

other species, although study conclusions have been rather controversial (Comin et al., 2013; Moya et al., 2013; Heimbürge et al., 2018).

2.2.2 Cortisol

Cortisol is considered to be an important biomarker of stress. This glucocorticoid hormone is released via stimulation of the HPA axis (Figure 2.1). Initially, the brain perceives an external stimulus as a stressor, which further initiates a cascade of events (Matteri et al., 2000). After the perception of the stressor, the cells within the paraventricular nucleus (PVN) in the hypothalamus of the brain release a hormone called corticotropin releasing hormone (CRH) (Papadimitriou and Priftis, 2009). Then, the CRH travels through the infundibulum, the hollow stalk that connects the hypothalamus and the posterior pituitary gland, to the pituitary gland, where the secretion of adrenocorticotropic hormone (ACTH) is stimulated. The ACTH is released into the blood stream, within which it travels to the adrenal cortex. Within the adrenal cortex, the ACTH binds to specific receptors that further stimulate the secretion of glucocorticoids, specifically cortisol into the bloodstream to be circulated throughout the body (Papadimitriou and Priftis, 2009). Cortisol is intercepted by many cells within the body that have specific receptors, allowing for this hormone to have a broad variety of effects on the body, such as metabolic, physiologic and immunologic responses (Buckingham, 2006). The release of corticosteroids is a slow process that begins with an initial increase of the concentration in the blood and continues with a prolonged response for about an hour after the stressor event termination (Veissier and Neindre, 1992).

The use of cortisol in studies related to acute cattle stress has been quite extensive. Several mediums, including serum (Bristow and Holmes, 2007), plasma (Buckham Sporer et al., 2008), saliva (Negrao et al., 2004; Chacón Pérez et al., 2004), urine (Morrow et al., 2000) and

feces (Palme et al., 2000) are used to assess cortisol concentrations pertaining to acute stress in cattle. The use of cortisol to quantify chronic stress in cattle is still considered a rather novel method. Despite the fact that cortisol concentration increases are observed after acute stress, the concentrations often exhibit variations throughout distress that may relate to chronic stress (McEwen, 1998; Lay and Wilson, 2004).

2.2.2.1. Blood

Using blood to assess metabolite concentrations is an application that has been extensively performed in research. Blood is usually further processed so that cortisol concentrations can be quantified from a serum or plasma medium. Cortisol measurement has been applied to multiple acutely stressful events with cattle, including branding (Schwartzkopf et al., 1997), castration (Coetzee et al., 2008), weaning (Lefcourt and Elsasser, 1995), dehorning (Stafford et al., 2003), disbudding (Stilwell et al., 2010), restraint (Grandin, 1997), disease incidence (Nahed, 2010), transport, lairage time and stunning efficiency (Chulayo et al., 2016). The determination of basal blood cortisol must be performed with the consideration of many factors that can affect concentration, such as circadian rhythms (Möstl and Palme, 2002), sex (Doornenbal et al., 1987), handling (Hemsworth et al., 2011), restraint (Szenci et al., 2011), lactation (Fukasawa et al., 2008) and degree of habituation or acclimation (Hopster et al., 1999).

Blood cortisol is mainly used to quantify acute stress as opposed to chronic stress. This is due to three important issues that affect the ability to measure chronic stress in blood cortisol concentrations (Rushen et al., 2009). The first issue is in relation to methodology and hormone release. Cortisol is released by the adrenal gland in a pulsatile secretion that can change related to the amount that is released in a matter of a few minutes or hours (Ladewig and Smidt, 1989). According to our current scientific body of knowledge, there are no confirmed events that these

specific cortisol release pulses take place near specific daily events (i.e. feeding or milking), which increases the number of samples that should be taken throughout a day to control for the time of day effects (Rushen et al., 2009). As a general rule, the accurate assessment of acute stress requires that blood samples be taken throughout the entire period of stress, usually every 15-20 min (Rushen et al., 2009). The second issue is related to the reliability of the samples due to the fact that the overall average concentration of cortisol will be used to make conclusions pertaining to the effect of the stress. In a study evaluating the effect of lying deprivation and social isolation in dairy cows, Munksgaard and Simonsen (1996) found no effect based on treatment but did find that certain times of the day resulted in increased ACTH concentration, as well as, a reduced number of ACTH peaks in the cattle that were deprived of laying down for 14 hours per day for 3 weeks. Additionally, tethered young bulls exhibited higher average plasma cortisol concentrations, though the pulsatile release duration and frequency increased as well (Ladewig and Smidt, 1989). This shows that a potential way to assess chronic stress may be to focus on the cortisol pulsatile release patterns as opposed to the average concentrations over time (Rushen et al., 2009). The third issue is the event of acclimatization to the stressor over an extended period of time. For example, Ladewig and Smidt (1989) observed that the cortisol secretion increased during initial tethering, but then disappeared after one month. The sensitivity of the HPA axis over a long duration of a specific stressor or combination thereof may have a critical impact on the plasma levels of cortisol.

2.2.2.2. Hair

Hair cortisol has potential to be a useful measurement tool of chronic stress (Burnard et al., 2017). The use of this medium has been explored in a multitude of species, including but not limited to rock hyrax (Koren et al., 2002), rhesus macaques (Davenport et al., 2006), domestic

dogs and cats (Accorsi et al., 2008), grizzly bears (Macbeth et al., 2010), sows (Bacci et al., 2014), sheep (Stubsjøen et al., 2015), rabbits (Peric et al., 2017), humans (Short et al., 2016), and coyotes (Schell et al., 2017). Due to the novelty of this stress assessment method and varying existing results, several areas are still deemed controversial or unknown, including cortisol incorporation into hair mechanisms, sampling techniques, and storage and analysis procedures.

The mechanism by which cortisol enters hair is one of ambiguity (Burnard et al., 2017). Three different mechanisms have been proposed as potential incorporation mechanisms, as shown in Figure 2.2 (Russell et al., 2012). The process is thought to resemble the mechanisms of drug incorporation into hair that consists of a multi-compartment model (Henderson et al., 1993; Boumba et al., 2006). Hypotheses have suggested that cortisol enters via the blood into the hair shaft from the medulla level using passive diffusion (Russell et al., 2012). Sebum and sweat secretions surrounding the outer cuticle may also contribute to cortisol entering the hair shaft (Pragst and Balikova, 2006; Raul et al., 2004), though Grass et al. (2015) reported that cortisol in sweat is an unlikely source of hair cortisol. Hair growth takes place in three specific phases: active phase (anagen), transition (catagen) and resting (telogen) (Harkey, 1993). The cortisol incorporation into hair takes place underneath the skin surface (Harkey, 1993) via passive diffusion from blood vessels in the anagen growing phase (Meyer and Novak, 2012). Therefore, a time delay occurs between the actual cortisol incorporation into the hair and skin surface emergence of the specific hair section (Montillo et al., 2014; Stalder and Kirschbaum, 2012). Nonetheless, hair has been deemed a stressful event “retrospective calendar” due to the fact that long hair samples can be cut into segments that each represent a particular measurement of time (Russell et al., 2012). The “shave-reshave” method is often performed to ensure that enough growing hairs are collected. This involves shaving a specific area initially and then re-shaving

the same area after a period of time to collect the regrown hair sample (Davenport et al., 2008; Meyer and Novak, 2012).

Hair sampling and cortisol analysis methodologies have been found to affect the cortisol concentration results. Cortisol concentration in hair has been found to be affected by age (González de la Vara et al., 2011), pregnancy (Braun et al., 2017b), season and weather change (Uetake et al., 2018), hair color (Burnett et al., 2014; Tallo-Parra et al., 2015), collection method (Moya et al., 2013), sampling location (Moya et al., 2013; Burnett et al., 2014). Others have found that hair color did not have an impact on hair cortisol concentration (Ghassemi Nejad et al., 2017; Nedic et al., 2017). Moya et al. (2013) compared multiple sampling locations in beef cattle. They found that clipping hair from the tail resulted in the highest cortisol concentrations. Given that the sampling site remains constant, comparison between subjects is usually reliable (Burnard et al., 2017). Various sample storage techniques have been used including aluminum foil (Braun et al., 2017a), plastic bags (Lockwood et al., 2017), and paper envelopes (Creutzinger et al., 2017). Hair can be affected by a “wash-out” affect related to the exposure to UV light; therefore, samples should be stored in a dark, dry place (Wester et al., 2016). Cattle hair samples have been found to have long-term storage stability for months and years (González de la Vara et al., 2011). Additionally, many different approaches have been taken during the hair sample analysis procedures. Studies have successfully obtained cortisol concentrations from hair that was ground up with a ball and mill (Burnett et al., 2014) and cut into fragments with a scissors (Braun et al., 2017b). A wash protocol with isopropanol is often used (Davenport et al., 2006). Hair cortisol has been quantified using liquid chromatography tandem mass spectrometry (LC-MS/MS) (Van den hauwe et al., 2005; Binz et al., 2016), enzyme immunoassay (EIA) (Moya et al., 2013; Creutzinger et al., 2017), radio immunoassay (RIA) (Comin et al., 2011), and enzyme-

linked immunosorbent assay (ELISA) (Nedic et al., 2017). Mass spectrometry is considered the “gold standard” for hair analysis as the methods are very specific and highly sensitive (Gow et al., 2010). A study comparing analysis methods for human hair found that EIA yielded an average 15% inflation of the results compared to LC-MS/MS (Kirschbaum et al., 2009).

A recent review about hair cortisol use for animal welfare assessment cumulated five general recommendations for sampling and analysis: 1. selection of animal subjects from the same age group, sex and reproductive state; 2. the same body region and color should be used for all hair samples; 3. the hair cortisol incorporation time delay should be taken into consideration; 4. External contaminations should be avoided; 5. the “shave-reshave” method should be used if possible (Heimbürge et al., 2019).

Additional studies have been performed using hair cortisol analysis as a chronic stress assessment in cattle. Two studies observed higher cortisol concentrations in cattle that were deemed to be clinically diseased compared to cows that were not (Comin et al., 2013; Burnett et al., 2015). Moreover, chronically ill cattle showed elevated hair cortisol concentrations versus acutely ill cattle (Braun et al., 2017a). Another study reported that healthy and non-healthy dairy cows did not show a difference in hair cortisol concentration (Tallo-Parra et al., 2018). An evaluation of beef cattle stocking density with hair samples from the tail switch showed that heifers housed in a higher density environment (14 m²/head vs. 25,000 m²/head) yielded higher cortisol concentrations (Schubach et al., 2017). However, stocking density did not impact hair or serum cortisol concentrations in an experiment involving prepartum dairy cows housed at 80% or 100% densities (Silva et al., 2016). Creutzinger et al. (2017) investigated the long-term stress effects of castration with hair cortisol. Calves were either surgically castrated with meloxicam, surgically castrated with saline, or sham castrated with saline. They found treatment differences

showing that 14 days after the procedure those calves that were surgically castrated with saline exhibited higher hair cortisol concentrations than those calves that were sham castrated (Creutzinger et al., 2017). Further research is needed to reach an extensive and consistent understanding of the implication of hair cortisol pertaining to an overall non-invasive method to quantify stress.

2.2.3 Infrared Thermography

Infrared thermography is a useful, noninvasive tool used to evaluate health, production and welfare related measures in cattle. Producers and veterinarians utilize IR to define normal animal temperature values that can further be used to detect animals that may be displaying abnormal temperatures, often related to the well-being state of the animal. Infrared thermography has been found useful when identifying cattle with subclinical foot lesions (Alsaad and Büscher, 2012), ear inflammation due to infected implant placement (Spire et al., 1999) and foot and mouth disease virus using coronary band thermal images (Rainwater-Lovett et al., 2009). Mammary skin temperature of dairy cows obtained via IR technology showed a strong, positive correlation with the severity of the mastitis case (Colak et al., 2008; Polat et al., 2010). Ocular thermography has been reported as a reliable indicator of bovine respiratory disease (Schaefer et al., 2007; Schaefer et al., 2012; Rekant et al., 2016) and bovine viral diarrhoea virus (Schaefer et al., 2004) before clinical signs are observed. Infrared thermography has also been used in cattle for reproduction (Menegassi et al., 2015), metabolism and nutrition (Montanholi et al., 2008; Montanholi et al., 2009). Researchers have shown that there is relationship between residual feed intake and other efficiency traits with IR of different body locations (Montanholi et al., 2009). Thermography can also be used to quantify bull testicular temperature to further assess sperm characteristics (Menegassi et al., 2015).

Generally, IR has been used to quantify acute stress, related to painful procedures or stressful events, although multiple studies have shown varying results. Studies involving calf castration (Stewart et al., 2010) and competition horses (Valera et al., 2012) have reported increases in the maximum ocular temperature in association with these painful or high exertion events. On the contrary, rapid decreases in eye temperature have been observed immediately after cautery disbudding (Stewart et al., 2008a) and after stressful cattle handling procedures (Stewart et al., 2008b). Stewart et al. (2008a, 2008b) mention that the drop in eye temperature may have been linked to sympathetic-related vasoconstriction via the HPA axis. Stewart et al. (2007) conducted a study with non-lactating dairy cows to investigate the link between eye temperature and the HPA axis as a potential way of detecting stress. They found that the exogenous stimulation of the HPA axis, via ACTH injection, alone does not cause eye temperature to increase; however, the temperature increased after catheterization. An animal may not display signs of stress unless there is a cognitive awareness that stress is occurring and being perceived as such (McMillan, 2005). Conversely, a study performed in horses found that salivary cortisol levels and eye temperature were correlated, possibly linked to an HPA response (Cook et al., 2001).

The use of IR thermography to quantify chronic stress is a relatively new approach. Ocular thermal images of piglets postweaning showed high and positive correlations with dorsal surface temperature and salivary cortisol during the first two weeks after weaning, which may indicate a possible connection between IR and chronic stress may exist (Pulido-Rodríguez et al., 2017). Herborn et al. (2018) reported that increased comb, face and eye temperature may be indicators of chronic stress in laying hens.

There are many factors that may affect the reliability and accuracy of an infrared thermal image that must be taken into consideration when utilizing the technology. Okada et al. (2013) found three important conditions that should be followed to obtain optimum IR pictures: 1. a constant distance should be determined between the camera and the object of interest that is consistently ensured, as well as, a focus setting that is within a 45 degree angle relative to the object; 2. extreme temperature ranges, including direct sunlight, high winds and elevated humidity should be avoided; 3. reliable image comparison and analysis should only be performed if all images were taken in a stable, ambient temperature with identical conditions. Other environmental conditions such as solar loading should also be controlled for in the experimental design (Church et al., 2013). Cardoso et al. (2015) reported that environmental factors affect eye and brain surface temperatures most. Essentially, the calculated surface temperature with the use of IR is a collaborative result of the amount of heat produced by the body, environmental factors (Soroko et al., 2016), and air movement velocity (Westermann et al., 2013). Another factor to consider is the varying types of subjects, especially concerning different animal species and breeds. In fact, black areas of skin usually result in warmer temperatures than adjacent white areas in cattle (Hellebrand et al., 2003). Temperatures have been shown to differ between clipped and nonclipped areas in dogs (Loughin and Marino, 2007) Even circadian rhythms have been shown to affect body temperatures (Stewart et al., 2005). The awareness of the external environment and subject related characteristics is crucial to ensure the accuracy of the collected thermal images.

2.2.4 Mobility Scoring

Mobility is considered the method of movement or ambulation for an animal. Lameness is an abnormal gait or stance, which results in hindered mobility (Van Nuffel et al., 2015;

Edwards-Callaway, 2017). Poor mobility or lameness can occur due to infectious disease (i.e. foot rot and digital dermatitis) and lesions related to claw horn disruption (von Keyserlingk, et al., 2009), as well as, injury and laminitis (Osterstock, 2009). Furthermore, laminitis is commonly observed in high concentrate/carbohydrate-rich nutritional programs and is believed to be linked to the onset of acidosis (Stokka et al., 2001). Lameness is generally considered to be a symptom rather than a disease, often associated with the presence of pain (Archer et al., 2010; Webster et al., 1986). Lameness has negative impacts on animal welfare, which further creates economic losses from reduced production and performance (Griffin et al., 1993; Tibbetts et al., 2006).

Mobility scoring systems have been developed to objectively assess and evaluate cattle lameness. Primary focus of cattle lameness quantification has taken place in the dairy cattle industry, though an increase in research involving beef cattle has recently occurred. Three defined 4-point scoring systems are currently used for finished cattle (Edwards-Callaway et al., 2017). The North American Meat Institute (NAMI) Mobility Scoring System is scored as follows: “1 = Normal - walks easily with no apparent lameness or change in gait; 2 = Exhibits minor stiffness, shortness of stride or a slight limp but keeps up with normal cattle in the group; 3 = Exhibits obvious stiffness, difficulty taking steps, an obvious limp or obvious discomfort, and lags behind normal cattle walking as a group; 4 = Extremely reluctant to move even when encouraged by a handler, described as statue-like” (NAMI, 2016). Another 4-point scoring system was created by Terrell and colleagues (2016), which included the scores of “0 = Normal – animal walks normally, no apparent lameness or change in gait; 1 = Mild lameness – animal exhibits shortened stride, may move head slightly side to side but no head bob; 2 = Moderate lameness – animal exhibits a limp, with an obviously identifiable limb or limbs affected and/or

head bob present when walking, limb still bears weight; 3 = Severe lameness – animal applies little to no weight to affected limb while standing or walking, animal reluctant or unable to move, while walking, animal’s head dropped, back arched, with head bob and limp detected.” A third system, known as the Step-Up Locomotion Scoring System, was developed in association with the previously mentioned authors from Terrell and colleagues (2016) and Zinpro Corporation (2016) as a part of the Step-Up Management Program for Beef Cattle. The four scoring categories include the following: “0 = Normal – animal walks normally with no apparent lameness or change in gait, hind feet land in a similar location to front feet; 1 = Mild lameness – animal exhibits short stride when walking, dropping its head slightly, animal does not exhibit a limp when walking; 2 = Moderate lameness – animal exhibits obvious limp, favoring affected limbs, which still bear weight, a slight head bob is present when the animal is walking; 3 = Severe lameness – animal applies little or no weight to affected limb and is reluctant or unable to move, while walking, the animal’s head is dropped, back arched, with head bob and limp detected” (Step-Up Locomotion Scoring System, 2016). Edwards-Callaway et al. (2017) recognized the fact that the two commonly used 4-point systems from Terrell and colleagues (2016) and Zinpro Corporation for beef cattle failed to include the comparison of a lame animal to its cohorts. The NAMI Mobility Scoring System includes the consideration of the lame animal’s ability to keep up with the group at a walking pace or not, which adds an important dynamic (Edwards-Callaway et al., 2017).

The National Beef Quality Audit included cattle mobility scoring for the first time in 2016. Using the NAMI scoring system, the 2016 audit conducted at various slaughter plants in the United States reported 96.8% of the evaluated cattle were scored as a 1, with the remaining 3.0% as a score of 2, 0.1% as a score of 3, and 0.02% as a score of 4 (Eastwood et al., 2017).

Another recent study performed by Lee et al. (2018) reported that 97.02% of cattle scored during an observational study performed at six slaughter facilities in the United States exhibited a score of 1 or normal mobility, based on the NAMI scoring system. The prevalence of cattle from both studies with abnormal mobility scores is relatively small, though the subset of animals, especially within large sample sizes, continues to highlight an animal welfare concern and demands further research and discussion (Lee et al., 2018). Frequently scoring cattle can serve as a “vital benchmark for animal welfare” (Eastwood et al., 2017) and allows for the tracking of progression and improvements over time. Measuring parameters such as mobility is essential so that “bad does not become normal” (Grandin, 2018). This phenomenon can occur as producers and employees become gradually accustomed to seeing lame cattle and then begin to consider the presence of lameness as normal and acceptable. The consistent use of one type of scoring system is important, especially considering comparability across facility audits over time (Grandin, 2017). Continuous monitoring of cattle lameness via mobility scoring is an important and easily attainable measurement that can help to further the understanding of an operation’s impact on cattle welfare and production efficiency.

2.3 Liver Abscesses

Liver abscesses have a gross appearance of being filled with pus, capsules of varying thickness, and an expansive range in size from a pinpoint to over 15 cm in diameter (Nagaraja and Chengappa, 1998). They are considered to be a “production disease” in the cattle industry. According to Rollin (2009), a production disease is considered a pathological condition in an animal that develops because of production environments and management. These diseases cause a “breakdown of the various metabolic systems” under high production and modern husbandry systems (Payne, 1972).

2.3.1 Etiology and Pathogenesis

The development of liver abscesses was attributed early on to the abrupt increase in high energy feed intake of cattle (Brown et al., 1973). Specifically, the amount and type of roughage level in the finishing diet affects liver abscess prevalence. As roughage level decreased in the diet, the incidence rate and severity of abscesses increased (Gill et al., 1979, Zinn and Plascienca, 1996). In a survey provided to 32 individual feedlots intended to determine which factors are associated with the development of liver abscesses in fed beef, Herrick et al. (2018a) concluded that liver abscesses are likely a multifactorial disease that are influenced by other factors besides nutrition after finding that more than 40% of the variation in the statistical model was unexplained. Nutrition is a major contributor to the development of liver abscesses; however, there are several other factors, such as, days on feed, cattle type, breed, gender, geographic location and season, that also influence the incidence (Reinhardt and Hubbert, 2015).

Liver abscesses are considered to be polymicrobial infections (Nagaraja and Chengappa, 1998). The primary causative bacterial agent is said to be *Fusobacterium necrophorum* (Nagaraja and Chengappa, 1998; Amachawadi and Nagaraja, 2016). However, recent literature has reported the presence of a wide variety of bacterial populations (Weinroth et al., 2017). The exact mechanism of liver abscess development is unknown. Though, it is widely recognized and accepted that ruminal acidosis is caused by the ruminal microbe fermentation of grain and resulting build up of organic acids (Nagaraja and Lechtenberg, 2007). The damage of the rumen lining and rumen inflammation due to acid presence weakens the rumen wall, which increases the likelihood of *F. necrophorum* colonization (Jensen et al., 1954). The bacteria enter the portal blood stream through rumen wall lesions and then travel to the liver (Smith, 1944). Once in the

liver, the bacteria colonize and create abscesses. The bacteria use several defense mechanisms to breach the rumen wall and reach the liver. Virulence factors, including leukotoxin and hemagglutinins, and endotoxic lipopolysaccharide (LPS), assist with attachment to the rumen epithelium and abscess formation in both the rumen wall and liver parenchyma (Tadepalli et al, 2009).

2.3.2 Production Impact

The overall prevalence of total liver abscesses can range from approximately 0% to greater than 70% for cattle in a given lot (Reinhardt and Hubbert, 2015). As reported in the 2016 National Beef Quality Audit, the overall liver abscess rate was 17.8% for steers and heifers and 20.7% for cows and bulls (Harris et al., 2018). Data consistently shows that liver abscesses are found more frequently in steers as compared to heifers, as well as, Holsteins more often than beef breeds (Amachawadi and Nagaraja, 2016). It is theorized that the higher prevalence in Holsteins could be linked to the increased days on feed, specifically with a high energy diet (Duff and McMurphy, 2007) and the greater total feed intake as compared to breed breeds (Hicks et al., 1994).

The presence of liver abscesses in cattle are a major economic liability for the producer, processing plant and consumer. Cattle with severe liver abscesses have been found to be associated with a reduction in carcass weight (Brown and Lawrence, 2010), decreased dressing percentage, (Rust et al., 1980), and reduced nutritional and production parameters, specifically decreased feed intake and lower final weight (Brink et al, 1980). The presence of liver abscesses decreases carcass value due to trim loss (Rezac et al., 2014; Herrick et al., 2018b). A decrease in average daily gain, along with other lung and rumen abnormalities and lesions are present with severely liver abscessed cattle (Rezac et al., 2014). In relation to the consumer's product quality,

diminished marbling was reported in association with severe liver abscess presence (Brown and Lawrence, 2010). However, mild or moderate liver abscess scores have been shown to have minimal or no impact on production (Davis et al., 2007; Nagaraja and Lechtenberg, 2007; Brown and Lawrence, 2010).

Cattle livers are commonly scored for the presence of abscesses using the Elanco Scoring System (“0 = No abscesses – a normal, healthy liver; A = One or two small abscesses, or up to two or four well-organized abscesses, which are generally under one inch in diameter, with the remainder of the liver appearing healthy; A+ = One or more large abscesses present, along with the inflammation of liver tissue surrounding the abscess, with portions of the diaphragm adhered to the surface of the liver that may have to be trimmed to separate the liver from the carcass”) (Elanco, 2019).

2.3.3 Welfare Implications

The presence of liver abscesses in cattle has been widely studied pertaining to topics such as nutrition, performance and economics, but there is very limited research that addresses well-being directly. One study has been performed related to the effect of grain characteristics on production, meat quality, behavioral and stress parameters of feedlot steers (Moya et al., 2015). Each crossbred steer was assigned to one of four treatment based on a 2 x 2 factorial design, involving one factor as type of grain (wheat or barley based) and another factor as the processing index (85% or 75%). They concluded that cattle fed a wheat-based diet as opposed to a barley-based diet yielded higher hair cortisol concentrations. Furthermore, cattle fed barley with an 85% processing index tended to exhibit a lower incidence of severe liver abscesses as opposed to other diet types. This suggests a possible indication that liver abscess presence may have an additional stressful impact on cattle based upon the fact that steers with a higher incidence of

severe liver abscesses yielded higher hair cortisol concentrations. This aforementioned conclusion was not stated specifically within Moya et al. (2015), which illustrates the need for further research on the topic of quantifying stress in cattle with liver abscesses.

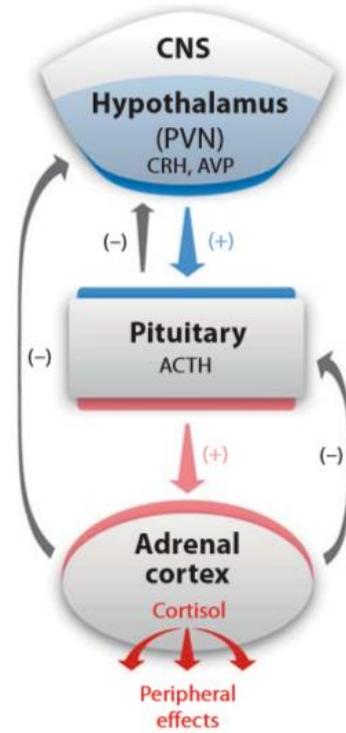


Figure 2.1 Schematic representation of the hypothalamic-pituitary-adrenal (HPA) axis to stress in relation to cortisol release. PVN: paraventricular nucleus; CRH: corticotropin releasing hormone; AVP: arginine vasopressin; ACTH: adrenocorticotrophic hormone. Adapted from Papadimitriou and Priftis (2009).

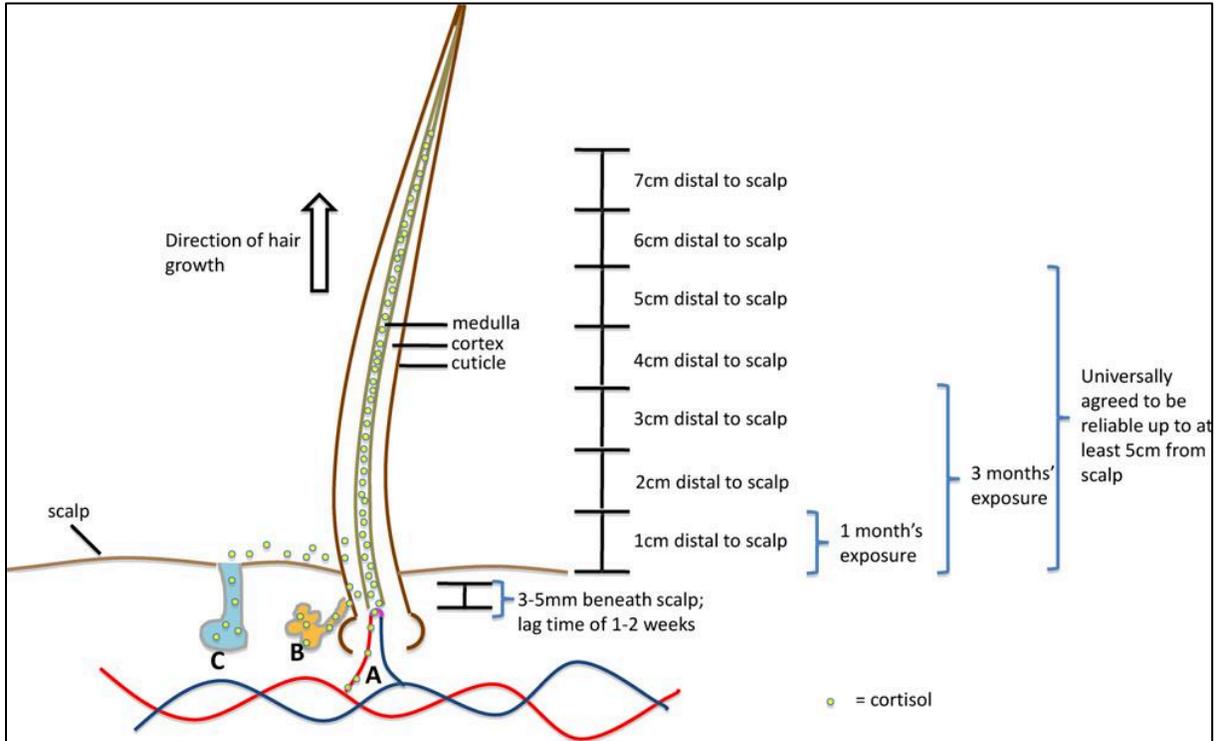


Figure 2.2 Three mechanisms have been proposed as methods of incorporation of cortisol in hair: A. blood, B. sebum, and C. sweat. Adapted from Russell et al. (2012).

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CHAPTER III

EVALUATION OF HAIR CHARACTERISTICS ON THE IMPACT OF HAIR CORTISOL CONCENTRATION IN BEEF CATTLE

SUMMARY

Hair cortisol is a novel biomarker of chronic stress. Multiple studies have been performed utilizing this physiological parameter; however, research is revealing controversial results and the need for further validation in cattle. The objective of this study was to determine the effect of hair color and length, as well as, animal age on hair cortisol concentration in beef feedlot steers. Nineteen beef crossbred steers generally used for nutrition trial research and housed in a small feedlot setting were used for this study. Seven of the steers ($1,043 \pm 6.8$ kg; approx. 9 years of age) were fitted with ruminal fistulas and duodenal cannulas. The other twelve steers (680 ± 4.5 kg; approx. 2.5 years of age) were fitted with only ruminal fistulas. One steer was euthanized due to health problems within the first week of the study. During routine fistula cleaning and restraint, hair samples from the right rump region of each steer were collected throughout a period of six weeks from six different areas and analyzed for cortisol concentrations. One pre-determined area was shaved each week for five weeks (Weeks 1-5). During week six, all five, previously shaved areas and an additional area was shaved to collect hair samples of various lengths. Hair length was recorded prior to the collection of each hair sample. Only data from the last week (Week 6) of collection were included in the analyses. For statistical analysis, steers were categorized into one of three groups: old with black hair (OB, $n = 3$); old with white hair (OW, $n = 3$); young with black hair (YB, $n = 12$). Older steers exhibited greater hair cortisol concentrations than younger steers ($P < 0.001$). The white hair of the old steers yielded higher

concentrations of hair cortisol as compared to black hair from young steers (OW = 10.89 ± 2.03 pg/mg and YB = 0.98 ± 0.11 pg/mg, respectively; $P < 0.001$). Hair cortisol concentration was not impacted by duration of growth ($P = 0.33$). However, cortisol concentrations exhibited a weak, positive correlation with hair length ($r = 0.33$, $P = 0.01$). Additionally, the average hair growth per week of beef steers in the winter months was calculated to be 0.90 mm. Further research should be performed to improve our understanding of the effect of hair characteristics, as well as, sampling methodologies and analysis techniques on hair cortisol concentrations.

INTRODUCTION

Physiological parameters are often used to quantify animal well-being. Cortisol is commonly measured to assess stress, whether acute or chronic, in many livestock species. This hormone can be measured using several different mediums – blood (Bristow and Holmes, 2007; Buckham Sporer et al., 2008), saliva (Negrão et al., 2004; Chacón Pérez et al., 2004), urine (Morrow et al., 2000) and feces (Palme et al., 2000). These parameters provide an acute, time-point measurement of cortisol concentration (Palme, 2019). The measured cortisol concentration when using these methods, specifically blood, can be affected by several factors, such as circadian rhythms (Möstl and Palme, 2002), handling (Hemsworth et al., 2011), restraint (Szenci et al., 2011) and degree of habituation or acclimation (Hopster et al., 1999).

Hair cortisol has potential to be a useful non-invasive, measurement tool of chronic stress (Burnard et al., 2017). Measuring cortisol in hair is thought of as a stressful event “retrospective calendar” representing a chronic or long-term period (Russell et al., 2012). The use of this medium has been explored in a multitude of species, including but not limited to rock hyrax (Koren et al., 2002), rhesus macaques (Davenport et al., 2006), domestic dogs and cats (Accorsi

et al., 2008), sows (Bacci et al., 2014), sheep (Stubsjøen et al., 2015), humans (Short et al., 2016), and coyotes (Schell et al., 2017). Studies have been performed with beef (Moya et al., 2013) and dairy cattle (Comin et al., 2011; González de la Vara et al., 2011); however, results have shown varying conclusions related to cattle. Factors such as hair color (Burnett et al., 2014; Tallo-Parra et al., 2015), collection method (Moya et al., 2013), sampling location (Moya et al., 2013; Burnett et al., 2014), age (González de la Vara et al., 2011), pregnancy (Braun et al., 2017b), season and weather change (Uetake et al., 2018) have been shown to affect cortisol concentration in hair to various degrees in cattle. Specifically related to beef cattle, hair cortisol measurements have been used to quantify stress related to castration (Creutzinger et al., 2017; Marti et al., 2018), excitability (Lockwood et al., 2017), and long-distance transport (González et al., 2012; Marti et al., 2017).

A relatively, small amount of research has been performed with hair cortisol quantification in beef cattle. The objective of this study was to determine the effect of hair characteristics (hair color and length) and animal age on hair cortisol concentration in beef feedlot steers.

MATERIALS AND METHODS

IACUC Protocol

Prior to the initiation of this study, animal use and associated procedures were approved by the Colorado State University (CSU) Institutional Animal Care and Use Committee (Protocol #'s 16-6550A and 17-7107A).

Animals

This study was conducted on 19 beef crossbred steers over the period of six weeks in the months of December 2017 and January 2018. The animals were housed in a small feedlot setting at CSU's Agricultural Research, Development, and Education Center (ARDEC, Fort Collins, CO) and fed a diet that met all NRC (National Research Council, 2016) requirements. Seven of the steers ($1,043 \pm 6.8$ kg; approx. 9 years of age) were fitted with ruminal fistulas and duodenal cannulas. The other twelve steers (680 ± 4.5 kg; approx. 2.5 years of age) were fitted with ruminal fistulas only. The fistula surgery for these twelve steers took place in September 2017. The surgeries for the 9-year-old steers took place in 2012. All fistulas were implemented for the purposes of nutrition research, not this particular study. Old steers with both a rumen fistula and duodenal cannula were housed together in two combined 10 head pens (12 m x 61 m). Young steers with only a rumen fistula were housed together in a pen of the same dimensions. One of the old steers with both a ruminal fistula and duodenal cannula was humanely euthanized during the second week of the study due to health concerns.

Animal handling

All animals were subjected to the same handling techniques and sampling methods. All samples were collected within the same timeframe each week during a once weekly 7 AM – 9 AM time period. Data were collected during normal routine handling for fistula cleaning to avoid creating additional stress. Animals were restrained in a hydraulic chute (Silencer, Silencer Hydraulic Chutes, Stapleton, NE) using low stress handling through a serpentine crowd pen and chute design.

Experimental Design

Hair samples and hair length measurements were collected over a period of six consecutive weeks. All steers were categorized into one of three cattle groups: old with black

hair (OB, n = 3); old with white hair (OW, n = 3); young with black hair (YB, n = 12). Old steers were approximately 9 years of age and young steers were approximately 2.5 years of age. No steers fit the demographic of young with white hair. Data collection occurred during the same day of each week. Each week, pre-determined areas on the right rump of each animal were shaved (Figure 3.1). Multiple measurements and samples were collected from each animal depending on the sampling week (Figure 3.2). During week one, the designated area number one was shaved on each animal; during week two, the designated area number two was shaved on each animal continuing with the same sampling scheme throughout week five. On the last collection day during week six, all six areas were shaved – five areas (S1-S5) were re-shaved and one area (S6) was newly shaved. Specific areas were shaved at variable times to collect hair samples at different lengths (L1-L6). In total, 11 samples were collected from each steer. Only the six samples from each steer collected during Week 6 were included in the data analysis as these were the samples that displayed different lengths of hair for each steer over specific growing periods.

Hair Measurement and Collection

Hair length measurements were taken at multiple time points to collect hair measurements at different lengths over the course of the six-week experimental period. Due to the poor functionality of digital calipers in cold temperatures, a transparent ruler (Golden Harvest Seeds, Minnetonka, MN) was used to measure hair length for weeks one through five. Nitrile gloves (VWR[®], Radnor, PA) were worn by all researchers who were in contact with the hair measurement and sampling. For week six, a calibrated, digital caliper (Neiko 01407A, Neiko Tools, Taiwan) was used to measure the hair length of each animal, along with the same previously used ruler for consistency. Measurements of hair length were recorded for the

appropriate area on the rump region, pertaining to location and experimental week. All caliper measurements were obtained and verbally read to another researcher for data recording. The hair color of the animal was also recorded.

After length measurement, hair samples were collected from each animal using a cordless livestock clipper (Andis Pro Clip Pulse Ion, Andis Company, Sturtevant, WI) fitted with an adjustable, detachable No. 40 blade. Small, square areas of hair, approximately 2.54 cm x 6.35 cm, were shaved from the rump region on the right side of the steer (Figure 3.3). The hair was shaved as close to the skin as possible. The clipper was cleaned and disinfected with 70% isopropyl alcohol (Equate, Bentonville, AR) between animals. Each hair sample was immediately placed in an individually labelled Ziploc[®] bag and stored in an opaque container. The cover of the container was kept closed during sample collection except to place the most recently collected hair sample into the container. Hair samples were stored in an opaque container in Ziploc[®] bags at room temperature until analysis to reduce exposure to light (Creutzinger et al., 2017). A total of 198 samples were collected over the course of the six-week period.

Lab Analysis

Hair samples from Week 6 of collection were used for analysis, representing a total of 108 samples with 6 samples from each steer. Each hair sample was removed from the plastic bag and cut into small fragments (approximately 5 mm) using scissors. The scissors were cleaned with methanol (Optima LC-MS grade, Fisher Scientific, Waltham, MA) after each sample. Hair was submerged in liquid chromatography mass spectrometry (LC-MS) grade water (Optima LC-MS grade, Fisher Scientific, Waltham, MA) and placed on a shaker (Reliable Scientific Rocking Shaker, Reliable Scientific Inc., Hernando, MS) for 4 hours at 37°C. To dry the hair, acetone

(Optima LC-MS grade, Fisher Scientific, Waltham, MA) was added, and samples were shaken at room temperature for two minutes. Next, samples were placed under a stream of nitrogen gas until completely dry. Cleaned, dry hair was weighed (20 ± 0.5 mg) into a 2.0 mL glass vial. Then, 1.5 mL of the internal standard methanol (100% methanol spiked with d4-Cortisol at 26.67 pg/mL) was added. Samples were sonicated for 16 hours at 50°C in a covered bath sonicator (Branson Ultrasonic Cleaner, Branson Ultrasonics, Danbury, CT). After sonication, hair was pelleted to the bottom of the tube via centrifugation (Model 5430, Eppendorf, Hauppauge, NY) at 3,000 x g for 20 minutes. The methanol supernatant was transferred to a 1.5 mL microfuge tube and incubated at -80°C for 1 hour to precipitate out proteins and other insoluble particulates. The samples were centrifuged at 18,000 x g for 20 min at 4°C. The supernatant was transferred again to a new tube and dried under nitrogen gas. Samples were resuspended in 100 uL of 100% methanol. Resuspended material was vortexed and centrifuged again at 18,000 x g for 5 min at 4°C. Finally, 80 uL of resuspension was transferred to a LC-MS vial insert. To make a representative quality control (QC) sample, 10 uL volume of each final extract was pooled together and aliquoted into three separate QC vials.

Sample analysis via liquid chromatography tandem mass spectrometry (LC-MS/MS) was performed using a Waters Classic Acquity UPLC coupled to a Waters Xevo TQ-S triple quadrupole mass spectrometer (Waters Corporation, Milford, MA). The calibration curve was constructed using washed test samples of cattle hair prepared as described above. A surrogate “light” standard of ^{13}C -Cortisol (Sigma-Aldrich, Inc., St. Louis, MO) was used, while the d4-Cortisol (Cerilliant Corporation, Round Rock, Texas) served as the true internal standard as performed by Binz et al. (2016). The pooled QC samples yielded a 9.54% variation. The level of

detection (LOD) was 0.145 pg/mg. Thirty samples contained cortisol concentrations that were unable to be detected.

Statistical Analysis

Data were analyzed with the software R version 3.4.1 (R Core Team, Vienna, Austria) in RStudio using the car (Fox and Weisberg, 2011), plyr (Wickham, 2011), lme4 (Bates et al., 2015) and emmeans (Lenth, 2019) packages. Linear mixed models fit by REML were created for the numerical response of hair cortisol concentration with cattle group type (including age and color demographics), duration of growth and hair length as predictor variables using the lmer function to account for repeated measures on steers. All models were subject to a type three analyses of variance using Kenward-Roger's method. For categorical variables, estimated marginal means (or least squares means) and Tukey's adjusted pairwise comparisons were calculated. Summary statistics and data graphing were performed to check normality. Hair cortisol concentrations were base 10 logarithm transformed to achieve normality and satisfy all model assumptions. Correlation between transformed hair cortisol values and hair length values was calculated accounting for repeated measures on animals (Bland and Altman, 1995). All cortisol concentrations considered to be below the LOD were analyzed as values equating to 0.5*LOD. Three samples yielded concentrations below the LOD. Values that were out of the range of three standard deviations plus or minus the mean were considered outliers and excluded from the analysis. The data contained three hair cortisol outliers, which were removed from the analysis. Significant differences were recognized at $\alpha \leq 0.05$.

RESULTS AND DISCUSSION

Our reported hair cortisol concentration values ranged from 0.0725 to 31.59 pg/mg. Other studies have found similar values on both ends of the value spectrum involving beef bulls (2.31 ± 0.176 pg/mg, Moya et al., 2013; 3.52 ± 0.49 pg/mg, Lockwood et al., 2017), weaned beef calves (2.36 ± 0.38 pg/mg; Marti et al., 2017), beef bull calves (5.16 ± 1.63 pg/mg; Creutzinger et al., 2017), lactating dairy cows of various parities (5.7 ± 1.7 pg/mg; Burnett et al., 2014), dairy cows ranging from 3 to 17 years of age (0.69 ± 0.45 pg/mg; Braun et al., 2017b) and 2-year-old dairy cows (12.15 ± 1.85 pg/mg; González de la Vara et al., 2011). Extremely high values were reported in 15-day-old heifers (114.5 ± 14.43 pg/mg; González de la Vara et al., 2011). Our results encompass a large range of values, which is unique when compared to past research. Several factors may have played a role in the greater variation of our values. Cortisol concentrations have been found to be higher in the clipped hair as compared to plucked hair (Moya et al., 2013). Other factors, including animal age, hair color and sampling location have been shown to impact hair cortisol concentrations as illustrated below.

In our study, the age of the steers significantly impacted the hair cortisol concentration, as reported in Table 3.1. Older steers with both black and white hair showed higher amounts of hair cortisol than younger steers with white hair ($P = < 0.001$). Our results seem to be the converse of other reported values. As mentioned previously, a study with dairy cattle reported that hair samples from 2-year-old cows yielded lower cortisol concentrations than samples from 15-day-old heifer calves (González de la Vara et al., 2011). The significant difference was mentioned to possibly be attributed to a parturition initiation pathway, known as the fetal pituitary adrenal axis, that stimulates increased serum cortisol concentrations during late stages of pregnancy (Flint et al., 1979; Kindahl et al., 2002). Tallo-Parra et al. (2018) found no correlations between

hair cortisol concentration and age in dairy cows. Other species have shown increases in cortisol concentrations from hair samples in relation to younger ages. For example, newborn foals showed higher hair cortisol as compared to 30 or 60 days old (Comin et al., 2012). The same observations have been reported in infant and juvenile comparisons with rhesus monkeys (Dettmer et al., 2014) and other primates (Fourie et al., 2016). These observations may be related to a reduced number of corticosteroid binding globulin concentrations present in infants, which yields greater concentrations of free plasma cortisol in humans (Grant et al., 2017). A decline in hair cortisol concentrations seems to be dependent upon age, though the time course is most likely species specific with possible increases occurring in later years of age (Heimbürge et al., 2018). Furthermore, the drastic difference in our results may have been related to the chronic presence of a ruminal fistula for the old steers versus the young steers. Though, all old steers in our study were in good health and not lame. Minimal research has been performed investigating the long-term impacts of ruminal or duodenal fistulas in cattle; however, two studies observed higher cortisol concentrations in cattle that were deemed to be clinically diseased compared to cows that were not (Comin et al., 2013; Burnett et al., 2015). Braun et al. (2017a) reported that chronically ill cattle showed elevated hair cortisol concentrations versus acutely ill cattle. Additional research is necessary across all species, especially cattle, to fully understand the impact of age on hair cortisol and possible stress associated with fistulas.

Hair color seems to have a reported variable impact on the overall concentration of cortisol in hair. In our study, the combined impact of age and color had a significant impact on hair cortisol concentrations across periods of growth ($P < 0.001$), in relation to the different areas shaved in Week 6 that were allowed various durations of growth (Figure 3.4). As previously discussed, cortisol concentrations were lower in YB as compared to OW ($P < 0.001$). Though, no

difference was observed between OB and OW ($P = 0.99$). In agreement with our findings, greater cortisol concentrations have also been observed in white cattle hair as compared to black hair (González-de-la-Vara et al., 2011; Cerri et al., 2012; Burnett et al., 2014). Conversely, one study reported the presence of higher cortisol concentrations in black hair in relation to white hair (Tallo-Parra et al., 2015). In a study on lactating Holstein cows under heat stress conditions, no significant differences in hair cortisol levels were reported related to the hair color, yet higher cortisol levels in black coat colors as compared to white coats were observed (Ghassemi Nejad et al., 2017). They noted that collecting hair samples that reflects the overall coat color of the animal may have an impact on measured cortisol values (Ghassemi Nejad et al., 2017). This may especially be important when collecting hair samples from multi-colored cattle, such as roan, speckled or spotted. Nedić et al. (2017) also found that hair color had no effect on hair cortisol. The effect of hair color on the concentration of hair cortisol in cattle requires more research to reach a concise conclusion as current research results are quite variable.

A weak, positive correlation was observed between hair cortisol concentration and hair length ($r = 0.33$, P -value = 0.01) (Figure 3.5). This shows that there is potential for hair length to have an effect on hair cortisol concentrations. Though, the concentration of cortisol in the hair was not impacted by the duration of growth in terms of weeks ($P = 0.33$). Cortisol is deposited into the hair shaft during the anagen growing phase (Meyer and Novak, 2012). The stage of growth must also be accounted for when sampling hair. The “shave-reshave” method is often performed to ensure that enough growing hairs are collected (Meyer and Novak, 2012). This involves shaving a specific area initially and then re-shaving the same area after an elapsed period of time to collect the regrown hair sample (Davenport et al., 2008; Meyer and Novak, 2012). We used this method to obtain samples that consisted of growing hair of different lengths

based on specific growing periods (ranging from one week to five weeks). Varying hair growth profiles can exist between individuals and may need to be accounted for given certain circumstances (Russell et al., 2012). Specific areas of the body may experience different rates of hair growth. Hair cortisol concentration have been suggested to be affected by season (Comin et al., 2011), due to the changing the rate of hair growth (Courtois et al., 1996). Additionally, there is great ambiguity concerning the distribution of cortisol along the length of the hair shaft. Studies involving rhesus macaques (Davenport et al., 2006) and dogs (Bennett and Hayssen, 2010) found no significant differences when comparing cortisol concentrations of the most proximal half and the most distal half of the hair shaft. These conclusions may not necessarily be able to be extended to the species of cattle, though if proven could impact interpretation and general hair cortisol analysis. As there are very few studies regarding the impact of length on hair cortisol, this should be a continued area of future research focus.

Our study only consisted of one general sampling area – the right rump region, though sampling location has been demonstrated to have an impact on the concentration of cortisol (Moya et al., 2013). Hair samples collected from the tail region of Angus cross beef bulls yielded higher cortisol concentrations as compared to other areas, including the hip, shoulder, neck and head (Moya et al., 2013). Cerri et al. (2012) agreed with the finding that tail hair was a suitable means to measuring cortisol levels over time because of the increased amounts of cortisol observed. Furthermore, white hair showed greater cortisol concentrations in the tail as compared to the hip or top line (Burnett et al., 2014). An increased rate of hair growth in the tail has been possibly attributed to the increased cortisol concentrations (Moya et al., 2013).

Finally, the average growth rate of beef cattle hair has not been extensively defined thus far. Using our measured values, the average growth rate of beef cattle hair was calculated to be

0.90 mm per week. Studies with dairy cattle have been used to determine the average hair growth rate of dairy cows to be approximately 0.6-1 cm/month (Schwertl et al., 2003) with specifically calculated rate of 0.04 ± 0.05 mm/d in the hip area (Burnett et al., 2014). As previously discussed, the rate of growth has also been shown to be influenced by the location (Moya et al., 2013). Burnett et al. (2014) found that hair in the hip and shoulder areas have similar growth rates, while the tail switch hair grows over ten times faster than the other areas in lactating dairy cows. Similar results were found in studies with beef cattle (Fisher et al., 1985; Schwertl et al., 2003). Other factors have also been proven to affect the growth rate of hair in cattle, including temperature (Berman and Volcani, 1961; Hayman and Nay, 1961), photoperiod (Johnson, 1981) and nutritional deficiencies (Martin et al., 1969). The hair growth rate can play a role in the resulting hair cortisol concentration.

Overall, we identify the fact that these data and reported conclusions are stemming from a rather small and specific cattle population. This further highlights the critical need for more research pertaining to cortisol quantification in the hair of beef cattle.

CONCLUSION

The data indicate that hair cortisol concentrations are affected by animal age and color characteristics in beef cattle. Older steers exhibited significantly greater hair cortisol concentrations than younger steers. White hair of older steers exhibited higher hair cortisol concentrations as compared to the black hair of younger steers. The duration of growth had no impact on the resulting hair cortisol concentration. Additional studies should be performed to assess the impact of fistula presence on the animal well-being related to chronic stress. Further

research is also necessary to understand how these factors and others may impact hair cortisol concentrations in beef cattle.

Table 3.1 Effect of cattle group in relation to age and hair color on hair cortisol concentration in beef feedlot steers. (N = 18)

Outcome	n	Cattle Group ¹			Pooled SEM	P-value
		OB (n = 3)	OW (n = 3)	YB (n = 12)		
Hair Cortisol (pg/mg) ²	18	7.57 ^a	10.89 ^a	0.98 ^b	1.15	< 0.001

^{a-b} Within rows, values with different superscripts differ (P < 0.05).

¹ Steers were categorized into one of three groups: OB = old with black hair (~9 years of age), n = 3; OW = old with white hair (~9 years of age), n = 3; YB = young with black hair (~2.5 years of age), n = 12.

² Means and pooled standard error are reported on the original scale, while the P-value is reported based on the base 10 logarithm transformed values that was used to obtain a normal distribution of data.

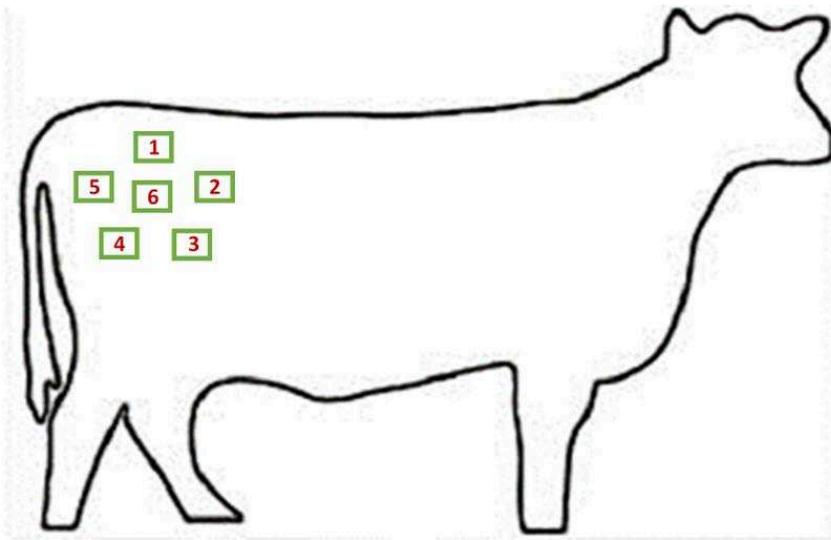


Figure 3.1 Diagram of hair sampling areas in the right rump region. Each individually labeled green box corresponds with a sampling site measuring approximately 2.54 cm x 6.35 cm in area. Image source: <http://clipart-library.com/outline-of-a-cow.html>.

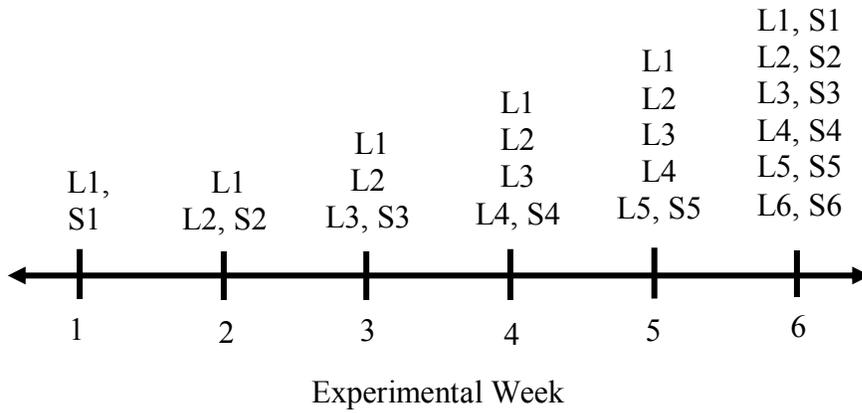


Figure 3.2 This timeline graphic outlines the measurements taken over the period of six weeks for each steer. Data collected are defined as follows: L# (1 – 6) = hair length for the specified area was measured in the rump region, S# (1 – 6) = hair sample for the specified area was collected.



Figure 3.3 The right rump region of an animal with all measurements and hair samples collected during Week 6.

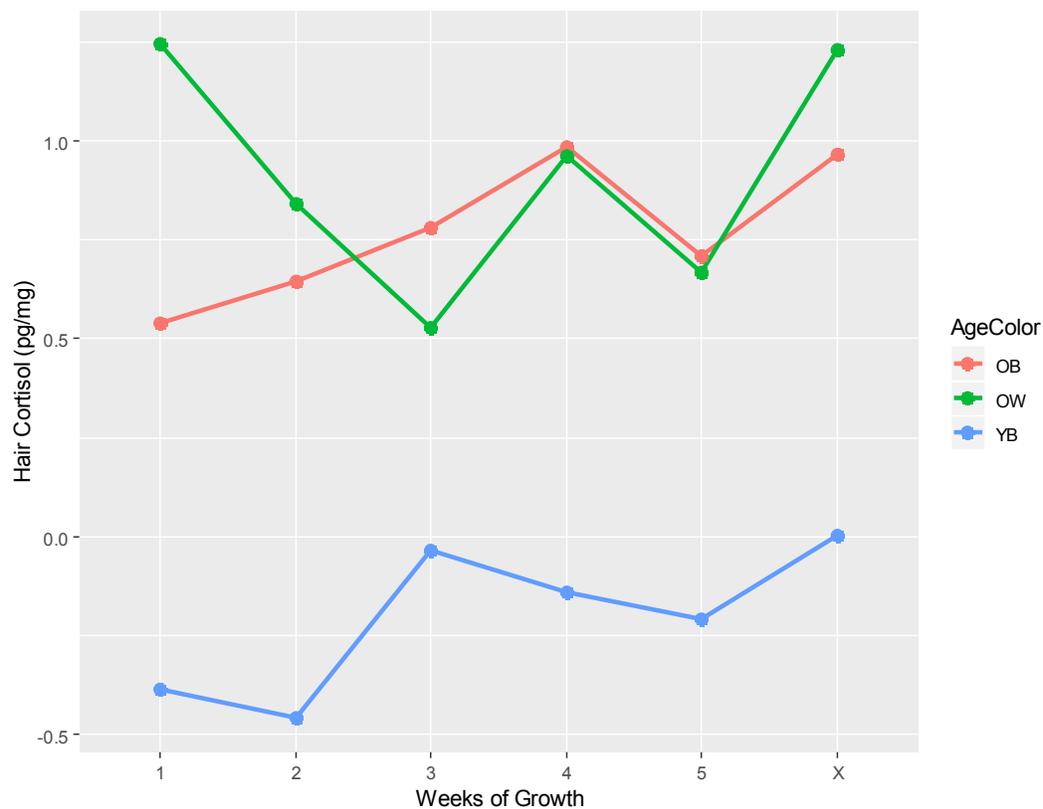


Figure 3.4 A plot between the average logarithm base 10 transformed hair cortisol concentrations and the weeks of growth for the samples shaved during Week 6 for each group of cattle (N = 18). Steers were categorized into one of three groups: OB = old with black hair (~9 years of age), n = 3; OW = old with white hair (~9 years of age), n = 3; YB = young with black hair (~2.5 years of age), n = 12. Weeks of growth were defined as follows: 1-5 = number of weeks each sample grew between the initial shave and re-shave sample collections, X = natural, previously unshaved hair.

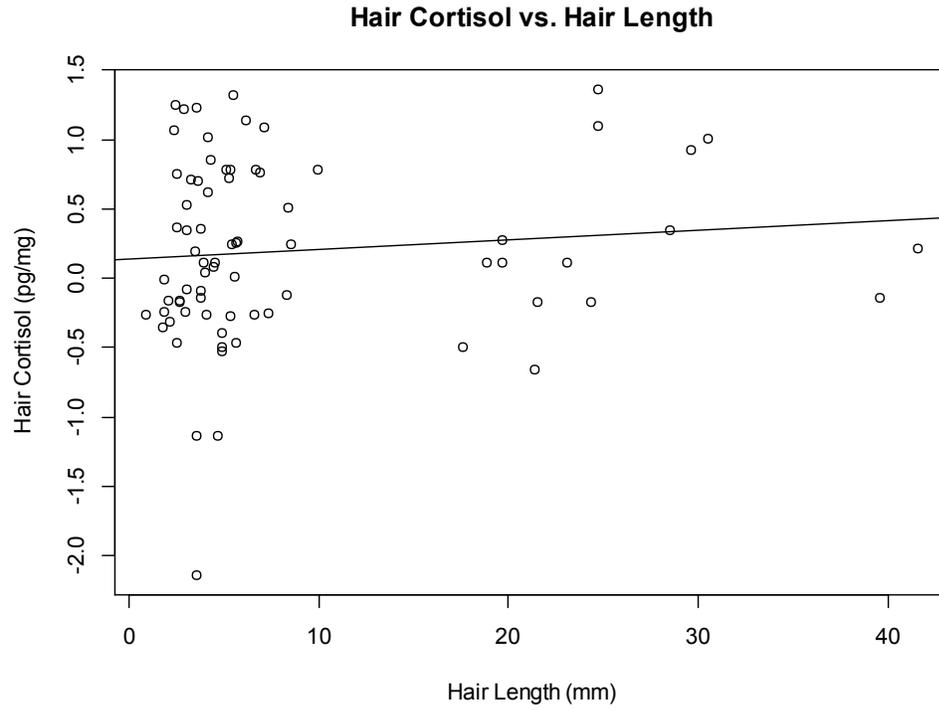


Figure 3.5 A scatterplot between base 10 logarithm transformed values for hair cortisol and hair length. A weak, positive correlation of $r = 0.33$ and $P = 0.01$.

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CHAPTER IV

IMPACT OF LIVER ABSCESS PRESENCE ON STRESS RELATED PHYSIOLOGICAL PARAMETERS ASSOCIATED WITH WELL-BEING IN FEEDLOT CATTLE

SUMMARY

Liver abscesses can affect cattle performance, though the impact on well-being is relatively unknown. The purpose of this study was to evaluate the relationship between liver abscess presence and stress-related parameters in beef breed feedlot cattle. Three hundred and sixty-three *Bos taurus* feedlot steers (675 ± 2.3 kg) were fed a steam flaked corn (53% to 70 %) based diet with corn silage (15% to 32%), and dried distiller's grain (8.5% to 9%). Each steer was allocated to one of three groups based on the liver abscess score assigned after slaughter. The liver abscess scoring groups were: no liver abscess presence (NLA; n = 316); mild liver abscess presence (MLA; n = 21) and severe liver abscess presence (SLA; n = 24). Two animals were unable to be assigned liver abscess scores. Two days prior to slaughter hair samples were collected from each animal and analyzed for cortisol concentrations. Additionally, during restraint, infrared thermography was used to quantify eye temperatures and a mobility score was assigned to each animal upon chute exit. During slaughter, exsanguination blood was collected from 115 of the 363 animals and analyzed for serum cortisol concentrations. Cattle were blocked by nutrition feeding treatments and data were analyzed using analyses of variance to determine differences in outcome variables between liver abscess score groups. All animals were included in the infrared analysis and 115 animals were included in the serum and hair cortisol analyses. Infrared thermography ($P = 0.55$), hair cortisol ($P = 0.96$), and serum cortisol ($P = 0.21$) were similar across all liver abscess scores. All animals exhibited normal mobility, thus statistical

analysis was not performed. The data indicate that under the conditions of this experiment, where adhesions to the body wall were not tabulated, liver abscesses did not impact measured stress-related outcomes. Additional research is necessary to understand the impact of liver abscess presence on other stress-related parameters associated with well-being in cattle.

Keywords: cattle, liver abscess, stress, cortisol

INTRODUCTION

Today's cattle management systems have increased efficiency, though some of the improvements have caused several different types of "production diseases" upon the industry. A production disease is considered a condition that is a departure from normal that is generally related to the management of an animal, oftentimes nutrition and feeding practices (Nagaraja and Chengappa, 1998; Andersen, 2003). The development of liver abscesses is an example of a production disease commonly observed in feedlot cattle after harvest. According to the 2016 National Beef Quality Audit, the overall liver abscess rate was 17.8% for steers and heifers and 20.7% for cows and bulls (Harris et al., 2018). Friesian and dairy crossbred bulls reportedly had a higher incidence of liver abscesses as compared to beef breed females (10.3% vs. 4.7% respectively) (Trotter and Gibbs, 2016). Reinhardt and Hubbert (2015) reported that the prevalence of total liver abscesses ranged from near 0% to greater than 70% of the livers from cattle in any given lot at slaughter. Cattle with severe liver abscesses have been associated with reduced nutritional and production parameters (Brink et al., 1990; Rezac et al., 2014) and a reduction in carcass weight (Brown and Lawrence, 2010). Minimal effects, if any, have been observed concerning cattle performance for those animals categorized as having mild to moderate liver abscesses (Davis et al., 2007; Nagaraja and Lechtenberg, 2007; Brown and

Lawrence, 2010); therefore, the effect of liver abscesses on cattle well-being tends to be marginalized.

Liver abscesses in feedlot cattle are a management created animal well-being problem that warrants the attention of the animal and veterinary science communities and the feedlot industry. Currently, most of the concern and research targeting liver abscesses is focused on economic losses to the beef industry associated with liver condemnations (Hicks, 2011), excess carcass trim (Herrick et al., 2018), and poor feedlot performance or threats to human health as related to the development of antibiotic resistance through extensive use of antimicrobial products that fall into a class of medically important to human health (Cameron and McAllister, 2016; Muller et al., 2018; Huebner et al., 2019). A recent study evaluated the impact of diet rations and feed inclusion rates with mention of stress-related parameters and liver abscess presence (Moya et al., 2015). Additionally, cattle with liver abscesses were reported to exhibit an increase in plasma cortisol paired with an increase in size of the adrenal gland (Wilson et al., 2002) and to have a tendency to exhibit lower biliary cortisol metabolites (Macdonald et al., 2017), which both were related to possible welfare implications. Studies demonstrating the effects of liver abscesses on objective and subjective measures of animal well-being are relatively rare.

The objective of this study was to evaluate the relationship between liver abscess presence and stress-related parameters in beef breed feedlot cattle, utilizing both physiological (hair and serum cortisol, ocular temperature) and behavioral measurements (mobility scoring). The ultimate goal of the study was to establish an initial understanding of the welfare state of cattle with liver abscesses so that management practices can be maintained or changed to allow for the production of cattle to be continually practiced in an efficient and sustainable manner.

MATERIALS AND METHODS

IACUC Protocol

Prior to the initiation of this study, animal use and associated procedures were approved by the Colorado State University (CSU) Institutional Animal Care and Use Committee (Protocol #17-7202A).

Animals, Facilities and Management

This study was conducted on 363 *Bos taurus* crossbred beef steers (675 ± 2.3 kg). The animals were housed in a small feedlot setting at CSU's Agricultural Research, Development, and Education Center (ARDEC) in Fort Collins, CO. Steers were housed in groups of 10 in research feedlot pens (6.01 m x 30.5 m). These animals were part of a separate study evaluating the effect of corn silage variety and diet concentration on feedlot performance and carcass merit in feedlot steers. The feeding trial was a 3 x 2 factorial design, one factor was the type of corn silage (conventional, floury type of brown mid rib, or traditional brown mid rib) and the other factor was the inclusion rate in the diet (15% or 30% of dietary dry matter). Cattle were fed one of 6 high concentrate steam flaked corn-based finishing diets (depending upon dietary treatment, dry matter ingredient and nutrient composition ranged from 52.69-70.49% steam flaked corn, 15.21-32.39% corn silage, 8.52-8.77% dried distiller's grains, and 5.57-5.77% supplement and contained 12.8-13.8% crude protein, 1.36-1.49 Mcal/kg net energy for gain, 50.1-59.7% starch, 12.3-16.7% ash free neutral detergent fiber, and 5.7-9.3% acid detergent fiber, respectively) for 130 days on feed. All finishing diets contained monensin (41.9 mg/kg dry matter, Rumensin, Elanco Animal Health, Greenfield, IN) and tylosin (12.1 mg/kg dry matter, Tylan, Elanco Animal Health). Ractopamine HCl (24.2 mg/kg dry matter, Optaflexx, Elanco Animal Health) was fed to all treatments the final 30 days in the feedlot. Dietary treatments were shown to have

no impact on liver abscess scores and other production-related response variables, with the exception of carcass yield grade (unpublished data). Yield grade has shown to be higher in grain-based diets when compared to silage-based diets (Young and Kauffman, 1978). Upon arrival at the feedlot, cattle were individually weighed, identified with a unique ear tag, vaccinated with a respiratory vaccine (Bovi-Shield Gold, Zoetis, Inc., Kalamazoo, MI), and a clostridial bacterin-toxoid (Ultra Choice 7, Zoetis, Inc.), treated for parasites via injection (Bimectin injectable, Bimeda US, Oakbrook Terrace, IL) and oral drench (Safe-Guard, Fenbendazole, Merck Animal Health, DeSoto, KS), and implanted with a long release implant intended for feedlot steers (Revalor XS, Merck Animal Health). In the final week of the feeding trial, all steers were weighed two days before transport to slaughter. Data were collected during this pre-slaughter weighing event. Two days after pre-slaughter weighing, all animals were transported via four compartment double-deck livestock trailers to a commercial cattle slaughter facility inspected by the USDA. All cattle were slaughtered following standard plant operating procedures.

Experimental design

After slaughter, each animal was assigned a liver abscess score by a trained observer, as described below. Scores were based on the 3-point Elanco Scoring System, which included scores of 0, A, and A+ (Elanco, 2019). Based on the liver abscess scores, cattle were assigned into one of three groups: No Liver Abscess (NLA; score of 0; n = 316); Mild Liver Abscess (MLA; score of A; n = 21) and Severe Liver Abscess (SLA; score of A+; n = 24). Two animals were unable to be assigned a liver abscess score and were excluded from the data analysis. Eleven steers were removed from the feeding trial, described above, based on the presence of horns, presence of *Bos indicus* or Holstein breed characteristics, and/or intake body weight (BW) at arrival (mean BW \pm 2 sd), but were still included in this analysis. During pre-slaughter

weighing, four different data measurements were taken for each animal: hair length, hair sample for hair cortisol analysis, ocular infrared thermal image, and mobility score. During slaughter, exsanguination blood was collected for cortisol analysis from specifically marked animals. Data collected during live weighing and slaughter procedures were then compared based on the assigned liver abscess score groups.

Live Animal Data Collection

Data were collected between 6:20 AM and 11:20 AM as cattle underwent normal routine handling for pre-slaughter weighing. Cattle were moved through the CSU ARDEC handling facility using low stress handling techniques and were restrained individually in a hydraulic chute (Silencer, Silencer Hydraulic Chutes, Stapleton, NE). All animals were subjected to the same handling techniques.

Hair Measurement and Collection

While restrained, the right rump region of each animal was brushed with a rubber curry comb (ConairPRO[®] Equine, Conair Corporation, Glendale, AZ) in a circular motion before any measurements or samples were collected to remove as many shedding hairs as possible. The curry comb was cleaned and disinfected with 70% isopropyl alcohol (Equate, Bentonville, AR) between animals. A calibrated, digital caliper (Neiko 01407A, Neiko Tools, Taiwan) was used to measure the hair length of each animal in the intended shaving area of the right rump region. Samples were collected from one consistent area on all animals as suggested by Tallo-Parra et al. (2017). Caliper measurements were obtained and verbally read to another researcher to be recorded; all tasks were consistently performed by the same researcher for each animal. Nitrile gloves (VWR[®], Radnor, PA) were worn by all researchers who were in contact with the hair measurement and sampling.

Hair samples were then collected from each animal using a cordless livestock clipper (Andis Pro Clip Pulse Ion, Andis Company, Sturtevant, WI) fitted with an adjustable, detachable No. 40 blade. One small, square area of hair, approximately 2.54 cm x 6.35 cm, was shaved from the rump region on the right side of each steer (Figure 4.1). The hair was shaved as close to the skin as possible. Each sample consisted of only one hair color. The clipper was cleaned and disinfected with 70% isopropyl alcohol between animals. Each hair sample was immediately placed in an individually labeled Ziploc[®] bag and placed into an opaque container. The cover of the container was kept closed during sample collection, except to place the most recently collected hair sample in the container. Hair samples were stored in an opaque container in Ziploc[®] bags in the dark at room temperature until analysis to reduce exposure to light (Wester et al., 2016; Creutzinger et al., 2017).

Infrared Thermography

An infrared (IR) camera (FLIR E85 Thermal Camera, FLIR Systems, Boston, MA) was used to collect ocular thermal images of each steer while restrained. Images were taken of the right eye from an approximate distance of 0.914 m as measured by the internal, calibrated laser beam feature of the IR camera in perpendicular alignment to the ocular area of interest (Figure 4.2). The measuring laser beam was placed rostrally to the eye to avoid direct laser contact with the animal's eye. All images were taken by the same researcher while standing immediately to the right of the chute head restraint.

Ocular thermography images were analyzed with FLIR Tools software (FLIR Systems, Boston, MA). For each image, the area of interest was selected via the circle tool in FLIR Tools to calculate the maximum temperature of the eye region (Figure 4.3). External temperature and relative humidity averages for the day of collection via a local weather station were entered into

the image analysis software since these factors have shown to impact eye temperature measurement (Church et al., 2014). Temperature data were recorded into a Microsoft Excel spreadsheet and used for statistical analysis. One value was unable to be calculated; therefore, was excluded from the analysis.

Mobility Scoring

Upon chute exit, a trained observer assigned each steer a mobility score using the North American Meat Institute (NAMI) Mobility Scoring System (1 = Normal, walks easily with no apparent lameness or change in gait; 2 = Able to keep up with normal cattle walking as a group and exhibits one or more of the following: minor stiffness, shortness of stride or a slight limp; 3 = Unable to keep up with normal cattle walking as a group, exhibits one or more of the following: obvious stiffness, difficulty taking steps, an obvious limp or discomfort; 4 = Statue-like, extremely reluctant to move even when encouraged by a handler; NAMI, 2016). The observer assigned scores while standing slightly ahead of and to the right side of the chute exit to obtain a clear vantage point.

Post-mortem Data Collection

Two days after the pre-slaughter sample collection, cattle were shipped approximately 35 minutes to a commercial slaughter facility. Upon arrival, cattle were unloaded into holding pens. After USDA antemortem inspection, cattle were moved through the handling facility and processed through a center-track conveyor restrainer. All cattle were stunned with a pneumatic captive bolt gun and subsequently shackled and hoisted onto the main rail. Upon determination of insensibility, exsanguination occurred followed by further dressing procedures.

Blood Collection

Exsanguination blood was collected for 115 animals out of the 363 total number of animals. Due to the constraints of sampling rate and line speed, approximately every third stunned animal was marked with livestock chalk (All-Weather Paintstik Livestock Marker, Laco Industries Inc, Elk Grove Village, IL) to designate the animal to be sampled. For each marked animal, exsanguination blood was collected into a plastic cup (9 oz clear plastic cups, Great Value, Bentonville, AR) approximately 5 seconds after exsanguination was performed. The whole blood was immediately transferred from the plastic cup into a 10 mL red top serum tube (Monoject™ Blood Collection Tubes, Covidien, Dublin, Ireland). Once collected, blood tubes were placed in a Styrofoam cooler on ice for transport back to a lab for further processing.

In the lab, samples were placed into a centrifuge (Beckman Model TJ-6, Beckman Coulter, Brea, CA) and spun at 2,000 x g at 4°C for 20 minutes. The serum layer was transferred to plastic microcentrifuge tubes (VWR®, Radnor, PA) via disposable plastic pipettes. Samples were stored at -80°C until analysis.

Liver Abscess Scoring

During the evisceration process, a trained observer examined livers and assigned a liver abscess score to each animal using the Elanco Scoring System (0 = No abscesses – a normal, healthy liver; A = One or two small abscesses, or up to two or four well-organized abscesses, which are generally under one inch in diameter, with the remainder of the liver appearing healthy; A+ = One or more large abscesses present with liver tissue inflammation surrounding the abscess and portions of the diaphragm adhered to the surface of the liver that may have to be trimmed to separate the liver from the carcass; Elanco, 2019). The proportion of liver abscess adherence to the body wall was not specifically denoted if a score of A+ was assigned. Scores were assigned while livers remained in the normal production line.

Hair Cortisol Analysis

Hair samples were selected for analysis based on the associated serum sample from the same individual steer, thus 115 serum samples were analyzed. Each hair sample was removed from the plastic bag, cut into small fragments (approximately 5 mm) using scissors, and transferred to a plastic microfuge tube (Denville Scientific Inc, Metuchen, NJ). The scissors were cleaned with methanol (Optima LC-MS grade, Fisher Scientific, Waltham, MA) after each sample. Hair was submerged in liquid chromatography mass spectrometry (LC-MS) grade water (Optima LC-MS grade, Fisher Scientific, Waltham, MA) and placed on a shaker (Reliable Scientific Rocking Shaker, Reliable Scientific Inc., Hernando, MS) for 4 hours at 37°C. To dry the hair, acetone (Optima LC-MS grade, Fisher Scientific, Waltham, MA) was added, and samples were shaken at room temperature for two minutes. Next, samples were placed under a stream of nitrogen gas until completely dry. Cleaned, dry hair was weighed (20 ± 0.5 mg) into a 2.0 mL glass vial (VWR[®], Radnor, PA). Then, 1.5 mL of the internal standard methanol (100% methanol spiked with d4-Cortisol at 26.67 pg/mL) was added. Samples were sonicated for 16 hours at 50°C in a covered bath sonicator (Branson Ultrasonic Cleaner, Branson Ultrasonics, Danbury, CT). After sonication, hair was pelleted to the bottom of the tube via centrifugation (Model 5430, Eppendorf, Hauppauge, NY) at 3,000 x g for 20 minutes. The methanol supernatant was transferred to a 1.5 mL microfuge tube and incubated at -80°C for 1 hour to precipitate out proteins and other insoluble particulates. The samples were centrifuged at 18,000 x g for 20 min at 4°C. The supernatant was transferred again to a new tube and dried under nitrogen gas. Samples were resuspended in 100 uL of 100% methanol. Resuspended material was vortexed and centrifuged again at 18,000 x g for 5 min 4°C. Finally, 80 uL of resuspension

was transferred to a LC-MS vial insert. To make a representative quality control (QC) sample, 10 μ L volume of each final extract was pooled together and aliquoted into three separate QC vials.

Sample analysis via liquid chromatography tandem mass spectrometry (LC-MS/MS) was performed using a Waters Classic Acquity UPLC coupled to a Waters Xevo TQ-S triple quadrupole mass spectrometer (Waters Corporation, Milford, MA). The calibration curve was constructed using washed test samples of cattle hair prepared as described above. A surrogate “light” standard of ^{13}C -Cortisol (Sigma-Aldrich, Inc., St. Louis, MO) was used, while the d_4 -Cortisol (Cerilliant Corporation, Round Rock, Texas) served as the true internal standard as performed by Binz et al. (2016). The pooled QC samples yielded a 10.79% variation. The level of detection (LOD) was 0.156 pg/mg. Three samples contained cortisol concentrations that were unable to be detected.

Serum Cortisol Analysis

For hormone extraction, frozen serum samples were thawed on ice. A 150 μ L aliquot of each sample was placed into a 2.0 mL microfuge tube (Denville Scientific Inc, Metuchen, NJ). A 1.5 mL of cold 1:1 Chloroform:Methanol (spiked with 2.5 ng/mL of Corticosterone- d_4 (Sigma-Aldrich, Inc., St. Louis, MO)) was added to each sample as a surrogate internal standard and vortexed at 4°C for 10 minutes. Samples were placed at -80°C for 30 minutes to facilitate protein precipitation. Precipitated proteins were collected via centrifugation (Model 5430, Eppendorf, Hauppauge, NY) 18,000 x g, 15 minutes at 4°C. Next, 1.6 mL of the supernatant was collected into a new microfuge tube and concentrated to dryness under nitrogen gas at room temperature. Samples were resuspended in 0.080 mL of 25% methanol, 75% water. Insoluble material was centrifuged out of solution and 0.075 mL was transferred into a glass insert and autosampler vial (VWR[®], Radnor, PA).

The LC-MS/MS analysis was performed using a Waters Classic Acquity UPLC coupled to a Waters Xevo TQ-S triple quadrupole mass spectrometer (Waters Corporation, Milford, MA). An 8 point standard curve of cortisol was generated by spiking in ^{13}C -Cortisol (Sigma-Aldrich, Inc., St. Louis, MO) into steroid-free bovine serum (0.15 mL) at concentrations between 0 – 1000 ng/mL. The LOD was 0.056 ng/mL.

Statistical Analysis

Data were analyzed with the software R version 3.4.1 (R Core Team, Vienna, Austria) in RStudio using the car (Fox and Weisberg, 2011), plyr (Wickham, 2011), and emmeans (Lenth, 2019) packages. Linear fit models were created for each numerical response (IR ocular maximum temperatures, hair cortisol concentrations, serum cortisol concentrations) with liver abscess score as a predictor variable using the lm function. Models that included hair cortisol concentration as the response variable also included hair color as a predictor variable. All models included a block by feeding trial treatment. All models were subject to an analysis of variance using the Anova function. For categorical variables, estimated marginal means (or least squares means) and Tukey adjusted pairwise comparisons were calculated. Summary statistics and data graphing were performed to check normality. Hair and serum cortisol concentrations were base 10 logarithm transformed to achieve normality and satisfy all model assumptions. All cortisol concentrations considered to be below the LOD were analyzed as values equating to $0.5 \times \text{LOD}$. Eight hair samples and four serum samples yielded concentrations below the LOD. Values that were out of the range of three standard deviations plus or minus the mean were considered to be outliers and excluded from the analysis. The data contained one hair cortisol outlier and two serum cortisol outliers, which were removed from the analysis. Three defined time groups pertaining to the time of hair sample collection (Group 1: samples taken from 6:20 AM – 8:00

AM, Group 2: samples taken from 8:01 AM – 9:41 AM, Group 3: samples taken from 9:42 AM – 11:22 AM) were used to test the effect of sampling time on hair cortisol concentration. Categorical data were analyzed with a Fisher's exact test to evaluate the relationship between hair color and liver abscess presence. Significant differences were recognized at $\alpha \leq 0.05$.

RESULTS

The demographic breakdown of the hair color and liver abscess scores for the cattle on trial are reported in Table 4.1. A majority of cattle were black with no liver abscesses present. The overall liver abscess rate was 12.4%.

Table 4.2 reports the impact of liver abscess presence on ocular thermography, hair cortisol and serum cortisol. There were no statistically significant effects on maximum ocular temperature, hair cortisol and serum cortisol ($P > 0.05$). Additionally, hair color was not statistically significant when compared across liver abscess scores ($P = 0.70$). All cattle were assigned a mobility score of 1, exhibiting normal mobility and thus statistical analyses were not performed.

No relationships were observed regarding hair cortisol and related metrics. Significant differences were not observed for the effect of hair color ($P = 0.52$), hair length ($P = 0.61$) or sampling time ($P = 0.80$) on hair cortisol concentration. Figure 4.4 shows the correlation between hair and serum cortisol concentrations. No linear relationship was observed ($r = 0.18$, $P = 0.06$) between the two values.

DISCUSSION

As the concern for the welfare of animals from the perspectives of scientists, producers and consumers rises, the impact of management practices on animal well-being is becoming a

focal point of interest. Liver abscesses have been a topic of research for animal scientists for many years with primary focus of abscess impact on nutrition, feed efficiency, and carcass quality (Smith, 1940; Brown et al., 1973, Rust et al., 1980; Brink et al., 1990). However, the influence that a metabolic production disease may have on the welfare state of animals, specifically cattle, has not been explored. In this study, we chose to observe and evaluate parameters of acute and chronic stress.

Infrared thermography is usually used to assess stress during acute stress-related events. The viability of this technology being used to quantify chronic stress is a relatively new approach. Ocular thermal images of piglets postweaning showed high and positive correlations with dorsal surface temperature and salivary cortisol during the first two weeks after weaning (Pulido-Rodríguez et al., 2017). Researchers indicated that a possible connection between IR and chronic stress may exist, though additional investigation is necessary. Herborn et al. (2018) reported that face, eye and comb (only of those birds subject to a mild weighing handling protocol) temperatures were elevated the day after different types of weekly handling occurred in laying hens. Furthermore, they found that comb temperature was higher six days after the last handling event in hens subjected to an invasive blood sampling handling protocol. These findings suggest that increased comb, face and eye temperature may be indicators of chronic stress in laying hens (Herborn et al., 2018).

In this study, ocular IR thermography was explored as a potential tool to assess chronic stress associated with liver abscess presence, even though a large majority of research uses IR thermography in more acute applications. The effect of stressful events or procedures on measured maximum ocular temperatures varies with research studies. Gomez et al. (2017) found no effect of a restraint event for claw trimming on maximum eye temperatures in dairy cows.

Studies involving calf castration (Stewart et al., 2010) and competition horses (Valera et al., 2012) have reported increases in the maximum ocular temperature in association with these painful or high exertion events. On the contrary, rapid decreases in eye temperature have been observed immediately after cautery disbudding (Stewart et al., 2008a) and after stressful cattle handling procedures, including shouting, waving objects close to the face, rump slaps, or electric prodding (Stewart et al., 2008b). Stewart et al. (2008a, 2008b) mention that the drop in eye temperature may have been linked to sympathetic-related vasoconstriction via the HPA axis. The lack of eye temperature differences in this study may be linked to the relatively unknown application of thermography to show chronic stress as compared to acute event related stress. More research is required to improve our understanding of ocular temperatures and long term, chronic stress in cattle.

Cortisol is considered a biomarker of stress. Hair is becoming utilized as a non-invasive measurement tool for chronic stress. The use of this medium has been explored in a multitude of species, including but not limited to rock hyrax (Koren et al., 2002), rhesus macaques (Davenport et al., 2006), domestic dogs and cats (Accorsi et al., 2008), grizzly bears (Macbeth et al., 2010), sows (Bacci et al., 2014), sheep (Stubsjøen et al., 2015), rabbits (Peric et al., 2017), humans (Short et al., 2016), and coyotes (Schell et al., 2017). Using hair as a matrix to measure cortisol is a valid method to monitor the exposure of an animal to environments and situations that will increase cortisol levels over time, which would be indicative of chronic stress (Comin et al., 2013). Creutzinger et al. (2017) reported that hair cortisol shows potential as a measurement of chronic stress in a study involving the castration of beef calves, during which a significant difference was reported between treatment groups at day 14. Moya et al. (2013) standardized the method of clipping hair as a method to measure cortisol in beef cattle hair as an indicator of

chronic stress. Other studies have reported the necessity for further research to truly conclude that hair cortisol is a measure of chronic stress in multiple species (Finkler and Terkel, 2010; Burnard et al., 2017; Casal et al., 2017).

Our reported hair cortisol concentration values ranged from 0.078 to 1.71 pg/mg with an overall mean of 0.47 ± 0.09 pg/mg. These results were similar to values reported in dairy cattle (0.73 ± 0.46 pg/mg; Braun et al., 2017). Other studies have found values that are substantially higher in beef bulls (2.35 ± 0.176 pg/mg; Moya et al., 2013), weaned beef calves (2.36 ± 0.38 pg/mg; Marti et al., 2017), beef bull calves (5.16 ± 1.63 pg/mg; Creutzinger et al., 2017) and 2-year-old dairy cows (12.15 ± 1.85 pg/mg; González de la Vara et al., 2011). The variability in hair cortisol concentration of cattle suggests that several factors, such as age (González de la Vara et al., 2011), hair color and sampling location (Burnett et al., 2014) may have an impact. Additionally, we analyzed our hair samples using LC-MS/MS, as performed in Braun et al. (2017), whereas other studies have commonly used enzyme immunoassay (Moya et al., 2013, Creutzinger et al., 2017, Marti et al., 2017), enzyme-linked immunosorbent assay (ELISA) (Marti et al., 2017) or radioimmunoassay (González de la Vara et al., 2011). Mass spectrometry is considered the “gold standard” for hair analysis as the methods are very specific and highly sensitive (Gow et al., 2010). A study comparing analysis methods for human hair found that EIA yielded an average 15% inflation of the results compared to LC-MS/MS (Kirschbaum et al., 2009). We prepared the hair samples by using a scissors to cut small fragments, which has been shown to produce significantly lower cortisol concentrations as compared to hair samples that were processed with a ball mill (Burnett et al., 2014). Similarly, to Braun et al. (2017), we believe that our measurement technique and hair preparation may have been linked to the observed differences in cortisol concentrations between this study and other studies. Further

research should be performed to create methodology validation and establish baseline hair cortisol values.

Studies have presented varying results regarding the levels of cortisol in hair related to chronic stress. Elevated serum cortisol levels in relation to acute adrenocorticotrophic hormone (ACTH) injection challenges, which test the reactivity of the adrenal gland to produce cortisol through stimulation of the hypothalamic-pituitary-adrenal (HPA)-axis, did not alter hair cortisol levels (Tallo-Parra et al., 2017). Serum cortisol concentrations are utilized as a single point in time measurements of acute stress, whereas a metric of chronic stress would reflect stress levels over long periods of time (Russel et al., 2012). Therefore, Tallo-Parra et al. (2017) concluded that the lack of change in the hair cortisol concentrations due to the acutely increased serum cortisol levels indicates the progress toward using hair cortisol as a chronic stress indicator. Ghassemi Nejad et al. (2019) found that hair cortisol concentrations increased after thermal-humidity exposure of high heat and humidity levels for 64 (Exp. 1) and 74 (Exp. 2) days when compared to other cortisol concentrations from cattle in the thermo-neutral zone. Healthy lactating dairy cows exhibited significantly lower hair cortisol concentrations than those clinically diseased; however, no difference was observed for those with subclinical disease (Burnett et al., 2015). The presence of liver abscesses could be considered a subclinical disease, being that most cattle do not show obvious clinical signs of this condition. Therefore, cattle with liver abscesses may not exhibit a significantly different hair cortisol concentration as compared to those without liver abscesses. In agreement with our results, Braun et al. (2017) found that hair cortisol concentrations of dairy cows did not differ between those with or without illness. Furthermore, hair cortisol was not demonstrated to be a biomarker for chronic lameness in dairy cows (Fischer-Tenhagen et al., 2017) and feedlot cattle (Marti et al., 2018). This may suggest

that the presence of liver abscesses does not create chronic stress due to previous hair cortisol validation studies.

In agreement with our findings, studies in cattle have found no effect of hair color on hair cortisol (Ghassemi Nejad et al., 2017; Nedić et al., 2017). Other findings have contrasted with our results. Greater cortisol concentrations were observed in white cattle hair as compared to black hair (González-de-la-Vara et al., 2011; Cerri et al., 2012; Burnett et al., 2014). On the contrary, Tallo-Parra et al. (2015) reported the presence of higher cortisol concentrations in black hair in relation to white hair, although did recognize possible hair sampling discrepancies (i.e. white hair samples were sampled from the same location, whereas black hair samples were homogenous in color, but not sample location – forehead vs. occipital crest). The majority of cattle in our study were black with a very limited number of non-black cattle present; further studies exploring a variety of hair colors would be beneficial to understand the impact of color on cortisol concentration.

Our reported serum concentrations ranged from 0.028 ng/mL to 1.079 ng/mL, which are somewhat similar to dairy heifer baseline values (approx. 2.3 to 5.2 ng/mL; Ghassemi Nejad et al., 2019). Other studies have reported values that are drastically different than our results, involving crossbreed beef cows subjected to handling and restraint (29.50 ng/mL; Bristow and Holmes, 2007), young crossbred beef heifers (16.8 to 59.8 ng/mL; Chen et al., 2016), and lactating dairy cows (8.69 ng/mL; Barragan et al., 2018). The large difference between our reported values and other studies may be linked to the fact that our values were obtained from exsanguination blood rather than live animal blood samples. Cattle were also subject to additional handling and restraint stress in a novel environment, which has been shown to increase cortisol concentrations (Grandin, 1997).

Studies have shown that serum cortisol becomes elevated during various disease and environmental stress related events, including retained placenta before parturition (Peter and Bosu, 1987), diagnosed lameness (O'Driscoll et al., 2017), longer transport distances (Chulayo et al., 2016) and general human presence and restraint, especially involving unhabituated cattle (Saco et al., 2007). Serum cortisol is generally associated with acute stress, as shown by marked time point increases pertaining to the occurrence of stressful events (Russell et al., 2012). The development of liver abscesses takes place over time, which indicates that any experienced stress would most likely be deemed chronic instead of acute. Thus, the relationship between serum cortisol and associations with acute stress may explain why differences in serum cortisol concentrations between liver abscess scores were not observed in our study. However, Macdonald et al. (2017) reported significantly higher plasma cortisol levels in bulls with liver abscesses as compared to those without abscesses when nine blood samples were taken at different time points (days 0.5, 1, 5, 18, 22, 26, 28 and 33 before slaughter) throughout the 56-day sampling duration during the finishing period. This result suggests that the presence of liver abscesses may induce a stress response related to a diseased state. Additionally, the researchers did not find differences between liver abscessed and non-abscessed cattle for serum cortisol concentrations from a blood sample that was collected at the time of slaughter after exsanguination (Macdonald et al., 2017). The various stress induced by the transportation, handling and restraint associated with slaughter may have affected these cortisol concentrations. Montanholi et al. (2013) observed variation in cortisol concentrations from samples that were collected during a stressful handling and restraint event. Therefore, the occurrence of pre-slaughter stress may have overridden the effects of liver abscess presence on the levels of serum cortisol.

Hair and serum cortisol concentration correlations have shown varying results from several studies. Our results indicate a correlation trend, though the coefficient indicates that the variables are non-linear. Lockwood et al. (2017) found no relationship between hair and serum cortisol concentrations. On the contrary, a significant positive association was reported between hair cortisol concentrations from the tail and hip regions to cortisol concentrations in saliva (Moya et al., 2013). They stated that hair cortisol likely represents a long-term average of plasma cortisol levels and provides information on stress responses over long periods of time.

Research with aims to understand the welfare implications of liver abscesses is a rather novel focus. To the authors' knowledge, this study and one other recent publication are among the small number of projects that have evaluated the impact of liver abscess presence on stress and well-being of cattle. Moya et al. (2015) studied the effect of grain type and processing index on multiple factors concerning mixed breed feedlot steers, specifically the stress response as measured by hair cortisol and carcass quality. They reported that cattle had greater hair cortisol when fed a wheat-based diet as opposed to a barley-based diet. Furthermore, cattle fed barley with an 85% processing index tended to exhibit a lower incidence of severe liver abscesses as opposed to other diet types. This suggests a possible indication that liver abscess presence may have an additional stressful impact on cattle based upon the fact that steers with a higher incidence of severe liver abscesses yielded higher hair cortisol concentrations. This aforementioned conclusion was not stated specifically within Moya et al. (2015), which illustrates the need for further research on the topic of quantifying stress in cattle with liver abscesses.

CONCLUSION

The data indicate that under the conditions of this experiment, liver abscesses did not impact measured stress-related outcomes. Additional research needs to be conducted to specifically study liver abscesses that have adhesions to the body wall. Future effort should also be focused on establishing benchmark values of hair cortisol concentration for other applications and validations such as hair sampling techniques, metabolite analysis methodology, and potential health indicators. These results warrant further research to be performed to investigate the impact of liver abscesses on the well-being of feedlot cattle, especially involving other stress-related parameters.

Table 4.1 Hair color and liver abscess presence demographics of the research cattle (N = 363)

Hair Color	Liver Abscess Presence ¹		
	NLA (n = 316)	MLA (n = 21)	SLA (n = 24)
Black	286	21	21
White	14	0	1
Red	17	0	2

¹Liver abscess presence: NLA: no liver abscess presence, score of 0; MLA: mild liver abscess presence, score of A; SLA: severe liver abscess presence, score of A+.

Table 4.2 Effect of the presence of liver abscesses on ocular infrared thermography, hair cortisol, serum cortisol and hair length of feedlot cattle (N = 363)

Outcome	n	Liver Abscess Presence ¹			Pooled SEM	P - value
		NLA	MLA	SLA		
Ocular Thermography (°C)	360	36.97	37.14	36.89	0.13	0.55
Hair Cortisol (pg/mg) ²	111	0.474	0.468	0.474	0.09	0.96
Serum Cortisol (ng/mL) ²	113	0.475	0.517	0.341	0.04	0.22

¹ Liver abscess presence: NLA: no liver abscess presence, score of 0; MLA: mild liver abscess presence, score of A; SLA: severe liver abscess presence, score of A+.

² Means and pooled standard error are reported on the original scale, while the P-value is reported based on the base 10 logarithm transformed values that was used to obtain a normal distribution of data.

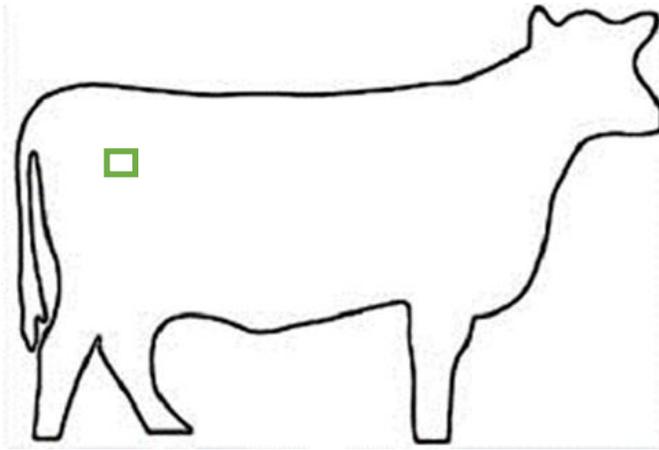


Figure 4.1 Diagram of the hair sampling area in the right rump region (the rectangle identifies the sampling area). The site measured approximately 2.54 cm x 6.35 cm. Image source: <http://clipart-library.com/outline-of-a-cow.html>



Figure 4.2 An image taken with an infrared FLIR E85 camera (FLIR Systems, Boston, MA) on the normal photo setting. The internal laser beam measuring device (circled in yellow) was placed rostrally to the eye to avoid direct laser to eye contact when measuring photo target distances.

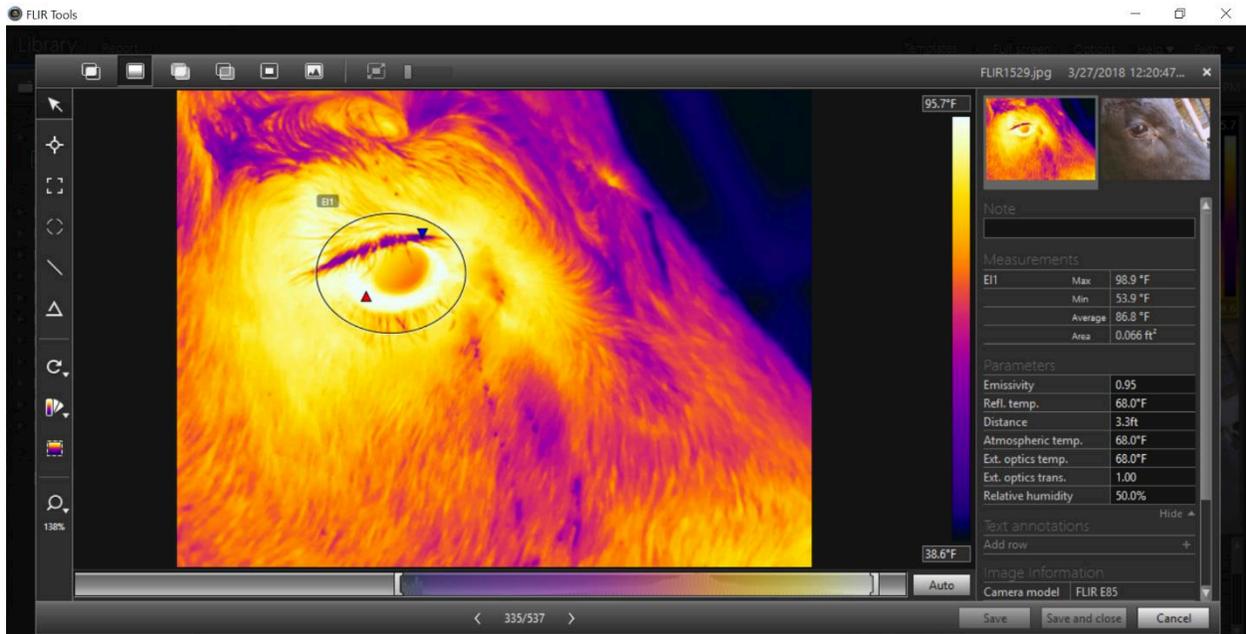


Figure 4.3 An ocular image taken with an infrared FLIR E85 camera (FLIR Systems, Boston, MA) uploaded to the FLIR Tools analysis software. A circle function tool was used to select the area of interest and measure the maximum ocular temperature.

A scatter plot of Hair Cortisol against Serum Cortisol

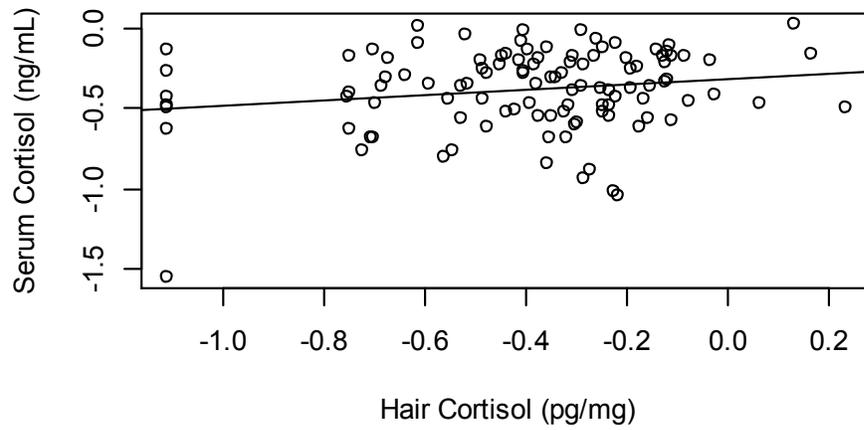


Figure 4.4 A scatterplot between base 10 logarithm transformed values for hair cortisol and serum cortisol (N = 111). The Pearson correlation is 0.1842 (P = 0.06).

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APPENDIX

CORTISOL ANALYSIS

Hair Cortisol

Sample Preparation

Hair samples were removed from the dark, room temperature storage conditions. Each hair sample was removed from the plastic bag and cut into small fragments (approximately 5 mm) using scissors. The scissors were cleaned with methanol (Optima LC-MS grade, Fisher Scientific, Waltham, MA) after each sample. Hair was submerged in LC-MS grade water (Optima LC-MS grade, Fisher Scientific, Waltham, MA) and placed on a shaker for 4 hours at 37°C. To dry the hair, 3-4 mL of acetone (Optima LC-MS grade, Fisher Scientific, Waltham, MA) was added, and samples were placed on a shaker at room temperature for 2 minutes. The washed hair was placed under a stream of N₂ until dry. Twenty mg (+/- 0.5 mg) of cleaned hair was weighed into a 2.0 mL glass autosampler vial. 1.5 mL of 100% methanol (Optima LC-MS grade, Fisher Scientific, Waltham, MA) spiked with d₄-Cortisol (Cerilliant Corporation, Round Rock, Texas) at 26.67 pg/mL was added. Samples were sonicated for 16 hours at 50°C in a covered bath sonicator. Care was taken that all samples were kept from light as much as possible.

After sonication, hair was pelleted to the bottom of the tube via centrifugation (Model 5430, Eppendorf, Hauppauge, NY) at 3000 x g for 20 minutes. Methanol supernatant was transferred to a 1.5 mL microfuge tube and incubated at -80°C for 1 hour. The samples were centrifuged at 18,000 x g for 20 min at 4°C. Samples were resuspended in 100 uL of 100% methanol. Resuspended material was vortexed and centrifuged again at 18K x g for 5 min at 4°C.

Finally, 80 uL of resuspension was transferred to a LC-MS vial insert. To make a representative quality control (QC) sample, 10 uL volume of each final extract was pooled together and aliquoted into 3 separate QC vials.

A standard calibration curve was constructed with the use of pooled test cattle hair samples and used as matched-matrix background. As stated in Binz, et al. (2016), 13C-Cortisol (Sigma-Aldrich, Inc., St. Louis, MO) was used as surrogate “light” standard while d4-Cortisol (Cerilliant Corporation, Round Rock, Texas) was used as the true internal standard. Once hair was pooled together, 20 mg of hair was weighed out into 9 separate glass vials. To this hair, 13C-Cortisol solutions were spiked at 0-100 ng/mL. Calibration points were extracted along with samples as described above. Final concentrations of curve points, after extraction and resuspension, was 0 – 25,000 pg/mL (100% Methanol). The final internal standard concentration was 400 pg/mL. The preparation procedure was performed in the same manner for both experiments.

Sample Analysis

Two separate analyses runs were performed for each experiment; therefore, analysis procedures are included in their entirety pertaining to Experiment 1 and Experiment 2.

Experiment 1

LC-MS/MS was performed on a Waters Classic Acquity UPLC coupled to a Waters Xevo TQ-S triple quadrupole mass spectrometer (Waters Corporation, Milford, MA). Chromatographic separations were carried out on a Waters HSS T3 UPLC column (2.1 mm x 50 mm, 1.7 µM). Mobile phases were 99.9% acetonitrile, 0.1% formic acid (B) and 10 mM Ammonium Formate with 0.1% formic acid (A). The analytical gradient was as follows: time = 0

min, 5% B; time = 1.0 min, 5% B; time = 3.0 min, 95% B; time 4.0 min, 95% B; time 4.1 min, 5% B. Total run time was 5 minutes. Flow rate was 350 $\mu\text{L}/\text{min}$ and injection volume was 10.0 μL . Samples were held at 6°C in the autosampler, and the column was operated at 45°C. The MS was operated in selected reaction monitoring (SRM) mode, where a parent ion is selected by the first quadrupole, fragmented in the collision cell, then a fragment ion selected for by the third quadrupole. Product ions, collision energies, and cone voltages were optimized for each analyte by direct injection of individual synthetic standards. Inter-channel delay was set to 3 ms. The MS was operated in negative ionization mode with the capillary voltage set to 2.2 kV. Source temperature was 150°C and desolvation temperature 550°C. Desolvation gas flow was 800 L/hr, cone gas flow was 150 L/hr, and collision gas flow was 0.2 mL/min. Nebulizer pressure (nitrogen) was set to 7 Bar. Argon was used as the collision gas. The pooled QC samples yielded a 9.54% variation. The level of detection (LOD) was 0.145 pg/mg.

Experiment 2

LC-MS/MS was performed on a Waters Classic Acquity UPLC coupled to a Waters Xevo TQ-S triple quadrupole mass spectrometer. Chromatographic separations were carried out on a Waters HSS T3 UPLC column (2.1 mm x 100 mm, 1.7 μM). Mobile phases were 99.9% acetonitrile, 0.1% formic acid (B) and 10 mM Ammonium Formate with 0.1% formic acid (A). The analytical gradient was as follows: time = 0 min, 5% B; time = 1.0 min, 5% B; time = 5.0 min, 95% B; time 6.0 min, 95% B; time 6.1 min, 5% B. Total run time was 10 minutes. Flow rate was 200 $\mu\text{L}/\text{min}$ and injection volume was 10.0 μL . Samples were held at 6°C in the autosampler, and the column was operated at 45°C. The mass spectrometer (MS) was operated in selected reaction monitoring (SRM) mode, where a parent ion is selected by the first quadrupole, fragmented in the collision cell, then a fragment ion selected for by the third quadrupole. Product

ions, collision energies, and cone voltages were optimized for each analyte by direct injection of individual synthetic standards. Inter-channel delay was set to 3 ms. The MS was operated in negative ionization mode with the capillary voltage set to 2.2 kV. Source temperature was 150°C and desolvation temperature 550°C. Desolvation gas flow was 800 L/hr, cone gas flow was 150 L/hr, and collision gas flow was 0.2 mL/min. Nebulizer pressure (nitrogen) was set to 7 Bar. Argon was used as the collision gas. The resulting pooled QC samples yielded a 10.79% variation. The LOD was 0.156 pg/mg.

Serum Cortisol

Sample Preparation

Frozen serum samples were thawed on ice and 150 µL of each sample was placed into a 2.0 ml polypropylene microfuge tube and kept on chilled on ice. One and one-half milliliter of cold (-80°C) 1:1 Chloroform Methanol, spiked with 2.5 ng/mL of Corticosterone-d4, was added to each sample and vortexed at 4°C for 10 minutes. Corticosterone-d4 was used as surrogate internal standard for serum extractions only. Samples were placed at -80°C for 30 minutes to facilitate protein precipitation. Precipitated proteins were collected via centrifugation 18,000 x g 15 minutes at 4°C. 1.6 mL of the supernatant was collected into a new polypropylene microfuge tube and concentrated to dryness under house N₂ at room temperature. Samples were resuspended in 0.080 mL of 25% methanol, 75% water. Insoluble material was centrifuged out of solution at max speed and 0.075 ml was transferred into a glass insert and autosampler vial.

An 8 point standard curve of Cortisol was generated by spiking in 13C-Cortisol into steroid-free bovine serum (0.15 mL) at concentrations between 0 – 1000 ng/mL. Each sample was extracted as above with Corticosterone-d4 labeled Chloroform:Methanol (1:1 v/v). Final concentrations of 13C-Cortisol back-calculated to ng/ml serum: 0 – 533 ng/ml.

Sample Analysis

LC-MS/MS was performed on a Waters Classic Acquity UPLC coupled to a Waters Xevo TQ-S triple quadrupole mass spectrometer. Chromatographic separations were carried out on a Waters HSS T3 UPLC column (2.1 mm x 100 mm, 1.7 μ M). Mobile phases were 99.9% acetonitrile, 0.1% formic acid (B) and 10 mM ammonium formate with 0.1% formic acid (A). The analytical gradient was as follows: time = 0 min, 5% B; time = 1.0 min, 95% B; time 5.0 min, 95% B; time 6.0 min, time 6.1 – 10 minutes, 5% B. Total run time was 10 minutes. Flow rate was 200 μ L/min and injection volume was 3.5 μ L. Samples were held at 6°C in the autosampler, and the column was operated at 45°C. The MS was operated in selected reaction monitoring (SRM) mode, where a parent ion is selected by the first quadrupole, fragmented in the collision cell, then a fragment ion selected for by the third quadrupole. Product ions, collision energies, and cone voltages were optimized for each analyte by direct injection of individual synthetic standards. Inter-channel delay was set to 3 ms. The MS was operated in positive ionization mode with the capillary voltage set to 3.0 kV and negative ion mode with capillary voltage set to 2.2kV. Source temperature was 150°C and desolvation temperature 550°C. Desolvation gas flow was 800 L/hr, cone gas flow was 150 L/hr, and collision gas flow was 0.2 mL/min. Nebulizer pressure (nitrogen) was set to 7 Bar. Argon was used as the collision gas. The resulting pooled QC samples yielded a 12.93% variation. The LOD was 0.056 ng/mL.