

THESIS

UTILIZATION OF ALLOSTATIC LOAD INDEX (ALI)

TO EVALUATE STRESS DURING WEANING OF BEEF CATTLE

Submitted by

Sara Meyer

Department of Animal Sciences

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Master's committee:

Advisor: Diego Manriquez Alvarez

Pablo Pinedo

Timothy Holt

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ABSTRACT

UTILIZATION OF ALLOSTATIC LOAD INDEX (ALI)

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Allostatic load index (ALI) has been utilized for years in human populations, and more recently in primate and wild animal populations, to predict the longevity and future health status of individuals. The purpose of this study was to apply the principles of allostatic load to assess beef cows' weaning stress as the principal factor. Two different weaning treatment groups, traditional abrupt or fence-line weaning strategies, were applied to cows. Stress levels were determined by measuring plasma cortisol, DHEA, TNF- α , and IL-6 concentrations through ELISA (enzyme-linked immunosorbent assay) over the course of a 64-day study period. Cows in the fence-line weaning treatment were found to have significantly decreased plasma TNF- α (2.83 pg/ml and 3.44 pg/ml \pm 0.30; p=0.04) levels and ALI scores (1.12 and 1.87 \pm 0.37; p=0.05) as compared to the abruptly weaned treatment group. The results from this study support that fence-line weaning of calves decreases the stress levels of cows following weaning. Additionally, there is evidence that cows with calves weaned via fence-line weaning have an increased longevity projection.

DEDICATION

I dedicate this work to my husband and son.

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CHAPTER 1: REVIEW OF THE LITERATURE

Introduction 1.1

Beef production systems require human intervention in the productive life of animals that might be subject to stress and impact later performance. Weaning of calves from their dams is required to continue the production cycle and it is key to maximize profitability, animal welfare, and the downstream efficiency of the beef supply. For the duration of this review, weaning will be defined as the physical separation of calf and cow, as well as the cessation of suckling.

Stress in animals has frequently been studied by observation of specific behaviors (Grandin, 1993; Watts and Stookey, 2000; Boland et al., 2008). Behavioral studies offer important insight into the state of an animal, yet it is difficult to set strict parameters for consistency across multiple studies. In consequence, objective studies to evaluate stress commonly involve analyzing plasma or serum cortisol levels (Cooke, 2016; Chase et al., 2017; Foote et al., 2017). One such method for objective evaluation of stress in animals, adapted from human research, is that of allostatic load. Allostatic load utilizes multiple blood biomarker concentrations to set a single score for an individual (Sterling and Eyer, 1988; Seeman, 1997; Edes et al., 2016b). Modification of current allostatic load principles for application in production animals could offer an effective tool to objectively determine stress levels in animals. Having clear physiologic indicators of stress in production animals would allow producers to predict possible physiological decline prior to the recording of decreased production parameters.

Beef Cattle Production 1.2

Beef cattle production is a segmented production system in which cattle can be purchased and sold at each step of the production cycle or retained in the case of larger companies. In the United

States, beef cattle production is divided into several segments: seedstock producers, commercial cow-calf production, yearling/stocker or background operations, finishing feedlot operations, packers, retailers and food service distributors, and consumers (Figure 1).

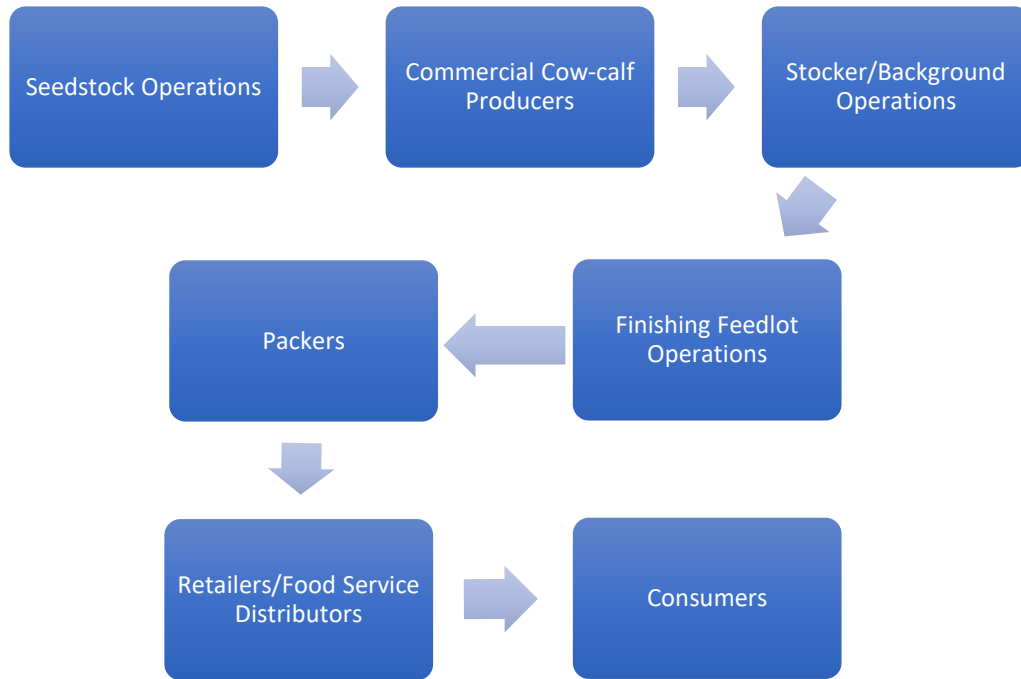


Figure 1. Beef cattle production segments (adapted from Field, 2007).

In brief, a seedstock producer’s goal is to generate animals with superior genetics to be utilized for breeding. The cattle raised in seedstock operations have been bred to possess high quality attributes related to conformation, growth, and health, which have been identified as critical production indices to the beef cattle industry (Ahola et al., 2019). Growth of the beef industry is achieved via interaction between cow-calf producers and seedstock producers. New pregnancies can be accomplished through natural or artificial conception methods. Natural conception involves the mating of a bull with a heifer or cow, whereas artificial conception methods include artificial insemination or embryo transfer. For much of the commercial beef industry, the production goal of cows is one calf per cow per year despite the production practices selected by each producer (Ahola et al., 2019).

Within the cow-calf production system, there are three different production cycles: spring calving herds, fall calving herds, and year-round calving herds. The USDA Animal and Plant Health Inspection Service (APHIS) conducted a survey from 1993-1997 to evaluate the percentage of producers with each type of production cycle. The results from this survey showed that close to 67% of beef calves are born in the early spring months, February through April (Feuz and Umberg, 2003). Calving seasons are typically dependent on the geographic location of a herd as well. The same APHIS survey found that production systems in the south were more likely to have two separate calving seasons, both fall and spring calving herds (Feuz and Umberg, 2003). In other regions of the country, a single calving season is more commonly adopted by producers.

On cow-calf operations, calves, after birth, will remain with the dam on pasture until weaning. Weaning occurs when the calves are approximately 6-7 months of age and average approximately 200-300 kg of body weight (Ahola et al., 2019). Newly weaned calves, not being retained as replacement females for the herd, are transported to one of three next subsystems in the production cycle: stocker operation, background feedlot, or finishing feedlot (Figure 1). Most calves produced will enter directly into a finishing feedlot where they will remain until market weight is reached. However, some producers opt to start calves in a stocker or backgrounding operation until they reach 450 kg, at which point they are then transported to the finishing feedlot (Ahola et al., 2019).

At weaning, depending on feed prices (Ahola et al., 2019), calves may bypass either the stocker or background operations and be transported directly to a finishing feedlot. These calves will typically be fed a growing diet for a period of 30-60 days prior to transitioning to the finishing diet. In the U.S., finishing diets for beef cattle are typically high concentrate diets containing large amounts of cereal grains or byproducts from human food and ethanol industries. Cattle remain in the finishing system for approximately 130-220 days until they reach an approximate live body weight of 680 kg body weight.

Once at market weight, the cattle are sold to a processing plant for harvest into products that subsequently enter different avenues within the food supply chain (Figure 1).

Weaning 1.3

Weaning is a stressful event for both calves and cows (Price et al., 2003; Haley and Stookey, 2005; Lambertz et al., 2014; Stěhulová et al., 2017; Rutherford et al., 2018). For cows, weaning is an annual process. The intention behind weaning calves from cows is to stop lactation and redirect that energy towards growth and development of their new fetus. Weaning in calves occurs between six and eight months of age. Following weaning, calves are often transported to a new environment. Commonly, transportation involves relocating to an alternate location where calves are mixed with calves from additional producers. Accompanying the mixing of calves is the establishment of a new social structure within the group. Hickey et al. (2003) conducted a study evaluating the effect of abrupt weaning on physiological mediators of stress and immune function. Reported results showed that disruption of the social group caused elevations in plasma cortisol, decreased leukocyte concentration, and decreased interferon- γ response. These responses were still evident at 7 days post weaning supporting evidence of altered immune function during a critical time in a calf's production cycle (Hickey et al., 2003).

In addition to the listed stressors, calves are subjected to a change in diet depending on which subsystem (stocker operation, background feedlot, or finishing feedlot) they enter, which has been shown to contribute to decreased dry matter intake (DMI; Hutcheson and Cole, 1986; Arthington et al., 2008). A study conducted by Arthington et al. (2008) found that steers weaned between 70-90 days of age had greater average DMI of concentrates compared to steers that were weaned without any introduction to a concentrate diet. Additionally, the early weaned steers and steers accustomed to creep feed had greater average daily gain (ADG) than steers weaned abruptly (Arthington et al., 2008).

During the lactational period of the cow, dams are rebred to maximize the goal of one calf per cow per year. Great amounts of stress in early gestational stages can inhibit adequate development of the placenta, thus causing potential damage to the new fetus (Ott et al., 2018). Within the early and mid-gestational period for a cow, the greatest stressor is likely to be the weaning of her current calf. Stress associated with weaning thus has the potential to be severe enough to cause poor or inadequate development of a fetus. The consequences of inadequate fetal development include loss of pregnancy or decreased calf birth weights (Ott et al., 2018). Ott et al. (2018) found that cows whose calves were abruptly weaned had decreased birth weights of their subsequent calves as compared to cows whose calves were late weaned or weaned utilizing nose flaps.

Weaning Strategies 1.4

Traditional weaning of beef calves involves the abrupt separation of the calf from the dam with calves being immediately transported to a new location. Handling of production animals is known to cause stress, which thus increases the appeal to traditional weaning. Grandin (1993) found that bulls and steers who were agitated upon initial handling were equally or increasingly agitated upon subsequent handling over a four-month study period. With traditional weaning, calves are handled a single time. During the handling, calves are separated, processed (vaccinated and castrated, if needed), and loaded onto a trailer for transportation. Lambertz et al. (2014) conducted a study to determine if weaning alone or processing calves at the time of weaning caused greater stress. The level of stress each calf was experiencing was determined by the amount of time each animal spent feeding, lying, and standing/walking (Lambertz et al., 2014). Their results indicated that calves were under greater stress from the practice of weaning alone, and that additional processing did not increase stress levels of the animals (Lambertz et al., 2014). The vast research that has been published on the stress associated with the handling of production animals supports the continued use of abrupt weaning. Additionally, producers have decreased labor costs by performing multiple tasks within a single handling period

(Moggy et al., 2017). However, the extreme stress reported to be associated with abrupt weaning has caused producers and researchers to develop alternative weaning strategies that decrease the induced stress on both cows and calves.

Another method of weaning utilized by producers is the two-stage weaning process. This involves the placement of a plastic nose flap on calves 4 to 7 days prior to separation from the dam. These flaps are designed to prohibit the calves from suckling while allowing them to remain with their dam. This strategy targets one of the many stressors associated with weaning by reducing the milk supply to the calf, thus making a dietary transition easier on the calf. Haley (2006) indicated that over 750 producers throughout Canada expressed interest in using the two-stage weaning process. Additionally, Haley (2006) observed that over 100,000 anti-suckling nose flaps were purchased from a single company during the same period of time. This suggests that the increased popularity of two-stage weaning is due to the supported decrease in stress both in calves and cows throughout the entire weaning process. Though most of the Haley's work was conducted with Canadian producers, there was at least one study conducted utilizing a cattle population within the United States (Haley et al., 2005).

Multiple studies across the world have been conducted to evaluate the level of stress in calves during the use of the nose flaps in the two-stage weaning process (Haley and Stookey, 2005; Boland et al., 2008; Enríquez et al., 2010; Clark et al., 2016). Calves using nose flaps prior to weaning were found to spend less time walking and more time lying than calves who were weaned traditionally. In addition, the calves that were traditionally weaned vocalized more frequently compared with those wearing nose flaps. Moreover, there is evidence that utilizing nose flaps during weaning decreases the stress in the calves and cows during process. A study conducted by Haley and Stookey (2005) compared three different treatment groups: abruptly weaned, two-stage weaning for 8 days, two-stage weaning for 4 days. Calves in both two-stage weaning groups were found to vocalize 97% less than those in the abruptly weaned group. Haley and Stookey (2005) also reported that cows had similar changes in stress

related behaviors to the calves. Cows whose calves wore nose flaps spent less time walking and more time lying, as well as they showed decreased vocalizations compared to cows whose calves were abruptly weaned.

Late weaning, though less common, is a weaning strategy still utilized by producers to improve calf performance without increasing labor input. Freeman et al. (2017) evaluated the impacts of late weaning (49 days post traditional weaning date), traditional weaning, and the use of nose flaps on calf behavior and productivity. Results from this study showed that calves weaned either traditionally or late had increased vocalizations and pacing as compared to those who wore the nose flaps prior to weaning. However, despite the apparent lesser stress inflicted on the calves wearing nose flaps during the weaning process, the late weaned calves and traditionally weaned calves had increased average daily gain (ADG) post weaning as compared to calves weaned with the two-stage weaning process (Freeman et al., 2017).

Another commonly utilized weaning method is fence-line weaning. This method requires little additional labor and involves placing newly weaned calves on the opposite side of a shared fence with their dams. Price et al. (2003) evaluated the performance and stress related behaviors of calves that were either fence-line weaned, totally separated from dams into a dry lot, or age-paired, non-weaned calves. Observational results showed that calves who had some access to their dams (fence-line and non-weaned) spent a greater amount of time eating and lying than those who were completely separated from the dams. In addition to observation, calf weights were recorded to assess the weaning treatment effects on short and long-term gain (Price et al., 2003). Calves were weighed at weaning then one time each week for a period of 10 weeks post weaning. Production data provided evidence that calves from the fence-line weaned treatment gained 10 kg more (mean = 21.4 kg) than the average for the other three totally separated groups (mean = 11.0 kg; Price et al, 2003). The average weight gain of calves in the non-weaned treatment group at week 2 post weaning was 20.0 ± 1.20 kg, and average

weight gain at 10 weeks post weaning was 64.8 ± 1.40 kg. The calves in the fence-line weaned treatment group had a 2 week post weaning average weight gain of 21.4 ± 1.05 kg, and a 10 week post weaning average weight gain of 50.0 ± 2.06 kg. Calves that were completely separated from their dams into a dry lot had 2 weeks post weaning average weight gains of 9.0 ± 1.05 kg and 10 weeks post weaning average weight gains of 37.2 ± 1.77 kg (Price et al., 2003).

A 2018 study conducted by Rutherford et al. (2018) examined cow behavior at weaning using a fence-line weaning strategy. Each cow was also assigned both a maternal aggression (MA) score (MA: 1 = flight response to 5 = fight response) and a mothering aptitude (MOM) score (MOM: 1 = calf abandonment, 2 = cow retreats quickly with calf, 3 = cow flees chasing calf, 4 = cow walks away with calf, 5 = cow stays in immediate area with calf) at calving. These researchers reported that first-calf heifers would remain closer to the shared fence line than multiparous cows. Additionally, cows who were assigned a MOM of 3/5 maintained the shortest distance to the fence despite their age (Rutherford et al., 2018). After data analysis, the authors concluded that if fence-line weaning were to be utilized, it should exceed a period of 5 days as the distance cows spend to the fence increases with each day post weaning (Rutherford et al., 2018).

Recent research has shown the combination of different weaning strategies with the goal of reaping multiple benefits from each individual strategy. A study from Morehead State University evaluated the effects of nose flap usage in addition to fence-line weaning. Calves were placed in one of three treatments: nose flaps placed 2 days prior to weaning, nose flaps placed 4 days prior to weaning, or no nose flap used. All calves were fence-line weaned on the same day no matter the assigned treatment group (Harrelson and Harrelson, 2017). Behavioral observations were collected beginning 6 days prior to weaning, daily, through 6 days post weaning. Calf activity was categorized as eating, suckling/attempting, walking, or inactive (lying, standing, or ruminating; Harrelson and Harrelson, 2017). Results showed calves that did not have a nose flap spent less time eating than calves with nose flaps

prior to weaning. However, during postweaning, calves without a nose flap were found to spend more time eating than calves with a nose flap in place 2 days prior to weaning. Harrelson and Harrelson (2017) reported that calves without nose flaps spent an increased amount of time walking postweaning as compared to both treatment groups of calves with nose flaps. The researchers concluded that the two-stage nose flap weaning strategy may indeed impact on stress related behaviors associated with weaning.

Despite the extensive research on alternative weaning strategies, traditional weaning, abrupt separation of calf and cow, remains common amongst producers in multiple countries (Moggy et al., 2017; Justice et al., 2024). A Canadian study found that producers do not utilize alternative weaning strategies due to the increased labor costs associated with the use of alternative weaning strategies. However, the researchers did additionally identify that cow-calf producers felt weaning was the most stressful event within a calf's life cycle (Moggy et al., 2017).

Limited quantitative research has been conducted to identify the stress associated with weaning from the cows' perspectives. A study conducted by Stěhulová et al. (2017) reported that the frequency of cow vocalization during and post weaning increased as the age of their calf at weaning decreased. Additionally, Stěhulová et al. (2017) reported that cows who were not pregnant at the time of weaning had increased vocalizations as compared to those who were pregnant. These same researchers reported that the cow's stress response associated with weaning was greatest within the first 24 hours post weaning as determined by frequency of vocalizations and time spent moving, thus suggesting that the initial separation between cow and calf may cause the most intense response.

Stress 1.5

Stress is defined as "the nonspecific response of the body to any demand" (Selye, 1976). When an organism undergoes stress, their welfare status changes, thus initiating biological processes to

combat the effects of altered homeostasis, or the stress response (Dhama et al., 2019). The stress response involves the neurological, immunological, and endocrine systems (Chu et al., 2020).

The biological stress response involves the activation of multiple interrelated systems. When an organism first perceives a stressor, the sensory systems of the brain will evaluate the stressor in relation to the previous stressors the organism has encountered (Fink, 2016). If the stressor is deemed to be of concern, the brain will activate the sympathetic-adrenal-medullary (SAM) axis. Activation of the SAM axis results in the release of catecholamines, epinephrine and norepinephrine (Dhama et al., 2019). These catecholamines cause an increased heart rate, respiratory rate, and divert energetic resources and blood supply from the gastrointestinal organs and the integumentary system to skeletal muscle (Fink, 2016).

In addition, the hypothalamic-pituitary-adrenal (HPA) axis is activated. Glucocorticoid release, in response to HPA activation, acts synergistically with catecholamines to increase blood glucose concentrations to support the organism's fight or flight response. Additionally, glucocorticoids allow the organism to record the response to a stressor for future perception; elevated glucocorticoid levels in an organism can improve their adaptation to a stressor so that future experiences with that stressor result in lower levels (Fink, 2016). Alternatively, glucocorticoids cause negative responses as well. In response to a stressor, glucocorticoids will inhibit the immune system thus decreasing the production of inflammatory cytokines (Fink, 2016). Short-term elevations of glucocorticoids allow for adaptation to stressors, whereas long-term elevations can result in cellular damage and system failure (Sapolsky, 2002; Goymann et al., 2004). Both glucocorticoids and catecholamines are commonly measured in the blood in the form of cortisol and epinephrine, respectively, to evaluate an organism's stress response (Sterling and Eyer, 1988; Goymann and Wingfield, 2004; Fink, 2016).

Stress Biomarkers 1.6

An organism's level of stress, and effects of stress on their body, can be measured utilizing biomarkers. Potential biomarkers to evaluate stress levels include heat shock proteins (HSPs), acute phase proteins (APPs), chemicals in bodily fluids, and physiologic markers of oxidative stress (Dhama, 2019). These biomarkers can be objectively measured to provide prognoses for long term effects of stress. For example, Arthington et al. (2008) measured plasma ceruloplasmin, an acute phase protein, concentrations in steers over a 30-day period to evaluate how weaning strategy affected feedlot performance. Results from the study showed that plasma ceruloplasmin concentrations were lower in abruptly weaned steers on day 0 as compared to early weaned steers (weaned at 70 to 90 days of age). However, the plasma concentrations significantly ($p < 0.05$) increased in the abruptly weaned steers on days 15 and 22 (Arthington et al., 2008).

Depending on their purpose, biomarkers can be placed in one of three classifications: diagnostic, prognostic, and therapeutic (Dhama et al., 2019). Diagnostic biomarkers assist in the detection of diseases, prognostic biomarkers are used to predict disease outcomes, and therapeutic biomarkers are used to measure the progress of an in-progress treatment (Dhama et al., 2019). Multiple biomarkers, in conjunction with each other, form a stress marker. Classic stress markers (heart rate, respiratory rate, body temperature) cause changes in hormone levels and pro-inflammatory cytokine concentrations, also referred to as physiologic biomarkers (Dhama et al., 2019). During a stress response, the release or change of one biomarker will influence the release or change in one or multiple other biomarkers due to the close interactions of all biomarkers (Dhama et al., 2019). Physiologically, this concept is important because the body's homeostatic mechanisms should work synergistically to return all systems back to normal following a stress response.

Cortisol 1.6.1

Cortisol is a primary mediator of physiological changes within an organism during stress. Three primary methods have been developed to measure cortisol levels in organisms: blood, hair, and saliva (Dhama et al., 2019). Plasma cortisol concentrations are most accurate as the HPA axis releases cortisol directly into the bloodstream during the stress response (Dhama et al., 2019). Salivary cortisol is primarily in the unbound form (Jung et al., 2014), and the concentrations found in saliva have been found to be significantly correlated with those in the blood (Ivkovic et al., 2015). In animal populations where blood, hair, and saliva may be difficult to obtain, feces can be collected and analyzed via glucocorticoid enzyme immunoassay (Goymann et al., 2004).

A study conducted by Peric et al. (2017) measured hormone levels in dairy cattle housed both indoors and tethered, as well as outdoors on pasture. Peric et al. (2017) reported that cortisol concentrations in cattle housed indoors were not significantly different between the first two months of the study, however, between the second and third months of the study, there was a significant increase in cortisol concentrations for the indoor housed cattle. The remaining study period (month 3 to 5) showed no further significant changes in cortisol concentrations. For the cattle housed on pasture the researchers found that cortisol concentrations increased during the first month, were similar to the concentrations at baseline during the second month, then increased again throughout the last month of the experimental period (Peric et al., 2017). Conclusions of the study inferred that the increases in cortisol for indoor housed cattle could be attributed to the increase in heat index during that specific period of time (Peric et al., 2017).

Sporer et al. (2008) evaluated how transportation stress altered blood physiology in beef cattle. The researchers looked at inflammation markers, steroid hormones, and measures of metabolism. Results showed that plasma cortisol concentrations were significantly elevated at the onset of

transportation as compared to 24 hours prior to transportation. Further, the plasma cortisol concentrations peaked at 4.5 hours post transportation, plateaued at 14.25 hours post transportation, then returned to basal levels between 24 and 48 hours post transportation (Sporer et al., 2008). As shown by Arthington et al. (2008), transportation stress directly affects average daily gain, feed efficiency, and dry matter intake. Decreased dry matter intake during a period of stress could result in increased risk of gastrointestinal disease or other health implications. These health implications can contribute to decreased longevity of cattle in the production system, and for cows an increased risk of long-term physiological decline.

Dehydroepiandrosterone (DHEA) 1.6.2

A steroid hormone that plays a role in multiple physiological processes is dehydroepiandrosterone (DHEA). DHEA contributes to immune system activation, is involved in lipid metabolism, and can have anti-inflammatory properties (Peric et al., 2017). During stress responses, DHEA acts as an HPA axis antagonist by suppressing inflammatory cytokines and protecting cells in the nervous system (Edes et al., 2016b).

In cattle, lameness studies have found that serum DHEA decreases in lame cattle and the cortisol:DHEA ratio is higher than in healthy cattle (Almeida et al., 2008). Peric et al. (2017) measured DHEA concentration in the hair of dairy cattle from two different housing options, indoor or pasture. The results showed that hair DHEA concentrations remained constant over the experimental period for cattle housed indoors and tethered. However, cattle that were housed on pasture and allowed to graze had increased DHEA concentrations during the experimental period (Peric et al., 2017). Cortisol:DHEA ratios of cattle housed indoors increased over the second half of the experimental period. For cattle housed on pasture the cortisol:DHEA ratio decreased over the first month of the experimental period then increased over the remaining three months (Peric et al., 2017).

Sporer et al. (2008) measured DHEA and cortisol:DHEA ratio in recently transported beef cattle. Their results showed that plasma DHEA concentrations decreased by 30% at 4.5 hours post transportation, when cortisol concentrations peaked, as compared to 24 hours prior to transportation. Cortisol:DHEA ratio followed a similar pattern as plasma cortisol concentrations with the peak ratio occurring at 4.5 hours post transportation (Sporer et al., 2008).

A study conducted by Gundlach et al. (2017) evaluated serum cortisol and DHEA concentrations in dairy cattle postpartum. Results from the study showed that cows with metritis had similar serum cortisol levels as healthy cows. However, DHEA concentrations were reported to be greater in cows with metritis and a subsequent leukopenia than in healthy cows or cows with metritis but a normal leukocyte count (Gundlach et al., 2017). With DHEA having anti-inflammatory properties, an elevation in serum DHEA concentrations likely indicates an active inflammatory process within the body.

Interleukin-6 (IL-6) 1.6.3

Interleukin-6 (IL-6) is a pleiotropic, pro-inflammatory cytokine involved with inflammation, immunity, and many other physiological processes (Callahan and Yates, 2014). IL-6 is both a diagnostic and prognostic biomarker. In humans, IL-6 has been shown to be a biomarker associated with cardiovascular disease, autoimmune disease, and hepatic regeneration (Tanaka et al., 2014; Narazaki and Kishimoto, 2018). In livestock, IL-6 is associated with many pro-inflammatory responses as triggered by disease, parturition, and stress.

A 2014 study conducted by Osorio et al. evaluated the biomarkers associated with inflammation, metabolism, and oxidative stress in peripartal dairy cows who were supplemented with different methionine feed additives. The hypothesis that methionine supplementation in cattle will increase triacylglycerol (TAG) export from the liver has been supported (Durand et al., 1992). The liver is the main target of IL-6 since hepatocytes express IL-6 receptors and gp130, a signaling molecule

(Narazaki and Kishimoto, 2018). Additionally, the liver is an active participant in acute phase responses which have been found to require the input of IL-6 (Narazaki and Kishimoto, 2018). It has been shown that in the liver of cows, the peripartal inflammatory response is triggered by pro-inflammatory cytokines IL-6 and tumor necrosis factor-alpha (TNF- α ; Kindt et al., 2007). Hepatic inflammation during the peripartal period could support the development of hepatic accumulation of TAGs thus increasing the cow's risk of developing ketosis (Osorio et al., 2013). The results of this study were that cows given a methionine supplement postpartum had a trend of lower plasma IL-6 concentrations as compared to cows not supplemented (Osorio et al., 2013). This trend allows for the assumption that providing supplemental Methionine will likely decrease the production of pro-inflammatory cytokines after calving. A decrease in pro-inflammatory cytokine production postpartum supports a shorter recovery for the cow, as well as a decreased risk of postpartum infections. Similarly, it could be assumed that decreased IL-6 levels postweaning would decrease the potential of inflammatory processes in the cow and provide improved support for the growing fetus.

Tumor Necrosis Factor-alpha (TNF- α) 1.6.4

TNF- α , also a pro-inflammatory cytokine, is essential for inducing the production of other cytokines such as IL-6. Additionally, TNF- α plays a role in the release of leukotrienes, nitric oxide, and platelet activating factor (Gruys et al., 2005). During times of metabolic need, TNF- α will initiate muscle catabolism mediated by glucocorticoids. Further physiological processes that result in the release of TNF- α include bacterial infections, endotoxin exposure, and viral infections at much lower concentrations (Gruys et al., 2005). When TNF- α concentrations are high in an organism, there is a very high probability that cellular damage has occurred.

Brodzki et al. (2015) evaluated pro-inflammatory cytokine levels in dairy cattle with subclinical endometritis. Results from this study found that serum TNF- α , IL-6, and IL-10 concentrations were

significantly ($p < 0.01$) higher in cows with subclinical endometritis as compared to healthy cows. Uterine lavages were also collected, and cytokine levels were tested. It was reported that serum concentrations of TNF- α and IL-6 were higher than concentrations in the uterine lavage. The researchers concluded that the elevated levels of cytokines in both the blood and uterine lavage were likely due to subclinical endometritis (Brodzki et al., 2015).

Though TNF- α plays many important roles in physiological processes, there has been limited research conducted analyzing TNF- α concentrations during periods of stress in beef cattle.

Summary of Biomarkers 1.7

Multiple studies have published data utilizing cortisol as a means of evaluating stress during weaning for both cows and calves (Hopster et al., 1995; Lynch et al., 2010; O'Loughlin et al., 2014; Cooke, 2016; Chase et al., 2017; Foote et al., 2017). Though cortisol can become elevated with handling, it still offers evidence of the stress response occurring for each individual animal. The other biomarkers have been utilized less when evaluating weaning stress with plasma TNF- α and DHEA recorded in select studies (Lynch et al., 2010; Cooke, 2016).

Utilization of multiple biomarker data recorded during stressful events and application of that information to weaning can offer a new level of insight into how the body physiologically and immunologically responds to the stress of weaning in cows.

Allostatic Load 1.8

Allostasis refers to an organism's natural ability to vary somatic system reactions in response to stress (Sterling and Eyer, 1988; Edes et al., 2016b). Unlike in homeostasis where physiological reactions are reflexive, allostatic variance is in response to perceived or anticipated stressors by each organism (Edes et al., 2016b). The fluctuations involved with allostasis are carried throughout multiple

physiological systems via nerves. Each allostatic activation creates a signaling cascade to target every necessary system's input to result in the return to homeostasis (Sterling and Eyer, 1988; Edes et al., 2016b).

Over time, the repetition of allostatic stimuli will result in multi-level cellular damage. This cellular damage can be measured by analyzing biomarkers of cellular function. The severity of the damage measured allows for the estimate of stress on an individual termed allostatic load (Sterling and Eyer, 1988; McEwen and Wingfield, 2003; Goymann and Wingfield, 2004). Allostatic load is unique to each individual organism and is influenced by behavioral, social, and biological variance (McEwen and Wingfield, 2003).

Allostatic load is determined by measuring physiological biomarkers in an individual, then summing those markers that are in dysregulation (Seeman et al., 1997). Individuals with a greater number of dysregulated biomarkers are considered to have a higher allostatic load. The biomarkers analyzed are selected based on their representation of cardiovascular, neuroendocrine, and metabolic functions (Seeman et al., 1997).

A 1997 study followed a cohort of participants from the MacArthur studies of successful aging that agreed to provide biological samples for analysis. The research group collected blood and urine samples, as well as conducting multiple interviews to gauge each individual's physical, psychological, and social health. Biomarkers chosen for analysis included systolic and diastolic blood pressure, waist-hip ratio, urine cortisol, norepinephrine and epinephrine, serum HDL, total cholesterol, total glycosylated hemoglobin and DHEA-S (Seeman et al., 1997). Results from this study show that a greater allostatic load is associated with poorer cognitive function and decreased memory performance in humans. Physical performance results were similar to those of cognitive function; the greater the allostatic load, the lower the physical performance and the more likely an individual would decline

further. The third factor evaluated was the likelihood of cardiovascular disease (CVD). Study results found not only that allostatic load is an adequate predictor of CVD, but also that individuals with a greater allostatic load are at an increased risk of developing CVD with age (Seeman et al., 1997).

Recently, these concepts have been applied to primates in captivity allowing for improved monitoring of longevity in captive populations. One study utilized serum from 27 Western Lowland Gorillas to analyze seven biomarkers for estimation of allostatic load. Following allostatic load estimation for each individual, Edes et al. (2016b) looked for association with age, sex, parturition, number of stressful events, and age at death. Results from this study show that allostatic load scores in females were significantly higher than those of the males, but without correlation to parity or number of offspring. Additionally, high allostatic load was positively associated with both age and number of stressful events (Edes et al., 2016b).

A second primate study evaluated the association between rearing history of captive gorillas and allostatic load. The classifications of rearing were by nursery, mother, or wild-caught (Edes et al., 2016a). An analysis of seven different biomarkers was performed to assign allostatic load scores to each individual animal. This study concluded that allostatic load was significantly associated with rearing history in gorillas with both age and sex as covariates. Furthermore, it was determined that wild-caught females had the greatest allostatic load score.

In 2003, Goymann and Wingfield conducted a study evaluating the correlation between allostatic load, social status and stress hormones. These researchers used free-ranging wild animals living in stable populations (Harris's hawk, wolf, African wild dog, Mountain gorilla, etc.) to minimize additional factors of environmental and breeding instability. Depending on species, plasma or urine samples were collected and analyzed for glucocorticoid concentrations, then mean concentrations were calculated for both dominant and subordinate status (Goymann and Wingfield, 2003). Analysis showed

that a greater allostatic load in dominants as compared to subordinates was correlated with increased glucocorticoid levels in the dominant individuals. Alternatively, when subordinate individuals had a greater allostatic load, dominant individuals had glucocorticoid levels similar to or lower than those in the subordinates (Goymann and Wingfield, 2003).

In domestic animals, specifically livestock species, there are a large number of stressors that can influence animal health and productivity. The application of these stressors is usually on a regular basis, which in the long-term, can directly impact the animals' longevity in a production system. Thus, allostatic load may offer a novel strategy to evaluate how stress affects multiple physiologic systems of an animal and it may help to predict an animal's expected performance and longevity.

Final Remarks 1.9

Based on current literature, the majority of research investigating the impacts of stress induced by weaning has been focused on the calf. Cows are the lifeline of the beef industry, and their overall health and longevity play a major role in the production of quality animals. However, due to the scarcity of objective methods to measure stress from the dams, there is a substantial lack of knowledge on stress level dynamics of the dams during the weaning process that may have an impact on the current gestation and on the subsequent cow and calf performance. Allostatic load has been proven to be a valuable tool for estimation of longevity in humans, primates and other wild animal species. Biomarker analysis tools are widely available for bovine samples, which would allow for similar protocols to those performed in previous allostatic load research. Though allostatic load has not been utilized in domesticated species, its application could alter the future of livestock production and how current protocols are modified in the years to come.

CHAPTER 2: EVALUATION OF TWO WEANING STRATEGIES ON COW BIOMARKER CONCENTRATIONS AND ALLOSTATIC LOAD INDEX

Introduction 2.1

Stress is defined as the “nonspecific response of the body to any demand” (Tan and Yip, 2018). A body’s physiologic response to stressors involves the neurologic, immunologic, and endocrine systems all working together to restore homeostatic balance (Chu et al., 2020). If an organism undergoes persistent stress, the body’s physiologic systems will begin to fail and cellular damage will occur (Sapolsky, 2002; Goymann and Wingfield, 2004). This cellular damage, in animals, can result in decreased performance or development of illness.

Stress associated with weaning in beef calves has been studied extensively (Price et al., 2003; Haley and Stookey, 2005; Freeman et al., 2016; Harrelson and Harrelson, 2017). Physical separation of calves from their dams has been shown to decrease dry matter intake (Hutcheson and Cole, 1986), impair digestive and immune function (Taylor et al., 2010), and affect the allocation of energy expenditure in calves (Haley and Stookey, 2005; Lambertz et al., 2014; Freeman et al., 2016). Traditionally, weaning involves abruptly removing the calves from the dams then transporting the calves to a new facility within the same day. This abrupt weaning method typically occurs when the calves are between 6-7 months of age. Lambertz et al. (2014) reported that calves experienced more stress, as determined by vocalization frequency, from the act of weaning alone as compared to additional handling and processing procedures. Thus, the process of abruptly weaning calves is a major stressor within their lifetime.

Alternative weaning strategies, such as fence-line weaning, have also been studied to compare the impact of weaning strategy on calf stress (Price et al., 2003; Boland et al., 2008; Enríquez et al.,

2010). Fence-line weaning involves physical separation of cows and calves with a shared fence-line that separates the two groups of animals allowing for vocal and nose to nose contact. Studies on fence-line weaning have reported that calves who are fence-line weaned spend a greater amount of time eating and lying than those who are abruptly weaned (Price et al., 2003; Harrelson and Harrelson, 2017). Additionally, fence-line weaned calves had greater average weight gain than abruptly weaned calves at both 2 weeks and 10 weeks post weaning (Price et al., 2003). In the same study, Price et al. (2003), also reported that the fence-line weaned calves had decreased vocalizations per hour than calves that were abruptly weaned, 216.7/hour/group vs. 434.6/hour/group respectively. Additionally, compared to all other weaning groups in the study, calves that were not weaned had the lowest vocalizations per hour at 0.1/hour/group (Price et al., 2003). Time spent eating and lying were significantly lower in calves that were abruptly weaned (23.7%/day and 16.0%/day, respectively) than calves in both the fence-line weaned (37.3%/day and 23.3%/day, respectively) and non-weaned (41.4%/day and 22.9%/day, respectively) groups. No difference was noted between the non-weaned and fence-line weaned groups for times spent eating and lying.

Limited research has been conducted evaluating how stress associated with weaning affects the dam. Stěhulová et al. (2017) reported that the greatest stress response, as determined by frequency of vocalization and time spent physically moving, occurred within the first 24 hours post weaning in cows. Factors reported to increase vocalizations in cows post weaning were the age of the calf and pregnancy status of the cow. Cows with younger calves (median age 201 days) had significantly increased vocalizations than cows with older calves at time of weaning. Additionally, cows that were open (nonpregnant) had increased ($p < 0.01$) vocalizations as compared to pregnant cows at the time of weaning (Stěhulová et al., 2017). Rutherford et al. (2018) evaluated dam behavior at weaning with the use of a fence-line weaning strategy. Cows were outfitted with a GPS collar that tracked their location within the pasture every 5 minutes. Their results showed that initially, first calf heifers remained a

greater ($P < 0.01$) mean distance away from the fence than multiparous cows. Over the five days of monitoring, all cows increased their distance away from the fence. Rutherford et al. (2018) concluded that fence-line weaning protocols should exceed a length of five days to minimize stress in the cows. Though behavioral research offers an insight into effects of stress on individual cows, biochemical analysis can offer a more detailed perspective about physiological changes and the effects on the body. Currently, there is limited biochemical data available supporting the stress cows undergo during the weaning process.

Allostatic load is the cumulation of physical and mental stressors underwent by an individual and the effects those stressors have on future physiological reactions (Sterling and Eyer, 1988). The concept of allostatic load has been used in humans for decades (Sterling and Eyer, 1988; Seeman, 1997; McEwen and Wingfield, 2003), but has only recently been utilized in animals. Allostasis refers to an organism's natural ability to vary somatic system reactions in response to stressors (Sterling and Eyer, 1988; Edes et al., 2016a; Edes et al., 2016b). Repetition of stressful stimuli over time results in multi-level cellular damage. This damage can be measured through the analysis of cellular function biomarkers. Following biomarker analysis, each biomarker is assigned a score (1 to 5) based on quartile ranking. The sum of each biomarker score results in a whole number which is assigned to each individual. This whole number represents an estimate of the long-term effects of stress on the body (Seeman, 1997).

Two separate studies conducted by Edes et al. (2016a,b) applied allostatic load principles to captively housed Western Lowland Gorillas. Allostatic load scores were determined by analyzing blood concentrations of albumin, cortisol, corticotropin-releasing hormone, dehydroepiandrosterone sulfate, glucose, interleukin-6, and tumor necrosis factor alpha. One of these studies reported that females had greater allostatic load scores as compared to males, as well as a positive association between allostatic load and age and number of stressful events (wounds, anesthetizations, transfers) (Edes et al., 2016b).

The second of the two studies evaluated how rearing history affected allostatic load in the gorillas. Results reported a significant association between allostatic load and rearing history when both age and sex were used as covariates during analysis; the greatest allostatic load was in wild-caught females (Edes et al., 2016a). The authors hypothesized that females face more stressors, both socially and physiologically, thus leading to increased allostatic load indexes.

Based on the existing literature, the application of allostatic load to livestock species could be a useful tool to evaluate how stressful management practices, such as weaning, affect the dams over time. It was hypothesized that cows whose calves were weaned abruptly would have a greater allostatic load as compared to cows with fence-line weaned calves. In consequence, the objective of this study was to compare allostatic loads for quantifying stress in beef cows subject to two weaning methods (abrupt or fence-line weaning).

Materials and Methods 2.2

Ethical Statement 2.2.1

All experimental procedures used within this study were approved by the Colorado State University's Institutional Animal Care and Use Committee (IACUC protocol ID #1167).

Animals 2.2.2

Ninety-one multiparous Angus crossbred cow with calves (182 total animals) were included in the study. Calves were born within a three-week period between March and April 2020. The day of weaning is referred to as day 0. The average (SD) calf age at the time of weaning was 159.3 ± 3.7 days of age (range= 146 to 166 days of age). The average (SD) calf body weight on day 0 was 220.6 ± 22.8 kilograms. Physiological demographics for cows in the study are reported in Table 1.

All animals were housed at the Eastern Colorado Research Center in Akron, CO for the 65-day study period. Throughout the duration of the study, animals had ad libitum access to fresh water. Feeding during the study period was as follows: all cows were range fed with ad libitum access to mineral supplementation, traditionally weaned calves were fed a total mixed ration for growing cattle once daily in feed bunks, fence-line weaned calves were range fed with ad libitum access to mineral supplementation and starter/grower feed offered loosely in tubs or in the form of molasses based mineral tubs.

Table 1. Comparison of dam characteristics at weaning.

Variable	Treatment		P - value
	Fence-line	Conventional	
Average age (years)	4.48±1.3	4.11±1.2	0.17
Average parity	3.48±1.3	3.11±1.2	0.17
Average body weight (kg)	578.11±64.2	574.25±66.0	0.78
Average body condition score	5.48±0.56	5.51±0.58	0.85

Treatments 2.2.3

Each cow-calf pair was assigned to one of two treatment groups: abrupt or fence-line weaning. Treatment groups were assigned randomly based on the calf identification (ID) number being odd or even. Calves with odd numbered IDs, and their respective dams, were placed into the fence-line weaned treatment group. Calves with even numbered IDs, and their respective dams, were placed into the abrupt weaned treatment group. Calves were weaned on September 8, 2020 (day 0).

Calves were separated from dams in a corral prior to weighing on day 0 of the experiment. For weighing, all calves were weighed followed by all cows using a Tru-Test (Cattlesoft, Inc., College Station, TX) in-chute scale. Calves averaged 159.3 ± 3.7 days of age and 220.6 ± 22.8 kilograms at weaning. Upon exiting the chute, calves were separated into two pens based on treatment group. The fence-line

treatment group had 44 calves and the abrupt treatment group had 47 calves. Calves in the abrupt treatment group were moved to an onsite feedlot, approximately 1 kilometer away from the dams, following weighing and sample collection. The onsite feedlot pens, approximately 680 sq meters in size, were set up with concrete feed bunks on a single side with metal slatted panels lining the other three sides. Stocking density of each feedlot pen was 25 head per pen (approximately 27.2 sq meters per head). A total of 4 pens were used with the fourth pen containing 16 animals.

The fence-line treatment group calves were moved following weighing and sample collection into a pasture that shared a fence-line with the dams allowing for visual, auditory, and nose-to-nose contact. All cows, abrupt and fence-line weaned treatment groups, were placed into one pasture located opposite the shared fence-line from the calves in the fence-line treatment group. Both pastures were of equal size, 546,326 square meters each. The stocking density was approximately 5,989.3 square meters per cow and 12,140.6 square meters per calf.

Sample Collection 2.2.4

Weaning (day 0) was performed at the chute following the collection of individual calf weight, serum, and plasma samples. Following calf weaning, dams body weight, body condition score (BCS; scale from 1 to 9), serum, and plasma samples were collected (Table 1).

Whole blood was collected via jugular venipuncture for all animals for a maximum total volume collected of 20 ml. Blood was evenly divided between one red top serum separator tube and two trace element K2 EDTA tubes (BD Vacutainer, Plymouth, England). Body condition scores were determined by palpation of the amount of tissue coverage over the ribs and tuber coxae. A scoring system of 1-9 was utilized for BCS (Thomas and Bailey, 2021) and scores were determined by the same individual at all time points.

Sample collection was repeated for the calves on days 1, 3, 7, and 29. Cow sample collection was repeated on days 1, 3, 7, and 64. During sample collection, calves in the abrupt treatment group were handled at facilities adjacent to the feedlot so no contact was made with the dams. Cows and fence-line treatment group calves were handled at remote facilities within the pasture, so visual and nose-to-nose contact with the dams was unavailable for a brief period of time during processing.

Serum separator tubes were left to coagulate at ambient temperature during processing and then transport to the laboratory (approximately 9 hours post collection). Potassium EDTA tubes were inverted approximately 10 times immediately following filling to prevent blood from coagulating. These tubes were also kept at ambient temperature during processing and transport to the laboratory. Tubes were stored in a walk-in cooler set at 4°C for 12-24 hours, then centrifuged at 2,000 x *g* for 10 minutes at 4°C. Following centrifugation, both serum and plasma samples were stored in 1-3 ml aliquots at -60°C until utilized for analysis. For this study, only samples from cows collected on days 0, 3, and 64 were analyzed and reported.

Biomarkers 2.2.5

Four biomarkers were measured in the cows for use in the calculation of an allostatic load index (ALI): cortisol, dehydroepiandrosterone (DHEA), tumor necrosis factor- alpha (TNF- α), and interleukin-6 (IL-6). The selection of biomarkers was based on previous ALI research in humans, primates, and other wildlife species (Seeman, 1997; Goymann and Wingfield, 2004; Edes et al., 2016a; Edes et al., 2016b; Outland, 2019). Cortisol analysis was performed using plasma samples collected at the following timepoints: days 0, 3. The timepoints selected for analysis of DHEA, IL-6, and TNF- α were days 0 and 64 (Table 2). Cortisol analysis timepoints were selected based on the rapid half-life of plasma cortisol (120 minutes with large hormone levels (Holland-Frei Cancer Medicine, 2003)).

Cortisol and DHEA concentrations were determined using quantitative, competitive enzyme-linked immunosorbent assay (ELISA) kits from Abnova (KA2275) and Novus Biologicals (NPB2-61256), respectively. The minimum detectable concentration for cortisol was 1.0 ng/ml. Analytical sensitivity for DHEA was 2.90 pg/ml; intra-assay variation was 6.4% and inter-assay variation was 8.4%. TNF- α and IL-6 levels were determined using DuoSet sandwich ELISA kits from R&D Systems (DY2279 and DY8190, respectively) specific to bovine species. Assay sensitivity range for TNF- α was 125.0- 8,000 pg/ml with a no cross-reactivity specificity of 50 ng/ml. Assay sensitivity range for IL-6 was 15.6- 1,000 pg/ml with no cross-reactivity specificity of 50 ng/ml. All kits were stored between 2-8°C prior to and following use. Assays were performed by one individual following the manufacturer's protocols provided in each assay kit.

Table 2. Biomarker sample collection dates for cows and data used in final statistical analysis.

Biomarker	Collection Timepoints (day of study) ¹	Timepoints Used in Analysis (day of study)
Cortisol	0, 1, 3, 7, 64	0, 3
DHEA ²	0, 1, 3, 7, 64	0, 64
TNF- α ³	0, 1, 3, 7, 64	0, 64
IL-6 ⁴	0, 1, 3, 7, 64	0, 64

¹Day 0 = day of weaning, day 64 = final day of study and 64 days post weaning

²Dehydroepiandrosterone.

³Tumor necrosis factor alpha.

⁴Interleukin 6.

Analysis 2.2.6

Allostatic Load Index Scoring 2.2.6.1

Biomarker (cortisol at day 3; TNF- α , IL-6, and DHEA at timepoint day 64) score assignments were determined in Microsoft Excel (Microsoft Office, Redmond, WA) by utilization of the sort and filter function to rank biomarker concentrations from smallest to largest. This ranking was performed on each biomarkers' complete data set (quartile population) which included all animals with a positive,

reportable value (cortisol: n = 82, DHEA: n = 45, IL-6: n= 33, TNF- α : n = 35) to ensure accurate quartile assignment prior to allostatic load index analysis.

Once sorted, the quartile formula was applied to the analyte concentration for separation into four quartiles. For IL-6 and TNF- α , a one-tailed scoring system was utilized for concentrations that were increased. Animals within quartiles one and two were given a score of 0, whereas animals in quartiles three and four were assigned a score of 1.

A one-tailed scoring system was utilized for decreased concentrations of DHEA giving animals in quartile one a score of one, and all other quartiles an assigned score of 0. For cortisol, a two-tailed scoring system was utilized for both increased and decreased concentrations. Animals that fell into both quartiles one and four were assigned a score of 1, and animals in quartiles two and three were given a score of 0.

Following the assignment of quartiles and individual biomarker scores, only animals that were found to have complete biomarker profiles (a positive, reportable value for cortisol on day 3 and all other biomarkers on day 64) were utilized for allostatic load index analysis. The total number of animals with complete biomarker profiles was 23 animals.

Statistical analysis 2.2.6.2

The total number of cow samples analyzed from day 0 and day 3 were 93 animals per sampling day. On day 64, only 48 cow samples were collected due to movement or culling of the other 45 animals prior to final sample collection by research housing operators. Incomplete data sets, as a result of data abnormalities (values greater than ± 3 standard deviations from the mean), were excluded from data analysis. A total of 25 animals, from the 48 sampled on day 64, were excluded from final data analysis. Due to the removal of samples throughout the experiment, only day 64 samples were analyzed for this study.

Cortisol, DHEA, TNF- α , and IL-6 concentrations were analyzed using a t-test assuming unequal variances in Microsoft Excel (Microsoft Office, Redmond, WA). Both a one-tailed and two-tailed approach were considered to encompass all possible effects. Three of the four biomarkers were scored based on one-tailed approaches, so a one-tailed analysis would likely yield the most accurate outcomes based on previous ALI research. However, all effects are valuable when determining allostatic load index, thus a two-tailed approach was also utilized for all data sets. Allostatic load index scores were also analyzed using the same t-test methods. Individual animal was considered the experimental unit for all response variables.

Treatment was considered a fixed effect. Response variables for the study were biomarker (cortisol, DHEA, TNF- α , IL-6) concentrations. Significance was determined at $p \leq 0.05$. Tendencies were determined at $p > 0.05$ but $p \leq 0.10$.

Some covariates that were collected but not reported in this study include cow age, calf sex, and calf age. These covariates were not included in the reported statistical model due to the lack of effect on the ALI data.

Results 2.3

Cortisol 2.3.1

The mean cortisol concentration for the population utilized to establish quartiles was 35.55 ± 2.73 ng/ml (Mean \pm SEM). Table 3 shows descriptive statistics from the population. For the fence-line treatment group, mean cortisol on day 64 post calf weaning was 45.15 ± 11.24 ng/ml. Mean cortisol concentration for the conventional weaning treatment group was 32.97 ± 6.86 ng/ml. Table 4 shows that there were no differences ($p > 0.05$) between treatment for plasma cortisol concentrations on day 64 post calf weaning.

DHEA 2.3.2

The mean DHEA concentration of the quartile population was 596.78 ± 85.89 pg/ml. As shown in Table 4, the mean DHEA concentration for the fence-line weaning treatment was 825.96 ± 258.08 pg/ml and 763.38 ± 124.71 pg/ml for the traditional weaning treatment. No difference was found between treatment groups ($p > 0.05$). Additional descriptive statistics regarding DHEA analysis for the population can be found in Table 3.

TNF- α 2.3.3

For the quartile population, the mean plasma TNF- α concentration was 3.20 ± 0.13 pg/ml. Other population descriptive statistics for TNF- α are reported in Table 3. Fence-line and traditional weaning treatment groups had mean TNF- α concentrations of 2.83 ± 0.21 pg/ml and 3.44 ± 0.26 pg/ml, respectively (Table 4). There was a main effect of treatment ($p = 0.04$) using a one-tailed distribution, and a tendency for effect ($p = 0.08$) using a two-tailed distribution.

IL-6 2.3.4

For the quartile population, the mean IL-6 concentration was 76.33 ± 15.76 pg/ml as reported in Table 3. The mean IL-6 concentration for the fence-line weaning treatment group was 76.59 ± 32.19 pg/ml, as presented in Table 4. For the traditional weaning treatment group, the mean IL-6 concentration was 87.95 ± 29.28 pg/ml. Additional descriptive statistics from the analysis are also reported in Table 3. No effect was found to exist between treatment groups for plasma IL-6 concentrations utilizing a one or two-tailed distribution ($p = 0.39$ and $p = 0.79$), respectively.

Allostatic Load Index 2.3.5

Table 3 shows the quartile population descriptive statistics for ALI. The mean total ALI for the population was 1.60 ± 0.24 . Fence-line treatment group animals had a mean ALI of 1.12 ± 0.29 and

traditional treatment group animals had a mean ALI of 1.86 ± 0.33 . Using a one-tailed distribution, there was a main effect ($p = 0.05$) of treatment on ALI. No effect was found when using a two-tailed distribution.

Table 3. Biomarker descriptive statistics from the cow population utilized to establish quartiles for individual score assignments.

Biomarker	n*	Min	Mean	Max	SEM
Cortisol, ng/ml ¹	82	1.01	35.55	117.87	2.73
DHEA, pg/ml ²	45	2.79	596.78	2246.72	85.89
TNF- α , pg/ml ³	35	1.98	3.20	5.11	0.13
IL-6, pg/ml ⁴	33	1.38	76.33	378.40	15.76
ALI ⁵	23	0	1.60	4	0.24

*Variation in n is due to animal movement and culling prior to final day of study. If quartiles were assigned based on only 23 animals, there would have been no variation of ALI scores.

¹Cortisol samples were collected on day 3 post weaning.

²Dehydroepiandrosterone. Samples were collected on day 64 post weaning.

³Tumor necrosis factor alpha. Samples were collected on day 64 post weaning.

⁴Interleukin 6. Samples were collected on day 64 post weaning.

⁵Allostatic load index. A total of 23 animals had complete data sets following sample analysis.

Table 4. Main effects of fence-line and conventional weaning on plasma concentration of cortisol on day 3 of study and TNF- α , DHEA, IL-6, and ALI on day 64 of study for 23 animals.

Biomarker	Treatment		SEM	P <	
	Fence-line (n=8)	Conventional (n=15)		Treatment, one-tailed	Treatment, two-tailed
Cortisol, ng/ml	45.15	32.97	6.08	0.18	0.37
TNF- α , pg/ml ¹	2.83	3.44	0.30	0.04	0.08
DHEA, pg/ml ²	825.96	763.39	31.28	0.41	0.83
IL-6, pg/ml ³	76.59	87.95	5.68	0.39	0.79
ALI ⁴	1.12	1.87	0.37	0.05	0.11

¹Dehydroepiandrosterone.

²Tumor necrosis factor alpha.

³Interleukin 6.

⁴Allostatic load index.

Discussion 2.4

Cows, both dairy and beef, undergo significant physiological stressors during their production lifespan. Beef cows gestate for approximately nine months then give birth to a calf that will then nurse for approximately six to seven months. During lactation, the cows are bred and expected to maintain a healthy pregnancy while also undergoing weaning of their current calves. As previously reported, the act of weaning calves is also stressful on the dams (Stěhulová et al., 2017; Rutherford et al., 2018). Weaning methods have been shown to influence the stress a cow undergoes during the weaning process via behavioral studies (Stěhulová et al., 2017; Rutherford et al., 2018). The current study offers support for the evidence that fence-line weaning is a lower stress method for the cows as opposed to the conventional, abrupt calf weaning methods.

Allostatic load has been shown to be a useful tool to assess stress and estimate disease risk in human medicine for many years. Allostatic load is calculated utilizing concentrations of between 7-10 plasma or urine biomarkers. These biomarkers can include glucocorticoids, neurosteroids, immune markers, and physiological health markers (Outland, 2019; Seeman, 1997; Sterling and Eyer, 1988). The current study reports on the use of four biomarkers (cortisol, DHEA, IL-6, TNF- α) to calculate allostatic load. Additionally, this study utilized a set population of animals over a predetermined timeframe which is different from other published ALI literature. Limitations in this study included a small sample size, a shortened sample time window, and fewer analyzed biomarkers as compared to other ALI literature. Despite the variation from other ALI literature, the findings of this study offer a valuable baseline for the application of allostatic load in response to weaning of beef cows and calves.

With a growing interest in animal longevity and production within the animal health industries, the potential application of allostatic load for the assessment of stress throughout production animal life cycles is an intriguing possibility. The application in captive primates has shown that females are more

likely to have increased stress levels, and thus increased allostatic load indices, due to their biologic capabilities (Maestriperi and Hoffman, 2011; Edes et al., 2016a; Edes et al., 2016b). The potential utilization of allostatic load in animal production would offer the ability to improve the advancement of production practices. These advancements could include, but are not limited to, selection of alternative weaning strategies in beef cattle, decreased handling events for processing of production animals, and alternative housing considerations for confined production animals.

No effect was found between the two treatment groups for cortisol. Similar to the findings of this study, two studies reported no significant changes in plasma cortisol levels of cows following conventional, abrupt, weaning (Hopster et al., 1995; Lynch et al., 2010). Hopster et al. (1995) reported that dairy cows had increased heart rates for the first two minutes following separation of their calves and increased vocalizations during the first five minutes post separation, yet plasma cortisol levels remained unchanged. Lynch et al. (2010) reported that both plasma cortisol and DHEA levels in beef cows were unchanged following weaning. Following 35 days on pasture, the cows were moved to an indoor housing system which resulted in unchanged plasma cortisol levels again, but decreased DHEA concentrations (Lynch et al., 2010). The current study found that cows in the abruptly weaned treatment group had lower DHEA concentrations than cows in the fence-line weaned treatment group, but the difference between the groups was not significant.

The reported results of this study disagree with physiological research in beef calves. Additionally, this study's physiological findings do not align with a majority of previously reported behavioral research in beef cows. Previous research has shown increased stress behaviors (vocalization frequency, frequency of movement, mean distance from shared fence-line) amongst cows whose calves were abruptly weaned as compared to those weaned via the fence-line weaning strategy (Stěhulová et al., 2017; Rutherford et al., 2018). Published weaning associated research in calves has shown elevated plasma cortisol levels following abrupt weaning in addition to stress behaviors. Chase et al. (2017)

reported that calves had increased cortisol levels at both 24 and 72 hours post abrupt weaning (36.59 ng/ml and 35.07 ng/ml, respectively). This study did not have similar results for plasma cortisol concentrations in cows. One possible explanation for the difference in results is the study's small sample size. Despite the results reported, it cannot be deduced that conventional weaning causes greater stress on the cow.

A main effect of weaning method was found for plasma TNF- α concentration, showing the fence-line group having lower concentrations compared to the conventionally weaned group. TNF- α is a proinflammatory cytokine released by macrophages during acute inflammation (Callahan and Yates, 2014). Stress upon the body, no matter the cause, results in an increase in inflammatory responses within the body. In this study, the elevation of TNF- α is associated with the weaning method as compared to animal handling since all animals in the study were handled similarly. Behavioral observations of stress, such as vocalization frequency, can be utilized to make connections between stressful events and physiological responses to stress. Lambertz et al. (2014) reported that vocalizations in beef calves were significantly affected by weaning time, day, and a weaning time x day interaction. Though no blood biomarkers were analyzed in the study, the increased vocalizations reported support that weaning is more stressful than handling or processing of the animals (Lambertz et al., 2014). This in turn, allows for this author's belief that the elevated levels of TNF- α reported on day 64 were likely not due to animal handling but instead due to weaning stress in the cows.

The current study found significant effects to support a variation in the allostatic load index in this population of beef cattle. The allostatic load index was found to be greater in the conventional treatment group as compared to the fence-line treatment group. This result provides the beef industry with evidence that fence-line weaning is not only less stressful for calves but is also a lower stress method for cows as well. Additionally, this study offers the introduction of a new tool that can be utilized to evaluate not only weaning associated stress, but stress collectively.

Collected covariates that were excluded from statistical analysis could offer additional support with an increased sample size as previous research in other species has reported. In primate ALI studies, age of the animal has been positively associated with higher allostatic load, and when applied as a covariate, there was a significant association between allostatic load and mortality risk (Edes et al., 2016b). Stěhulová et al. (2017) reported that there was a correlation between calf age and vocalization frequency of the cows; the younger the calf at weaning the more the cow vocalized. The same study also reported that calf sex had no effect on cow behavior following weaning (Stěhulová et al., 2017).

Allostatic load is influenced by stressful events that can include handling, social structure, administration of treatments, anesthetic events, and injury in addition to applied production practices (Goymann and Wingfield, 2004; Edes et al., 2016a). Beef cattle systems tend to have fewer of these stressful events as compared to other livestock production systems, such as dairy and swine industries. In both the dairy and swine industries, animals are handled daily for routine processes such as breeding, heat checking, and milking (dairy specific). In dairy systems, cows have been reported to show little to no behavioral signs of stress when being handled for routine daily milking (Lindahl et al., 2016). Lewis et al. (2005) reported no differences in heart rate amongst pigs handled in five different ways.

When applying allostatic load principles in dairy systems, some differences must be noted from beef systems. Weaning in dairy systems commonly involves the separation of calf and dam prior to the occurrence of suckling. Due to this weaning practice, it would be better to evaluate this as a stressful event as compared to a single event as reported in this study. An additional difference to note is the difference in lactation between beef and dairy cattle. Dairy cattle often undergo increased administration of treatments due to mastitis infection, whereas mastitis in beef cattle is not as closely monitored (Kibebew, 2017). These noted differences would support a study design in dairy systems more similar to those reported in non-human primates where the variables would include the number of stressful events and age of the animals (Edes et al., 2016b).

Application of allostatic load principles in swine populations would include age/parity, BCS, number of stressful events, and housing design. Additionally, weaning could be utilized in swine populations as well. The weaning process is similar to that of cattle, in that piglets are abruptly separated from the dam, transported to a new location, mixed with other animals, and started on a new diet (Martínez-Miró et al., 2016). However, the lactation period is much shorter than in cattle, thus utilization of weaning as a predetermined stressful event, or fixed effect, would be more useful than selecting it as a stand-alone stressor.

Further application of allostatic load to animal production populations would benefit from additional biomarkers evaluated. Published primate and wildlife studies evaluated seven or greater biomarkers to include in the index score (Goymann and Wingfield, 2003; Edes et al, 2016a; Edes et al, 2016b). Human allostatic load studies analyzed ten biomarkers across various physiological processes for inclusion in the final allostatic load index score (Seeman, 1997). Increased biomarker numbers along with increased population numbers would elevate the potential for improved statistical outcomes of allostatic load index applications in production animal systems.

Though significant effects were found for ALI in this population of cattle, there is potential for improvement to better support the concept in future studies. If repeated in beef cattle, the total number of cows sampled could increase to encompass multiple groups of similar treatments across an individual system. The repeated study could be set up for 385 cows (as determined by a power of 80% calculation with 95% confidence interval and 5% margin of error) sorted into two groups per treatment (4 study groups total). It would also be encouraged to follow the cattle for a greater length of time; from their first breeding through their third or fourth parity. The increased timeframe would offer individual parity variation, ability to track and annotate predetermined stressful events, and increased time periods between handling of animals. An additional production statistic that would be of value in future studies is the breed back percentage of the cow population. Ott et al. (2018) stated that excessive

amounts of stress during early gestation can inhibit the proper development of the placenta. Damage to the placenta during early development can result in loss of early pregnancy for the cow. Inclusion of this data would allow for the prediction of a cow to breed back annually or offer insight into early culling due to a potentially increased allostatic load index.

Conclusions 2.5

The findings of this study offer preliminary evidence that allostatic load index could be a useful tool for the beef industry. Though conducting the testing necessary to determine the allostatic load index is both time and financially consuming, the benefits of the analysis could prove to be a worthy investment for producers.

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