EFFECTS OF ZILPATEROL HYDROCHLORIDE ON MOBILITY, GENERAL ANIMAL WELL-BEING AND THE THERMOREGULATORY RESPONSE OF FEEDLOT STEERS AND HEIFERS DURING MODERATE HEAT STRESS

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A Thesis

In

ANIMAL SCIENCE

Submitted to the Graduate Faculty
of Texas Tech University in
Partial Fulfillment of
the Requirements for
the Degree of

MASTER OF SCIENCES

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ACKNOWLEDGEMENTS

I consider myself extremely blessed to have spent the last four years at Texas Tech completing my B.S. and working toward my Master of Science degree. The opportunities that I have had to grow and learn have been truly invaluable. I trust the skills and character that I have developed here will serve as my foundation in future endeavors. This academically challenging, research oriented program of study has pushed me to broaden my scope of thought; for that I am grateful. I would be remiss not to mention the newfound friendships that have enriched my life.

First and foremost, I want thank God for blessing me with salvation, health and the opportunities I have been afforded. My faith has help me through trying times and the Lord is truly the cornerstone of my life. I am tremendously thankful to my family for their endless love, encouragement and support. A lifetime of love and guidance from my family has shaped the man that I am today.

Thank you to my major professors, Dr. Ryan Rathmann and Dr. Bradley Johnson, for their leadership in my pursuit of this degrees. I am grateful to have had the opportunity to work with respected leaders in their fields. Not only have I benefited from their knowledge and perspective; but more importantly, I have been blessed to learn from their character and honorable way of living. I also want to extend thanks to Dr. Sara Trojan for serving on my committee and teaching many of my graduate courses. I greatly enjoyed each and every class that I was able to take part in and gained valuable knowledge. The support of the Burnett and Beef Center crews is greatly appreciated, as

they ensured the success of my feeding trial. I am forever indebted to the graduate and undergraduate students that helped me collected data throughout a lengthy project.

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CHAPTER I

INTRODUCTION

Human demand for animal protein is expected to double by the year 2050; thus, livestock must be produced more efficiently considering limited water and arable land. In order to address issues of global food security, the livestock industry has embraced the use of efficiency enhancing technologies. These technologies include, but are not limited to: anabolic steroids, ionophores, direct-fed microbials and beta-adrenergic agonists (**β-AA**). Growth enhancing technologies are beneficial to sustainability and they do not pose a threat to the health of the consumer. However, the welfare of animals treated with these compounds has become an area of increasing interest.

Beta-adrenergic agonists have been established as an effective growth promoting technology. These compounds have been approved for use in several countries; the FDA has approved two β-AA for use in feedlot cattle: zilpaterol hydrochloride (**ZH**) and ractopamine hydrochloride. Zilpaterol hydrochloride may be fed at the dietary level of 8.33 mg/kg of dry matter (**DM**) at a level of 60 to 90 mg/animal/d for the last 20 to 40 d of the finishing phase, followed by a 3 d withdrawal period. Ractopamine hydrochloride has been approved to be fed at a dietary concentration of 10 to 30 mg/kg of DM at a level of 70 to 400 mg/animal/d for the final 28 to 42 d of the finishing phase. No withdrawal period is required for RH fed cattle. The FDA has approved these β-AA to be fed in combination with monensin, tylosin and melengestrol acetate.

Recent anecdotal reports have claimed that β-AA increase mortality, morbidity, behavioral agitation, heat stress and lameness in feedlot cattle. Few of these claims have been substantiated with any experimental evidence. Though proof of causal association

may not be present, these claims still impact the opinions of consumers and those involved in the agricultural industry. Consequently, there is a need for experimental validation of the effects of beta-adrenergic agonists on animal well-being. The objective of this review is to detail the β-AA mode of action, describe the characteristics of ZH fed beef cattle and evaluate potential effects of ZH feeding on cattle well-being.

CHAPTER II

LITERATURE REVIEW

B-AA MODE OF ACTION

Beta-adrenergic agonists belong to a class of chemicals known as phenethanolamines. Beta-adrenergic agonists are synthetic analogs to endogenous catecholamines, sharing distinct chemical and pharmacological properties with dopamine, norepinephrine and epinephrine (Bell et al., 1998). The effects of β-AA are generally characterized by consistent improvements in carcass weight gain, carcass cutability and feed efficiency in beef cattle (Avendaño-Reyes et al., 2006; Winterholler et al., 2007; Vasconcelos et al., 2008; Rathmann et al., 2012). These outcomes are derived from the ability of β-AA to bind and stimulate beta-adrenergic receptors (**β-AR**) on cell surfaces, resulting in a pronounced increase in protein accretion, decreased protein degradation and increased lipolysis (Mersmann et al., 1998). These reciprocal effects have resulted in the general classification of β-AA as repartitioning agents.

The β-AR is characterized by seven relatively hydrophobic transmembrane domains that anchor the receptor in the plasma membrane (Mersmann, 1998). When β-AA bind to β-AR the agonist-receptor complex activates the G_s protein subunit (Mersmann, 1998). The α-subunit of the Gs protein then activates adenylyl cyclase; this enzyme is critical in the production of cyclic adenosine monophosphate (**cAMP**), one of the primary intracellular signaling molecules (Mersmann, 1998). Next, cAMP binds to the regulatory subunit of protein kinase A to release the catalytic subunit that then phosphorylates a number of intracellular proteins (Mersmann, 1998). The cAMP

response element binding protein (**CREB**) is phosphorylated by protein kinase A; the CREB then binds to a cAMP response element in the regulatory part of a gene and stimulates the transcription of that gene (Mersmann, 1998). Phosphorylation increases the transcriptional activity of the CREB, providing the mechanism for β-AR agonist-mediated transcription of a number of genes in the mammalian cell (Mersmann, 1998).

Indefinite activation of the β-AR must be avoided by either removal or degradation of the β-AA or inactivation of the receptor (Mersmann, 1998). Several mechanisms are possible. After binding of the agonist, the β-AR may be phosphorylated by a specific kinase, which would inactivate the receptor (Mersmann, 1998). Protein kinase A may instigate phosphorylation of the receptor (Mersmann, 1998). Finally, the β-AR may regress from the plasma membrane if exposed to chronic β-AA stimulation (Mersmann, 1998; Ostrowski et al., 1992; Schwinn et al., 1992; Strosberg, 1992; Kobilka and Hoffman, 1995). The β-AR can be grouped into three discrete subtypes (**β_1-AR, β_2-AR and β_3-AR**) and these receptors are present on most mammalian cells. The ZH ligand exhibits greater affinity to bind to the β_2-AR, which is the predominant subtype found in the skeletal muscle cells and adipocytes of cattle (Sillence and Mathews, 1995). The downstream effects of β-AA binding to β-AR in skeletal muscle cells and adipocytes have been fully described (Chung and Johnson, 2007). However, the ubiquitous nature of the β-AR gives way to the possibility for ZH to bind and alter physiological activity of a wide range of tissues that have not been evaluated with respect to β-AA activity.

CHARACTERISTICS OF ZH FED CATTLE

Feedlot and Carcass Performance

Feeding ZH to cattle at the end of the finishing period has resulted in improved average daily gain (**ADG**) and final body weight (**BW**) (Delmore et al., 2010; Vasconcelos et al., 2008; Elam et al., 2009). Decreased DM intake and improved feed efficiency have been demonstrated during ZH feeding (Vasconcelos et al., 2008). Increases in hot carcass weight (**HCW**) of ZH fed cattle are even more dramatic than changes in BW. Traditionally, an increase of approximately 15 kg in HCW has been reported in ZH fed cattle (Delmore et al., 2010). The greater increase in HCW relative to BW results in significantly improved dressing percentage (**DP**). The increase in DP is approximately 1.5 to 2.0 percentage units due to ZH administration (Delmore et al., 2010). Furthermore, it is hypothesized that ZH feeding promotes mobilization of non-carcass components; these changes are thought to result in greater transfer of nutrients to the carcass (Delmore et al., 2010).

Studies have evaluated carcass cutability of ZH fed cattle. Increased subprimal yields are associated with ZH feeding. Rathmann et al. (2009) reported ZH related improvements in carcass cutout; specifically, 22 of the 33 subprimal yields evaluated were increased with ZH feeding. Also, Rathmann et al. (2009) demonstrated that cattle fed ZH had a greater proportion of protein and moisture when compared to control. Furthermore, Kellermeier et al., (2009) showed ZH related improvements in HCW, longissimus muscle area, yield grade and subprimal yield.

Feeding ZH has also been associated with changes in adipose related carcass traits. Decreased back fat thickness, marbling score and empty body fat percentage have been reported in various studies (Delmore et al., 2010). As a result, the proportions of USDA choice carcasses have been shown to decrease in some groups of cattle (Kellermeier et al., 2009). Plus, there is a reduction in the proportion of yield grade 3 and 4 carcasses (Delmore et al., 2010).

Tenderness and Consumer Acceptability

Warner-Bratzler shear force (**WBSF**) is a commonly used predictor of consumer perceived tenderness. Studies that evaluate WBSF with respect to ZH steaks have shown a 1.10- to 1.70-kg increase in 7-d-postmortem-aged LM steaks, a 0.40 to 1.30 kg increase in 14-d-postmortem-aged steaks, and a 0.30 to 1.40-kg increase in 21-d-postmortem-aged steaks when compared to control (Delmore et al., 2010; Hilton et al., 2009; Kellermeier et al., 2009). Recent studies have demonstrated that steaks from ZH fed cattle have a normal aging response (Delmore et al., 2010; Brooks et al., 2009; Hilton et al., 2009; Kellermeier et al., 2009; Rathmann et al., 2009). Although ZH feeding significantly increases WBSF, when typical industry aging protocols are observed, the difference between perceived tenderness of ZH and control steaks begins to diminish (Holmer et al., 2009).

Additional research has focused on other parameters of consumer acceptability. Hilton et al. (2009) did not detect perceived differences in juiciness, flavor, overall quality or overall acceptability of ZH fed steaks when compared to control. Mehaffey et al. (2009) did not observe differences in palatability when comparing ZH and control

USDA Select steaks after a 21-d aging period. Collectively, consumer palatability research suggests that overall acceptability of steaks from carcasses of ZH-fed cattle are similar to steaks from control cattle even in light of these changes in WBSF (Delmore et al., 2010).

POTENTIAL EFFECTS OF ZH FEEDING ON CATTLE WELL-BEING

The ubiquitous distribution of β-AR in bovine cells provides a basis for hypotheses that implicate β-AA in the modification of the inherent physiological function of various tissues in feedlot cattle. Cattle lameness, metabolic stress and impaired thermal tolerance have been cited as concerns associated with ZH feeding (Loneragan, 2014). The following sections will investigate potential effects of ZH on these parameters of cattle welfare.

Cattle Lameness

Lameness is a particularly important issue that influences the perceived welfare and performance of feedlot cattle. The ability of cattle to ambulate is related to many factors; often, it can be difficult to visually ascertain the cause of lameness. Bovine laminitis is one of the most common causes of bovine lameness. This disease is multifactorial in origin and may result from the release of endotoxins, activation of a cascade of inflammatory mediators and the disruption of microcirculation leading to degeneration of the laminar-suspending structures (Stokka et al., 2001). Laminitis is

generally associated with a dietary insult of rapidly fermentable carbohydrates that elicits systemic alterations.

Furthermore, vasoactive substances are known to alter hemodynamics and impact the intrinsic characteristics of the bovine digit (Nocek, 1997). Inflammation of the pastern and hoof is a clinical sign of bovine laminitis (Nocek, 1997). Swelling of the laminar tissue relative to the total width of the dermis can lead to mechanical damage of the structures that support the distal phalanx bone and connect to the horn of the claw (Nocek, 1997).

Eisemann et al. (1998) demonstrated that β-AA treatment can increase blood flow to the hindquarter of steers and Belloli et al. (2004) confirmed β-AR mediated vasodilation with respect to β_2- agonists; consequently, a hypothesis can be formed that would link ZH supplementation to alterations in the integrity of the bovine hoof. Thus, providing a basis for the exploration of the influence ZH may exert on hoof health and mobility of feedlot cattle.

Increasing credence should be given to studies that use objective measures, such as magnetic resonance imaging (**MRI**) to evaluate bovine lameness. The use of MRI technology has evolved into a powerful diagnostic imaging tool since its practical application in the late 1970's (Rothschild et al., 1990). Magnetic resonance imaging eliminates the need for ionizing radiation and provides significant advantages including: excellent inherent soft tissue contrast, multiplanar imaging capabilities and direct depiction of bone marrow, ligaments, tendons and cartilage (Reicher et al., 1985). The use of MRI is a common diagnostic tool in equine because definitive changes that occur

with the initial active stage of laminitis are detectable by using high field strength magnets (Arble et al., 2009).

Metabolic Stress

Beta-adrenergic agonist are potent metabolic modifiers. The effects of β-AA on the metabolism of protein and adipose tissue have been described thoroughly. However, the potential of β-AA to modify the physiological function of other tissues has not been fully elucidated. Research has demonstrated that β-adrenergic agonists reduce the frequency and intensity of ruminal contractions (Ruckebusch et al., 1983; McIntyre et al., 1992). These gut contractions are a critical aspect of ruminal digestion. Ruminal contractions also allow for eructation of ruminal gases; inhibition of eructation leads to digestive bloat, resulting in mortality (Walker and Drouillard, 2012). Data also suggests that β-AA increase absorption in the digestive tract (Walker and Drouillard, 2012; McIntyre et al., 1992). Aschenbach et al., 2002 found that a β_2-AA increased glucose uptake via sodium-glucose-linked transporter.

Beta-adrenergic agonists have been shown to increase creatine phosphokinase (**CPK**) in blood when the recommended dosage of zilpaterol hydrochloride was exceeded (FDA, 2006). Myocardial disease is often associated with an elevated concentration of serum creatine phosphokinase. Serum CPK concentration can be related to damage of striated muscle, cardiac tissue or both (Loneragen, 2014). Thus, diagnosis is dependent upon the particular isoform of CPK detected in serum (Loneragen, 2014).

Heat Stress

Across the United States, economic losses in the livestock industry attributable to heat stress are estimated to be between $1.69 and $2.36 billion; the beef industry is thought to contribute greater than $370 million to that total (St-Pierre et al., 2003). The semi-arid climate of the High Plains lends itself to persistent drought conditions and sustained high ambient temperatures, especially during the summer months. Accordingly, cattlemen are faced with a variety of challenges in maintaining efficient beef production when cattle are stressed by adverse environmental conditions. The negative effects associated with heat stress will likely become an increasing concern in the future. If current predictions hold true, the climate of the Earth will continue to warm and the population will proliferate at an astounding rate. In order to feed a growing populace, the livestock industry will need to enhance production through the improvement of genetics and the use of technology. However, basal metabolic heat has been shown to increase along with improvements in lean tissue accretion (Brown-Brandle et al., 2004). This catch-22 has led to an urgent need for a more in-depth understanding of the biology and mechanisms of how heat stress compromises livestock performance.

Livestock productivity is most efficient within a narrow window of environmental conditions. Metabolizable energy, which is generally partitioned between production and maintenance, must be diverted to assure thermal balance when an animal's internal environment ventures outside the "thermoneutral zone" (Collier and Zimbelman, 2007). Heat stress is characterized by a negative balance between the net amount of energy liberated from cattle to the environment and the amount of internal heat generated through a variety of metabolic processes. An array of environmental factors may

influence the degree to which cattle are stressed by heat; these include: 1) ambient temperature, 2) humidity, 3) air movement and 4) solar radiation. Furthermore, specific animal factors can modify the ability of cattle to cope with the aforementioned stressors. Examples include: 1) breed type, 2) hide color, 3) hair thickness, 3) extent of fat deposition, 4) mass to surface area ratio, 5) health status, 6) degree of acclimation and 7) ration composition (Mader and Davis, 2004; Dye-Rose et al., 2009). When cattle are forced outside the thermoneutral zone, it is well documented that feedlot performance is significantly compromised.

Heat stress is typically quantified in terms of intensity and duration. For more than four decades, the temperature humidity index (**THI**) has been the standard used to assess the degree of intensity to which cattle experience thermal stress. This index forms the basis for the Livestock Weather Safety Index (Livestock Conservation Incorporated, 1970). Recent data has shown that high producing dairy cattle may begin to exhibit signs of diminishing milk production when the THI reaches 68 (Zimbleman et al., 2009). However, beef cattle perform at a lower level of production and they generate less metabolic heat; accordingly, their threshold for heat stress is generally thought to be around a THI level of 75.

Recently, some experts have stated that the THI underestimates the extent of heat stress because it does not account for solar radiation or wind speed (Gaughan et al., 2008). Mader et al., (2010) developed a comprehensive climate index (**CCI**) to estimate environmental heat load. The index treats ambient temperature as the basis for the index and provides adjustments for relative humidity, wind speed and solar radiation (Mader et

al., 2010). In addition, Mader et al., (2010) established thresholds for the CCI and classified the levels of thermal stress as: no stress, mild, moderate, severe, extreme and extreme danger.

The duration of heat stress also plays a critical role in thermal regulation. This is simply defined by the amount of time that cattle spend outside the thermoneutral zone. Additionally, if there is insufficient night cooling, cattle may accumulate body heat that carries over to subsequent days (Gaughan et al., 2008). Initially, cattle will respond to heat stress by attempting to dissipate heat (i.e. sweating and panting); eventually, their response will gradually shift toward physiological adjustments targeted at reducing metabolic heat load (Gaughan et al., 2008).

Cattle that are exposed to heat stress express an altered endocrine profile that is defined by reciprocal changes in circulating hormones (Collier et al., 2005). Furthermore, intracellular signaling pathways are modified in order to aide in the dissipation of heat (Collier et al., 2008). The net result of this homeorhetic response is a highly coordinated process that allows the animal's body to prioritize acclimation and survival. In contrast to evolutionary adaptation, heat stress acclimation is a within lifetime phenotypic adaptation by the animal which widens its dynamic regulatory range of body temperature (Horowitz, 2001). In a post-acclimated state, an animal would have a reduced heart rate, a more efficient metabolism, a lower body temperature and a lowered body temperature threshold for subsequent activation of heat dissipation effectors (i.e. more heat endurance).

An animal's response to heat stress is believed to occur in a biphasic pattern dependent upon time (Horowitz et al., 1996; Horowitz, 2001). Short-term heat acclimation (**STHA**) is defined by modifications in cellular signaling pathways (i.e. cellular reprogramming) driven by hormonal regulation (Collier and Zimbelman, 2007). During STHA, there is a transient acceleration of autonomic excitability, which compensates for the impaired responsiveness of post-synaptic thermoregulatory effectors. Initially, this appears to be at odds with the creation of a homeostatic state; but, this modification is necessary to establish long-acting protective mechanisms in cells. Long-term heat acclimation (**LTHA**) is characterized by the expression of heat shock proteins (**HSP**) which serve as agents of cytoprotection.

Heat shock proteins belong to a highly conserved family of molecular chaperons and act to maintain homeostasis when stressors are present (Hecker and McGarvey, 2011; Iwaki et al., 1993; Gaughan et al., 2012). These proteins are generally named according to their molecular weight, expressed in kilodaltons. The HSPs may be released intracellularly or extracellularly in an inducible form in response to stress (Gaughan et al., 2012). Large HSPs, like HSP70, are expressed at elevated concentrations during heat stress; however the source of these proteins has not been fully elucidated (Gaughan et al., 2012; Hom et al., 2012). Shapiro et al. (1986) proposed that the source may be from damaged intestinal cells. Transient heat stress can induce redirection of blood to the periphery for enhanced heat dissipation; concurrently, blood flow to the intestines is reduced (Gaughan et al., 2012; Cronje, 2007). If exposure to a thermal challenge persists, intestinal barrier integrity may be compromised leading to an increase in

intestinal permeability (Gaughan et al., 2012; Doklandy et al., 2006; Lambert, 2009). This facilitates the penetration of endotoxins, thereby causing an inflammatory response (Gaughan et al., 2012; Shapiro et al., 1986; Lambert, 2009). Extracellular HSP70 has important functions in pro-inflammatory immune response (Pockley, 2003); therefore, changes in eHsp70 may be an indication of cellular damage within the intestines (Gaughan et al., 2012; Doklandy et al., 2006). Small HSPs, like HSP27, are less conserved among species (Arrigo and Landry, 1994). Data suggest that small heat shock proteins serve homeostatic functions at the level of signal transduction (Arrigo and Landry, 1994). These proteins are involved in the maintenance of microfilament integrity, the control of cell division/differentiation, development of the structural components of cells and repair of stress cells (Arrigo and Landry, 1994).

Furthermore, a variety of alterations in cattle's systemic response to heat stress lead to a reprioritization of fuel preference. Recent data has revealed several novel findings related to the ability of heat-stressed cattle to direct metabolic and fuel selection priorities independent of nutrient intake or energy balance (Baumgard and Rhoads, 2012). Post-absorptive changes in the nutrient metabolism of heat-acclimated cattle have been well documented. Generally, there is a reduction in basal plasma non-esterified fatty acids (**NEFA**) despite a significant reduction in feed intake (Shwartz et al., 2009). Furthermore, many studies show that glucose is preferentially used to meet the energetic demands of the associated metabolic processes during the heat stressed state (Baumgard and Rhoads, 2007). Protein metabolism is also altered in the heat-stressed state. Muscle protein synthesizing machinery and RNA/DNA synthesis capacity are reduced; there is a

simultaneous increase in skeletal muscle catabolism (Streffer, 1982; Wheelock et al., 2010).

Data suggests that the principal metabolic downfall of heat-stressed cattle is the inability to utilize "glucose sparing" pathways to satisfy the energetic needs of production. Cattle tend to become less "metabolically flexible" because their inability to mobilize adipose tissue forces an increased reliance on glucose substrates. These factors combine with decreased dry matter intake and a reduction in protein turnover efficiency to seriously compromise productivity (Baumgard and Rhoads, 2007).

Recent speculation within the feedlot industry has led to concerns about the influence of β-AA feeding on the ability of cattle to cope with heat stress, especially in heifers. Mersmann (1998) theorized that the generally accepted mode of action for β-AA may be extremely entangled with some or even most of the ultimate effects resulting from secondary events caused by hormonal or physiological responses of numerous tissues to β-AA administration. Beta-adrenergic agonists have been associated with increased heart rates, respiration rates and blood flow to muscle tissue (Eisemann et al., 1988; Bruckmaier and Blum, 1992). When effects were detected, they were generally the most pronounced within the first few days of treatment. Recent studies have directed focus toward evaluating the thermoregulatory response of ZH fed cattle.

CONCLUSION

The efficacy of utilizing growth promoting technologies in beef production systems cannot be denied. However, concerns about the well-being of animals treated with metabolic modifiers have arose in recent dialogues. Dissenting opinions about animal well-being have been voiced not only by welfare activists, but debate has also occurred within the food production industry. Consequently, society must now weigh the value underlying current conventional production practices against potential concerns relative to animal welfare. In doing so, one must keep in mind that experimental evidence provides the strongest basis for making informed decisions.

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CHAPTER III

MAGNETIC RESONANCE IMAGING PROVIDES OBJECTIVE DIAGNOSIS OF HOOF HEALTH IN FEEDLOT CATTLE SUPPLEMENTED WITH ZILPATEROL HYDROCHLORIDE

ABSTRACT

The objectives of the current study were to evaluate the effects of zilpaterol hydrochloride on the anatomy of the lateral and medial bovine claw through the use of magnetic resonance imaging (MRI) and to determine if any correlations exist between potential anatomical differences and objectively measured movement of cattle. Black-hided feedlot steers and heifers (n = 96) were arranged in a complete randomized block design with predicted empty body fat percentage serving as the blocking factor. Exit velocity of cattle leaving the chute on day 20 of the treatment period was measured. Hooves were collected post-mortem from a subsampled set of cattle (n = 45) and initially evaluated for hoof and pastern circumference. Magnetic resonance imaging was used to capture a variety of measures in the lateral and medial claw. Zilpaterol hydrochloride treated cattle did not differ from control with respect to gross anatomical measures of the hooves ($P \geq 0.09$). Magnetic resonance imaging measurements revealed that treated cattle displayed thinner lamina in the dermal layers of both claws, with the most pronounced effects found in the distal portion of the hoof ($P < 0.01$). Treated cattle exhibited reduced sole depth in the mid and proximal portions of the lateral claw ($P \leq 0.04$) independent of a change in the depth of the digital cushion ($P \geq 0.13$). Characterization scores of hoof tissues did not differ between treatments ($P \geq 0.44$). Measures of the hoof did not correlate with movement of the cattle ($P \geq 0.06$).

Collectively, these data provide experimental evidence that ZH supplementation does not increase the likelihood of pathology in the hoof under the prevailing conditions within this study.

INTRODUCTION

Beta-adrenergic agonists (**β-AA**) belong to a class of chemicals known as phenethanolamines. These compounds have been approved for use in several countries; the FDA has approved two β-AA (zilpaterol hydrochloride and ractopamine hydrochloride) for use in feedlot cattle. Beta-adrenergic agonists are synthetic analogs to endogenous catecholamines, sharing distinct chemical and pharmacological properties with dopamine, norepinephrine and epinephrine (Bell et al., 1998). The effects of β-AA are generally characterized by consistent improvements in carcass weight gain, carcass cutability and feed efficiency in beef cattle(Avendaño-Reyes et al., 2006; Winterholler et al., 2007; Vasconcelos et al., 2008; Rathmann et al., 2012). These outcomes are derived from the ability of β-AA to bind and stimulate beta-adrenergic receptors (**β-AR**) on cell surfaces, resulting in a pronounced increase in the accretion of skeletal muscle and a decreased accretion of fat (Mersmann et al., 1998). The β-AR can be grouped into three discrete subtypes (β_1-AR, β_2-AR and β_3-AR) and these receptors are present on most mammalian cells. However, the distribution of subtypes and proportion of each varies among tissues in a given species (Mersmann et al., 1998).

Zilpaterol hydrochloride (**ZH**) is a particularly potent β-AA that has been widely used in commercial cattle feeding operations since its approval by the FDA in August of

2006. The ZH ligand exhibits greater affinity to bind to the β_2-AR, which is the predominant subtype found in the skeletal muscle cells and adipocytes of cattle (Sillence and Mathews, 1995). The downstream effects of β-AA binding to β-AR in skeletal muscle cells and adipocytes have been fully described (Chung and Johnson, 2007). However, the ubiquitous nature of the β-AR gives way to the possibility for ZH to bind and alter physiological activity of a wide range of tissues that have not been evaluated with respect to β-AA activity.

Recent anecdotal reports have attempted to link the use of ZH in feedlot cattle to limited mobility and hoof disease (laminitis). Since the effects of β-AA on a variety of bovine tissues have not been completely elucidated, the aforementioned hypothesis warrants investigation. The objectives of the study at hand were to evaluate the effects of ZH feeding on 1) the anatomy of the lateral and medial bovine claw through the use of magnetic resonance imaging (**MRI**) and 2) to determine if any correlations exist between potential anatomical differences and the actual, objectively measured movement of the cattle.

MATERIALS AND METHODS

All experimental procedures involving the use of animals were reviewed and approved by the Texas Tech University Animal Care and Use Committee (ACUC # 13059-07). The experiment was conducted at the Texas Tech University Beef Cattle Center located approximately 9.7 km east of New Deal, TX.

Cattle

On August 2, 2013, black-hided steers and heifers (n = 96) were delivered to the Texas Tech University Beef Center in New Deal, TX from a nearby commercial feedlot. Cattle were estimated to be 60 days from the projected time of harvest. The day of arrival, cattle were sorted by sex into two large pens and offered a 70% concentrate diet at 70% of the 5-day average dry matter intake prior to shipping. Before arriving at Texas Tech University, steers and heifers had been on a finishing ration at the commercial feedlot for 83 and 79 days, respectively. Steers were implanted with Revalor-XS (Merck Animal Health; Summit, NJ) on May 4, 2013 and heifers were implanted with Revalor-200 (Merck Animal Health; Summit, NJ) on May 13, 2013. Initial processing (on the morning of August 6, 2013) included: 1) individual identification by ear tag; 2) measurement of body weight (Silencer Chute, Moly Manufacturing, Lorraine, KS); 3) real-time ultrasound scanning by a certified technician (Aloka 500-V instrument with a 17-cm 3.5 MHz transducer; Walllingford, CT); 4) assignment of chute temperament scores as described by Grandin et al., (1993); and 5) determination of exit velocity (**EV**) from the chute as described by Curley et al., (2006). Exit velocity was measured as an objective indicator of temperament and mobility; briefly, the rate of speed of cattle traversing a distance of 1.93 m after exiting the head gate was determined using 2 infrared sensors (FarmTek Inc., North Wylie, TX) and velocity was calculated in the following manner: [velocity = distance (m)/time (s)]. Chute temperament scores and EV were standardized and used to determine a temperament index, which allocated twice as much weight to the objective measurement (EV) as to the subjective (chute temperament score).

Real-time ultrasound data was incorporated into an equation described by Guiroy et al., (2001) in order to determine the predicted empty body fat percentage (**pEBF%**) of the cattle. Steers (n = 48; BW = 520 ± 30.4 kg; EBF % = 26.2 ± 1.9) and heifers (n = 48; BW = 466 ± 29.5 kg; EBF % = 26.7 ± 1.7) were blocked within sex by pEBF% in a complete randomized block design and randomly assigned to pen (2 pens/block; 4 head/pen) and treatment (6 pens/treatment). Within each pen, two cattle were selected for more intensive sampling and designated as "sub-sample cattle" on the basis of the most intermediate temperament index scores. Treatments were as follows: 1) control heifers, 2) ZH supplemented heifers, 3) control steers and 4) ZH supplemented steers.

Cattle were placed in 3 X 9.1 m dirt surface pens with concrete aprons at the bunk and around the water source. The concentrate level of the ration delivered was stepped up by increasing the concentration 5% every 5 days for 20 days during the acclimation period. The 90% concentrate finisher ration is detailed in Table 1. This ration was a typical feedlot diet containing premixes made at the Texas Tech University Burnett Center Feed Mill in a paddle type mixer (Marion Mixers, Inc.; Marion, IA). The supplement premix included standard trace minerals, vitamins, monensin (Tylan; Elanco Animal Health; Greenfield, IN) and tylosin (Rumensin 90; Elanco Animal Health; Greenfield, IN). Heifers were supplemented with melengestrol acetate (MGA; Pfizer; New York, NY) (.4 mg/head/day) to suppress estrus. Feed was mixed and delivered daily in a drag type feed wagon (Rotomix; Dodge City, KS). Cattle were fed once daily (0800 to 0900 h) and feed delivery was adjusted to provide ad libitum access to feed while reducing wasted feed. On day 0 of the treatment period, feed refusals were

collected and weighed to determine dry matter intake. A body weight measurement was also collected on the cattle. For the remainder of the 20 day treatment period, a ZH premix (Zilmax; Merck Animal Health; Summit, NJ) was incorporated into the diets of the appropriate treatments to deliver approximately 75 mg/head/day of the compound.

Collection of hooves

On day 20 of the ZH treatment period, feed refusals were again collected and weighed. Cattle were sent through the chute, an individual body weight was captured and EV was recorded after exiting the head gate. Following a 4 day withdrawal period, cattle were shipped to a commercial abattoir. The back left hoof was collected from the sub-sample cattle (n = 45) immediately following exsanguination at the harvest facility. Hooves were uniquely identified, placed on ice and transported to an off-site location for thorough cleaning.

Following cleaning, the hooves were photographed and gross anatomical measurements were taken with a flexible tape measure (Executive Thin Line Pocket Tape; Apex Tool Group, LLC; Sparks, MD); these measures included hoof and pastern circumference. Hoof circumference was measured at the widest portion of each hoof. Pastern circumference was evaluated at the mid-point between the coronary band and the dew claws. Hooves were then placed in durable plastic bags (Boneguard Bags; Sirane, Ltd.; Telford, United Kingdom), vacuum sealed and frozen to maintain the integrity of the tissue.

Magnetic Resonance Imaging

Images were captured at the Texas Tech Neuroimaging Institute by a board certified technician using an advanced MRI instrument (Magnetom Skyra 3T; Siemens AG; Munich, Germany). Hooves were set out to thaw 12 hours before imaging. A custom brace was constructed to contain the hooves within the MRI machine. The parameters of the scanning procedure are detailed in Table 1. Images were saved and sent to a board certified radiologist that was blinded to treatments for interpretation (Certified by the American College of Veterinary Radiology; Images evaluated by Animal Imaging; Irving, TX).

Both the lateral and medial claws were evaluated separately using objective and subjective criteria. In the transverse plane, measurements of the dorsal soft tissues of the hoof were obtained at three different points (distally, near the toe; at the mid-portion between the toe and the coronary band; proximally, distal to the coronary band). The measurements included the thickness of the lamina, corium and total dermis (Figure 1). These measurements were used to calculate a ratio of the laminae relative to the total dermal tissue. Arble et al., (2009) demonstrated that this ratio could be used to accurately diagnose laminitis in equine. The calculation for the laminitis diagnosis ratio (**LDR**) was conducted in the following manner: [LDR = width of the lamina (mm)/ total width of the dermis (mm)]. Sole depth was also evaluated at three different sites (toe, mid and heel) using a sagittal plane image, acquired along midline of each claw. A sagittal plane 3D T1 MPRAGE was used for these measurements, as this sequence could be corrected for slice plane errors and oriented along midline of each claw (Figure 2). Depth measurements of

the three different portions of the digital cushion (axial, middle and abaxial) were acquired in the transverse, coronal and sagittal planes. The total thickness of the digital cushion included the peripheral layer of connective tissue and fat. On the transverse plane fat suppressed images, the central hypointense area of digital cushion fat was measured separately. Scores were subjectively assigned to characterize the appearance of the lamina (Figure 3) and distal phalanx in both claws on the following basis: 1 = normal character; 2 = mildly irregular; 3 = moderately irregular. The deep digital flexor tendon was also assessed for any abnormalities and graded as 1 = normal hypointense tendon, 2 = mildly abnormal tendon, 3 = moderately abnormal tendon and 4 = markedly abnormal tendon.

Histopathology

Four hooves were shipped frozen to the University of Arizona Veterinary Diagnostic Laboratory in Tucson, AZ. Hooves were selected from the steer group and represented control (n = 2) and ZH treatment (n = 2). The objective was to explore techniques to evaluate the laminae of the digits to determine whether the histopathology could be revealing of any significant changes. The hooves, while still frozen, were sectioned with a band saw so that approximately 0.5 cm slices of the hoof were obtained near the mid-portion of the toe. These slices were then fixed in 10% formalin for a prolonged period to ensure complete fixation. The slices were then sectioned into segments and the lamellar tissue was separated from the bone on the inside and the keratinized hoof on the outside. These small sections of lamellar region were then sectioned and the sections were stained with hematoxylin and eosin. The steers were identified by a unique number and the pathologist was blinded to treatment.

Statistical Analyses

Magnetic resonance imaging data were analyzed as a completely randomized block design using a commercially available statistical analysis software (SPSS Statistics 22.0; IBM; Armonk, NY). Individual animal served as the experimental unit. Initially, data were analyzed to assure that the assumptions of the model were satisfied. The assumptions of normality of errors, homogeneity of variances and sphericity were evaluated using Shapiro Wilk's test, Bartlett's test and Mauchely's test, respectively. The fixed effects included treatment, sex and the interaction thereof. Block was included as a random effect. Count data obtained from the characterization scores were analyzed using Fisher's exact test for count data to determine if the collective distribution differed between treatments. The relationships between exit velocity and MRI variables were explored using Pearson's product-moment correlation coefficients. For all analyses, P – values less than or equal to 0.05 were considered significant; P – values between 0.05 and 0.10 were determined to be tendencies.

RESULTS

Gross anatomical measurement of hooves are presented in Table 2. A significant treatment*sex interaction was not detected for either variable ($P \geq 0.88$). Zilpaterol hydrochloride tended to reduce hoof circumference ($P = 0.09$), but it did not influence pastern circumference ($P = 0.44$). Highly significant gender effects were found for hoof

and pastern circumference. Relative to steers, heifers exhibited lesser circumferences in both areas ($P < 0.01$).

Results obtained from hoof measurements are presented for the lateral and medial claw in Tables 3-6. The only significant treatment*sex interactions detected were in the abaxial fat portion of the digital cushion in the lateral claw and the corium of the mid dermal layer in the medial claw; however, simple effects were not different for these parameters ($P \geq 0.07$). Consequently, main effects will be discussed for the remainder of the response variables.

The effect of ZH impacted several variables in the dermal layers and sole of the lateral claw. In the distal dermal layer, ZH decreased the thickness of the lamina ($P < 0.01$) and total dermis ($P < 0.01$) and tended to decrease the width of the corium ($P = 0.09$). In the mid dermal layer, ZH decreased the thickness of the lamina ($P = 0.04$) and tended to decrease the width of the total dermis. Zilpaterol hydrochloride decreased depth of the sole in the mid ($P = 0.02$) and proximal areas ($P = 0.04$). Sex influenced hoof measures in the lateral claw. Relative to steers, heifers displayed decreased thickness of the lamina in the distal dermal layer ($P = 0.01$), abaxial portion of the digital cushion in the sagittal plane ($P = 0.05$) and in all regions of the sole (distal, $P < 0.01$; mid, ($P = 0.03$); proximal, ($P = 0.02$)).

In the medial claw, ZH reduced the thickness of the lamina in the distal dermal layer ($P = 0.01$) and the mid dermal layer ($P < 0.01$). Zilpaterol hydrochloride reduced the LDR in the mid dermal layer ($P = 0.01$). Zilpaterol hydrochloride also reduced the width of the axial digital cushion when evaluated in the coronal plane ($P = 0.01$).

Relative to the sex effect, heifers exhibited a reduced thickness of the total dermis in the mid dermal layer ($P = 0.05$); their sole was thinner in the distal ($P = 0.01$) and mid regions ($P = 0.04$).

Evaluation of the characterization scores for the lamina, distal phalanx and the deep digital flexor tendon are presented in Tables 7 and 8. No significant effects due to ZH treatment were detected in either claw ($P \geq 0.72$). In terms of the sex effect, a tendency was revealed for heifers to exhibit poorer lamina scores relative to steers in the lateral claw ($P = 0.08$); however, no differences were observed for the distal phalanx bone or the deep digital flexor tendon ($P \geq 0.44$).

Correlation analyses are presented in Table 9. When MRI measurements of hooves were considered independent variables and correlated with EV, no significant slopes were detected. Collectively, the correlation coefficients do not indicate a strong relationship between the physiological parameters measured and the apparent movement of cattle leaving the chute.

Evaluation of histological sections of hooves did not reveal any notable differences attributable to ZH treatment. The lack of discernable differences in cellularity indicate that none of the sections exhibited any evidence of inflammatory change. One must make the assumption that inflammatory cells may not be differentiated after freezing; nonetheless, there should be nuclear remnants. This is a preliminary report pending further discussion on possible options relative to the histopathology of bovine hooves.

DISCUSSION

Lameness is a particularly important issue that influences the perceived welfare and performance of feedlot cattle. Bovine laminitis is one of the most common causes of bovine lameness. This disease is multifactorial in origin and may result from the release of endotoxins, activation of a cascade of inflammatory mediators and the disruption of microcirculation leading to degeneration of the laminar-suspending structures (Stokka et al., 2001). Laminitis is generally associated with a dietary insult of rapidly fermentable carbohydrates that elicits systemic alterations. Furthermore, vasoactive substances are known to alter hemodynamics and impact the intrinsic characteristics of the bovine digit (Nocek, 1997). Eisemann et al., (1988) demonstrated that β-AA treatment can increase blood flow to the hindquarter of steers and Belloli et al., (2004) confirmed β-AR mediated vasodilation with respect to β_2- agonists; consequently, a hypothesis can be formed that would link ZH supplementation to alterations of the bovine hoof. Thus, providing a basis for the exploration of the influence ZH may exert on hoof health and mobility of feedlot cattle.

Inflammation of the pastern and hoof is a clinical sign of bovine laminitis (Nocek, 1997). In the case at hand, gross anatomical measures indicate negligible differences between ZH treated and control cattle relative to hoof and pastern circumference. A tendency was detected for reduced pastern circumference in the ZH treated group. Reduced pastern circumference would indicate a lack of inflammation and potentially

healthy blood flow to the lower extremities. The observed sex difference could be explained by variation in the final body weight.

The MRI measures collected in this study provide strong, objective indications of physiological differences among hooves. The use of MRI technology has evolved into a powerful diagnostic imaging tool since its practical application in the late 1970's (Rothschild et al., 1990). Magnetic resonance imaging eliminates the need for ionizing radiation and provides significant advantages including: excellent inherent soft tissue contrast, multiplanar imaging capabilities and direct depiction of bone marrow, ligaments, tendons and cartilage (Gonzales-Sangues et al., 2002). The use of MRI is a common diagnostic tool in equine because definitive changes that occur with the initial active stage of laminitis are detectable by using high field strength magnets (Arble et al., 2009).

Zilpaterol hydrochloride treatment reduced the thickness of the lamina in both claws; the effect was most pronounced distally. Inflammation of the laminar tissue relative to the total width of the dermis can lead to mechanical damage of the structures that support the distal phalanx bone and connect to the horn of the claw (Noceck et al., 1997). The results presented herein indicate that ZH treatment may actually be beneficial in reducing inflammation of the hoof supporting structures.

The sole depth of ZH treated cattle was reduced in the mid and distal portions of the lateral claw, but this result was not detected in the medial claw. It has been established that cattle normally distribute more weight on the lateral claw (Gonzales-Sangues et al., 2002); therefore, it can be assumed that differences would be observed

with greater magnitude in the lateral claw. No differences were detected relative to the fat or connective tissue thickness of the digital cushion in the lateral claw, presumably the reduced sole depth is primarily attributable to the thinner laminar tissue.

Characterization scores of the lamina, distal phalanx bone and deep digital flexor tendon did not reveal any differences with respect to ZH treatment; in fact, distributions of scores were near identical in both claws. Thus, ZH treatment did not impact diagnosis of mechanical damage in the tissues evaluated. A sex-related tendency was detected indicating an increase in irregular lamina within the lateral claw of the heifer group. These data suggest inherent variation among heifer contemporaries not associated with ZH treatment.

When the observed measures of hoof anatomy were correlated with an objective measure of movement, no significant relationships were detected. Exit velocity has been established as a reliable predictor of cattle movement and temperament (Burdick et al., 2011). Since the differences detected among cattle hooves were not directly correlated with the ability of cattle to move, one can assume that the anatomical differences observed were not of biological significance.

Conclusively, reducing the incidence of lameness in feedlot cattle is of considerable importance to animal welfare and performance. The ability of cattle to ambulate is related to many factors; often, it can be difficult to visually ascertain the cause of lameness. Diagnostic tools, such as MRI, provide objective information that can be used to establish causal relationships. Collectively, these data provide experimental evidence that ZH supplementation does not increase the likelihood of pathology in the hoof under

the prevailing conditions within this study. In fact, the ability of ZH to induce vasodilation may positively influence health of the digits and mitigate the destruction of laminar structure.

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Table 3.1 As-fed composition of 90% concentrate finishing diet.

Ingredient	%, AF
Corn Grain, Steam Flaked	54.05
Alfalfa Hay, Mid Bloom	8.48
Sweet Bran, WCGF	32.23
Tallow	2.27
Urea	0.32
Limestone	1.02
TTU Supplement[1]	1.63

[*]A 0.5% ground corn based zilpaterol hydrochloride premix was substituted for steam flaked corn to provide 75 mg/head/day for the 20 day treatment period followed by a 3 day withdrawal.
[1]Provides 29.9 g/ton Rumensin and 10.0 g/ton Tylan

Table 3.2 Parameters of MRI images

		Parameter[1]							
Image	**Orientation**	**Total time (sec)**	**FOV (mm)**	**TR (ms)**	**TE (ms)**	**Flip angle (degrees)**	**B/W (Hz/Px)**	**Scan matrix (mm)**	**Slices**
Scout	All Planes	14							
T1 MPRAGE	Sagittal	266	200	1900	2.49	9	180	.9x.9x.9	128
T2 TRANS Fatsat	Transverse	197	200	5000	64	150	217	.6x.6x2.5	28
T2 SAG	Sagittal	237	200	6050	64	150	217	.6x.6x2.5	36
T2 COR Fatsat	Coronal	324	128	8050	69	150	217	.4x.4x2.5	45
PD TRANS Fatsat	Transverse	162	200	4110	32	150	217	.6x.6x2.5	28
PD SAG Fatsat	Sagittal	240	200	5290	32	150	217	.6x.6x2.5	36
PD COR Fatsat	Coronal	324	128	7020	34	150	217	.4x.4x2.5	45

[1]Parameters are defined as follows: Total time = total amount of time to capture the image; FOV = field of view; TR = repetition time; TE = echo time; Flip angle = angle of net magnetization rotation relative to the main magnetic field direction; B/W = Band Width; Scan matrix = dimensions of the slice; Slices = planar regions of the images.

Table 3.3 Effects of zilpaterol hydrochloride (**ZH**) and sex on gross anatomical measurements of back, left hooves in feedlot cattle.

	Treatment[1]			Sex				
	CTRL	ZH	*P*-value	Heifers	Steers	*P*-value	SEM[2]	TRT*SEX
Cattle, n	21	24		22	23			
Hoof circumference[*]	32.45	31.72	0.09	30.97	33.20	< 0.01	0.30	0.88
Pastern circumference[*]	27.60	27.28	0.44	26.54	28.33	< 0.01	0.30	0.89

[*]Measurements reported in centimeters.
[1]Treatment diets were formulated to contain no ZH (Control) or ZH (zilpaterol hydrochloride; 75 mg/hd/day; Zilmax, Merck Animal Health, Summit, NJ) for the final 20 d before slaughter with a 4 d withdrawal.
[2]Standard error of treatment means.

Table 3.4 Effects of zilpaterol hydrochloride (**ZH**) and sex on dermal layer thickness quantitative traits in the lateral claw of the back, left hoof[1]

	Treatment[2]			Sex				
	CTRL	ZH	*P*-value	Heifers	Steers	*P*-value	SEM[3]	TRT*SEX
Cattle, n	21	24		22	23			
Distal Dermal Layer Thicknesses								
Lamina[*]	2.14	1.89	<0.01	1.93	2.09	0.01	0.45	0.37
Corium[*]	2.73	2.52	0.09	2.60	2.65	0.66	0.90	0.39
Total dermis[*]	4.87	4.40	<0.01	4.52	4.74	0.11	0.10	0.22
Laminitis diagnosis ratio[4]	0.44	0.43	0.34	0.43	0.44	0.26	0.01	0.94
Mid Dermal Layer Thicknesses								
Lamina[*]	1.93	1.79	0.04	1.84	1.88	0.61	0.05	0.44
Corium[*]	3.05	2.91	0.29	2.89	3.06	0.19	0.10	0.65
Total dermis[*]	4.97	4.70	0.07	4.73	4.94	0.18	0.11	0.47
Laminitis diagnosis ratio[4]	0.39	0.38	0.55	0.39	0.38	0.48	0.01	0.99
Proximal Dermal Layer Thicknesses								
Lamina[*]	2.03	1.97	0.47	1.92	2.08	0.07	0.06	0.18
Corium[*]	3.77	4.07	0.38	3.84	3.94	0.72	0.20	0.29
Total dermis[*]	5.97	5.80	0.51	5.76	6.01	0.34	0.19	0.14
Laminitis diagnosis ratio[4]	0.36	0.33	0.18	0.34	0.35	0.43	0.01	0.89

[*]Measurement reported in millimeters.

[1]Hooves were collected immediately post-mortem, frozen and thawed at a later date. Magnetic resonance images were collected using a Siemens Magnetom Skyra 3T. Images were submitted to a board certified radiologist blinded to treatments for evaluation.

[2]Treatment diets were formulated to contain no ZH (Control) or ZH (zilpaterol hydrochloride; 75 mg/head/day; Zilmax, Merck Animal Health, Summit, NJ) for the final 20 d before slaughter with a 4 d withdrawal.

[3]Standard error of treatment means.

[4]Laminitis diagnosis ratio = lamina thickness/total dermis thickness. Total dermis thickness is lamina thickness plus corium thickness. The greater the ratio the greater the indication that laminitis may exist because the lamina would be inflamed. Equine practitioners consider a ratio ≥ 0.70 as a clinical sign of laminitis[12]. Ratios in bovine have not been established.

Table 3.5 Effects of zilpaterol hydrochloride (**ZH**) and sex on quantitative traits in the lateral claw of the back, left hoof[1]

	Treatment[2]			Sex				
	CTRL	ZH	*P*-value	Heifers	Steers	*P*-value	SEM[3]	TRT*SEX
Cattle, n	21	24		22	23			
Sole Depth								
Distal[*]	10.12	9.48	0.30	8.87	10.73	0.00	0.44	0.72
Mid[*]	13.57	11.81	0.02	11.83	13.56	0.03	0.55	0.30
Proximal[*]	15.97	14.06	0.04	13.90	16.13	0.02	0.66	0.79
Transverse Plane of Digital Cushion								
Axial fat + connective tissue[*]	5.56	5.32	0.38	5.37	5.50	0.64	0.20	0.51
Middle fat + connective tissue[*]	5.34	5.19	0.60	5.10	5.42	0.26	0.21	0.96
Abaxial fat + connective tissue[*]	6.46	6.11	0.20	6.16	6.41	0.35	0.19	0.44
Axial fat[*]	3.57	3.59	0.95	3.80	3.36	0.09	0.19	0.90
Middle fat[*]	2.53	2.42	0.66	2.47	2.48	0.96	0.18	0.56
Abaxial fat[*]	3.91	3.67	0.27	3.84	3.74	0.65	0.15	0.02
Coronal Plane of Digital Cushion								
Axial[*]	5.57	5.70	0.67	5.50	5.77	0.37	0.21	0.45
Middle[*]	4.95	4.76	0.61	4.88	4.87	0.88	0.28	1.00
Abaxial[*]	7.45	6.97	0.13	7.09	7.34	0.43	0.23	0.65
Sagittal Plane of Digital Cushion								
Axial[*]	8.58	8.89	0.40	8.44	9.02	0.12	0.27	0.86
Middle[*]	4.17	4.01	0.53	3.86	4.32	0.08	0.19	0.34
Abaxial[*]	6.81	6.71	0.73	6.48	7.05	0.05	0.21	0.26

[*]Measurement reported in millimeters.

[1]Hooves were collected immediately post-mortem, frozen and thawed at a later date. Magnetic resonance images were collected using a Siemens Magnetom Skyra 3T. Images were submitted to a board certified radiologist blinded to treatments for evaluation.

[2]Treatment diets were formulated to contain no ZH (Control) or ZH (zilpaterol hydrochloride; 75 mg/head/day; Zilmax, Merck Animal Health, Summit, NJ) for the final 20 d before slaughter with a 4 d withdrawal.

[3]Standard error of treatment means.

Table 3.6 Effects of zilpaterol hydrochloride (**ZH**) and sex on the dermal layer thickness in the medial claw of the back, left hoof[1]

	Treatment[2]			Sex				
	CTRL	ZH	*P*-value	Heifers	Steers	*P*-value	SEM[3]	TRT*SEX
Cattle, n	21	24		22	23			
Distal Dermal Layer Thicknesses								
Lamina[*]	1.94	1.72	0.01	1.82	1.83	0.88	0.06	0.37
Corium[*]	2.17	2.06	0.56	2.15	2.08	0.69	0.14	0.32
Total dermis[*]	4.11	3.78	0.14	3.97	3.91	0.78	0.16	0.61
Laminitis diagnosis ratio[4]	0.48	0.46	0.21	0.47	0.47	0.75	0.01	0.16
Mid Dermal Layer Thicknesses								
Lamina[*]	1.89	1.65	<0.01	1.70	1.83	0.09	0.05	0.52
Corium[*]	2.48	2.48	0.98	2.37	2.59	0.09	0.09	0.04
Total dermis[*]	4.37	4.12	0.15	4.08	4.42	0.05	0.12	0.08
Laminitis diagnosis ratio[4]	0.43	0.40	0.01	0.42	0.42	0.93	0.01	0.09
Proximal Dermal Layer Thicknesses								
Lamina[*]	2.04	2.02	0.82	1.99	2.08	0.27	0.06	0.96
Corium[*]	3.32	3.23	0.60	3.34	3.22	0.50	0.12	0.06
Total dermis[*]	5.36	5.26	0.62	5.33	5.30	0.89	0.15	0.13
Laminitis diagnosis ratio[4]	0.38	0.39	0.74	0.38	0.39	0.15	0.01	0.08

[*]Measurement reported in millimeters.

[1]Hooves were collected immediately post-mortem, frozen and thawed at a later date. Magnetic resonance images were collected using a Siemens Magnetom Skyra 3T. Images were submitted to a board certified radiologist blinded to treatments for evaluation.

[2]Treatment diets were formulated to contain no ZH (Control) or ZH (zilpaterol hydrochloride; 75 mg/head/day; Zilmax, Merck Animal Health, Summit, NJ) for the final 20 d before slaughter with a 4 d withdrawal.

[3]Standard error of treatment means.

[4]Laminitis diagnosis ratio = lamina thickness/total dermis thickness. Total dermis thickness is lamina thickness plus corium thickness. The greater the ratio the greater the indication that laminitis may exist because the lamina would be inflamed. Equine practitioners consider a ratio ≥ 0.70 as a clinical sign of laminitis[12]. Ratios in bovine have not been established.

Table 3.7 Effects of zilpaterol hydrochloride (**ZH**) and sex on quantitative traits in the medial claw of the back, left hoof[1]

	Treatment[2]			Sex				
	CTRL	ZH	*P*-value	Heifers	Steers	*P*-value	SEM[3]	TRT*SEX
Cattle, n	21	24		22	23			
Sole Depth								
Distal[*]	9.82	9.62	0.65	9.17	10.27	0.01	0.30	0.46
Mid[*]	11.42	10.97	0.39	10.64	11.75	0.04	0.38	0.72
Proximal[*]	14.33	13.36	0.14	13.53	14.16	0.33	0.46	0.26
Transverse Plane of Digital Cushion								
Axial fat + connective tissue[*]	4.86	4.62	0.38	4.80	4.68	0.65	0.20	0.42
Middle fat + connective tissue[*]	4.76	4.49	0.28	4.39	4.86	0.07	0.18	0.89
Abaxial fat + connective tissue[*]	5.80	5.65	0.51	5.50	5.95	0.06	0.17	0.61
Axial fat[*]	3.09	2.76	0.14	2.82	3.03	0.34	0.16	0.33
Middle fat[*]	2.06	2.08	0.93	2.08	2.07	0.95	0.15	0.65
Abaxial fat[*]	3.43	3.30	0.53	3.18	3.55	0.10	0.16	0.52
Coronal Plane of Digital Cushion								
Axial[*]	4.99	4.30	0.01	4.70	4.58	0.64	0.18	0.41
Middle[*]	4.61	4.40	0.41	4.35	4.66	0.24	0.19	0.67
Abaxial[*]	7.10	6.73	0.16	6.74	7.10	0.18	0.19	0.50
Sagittal Plane of Digital Cushion								
Axial[*]	8.81	8.51	0.44	8.53	8.78	0.51	0.28	0.39
Middle[*]	3.83	3.77	0.83	3.65	3.95	0.27	0.20	0.41
Abaxial[*]	5.66	5.65	0.99	5.37	5.94	0.06	0.22	0.90

[*]Measurement reported in millimeters.

[1]Hooves were collected immediately post-mortem, frozen and thawed at a later date. Magnetic resonance images were collected using a Siemens Magnetom Skyra 3T. Images were submitted to a board certified radiologist blinded to treatments for evaluation.

[2]Treatment diets were formulated to contain no ZH (Control) or ZH (zilpaterol hydrochloride; 75 mg/head/day; Zilmax, Merck Animal Health, Summit, NJ) for the final 20 d before slaughter with a 4 d withdrawal.

[3]Standard error of treatment means.

Table 3.8 Effects of zilpaterol hydrochloride (**ZH**) and sex on the characterization of the lamina, distal phalanx and deep digital flexor tendon in the lateral claw of the back, left hoof[1]

	Treatment[2]			Sex		
	CTRL	ZH	*P*-value[3]	Heifers	Steers	*P*-value[3]
Cattle, n	21	24		22	23	
Character of the lamina						
Normal	16	20	0.86	15	21	0.08
Mildly irregular	3	3		4	2	
Moderately irregular	2	1		3	0	
Character of the distal phalanx bone						
Normal	21	23	0.99	22	22	0.99
Mildly irregular	0	1		0	1	
Moderately irregular	0	0		0	0	
Character of the deep digital flexor tendon						
Normal	21	24	1.00	22	23	1.00
Mildly irregular	0	0		0	0	
Moderately irregular	0	0		0	0	

[1]Hooves were collected immediately post-mortem, frozen, and thawed at a later date. Magnetic resonance images were collected using a Siemens Magnetom Skyra 3T. Images were submitted to a board certified radiologist blinded to treatments for evaluation.

[2]Treatment diets were formulated to contain no ZH (Control) or ZH (zilpaterol hydrochloride; 75 mg/head/day; Zilmax, Merck Animal Health, Summit, NJ) for the final 20 d before slaughter with a 4 d withdrawal.

[3]A Fisher's Exact Test for Count Data was conducted to determine if the collective distribution differed between treatments.

Table 3.9 Effects of zilpaterol hydrochloride (**ZH**) and sex on the characterization of the lamina, distal phalanx and deep digital flexor tendon in the medial claw of the back, left hoof[1]

	Treatment[2]			Sex		
	CTRL	ZH	*P*-value[3]	Heifers	Steers	*P*-value[3]
Cattle, n	21	24		22	23	
Character of the lamina						
Normal	18	21	0.99	18	21	0.44
Mildly irregular	1	1		2	0	
Moderately irregular	2	2		2	2	
Character of the distal phalanx bone						
Normal	20	23	0.72	21	22	0.99
Mildly irregular	0	1		0	1	
Moderately irregular	1	0		1	0	
Character of the deep digital flexor tendon						
Normal	21	24	1.00	22	23	1.00
Mildly irregular	0	0		0	0	
Moderately irregular	0	0		0	0	

[1]Hooves were collected immediately post-mortem, frozen, and thawed at a later date. Magnetic resonance images were collected using a Siemens Magnetom Skyra 3T. Images were submitted to a board certified radiologist blinded to treatments for evaluation.
[2]Treatment diets were formulated to contain no ZH (Control) or ZH (zilpaterol hydrochloride; 75 mg/head/day; Zilmax, Merck Animal Health, Summit, NJ) for the final 20 d before slaughter with a 4 d withdrawal.
[3]A Fisher's Exact Test for Count Data was conducted to determine if the collective distribution differed between treatments.

Table 3.10 Correlation of MRI measures of hooves with the exit velocity of cattle leaving the chute.

	Treatment[1]			
	CTRL	ZH	*P*-value[2]	PPM Coefficient[3]
Dependent Variable:				
Exit Velocity[4]	3.37	3.20	0.71	
Independent Variables:				
Hoof circumference, cm	32.51	31.72	0.58	-0.08
Pastern circumference, cm	27.64	27.28	0.81	0.04
Lateral Claw				
Distal laminitis diagnosis ratio	0.44	0.43	0.52	0.21
Mid laminitis diagnosis ratio	0.39	0.38	0.18	0.10
Proximal laminitis diagnosis ratio	0.36	0.33	0.07	0.27
Distal sole depth, mm	10.12	9.48	0.67	-0.07
Mid sole depth, mm	13.57	11.81	0.25	-0.18
Proximal sole depth, mm	15.97	14.06	0.52	-0.10
Medial Claw				
Distal laminitis diagnosis ratio	0.48	0.46	0.20	-0.20
Mid laminitis diagnosis ratio	0.43	0.40	0.72	-0.06
Proximal laminitis diagnosis ratio	0.38	0.39	0.79	0.04
Distal sole depth, mm	9.82	9.62	0.06	-0.29
Mid sole depth, mm	11.42	10.97	0.25	-0.18
Proximal sole depth, mm	14.33	13.36	0.22	-0.19

[1]Treatment diets were formulated to contain no ZH (Control) or ZH (zilpaterol hydrochloride; 75 mg/head/day; Zilmax, Merck Animal Health, Summit, NJ) for the final 20 d before slaughter with a 4 d withdrawal. The values presented indicate pooled treatment means.
[2]*P* – values indicate the significance of the slope between the various independent variables and the dependent variable (EV).
[3]PPM Coefficient = Pearson's product-moment correlation coefficient.
[4]Exit velocity from the chute was captured on d 20 of the ZH treatment period.

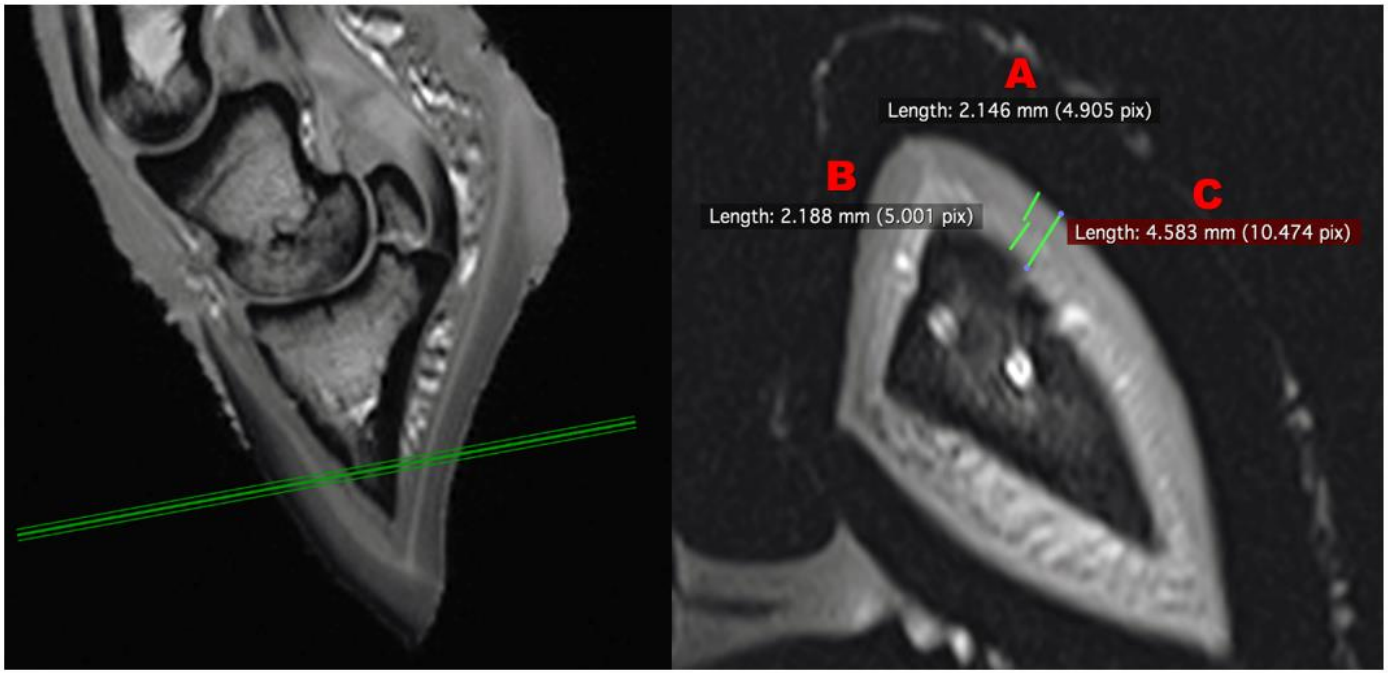

Figure 1 – Distal lamellar layers of the lateral, bovine claw delineated as the lamina (A), corium (B) and total dermis (C).

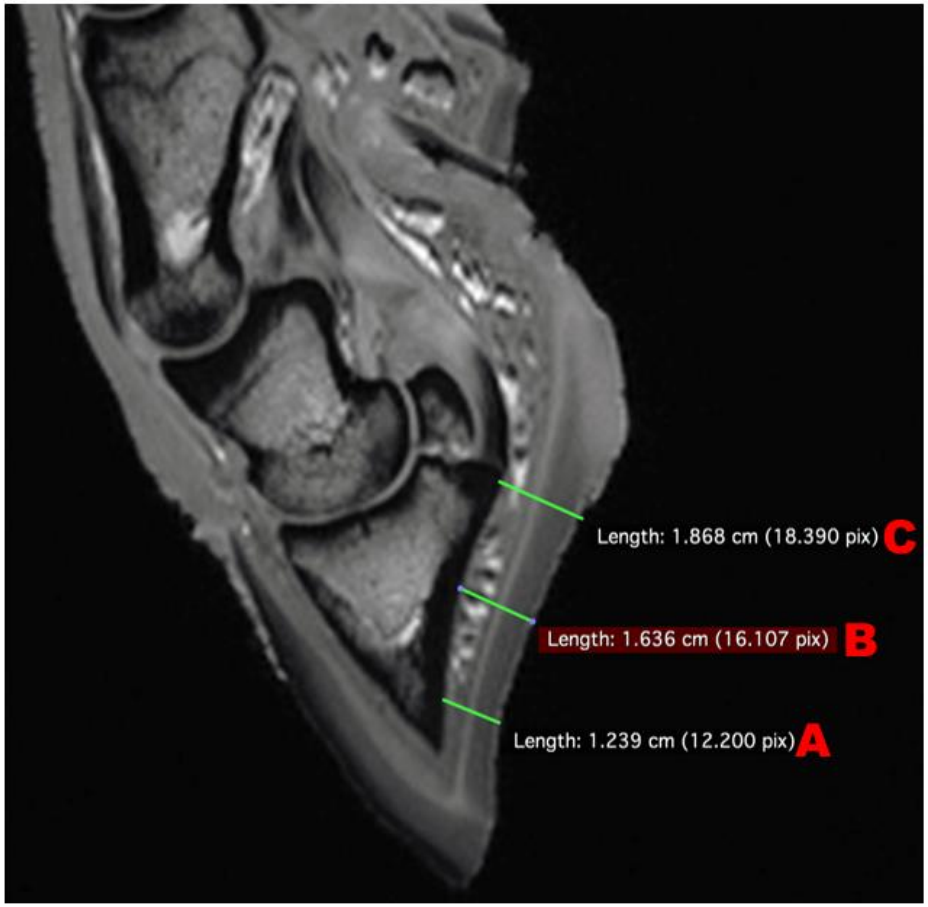

Figure 2 – Sole depth of the lateral, bovine claw measured at the distal (A), mid (B) and proximal areas (C) of the distal phalanx bone.

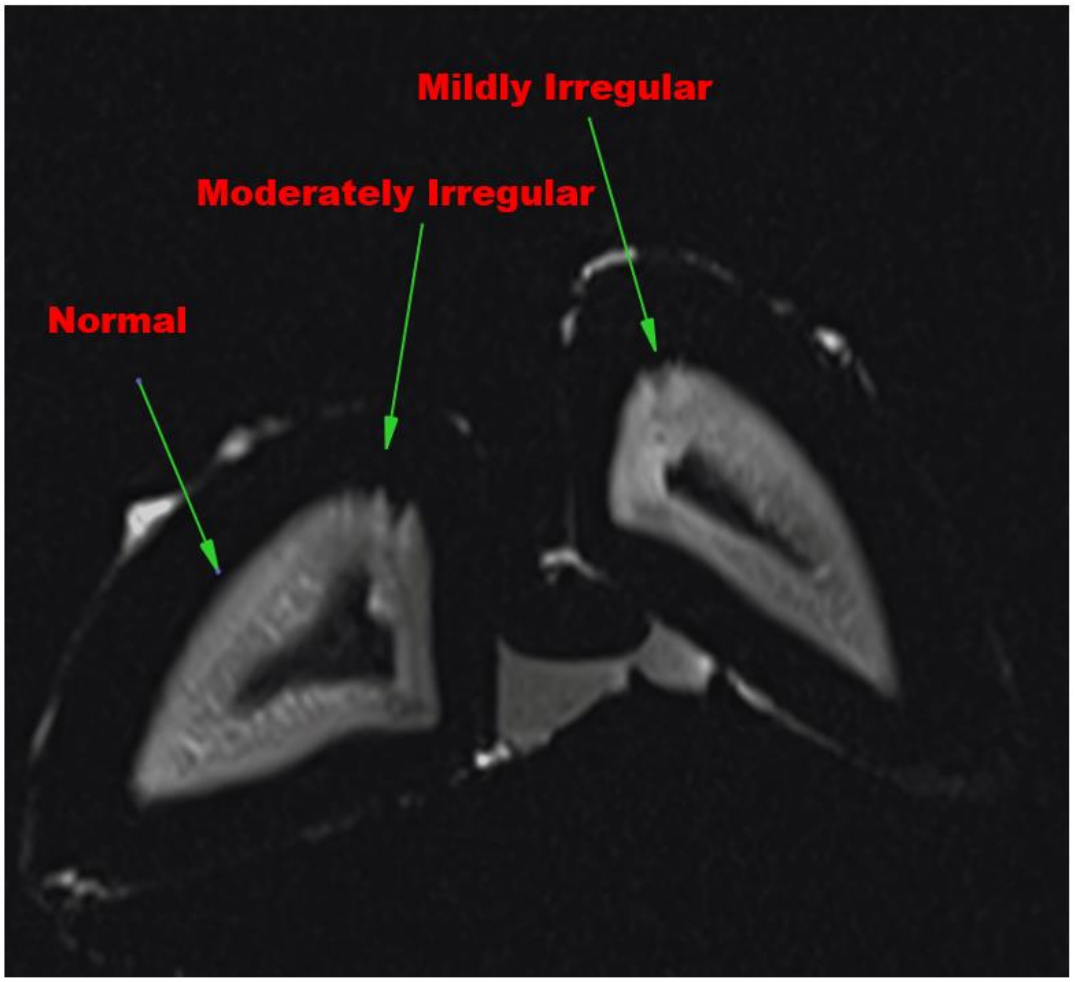

Figure 3 – Characterization of the lamina integrity in the lateral, bovine claw.

CHAPTER IV

ZILPATEROL HYDROCHLORIDE HAS MINIMAL EFFECTS ON THE WELL-BEING OF FEEDLOT CATTLE

ABSTRACT

The objectives of this study were to determine the effects of zilpaterol hydrochloride (**ZH**) feeding on cattle well-being by evaluating: 1) mobility and chute temperament 2) various indicators of physiological/metabolic stress and 3) post-mortem histopathology. Steers and heifers (n=96) were sourced from a commercial feedlot and transported to the Texas Tech University Beef Center. Cattle were weighed and scanned using real-time ultrasound. Resulting data were used to predict empty body fat percentage **(pEBF %)**. Steers (n=48; BW = 520 ± 30.4 kg; pEBF % = 26.2 ± 1.9) and heifers (n=48; BW = 466 ± 29.5 kg; pEBF % = 26.7 ± 1.7) were blocked within gender by pEBF % in a complete randomized block design and randomly assigned to pen (2 pens/block; 4 hd/pen) and treatment (6 pens/treatment). Movement differences were objectively assessed (d 0, 5, 10, 15 and 20) with several measures of mobility: exit velocity from chute **(EV)**, velocity traveling to the working area **(VT)** and velocity returning to the home pen **(VF)**. Individual locomotion scores **(LS)** were recorded based on a 1 to 4 scale (1 = no lameness, 4 = severe lameness). Venous blood was collected via jugular venipuncture (d 0, 5, 10, 15 and 20) and analyzed for various markers of physiological/metabolic stress. Skeletal muscle and cardiac tissue were evaluated for gene expression of creatine phosphokinase (**CPK**) using PCR. Cattle fed ZH displayed a TRT*TIME interaction for EV ($P = 0.03$) driven by an increase at d 5 (P = 0.03). Pooled treatment effects were not significant for VT or VF ($P \geq 0.31$). The proportion of cattle

with locomotion scores indicating sufficiently sound movement (LS = 1&2) versus cattle that were moderate or severely lame (LS = 3&4) was not different between treatment groups ($P = 0.24$). Blood potassium concentration increased with ZH treatment ($P < 0.01$). A TRT*SEX interaction was detected for blood ionized calcium ($P = 0.03$) indicating a ZH related reduction in both genders, but the magnitude of the effect was greater in steers. The concentration of serum NEFA was increased in ZH treated cattle ($P = 0.03$). Treatment effects were not detected for CPK in serum ($P = 0.70$) or local tissue ($P \geq 0.13$). No treatment effects were detected in the histopathology analysis ($P \geq 0.50$). Collectively, these data suggest that ZH supplementation does not impair cattle soundness of movement or apparent well-being; however, notable changes occur in terms of metabolic parameters in the blood.

Key words: cattle, zilpaterol hydrochloride, well-being

INTRODUCTION

Beta-adrenergic agonists (**β-AA**) are synthetic analogs to endogenous catecholamines, sharing distinct chemical and pharmacological properties with dopamine, norepinephrine and epinephrine (Bell et al., 1998). The effects of β-AA are generally characterized by consistent improvements in carcass weight gain, carcass cutability and feed efficiency in beef cattle (Avendaño-Reyes et al., 2006; Winterholler et al., 2007; Vasconcelos et al., 2008; Rathmann et al., 2012). These outcomes are derived from the ability of β-AA to bind and stimulate beta-adrenergic receptors (**β-AR**) on cell surfaces, resulting in a pronounced increase in the accretion of skeletal muscle and a decreased accretion of fat (Mersmann, 1998). These receptors are present on most mammalian cells. However, the distribution of subtypes and proportion of each varies among tissues in a given species (Mersmann, 1998).

Zilpaterol hydrochloride (**ZH**) is a β-AA that primarily binds to the β_2-AR, which is the predominant subtype found in the skeletal muscle cells and adipocytes of cattle (Sillence et al., 1995). The β-AA mode of action in skeletal muscle cells and adipocytes has been fully described (Chung and Johnson, 2007). However the ubiquitous nature of the β-AR gives way to the possibility for ZH to alter physiological activity of a wide range of tissues that have not been evaluated with respect to β-AA activity.

Recent anecdotal reports have attempted to link the use of ZH in feedlot cattle to limited mobility, increased incidence of morbidity and ultimately higher rates of mortality. Since the effects of β-AA on a variety of bovine tissues have not been fully elucidated, the aforementioned hypothesis warrants investigation. The objectives of this study were to determine the effects of ZH feeding on general cattle well-being by

evaluating: 1) mobility and chute temperament 2) various circulating and local indicators of physiological stress/metabolic status and 3) post-mortem histopathology of feedlot cattle.

MATERIALS AND METHODS

All experimental procedures involving the use of animals were reviewed and approved by the Texas Tech University Animal Care and Use Committee (ACUC # 13059-07). The experiment was conducted at the Texas Tech University Beef Cattle Center located approximately 9.7 km east of New Deal, TX.

Cattle and management

On August 2, 2013, black-hided steers and heifers (n = 96) were delivered to the Texas Tech University Beef Center in New Deal, TX from a nearby commercial feedlot. Cattle were estimated to be 60 days from the projected time of harvest. Before arriving at Texas Tech University, steers and heifers had been on a finishing ration at the commercial feedlot for 83 and 79 days, respectively. Steers were implanted with Revalor-XS (Merck Animal Health; Summit, NJ) on May 4, 2013 and heifers were implanted with Revalor-200 (Merck Animal Health; Summit, NJ) on May 13, 2013.

The day of arrival, cattle were sorted by sex into two large pens and offered a 70% concentrate diet at 70% of the 5-day average dry smatter intake prior to shipping. Initial processing (on the morning of August 6, 2013) included: 1) individual identification by ear tag; 2) measurement of body weight [(Silencer Chute, Moly Manufacturing, Lorraine, KS, mounted on Avery Weigh-Tronix load cells, Fairmount, MN; readability ± 0.45 kg); scales were calibrated with 454 kg of certified weights

(Texas Department of Agriculture) before use]; 3) real-time ultrasound scanning by a certified technician(Aloka 500-V instrument with a 17-cm 3.5 MHz transducer); 4) assignment of chute temperament scores (**CS**) as described by Grandin (1993); and 5) determination of exit velocity (**EV**) from the chute as described by Curley (2006). Exit velocity was measured as an objective indicator of temperament and mobility; briefly, the rate of speed of cattle traversing a distance of 1.93 m after exiting the head gate was determined using 2 infrared sensors (FarmTek, Inc.; North Wylie, TX) and velocity was calculated in the following manner: [velocity = distance (m)/time (s)]. Chute temperament scores and EV were standardized and used to determine a temperament index, which allocated twice as much weight to the objective measurement (EV) as to the subjective (CS).

Real-time ultrasound data was incorporated into an equation described by Guiroy (2001) to determine the predicted empty body fat percentage of the cattle (**pEBF%**). In order to reduce noise within the data related to inherent compositional differences among cattle, pEBF% was designated as the blocking factor. Steers (n = 48; BW = 520 ± 30.4 kg; EBF % = 26.2 ± 1.9) and heifers (n = 48; BW = 466 ± 29.5 kg; EBF % = 26.7 ± 1.7) were blocked within sex by pEBF% in a complete randomized block design and randomly assigned to pen (2 pens/block; 4 hd/pen) and treatment (6 pens/treatment). Within each pen, two cattle were selected for more intensive sampling and designated as "sub-sample cattle" on the basis of the most intermediate temperament index scores. Treatments were as follows: 1) control heifers, 2) ZH supplemented heifers, 3) control steers and 4) ZH supplemented steers.

Cattle were placed in 3 X 9.1 m dirt surface pens with concrete aprons at the bunk and around the water source. The concentrate level of the ration delivered was stepped up by increasing the concentration 5% every 5 days for 20 days during the acclamation period. The 90% concentrate finisher ration is detailed in Table 1. This ration was a typical feedlot diet containing premixes made at the Texas Tech University Burnett Center Feed Mill in a paddle type mixer (Marion Mixers, Inc; Marion, IA). The supplement premix included standard trace minerals, vitamins, tylosin (Tylan, Elanco Animal Health; Greenfield, IN) and monensin (Rumensin 90; Elanco Animal Health; Greenfield, IN). Heifers were supplemented with melengestrol acetate (.4 mg/head/day) to suppress estrus (MGA; Pfizer; New York, NY). Feed was mixed and delivered daily in a drag type feed wagon (Rotomix; Dodge City, KS). Cattle were fed once daily (0800 to 0900 h) and feed delivery was adjusted to provide ad libitum access to feed while reducing wasted feed. Daily health observations were made at the time of feed delivery. Prior to the ZH period, some cattle were treated for hoof root due to abnormally wet conditions in the month of August. Sulfamethazine boluses (Sustain III™; Durvet; Blue Springs, MO) and liquamycin injections (LA 200®; Pfizer; New York, NY) were used to treat tender-footed cattle.

On d 0 of the treatment period, feed refusals were collected and weighed to determine dry matter intake. A body weight measurement was also collected on the cattle. For the remainder of the 20 d treatment period, a ZH premix (Zilmax; Merck Animal Health; Summit, NJ) incorporated with ground corn was included in the ration of the appropriate treatments to deliver approximately 75 mg/hd/d of ZH. On d 21, feed bunks were swept and feed refusals were weighed. Based on DMI, cattle in the

appropriate treatment groups received approximately 73.7 ± 13.9 mg/hd/d of ZH. Following a 4 d withdrawal period, cattle were individually weighed and shipped to a commercial abattoir for harvest.

Mobility and temperament measures

Exit velocity was captured on d 0, 5, 10, 15 and 20 of the study using the aforementioned procedures. Additionally, movement of each pen was quantified at the same time points as they traveled to and from the working area. A single handler carrying a digital timer (480 Tough Timer Stopwatch; Sportline, Inc.; Elford, NY) pushed cattle from their home pen to a common point; the timer was started when the last animal broke the threshold. Cattle traversed a distance of 260 m and the timer was stopped when the last animal crossed a predetermined point at the beginning of the working area. Cattle were pushed through the tub and lead-up and assigned a CS by a trained, unbiased evaluator (prior to restraint in the head gate) on d 0 and 20 based on the previously noted criterion. Following processing, cattle were released from the chute and placed in a holding pen. The same handler removed cattle from the holding pens and timed their movement (travelling 170 m) between two set points as they returned to the home pen area.

Locomotion scores (**LS**) were obtained by a treatment blinded, trained evaluator as the cattle left the chute on the day of shipping. Scores were assigned on a concrete surface and based on the scale developed by Sprecher et al., (1996). 1 = (Normal) Stands and walks normally with level back; makes long, confident strides; 2 = (Mildy lame) Stands with flat back, but arches when walks. Gait is slightly abnormal; 3 = (Lame) Stands and walks with an arched back and short strides with one or more legs. Slight

sinking of dew-claws in limp opposite to the affected limb may be evident; 4 = (Severely lame) Arching of back is pronounced. Cattle are reluctant to move, with almost complete weight transfer off the affected limb.

Blood collection and analysis

Cattle were processed through the chute (d 0, 5, 10, 15 and 20) and two venous blood samples were collected from the designated sub-sampled animals via jugular venipuncture. One sample was collected in a sodium heparinized tube containing an anticoagulation agent (Green Top BD Vacutainer; Becton, Dickinson and Company; Franklin Lakes, NJ). This sample was inverted several times, placed on ice and transported to an on-site station for prompt analysis. Within 10 minutes of collection, a GEM Premier 3000 Blood Gas Analyzer (Instrumentation Laboratory; Lexington, MA) was used to measure blood partial pressure of carbon dioxide (**pCO_2**), partial pressure of oxygen (**pO_2**), oxygen saturation (**O_2Sat**), pH, hematocrit percentage (**Hct**), sodium (**Na^+**), potassium (**K^+**) and ionized calcium (**iCa**). A second sample was also collected in a silicone coated tube containing a clot activation agent (Red Top BD Vacutainer; Becton, Dickinson and Company; Franklin Lakes, NJ). These samples were placed on ice and transported to the Texas Tech University laboratory. Serum was isolated by centrifuging samples for 20 minutes at temperature of 4^{o}C and a speed of 1,000 G. Aliquots of serum were then frozen for subsequent analysis. The concentration of creatine phosphokinase (**CPK**), serum urea nitrogen (**SUN**) and NEFA were determined using ELISA kits.

Tissue collection and histopathology

When cattle were harvested at the commercial abattoir, Texas Tech University meat lab personnel collected a variety of tissue samples. Within 10 min post-mortem, 10-g samples of skeletal muscle (longissimus dorsi), heart, liver, lung and kidney tissue were collected, placed in sealable plastic bags (Whirl-Pack; Nasco, LLC; Stamford, CT) and snap frozen in liquid N (n = 45 for each tissue). Samples were placed on dry ice, transported to Texas Tech University and frozen at -80^{o}C for subsequent analysis. Additionally, another 10-g sample of heart, liver, lung and kidney tissue was collected in the same manner, placed on ice and transported to the Texas Veterinary Medical Diagnostic Laboratory (Amarillo, TX) for prompt histopathology analysis of the fresh tissues. Evaluators were blinded as to the treatment of the uniquely identified tissues. A complete report was generated for each sample that documented any histologic changes. For the purposes of this investigation, findings were categorized as either a) no significant pathology or b) abnormalities present.

RNA isolation and real-time quantitative PCR

Ribonucleic acid from muscle and heart tissue were isolated with ice-cold buffer containing TRI Reagent (Sigma, St. Louis, MO). Approximately 0.5g of frozen tissue was homogenized with TRI Reagent at a ratio of 0.5:1 grams of tissue to mL reagent. The homogenate was then pipetted into 2 microcentrifuge tubes (1 mL sample per tube), 200 µL chloroform was added to each tube, vortexed for 30 s and incubated for 5 min. The sample was then centrifuged at 15,000 x *g* for 15 min, separating the sample into 3 layers. The top supernatant layer was pipetted off and placed into new microcentrifuge tubes. Two hundred-fifty µL of ice cold isopropyl alcohol was added to

the supernatant, shaken and incubated for 10 min on the bench top. The samples were then centrifuged at 15,000 x *g* for 10 min. The supernatant was poured off, the RNA pellet at the bottom of each tube was allowed to dry and 500 µL of 75% ethanol was added to each tube to rinse and suspend the RNA pellet. Tubes were then placed in a -80°C freezer. One tube was then removed from the freezer and thawed on ice. Tubes were then centrifuged at 15,000 x *g* for 10 min, ethanol was poured off and the pellet was air dried. Thirty µL of nuclease free water was then added to each tube to dissolve the RNA pellet. The concentration of RNA was determined with a UV-Vis spectrophotometer at an absorbance of 260 nm, using a NanoDrop 1000 (NanoDrop products, Wilmington, DE). Samples were then treated with DNAse to remove any DNA contaminants using a DNA-free kit (Life Technologies, Grand Island, NY). The RNA was then subjected to reverse-transcription, and cDNA was produced. The cDNA was then used for real-time quantitative reverse transcription-PCR (RT-qPCR) to measure the quantity of creatine phosphokinase (**CPK**) mRNA relative to the quantity of Ribosomal protein subunit 9 mRNA in total RNA isolated from muscle and heart tissue. Specifications for the primers and probes used for each gene are detailed in Table 1. Assays were performed in the GeneAmp 7900HT Sequence Detection System (Applied Biosystems, Life Technologies) using thermal cycling parameters recommended by the manufacturer (40 cycles of 15 s at 95°C and 1 min. at 60°C).

Statistical Analyses

All continuously distributed data were analyzed as a completely randomized block design using the PROC GLIMMIX procedure of SAS (SAS 9.3; SAS Inst.; Cary, NC). Individual animal served as the experimental unit for the majority of analyses and

pen was the experimental unit for the group movement measures. The fixed effects included treatment, sex and the interaction thereof. The pEBF% blocking component was included as a random effect. Mobility and blood data that were collected at 5 d increments during the ZH treatment period were evaluated as repeated measures; the proper covariance structure for each analysis was selected based on fit statistics. The assumptions of normality of errors, homogeneity of variances and sphericity were evaluated for each fixed effect using Shapiro Wilk's test, Bartlett's test and Mauchely's test, respectively. Values greater than 3 standard deviations from the mean were considered outliers and removed from the analysis. Count data (LS, CS and histopathology) were analyzed using Fisher's exact test for count data (SPSS Statistics 22.0; IBM; Armonk, NY) to determine if the collective distribution differed between treatments. For all analyses, *P* – values less than or equal to 0.05 were considered significant; *P* – values between 0.05 and 0.10 were determined to be tendencies.

RESULTS

Feedlot performance and carcass data for the cattle used in the current study are reported by Ragland et al. (2014). Briefly summarized, ZH fed heifers exhibited a numerical increase of 7.68 kg in HCW ($P = 0.41$) relative to control heifers. A numerical increase in HCW of 6.09 kg ($P = 0.50$) was also detected when comparing steers fed ZH to control. Heifers fed ZH displayed a 1.43 percent increase ($P = 0.04$) in dressing percentage relative to control. Steers fed ZH demonstrated a tendency for a 1.2 percent

increase ($P = 0.06$) in dressing percentage relative to control. Heifers fed ZH exhibited a numerical increase of 2.45 cm^2 of REA ($P = 0.34$) relative to control heifers. Steers fed ZH expressed a tendency for an increase of 4.27 cm^2 of REA ($P = 0.10$) relative to control steers. The ZH related carcass effects in the study at hand are of lesser magnitude than previously published data; however, this difference can be explained by stress imposed on cattle when working them through the chute 5 times during the ZH treatment period.

Results obtained from the objectively measured mobility variables are presented in Table 2. The TRT*SEX interaction was insignificant for EV ($P = 0.23$). However, a TRT*DAY interaction was detected ($P = 0.03$) for exit velocity from the chute. These data indicate that ZH cattle exhibited an increase in exit velocity on d 5 ($P = 0.03$) and returned to baseline levels by d 20. However, the exit velocities did not differ due to treatment when pooled across all time points ($P = 0.23$). Heifers charted greater exit velocities relative to steers at d 0, d 10, d 20 ($P = 0.02$) and tended to show an increase at d 5 and d 15 ($P = 0.06$). Collectively, pooled exit velocity estimates indicate that heifers leave the chute more rapidly than steers ($P = 0.01$). In terms of pen movement, a tendency for a TRT*DAY interaction was detected when cattle were traveling to the working area ($P = 0.06$). Cattle in the ZH group were faster traveling to the working area on d 0 ($P = 0.03$). On d 20, ZH cattle tended to travel more slowly to the working area ($P = 0.07$); however, treatment groups did not differ for the entire period ($P = 0.76$). Treatment groups exhibited similar velocities as they returned to the home pen at each day and for the entire period ($P \geq 0.18$). Heifers traveled to the working area and

returned to the home pen more rapidly than steers when data were pooled across days [(P = <0.01) and (P = 0.01), respectively].

Data obtained from the subjective assessment of CS and LS are presented in Tables 3 and 4. Treatment groups did not differ when the baseline CS was captured on d 0 (P = 0.19). On d 20, ZH treated cattle exhibited elevated CS relative to control (P = 0.01). This result was a product of an increased proportion cattle in the slightly restless (2) and restless (3) classifications. There was a tendency for increased LS (LS $\geq$ 2) in ZH treated cattle (P = 0.09). However, the proportion of cattle with LS indicating sufficiently sound movement (LS = 1&2) versus cattle that were moderately or severely lame (LS = 3&4) did not differ between treatment groups (P = 0.24).

Analysis of analysis of blood gas, pH, hematocrit concentration and electrolytes did not produce any significant TRT*TIME interactions ($P \geq 0.17$), so pooled results are presented in Table 5. Cattle in the ZH treatment group expressed a tendency for decreased pO_2 (P = 0.08) relative to control. ZH treatment significantly increased K^+ concentration ($P < 0.01$). The concentration of iCa was reduced in both genders; but, a significant TRT*SEX interaction (P = 0.04) revealed that ZH decreased iCa with a greater magnitude in steers relative to heifers (Figure 1). Treatment groups did not differ with respect to pCO_2, O_2Sat, pH, Hct or Na^+ ($P > 0.14$). Evaluation of the sex effect revealed a lesser Hct concentration among heifers (P = 0.02); tendencies for lower pH and greater K^+ concentration were also detected (P = 0.10).

Results from the analysis of serum are presented in Table 6. Cattle that were treated with ZH expressed a greater concentration of NEFA relative to control (P = 0.03). Steers also exhibited a greater concentration of NEFA relative to heifers (P = 0.05).

Neither treatment, nor sex differed with respect to serum measurements of CPK ($P \geq 0.38$). Serum urea nitrogen was decreased with ZH treatment ($P = 0.28$). Heifers exhibited a greater concentration of SUN relative to steers ($P < 0.01$). Real-time quantitative reverse transcription-PCR data is presented in Table 7. Relative mRNA concentration of CPK did not differ in muscle tissue due treatment ($P = 0.30$) or sex ($P = 0.28$). Furthermore, expression of CPK did not differ in heart tissue due to treatment ($P = 0.13$) or sex ($P = 0.40$).

Histopathology data revealed little variation in the apparent health of the cattle. Of the four tissues evaluated (liver, lung, kidney and heart), only 3 distinct types of pathology were noted. In the kidney, minimal interstitial nephritis was noted in 14 cases, mild pneumonia was discovered in lung tissue once, subacute hepatitis was present in 2 livers and no significant pathology was found in heart tissue. Since a low number of pathological finding were present in the majority of tissues, the analysis was grouped as a pooled effect. Pathology of tissues was not increased due to treatment ($P = 0.50$) (Figure 2). In fact, ZH treated cattle exhibited a numerically lower frequency of pathological findings. Additionally, the effect of sex did not impact the histopathology analysis ($P = 0.70$).

DISCUSSION

Exit velocity has been established as a reliable predictor of cattle movement and temperament (Burdick et al., 2011). In the present study, evaluation of a significant

TRT*SEX interaction indicated that ZH treated cattle, relative to control, left the chute more rapidly on d5 of the study and returned to near identical EV by d20. Bernhard et al. (2014) reported no differences in EV between cattle that were fed naturally and cattle that were fed in a conventional manner with ZH treatment. However, Bernhard only measured EV on d 0, 10 and 20 during the ZH treatment period; thus, they could have potentially overlooked the d 5 spike in EV detected within this study. From a practical standpoint, neither study indicated that cattle are limited in terms of mobility prior to shipping.

Analysis of the velocity of cattle traveling to the working area revealed a difference at d 0; but, since no treatment had been applied, one can assume that inherent variation was present in the cattle. Although a tendency for slower movement of ZH cattle was detected on d 20, treatments did not differ for the entire period and thus significant variation in movement cannot be assumed. In a comparable study, Bernhard et al. (2014) reported slower movement of ZH fed cattle from the home pens to the working facility and Samuelson et al. (2014) reported no differences in pen movement of ZH fed cattle. ZH treated cattle did not differ in terms of the rate at which they returned to the home pen in the present study, although they did return at a numerically faster rate than when traveling to the working area. Similar results were also observed by Bernhard et al. (2014).

Subjective evaluation of chute temperament revealed a significant increase in CS of ZH treated cattle on d 20. Alternatively, Bernhard et al. (2014) detected a significant reduction in CS of ZH treated cattle on d 20. In light of these contradictory results, it is difficult to draw definitive conclusions about the chute temperament of cattle fed ZH. A

tendency for greater LS was detected for ZH fed cattle, primarily driven by an increase in the proportion of cattle receiving a LS of 2. When cattle defined as sufficiently sound (LS = 1&2) or moderately to severely lame (LS = 2&3), no differences were detected. Bernhard et al. (2014) also showed no difference in the mobility score of ZH treated cattle when grouped as "normal" and "abnormal" in terms of movement. These results indicate that ZH cattle are not predisposed to compromised soundness.

In the present study, differences detected in terms of blood gas, pH, hematocrit concentration and electrolytes remained within published reference ranges for clinically normal bovine (Table 8). Analysis of blood gas variables revealed a tendency for reduced partial pressure of oxygen in ZH fed cattle. These results suggest that ZH fed cattle tend to have greater tissue uptake of oxygen. Eisemann et al. (1988) reported greater tissue uptake of oxygen in the hindquarters of steers supplemented with the more potent β-AA, clenbuterol. Additionally, ZH fed cattle demonstrated an increase in the concentration of K^+. Bernhard et al. (2014) agreed that ZH supplementation increases blood K^+ concentration. Blood iCa concentration was reduced with ZH supplementation, particularly among steers. This result could partially explain the well-known decrease in meat tenderness associated with ZH feeding. Serum iCa has been associated with activation of calpain activity, which would promote increased tenderness during the post-mortem aging process (Swanek et al., 1999). Thus, a decrease in blood calcium could potentially inhibit calpain related tenderness mechanisms during the aging process. Using a similar instrument to measure blood variables, Bernhard et al. (2014) reported a numerical decrease in the concentration of iCa in ZH fed cattle, but failed to demonstrate significance.

Serum NEFA was elevated in the ZH treatment group. The results are not surprising considering β-AA mode of action. Elevation of NEFA concentration coincides with activation of the adipocyte lipolytic systems, associated with decreased fat in cattle fed a β-AA (Mersmann, 1998). Carroll et al. (2014) reported that ZH supplemented cattle mobilized greater amounts of NEFA when responding to a combined corticotropin releasing hormone, vasopressin challenge. Additionally, SUN was decreased due to ZH treatment and expressed at a lesser concentration in steers. This suggests that ZH treated cattle, especially steers, exhibit a net decrease in muscle tissue protein turnover which relates to more efficient protein deposition. Bryant et al. (2010) observed similar results when cattle were supplemented with RH and Parr et al., (2014) agreed that SUN concentration is decreased when cattle are fed ZH. Serum evaluations revealed no difference due to treatment in terms of the concentration of CPK. Additionally, gene expression of CPK did not differ between treatments in neither muscle nor heart tissue. Creatine phosphokinase is considered a metabolic marker of myocardial disease and has been implicated as a potential indicator of metabolic stress in cattle supplemented with ZH (Loneragan et al., 2014). When ZH was supplemented at a concentration greater than the approved dosage, an increase in CPK has been seen (FDA, 2006). However, under the prevailing conditions within the current study, CPK concentrations do not provide evidence of any association between ZH and heart disease. Furthermore, histopathology analysis did not reveal any treatment related differences in heart tissue.

CONCLUSIONS

The economic benefits of implementing β-AA supplements and other growth technologies into cattle feeding regimens are undeniable, particularly when one considers societal concerns about food security and sustainability (Johnson et al., 2013). However, increasing social awareness of animal welfare has placed many of these technologies under scrutiny. Duncan (1996) has described animal welfare as a product of feelings experienced by animals, specifically, the absence of negative feelings and the presence of positive feelings. It is difficult to objectively and scientifically assess animal feelings; nonetheless, studies can be designed to evaluate behavioral, metabolic and health responses of cattle in order to answer welfare related questions. Moreover, it can be challenging to ascertain which distinct factors of the environment animals perceive as stressful, especially since development history and prior experience play a role in behavior (McEwen et al., 1997). The research at hand provides an excellent model to study cattle well-being and make sound conclusions relative to the effects of ZH. Collectively, these data suggest that ZH feeding does not impose a threat to cattle well-being when utilized with proper management techniques.

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Table 4.1 As-fed composition of 90% concentrate finishing diet.

Ingredient	%, AF
Corn Grain, Steam Flaked	54.05
Alfalfa Hay, Mid Bloom	8.48
Sweet Bran, WCGF	32.23
Tallow	2.27
Urea	0.32
Limestone	1.02
TTU Supplement[1]	1.63

[*]A 0.5% ground corn based zilpaterol hydrochloride premix was substituted for steam flaked corn to provide 75 mg/head/day for the 20 day treatment period followed by a 3 day withdrawal.
[1]Provides 29.9 g/ton Rumensin and 10.0 g/ton Tylan

Table 4.2 Sequences of bovine-specific PCR primers and TaqMan probes utilized for determination of mRNA concentration of creatine phosphokinase (CPK) and ribosomal protein S9 (RPS).

Item	Sequence (5' to 3')
CPK (Accession # AB003307)	
Forward	CACCTCTCCCCAACAAGCA
Reverse	GGTCCTGGGAGTCTTGAGTCAT
TaqMan Probe	6FAM-TAACTCCCGTCCCCAGTG-TAMRA
RPS9 (Accession # DT860044)	
Forward	GAGCTGGGTTTGTCGCAAAA
Reverse	GGTCGAGGCGGGACTTCT
TaqMan Probe	6FAM-ATGTGACCCCGCGGAGACCCTTC-TAMRA

Table 4.3 Effects of zilpaterol hydrochloride (ZH) and sex on various objective measures of mobility.

	Treatment[1]			Sex					
Item[3]	CTRL	ZH	*P*-value	Heifers	Steers	*P*-value	SEM[2]	TRT*SEX	TRT*DAY
Cattle, n	46	46		45	47				
Exit velocity (m/sec)									
Pooled	3.15	3.32	0.23	3.41	3.06	0.01	0.130	0.70	0.03
d 0	2.87	2.99	0.43	3.12	2.74	0.02	0.156		
d 5	3.17	3.59	0.01	3.53	3.22	0.06	0.162		
d 10	3.30	3.47	0.31	3.57	3.20	0.02	0.156		
d 15	3.20	3.37	0.27	3.43	3.13	0.06	0.158		
d 20	3.23	3.17	0.71	3.40	3.00	0.02	0.158		
Pens, n	12	12		12	12				
Velocity traveling to working area (m/sec)									
Pooled	1.88	1.86	0.76	2.03	1.71	<0.01	0.090	0.46	0.06
d 0	1.58	1.76	0.03	1.76	1.58	0.03	0.076		
d 5	1.77	1.84	0.54	1.90	1.72	0.04	0.108		
d 10	1.94	1.99	0.66	2.14	1.79	<0.01	0.096		
d 15	1.75	1.85	0.53	1.84	1.76	0.61	0.155		
d 20	2.37	1.84	0.07	2.50	1.71	0.01	0.274		
Velocity returning to home pen (m/sec)									
Pooled	2.64	2.89	0.31	3.09	2.45	0.01	0.239	0.74	0.70
d 0	2.29	2.51	0.42	2.64	2.17	0.10	0.276		
d 5	2.73	3.11	0.18	3.15	2.70	0.11	0.279		
d 10	2.91	2.98	0.78	3.31	2.58	0.01	0.278		
d 15	2.77	2.95	0.53	3.23	2.49	0.01	0.283		
d 20	2.50	2.89	0.18	3.09	2.30	0.01	0.283		

[1]Treatment diets were formulated to contain no ZH (CTRL) or ZH (75 mg/hd/d of ZH; Zilmax, Merck Animal Health, Summit, NJ) for the final 20 d before slaughter with a 4 d withdrawal.

[2]SEM = Standard error of treatment means.

[3]Exit velocity is the rate of cattle traveling 1.93 meters when leaving the chute. Velocity traveling to the working area represents the amount of time it took cattle to travel 260 meters as they were pushed to the working area; velocity returning to the home pen represents the amount of time that it took cattle to travel 170 meters as they were pushed to the home pens.

Table 4.4 Effects of zilpaterol hydrochloride (ZH) on the chute temperament score of feedlot cattle at the beginning and end of the treatment period.

	Treatment[1]		
	CTRL	ZH	*P*-value (1 and 2 vs. ≥ 3)[2]
Cattle, n	45	43	
Chute Score d 0[3]			0.19
Calm, 1	2	6	
Slightly restless, 2	16	20	
Restless, 3	14	14	
Agitated, 4	7	3	
Severely agitated, 5	2	0	
Chute Score d 20[3]			0.01
Calm, 1	30	17	
Slightly restless, 2	7	8	
Restless, 3	4	18	
Agitated, 4	0	0	
Severely agitated, 5	0	0	

[1]Treatment diets were formulated to contain no ZH (CTRL) or ZH (75 mg/hd/d of ZH; Zilmax, Merck Animal Health, Summit, NJ) for the final 20 d before slaughter with a 4 d withdrawal.

[2]P-values were obtained using Fisher's exact test for count data.

[3]Chute temperament scores were based on the scale developed by Grandin (1993). 1 = calm, no movement; 2 = slightly restless; 3 = squirming, occasionally shaking the squeeze chute; 4 = continuous, very vigorous movement and shaking of the squeeze chute; 5 = rearing, twisting of the body and struggling violently.

Table 4.5 Effects of zilpaterol hydrochloride (ZH) on the locomotion score of feedlot cattle.

	Treatment[1]			
	CTRL	ZH	*P*-value (1 vs. ≥ 2)[2]	*P*-value (1 and 2 vs. 3 and 4)[2]
Cattle, n	45	43		
Locomotion Score[3]			0.09	0.24
Normal, 1	43	36		
Mildly lame, 2	2	5		
Lame, 3	0	1		
Severely lame, 4	0	1		

[1]Treatment diets were formulated to contain no ZH (CTRL) or ZH (75 mg/hd/d of ZH; Zilmax, Merck Animal Health, Summit, NJ) for the final 20 d before slaughter with a 4 d withdrawal.
[2]P-values were obtained using Fisher's exact test for count data.
[3]Locomotion scores were obtained by an unbiased, trained evaluator as the cattle left the chute on the day of shipping. Scores were assigned on a concrete surface and based on the scale developed by National Dairy Farm Program Care Manual, (Appendix D & E). 1 = (Normal) Stands and walks normally with level back; makes long, confident strides; 2 = (Mildy Lame) Stands with flat back, but arches when walks. Gait is slightly abnormal; 3 = (Lame) Stands and walks with an arched back and short strides with one or more legs. Slight sinking of dew-claws in limp opposite to the affected limb may be evident; 4 = (Severely Lame) Pronounced arching of back. Reluctant to move, with almost complete weight transfer off the affected limb.

Table 4.6 Effects of zilpaterol hydrochloride (ZH) and sex on blood gas, pH, hematocrit concentration and electrolytes in black-hided cattle during the finishing phase[1].

	Treatment[2]			Sex				
Item[3]	CTRL	ZH	*P*-Value	Steers	Heifers	*P*-Value	SEM[5]	TRT*SEX
Cattle, n	21	24		23	22			
pCO_2, mmHg	39.47	41.46	0.24	41.62	39.31	0.18	1.64	0.42
pO_2, mmHg	38.98	36.37	0.08	37.76	37.59	0.91	1.42	0.94
O_2Sat, %	71.40	66.95	0.16	70.54	67.81	0.39	3.07	0.51
pH	7.40	7.42	0.27	7.42	7.39	0.10	0.02	0.41
Hct, %	42.02	43.16	0.14	41.65	43.53	0.02	0.74	0.99
Na^+, mmol/L	142.81	142.82	0.99	142.57	143.05	0.33	0.48	0.45
K^+, mmol/L	4.37	4.74	<0.01	4.50	4.61	0.10	0.07	0.33
iCa, mmol/L[4]	1.18	1.13	<0.01	1.16	1.15	0.55	0.01	0.04

[1]Cattle were bled via jugular venipuncture on d 5, 10, 15 and 20 of the ZH treatment period. Samples were collected in sodium heparinized blood tubes containing an anticoagulation agent, inverted several times and transported to an on-site station for prompt analysis with a GEM Premier 3000 Blood Gas Analyzer.
[2]Treatment diets were formulated to contain no ZH (CTRL) or ZH (75 mg/hd/d; Zilmax, Merck Animal Health, Summit, NJ) for the final 20 d before slaughter with a 4 d withdrawal.
[3]pCO_2 is partial pressure of CO_2, pO_2 is partial pressure of O_2, O_2Sat is calculated O_2 saturation, Hct is hematocrit concentration and iCa is ioinized Ca.
[4]A TRT*SEX interaction was detected. Simple effects are shown in Figure 1.
[5]Standard error of treatment means.

Table 4.7 Effects of zilpaterol hydrochloride and sex on serum concentrations of creatine phosphokinase (CPK), NEFA and serum urea nitrogen (SUN)[1].

	Treatment[2]			Sex				
Item	CTRL	ZH	*P*-Value	Steers	Heifers	*P*-Value	SEM[3]	TRT*SEX
CPK, μEq/L	24.33	25.33	0.70	25.97	23.69	0.38	1.89	0.12
NEFA, μEq/L	294.86	295.00	0.03	295.11	294.89	0.05	0.11	0.49
SUN, mg/dL	7.80	7.65	0.04	7.44	8.01	<0.01	0.05	0.13

[1]Cattle were bled via jugular venipuncture on d 0, 5, 10, 15 and 20 of the ZH treatment period. No TRT*DAY interactions were detected ($P \geq 0.24$), thus pooled main effects are reported. Samples were collected in a silicone coated tube containing a clot activation agent. The concentration of CPK, NEFA and SUN were determined using ELISA kits.
[2]Treatment diets were formulated to contain no ZH (CTRL) or ZH (75 mg/hd/d; Zilmax, Merck Animal Health, Summit, NJ) for the final 20 d before slaughter with a 4 d withdrawal.
[3]Standard error of treatment means.

Table 4.8 Effects of zilpaterol hydrochloride and sex on relative mRNA concentration of creatine phosphokinase (CPK) in skeletal muscle and cardiac tissue of black-hided cattle[1].

	Treatment[2]			Sex				
Item	CTRL	ZH	*P*-Value	Steers	Heifers	*P*-Value	SEM[3]	TRT*SEX
Muscle CPK	5.12	13.63	0.30	4.64	12.98	0.28	6.38	0.70
Heart CPK	40.46	127.33	0.13	61.90	113.16	0.40	46.95	0.83

[1]Relative abundance of CPK was determined using Real-Time PCR. Genes were normalized with the RPS9 endogenous control gene by using the change in cycle threshold (ΔCT).
[2]Treatment diets were formulated to contain no ZH (CTRL) or ZH (75 mg/hd/d; Zilmax, Merck Animal Health, Summit, NJ) for the final 20 d before slaughter with a 4 d withdrawal.
[3]Standard error of the treatment means.

Table 4.9. Published reference ranges for blood gas, pH, hematocrit concentration and electrolytes in beef cattle[1].

Item[2]	Range
pCO_2, mmHg	34-45
pO_2, mmHg	30-45
O_2Sat, %	57-81
pH	7.35-7.45
Hct, %	35-45
Na+, mmol/L	132-152
K+, mmol/L	3.9-5.8
iCa, mmol/L	1.2-1.6

[1]Reference values were taken from Jackson, P.G. and P.D. Cockcroft. Clinical examination of farm animals.

[2] pCO_2 is partial pressure of CO_2, pO_2 is partial pressure of O_2, O_2Sat is calculated O_2 saturation, Hct is hematocrit concentration and iCa is ioinized Ca.

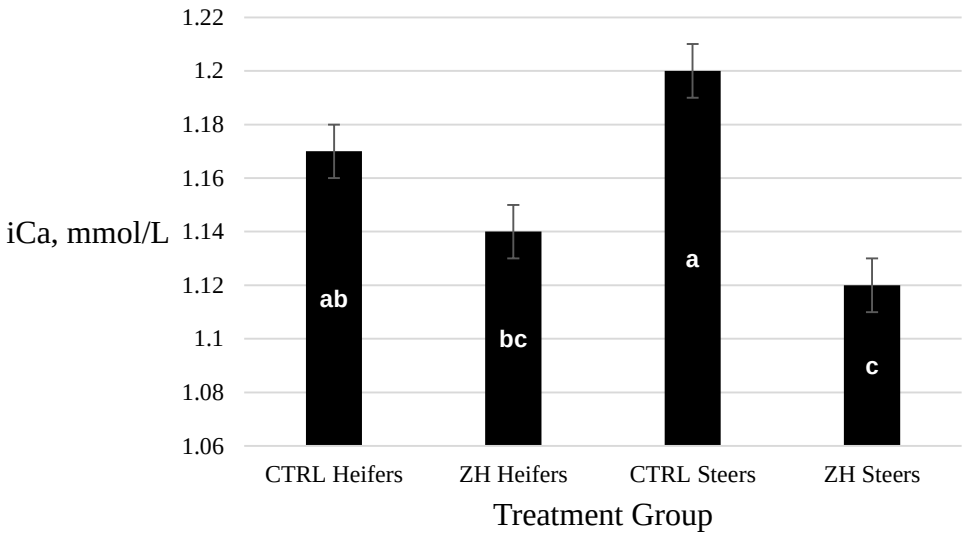

Figure 4.1 Cattle were bled via jugular venipuncture on d 5, 10, 15 and 20 of the ZH treatment period. Samples were collected in sodium heparinized blood tubes containing an anticoagulation agent, inverted several times and transported to an on-site station for prompt analysis with a GEM Premier 3000 Blood Gas Analyzer. Treatment diets were formulated to contain no ZH (CTRL) or ZH (75 mg/hd/d; Zilmax, Merck Animal Health, Summit, NJ) for the final 20 d before slaughter with a 4 d withdrawal. The concentration of ionized calcium is represented by iCa and expressed in mmol/L. A TRT*SEX interaction was detected ($P = 0.04$). Treatment groups that do not share a common letter are different ($P \leq 0.05$).

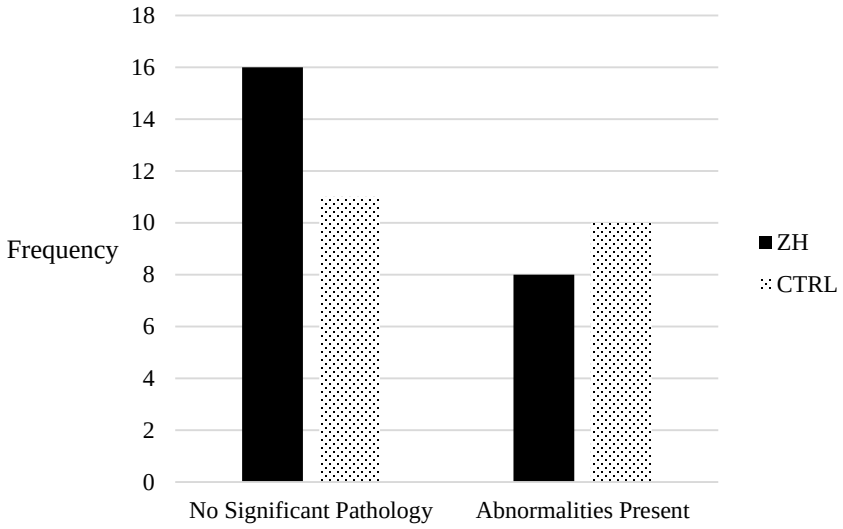

Figure 4.2 10-g samples of heart, liver, lung and kidney tissue was collected post-mortem, placed on ice and transported to the Texas Veterinary Medical Diagnostic Laboratory for prompt histopathology analysis of the fresh tissues. Pearson's Chi-squared test with a Yates' continuity correction was used to determine if differences exist in the distribution of pathological findings among treatment groups. No significant differences were detected ($P = 0.50$). Abnormalities include: minimal interstitial nephritis, mild pneumonia and subacute hepatitis.

CHAPTER V

THE EFFECTS OF ZILPATEROL HYDROCHLORIDE ON VARIOUS PHYSIOLOGICAL INDICATORS OF THERMAL REGULATION IN BLACK-HIDED FEEDLOT STEERS AND HEIFERS DURING MODERATE HEAT STRESS

ABSTRACT

The objective of this study was to determine the effects of zilpaterol hydrochloride (**ZH**) on thermal regulation of black-hided feedlot steers and heifers during moderate heat stress. Steers and heifers (n=96) were sourced from a commercial feedlot and transported to the Texas Tech University Beef Center. Cattle were weighed and scanned using real-time ultrasound. Resulting data were used to predict empty body fat percentage **(pEBF %)**. Steers (n=48; BW = 520 ± 30.4 kg; pEBF % = 26.2 ± 1.9) and heifers (n=48; BW = 466 ± 29.5 kg; pEBF % = 26.7 ± 1.7) were blocked within gender by pEBF % in a complete randomized block design and randomly assigned to pen (2 pens/block; 4 hd/pen) and treatment (6 pens/treatment): 1) control heifers (HC), 2) ZH heifers (HZ), 3) control steers (SC), and 4) ZH steers (SZ). During the ZH treatment period the climatic conditions were: mean maximum temperature, 29.67 °C; mean minimum temperature, 15.18 °C; mean relative humidity, 60.69%; mean wind velocity, 8.37 km/h. On d 0 of ZH treatment, cattle were fitted with an indwelling rectal temperature probe. Rectal temperatures **(RT)** were recorded throughout the treatment period. Panting scores **(PS)** were assigned to cattle every other day from 1500 to 1700 h during the ZH treatment period. Blood was collected via jugular venipuncture on d 0, 5, 10, 15 and 20 of the treatment period and analyzed for heat shock protein 70 (**HSP70**). Real-time quantitative PCR was used to measure local heat shock protein 27 (**HSP27**) in

heart, liver, lung, kidney and skeletal muscle. Repeated measures analysis of RT revealed a TRT*SEX interaction ($P < 0.01$). The HC group recorded higher RT relative to the HZ group ($P < 0.01$). Alternatively, the SC group recorded lower RT relative to the SZ group ($P < 0.01$). Although differences were detected for RT, the marginal effect size may be insufficient to deduce biologically significant implications. Chi-squared analysis was used to analyze the distribution of PS. No differences were detected in PS between treatments at any single point of data collection or for the entire treatment period ($P \geq 0.30$). Supplementation of ZH did not alter concentration of serum HSP70 or HSP27 mRNA abundance ($P \geq 0.25$). Collectively, the present study does not provide compelling evidence to suggest that ZH treated, black-hided cattle of either sex have more difficulty coping with moderate heat stress relative to their control counterparts.

Key words: cattle, zilpaterol hydrochloride, heat stress

INTRODUCTION

In order to feed a growing populace, the livestock industry will need to enhance production through the improvement of genetics and the use of efficiency enhancing technologies. Beta-adrenergic agonists (**β-AA**) belong to a class of growth promotants that elicit improvements in carcass weight gain, carcass cutability and feed efficiency in beef cattle (Avendaño-Reyes et al., 2006; Winterholler et al., 2007; Vasconcelos et at., 2008; Rathmann et al., 2012). These outcomes are derived from the ability of β-AA to bind and stimulate beta-adrenergic receptors on cell surfaces, resulting in a pronounced increase in the accretion of skeletal muscle and a decreased accretion of fat (Mersmann, 1998). Many of these technologies increase efficiency by promoting lean tissue deposition; however, increases in the rate of protein accretion are associated with greater internal heat production (Brown-Brandt et al., 2004).

Mersmann (1998) theorized that the generally accepted mode of action for β-AA may be extremely entangled with some or even most of the ultimate effects resulting from secondary events caused by hormonal or physiological responses of numerous tissues to β-AA administration. Beta-adrenergic agonists have been associated with increased heart rates, respiration rates and blood flow to muscle tissue (Eisemann et al., 1988; Bruckmaier and Blum, 1992). When effects were detected, they were generally the most pronounced within the first few days of treatment. Recent speculation within the feedlot industry has led to concerns about the influence of β-AA feeding on the ability of cattle to cope with heat stress, especially in heifers. Thus, the objective of this experiment was to examine the effects of zilpaterol hydrochloride on various indicators

of thermal regulation in black-hided feedlot steers and heifers during moderate heat stress.

MATERIALS AND METHODS

All experimental procedures involving the use of animals were reviewed and approved by the Texas Tech University Animal Care and Use Committee (ACUC # 13059-07). The experiment was conducted at the Texas Tech University Beef Cattle Center located approximately 9.7 km east of New Deal, TX.

Cattle and management

On August 2, 2013, black-hided steers and heifers (n = 96) were delivered to the Texas Tech University Beef Center in New Deal, TX from a nearby commercial feedlot. Cattle were estimated to be 60 d from the projected time of harvest. Before arriving at Texas Tech University, steers and heifers had been on a finishing ration at the commercial feedlot for 83 and 79 days, respectively. Steers were implanted with Revalor-XS (Merck Animal Health; Summit, NJ) on May 4, 2013 and heifers were implanted with Revalor-200 (Merck Animal Health; Summit, NJ) on May 13, 2013.

The day of arrival, cattle were sorted by sex into two large pens and offered a 70% concentrate diet at 70% of the 5-d average dry smatter intake prior to shipping. Initial processing (on the morning of August 6, 2013) included: 1) individual identification by ear tag; 2) measurement of body weight [(Silencer Chute, Moly Manufacturing, Lorraine, KS, mounted on Avery Weigh-Tronix load cells, Fairmount, MN; readability ± 0.45 kg); scales were calibrated with 454 kg of certified weights

(Texas Department of Agriculture) before use]; 3) real-time ultrasound scanning by a certified technician(Aloka 500-V instrument with a 17-cm 3.5 MHz transducer); 4) assignment of chute temperament scores (**CS**) as described by Grandin (1993); and 5) determination of exit velocity (**EV**) from the chute as described by Curley (2006). Exit velocity was measured as an objective indicator of temperament and mobility; briefly, the rate of speed of cattle traversing a distance of 1.93 m after exiting the head gate was determined using 2 infrared sensors (FarmTek, Inc.; North Wylie, TX) and velocity was calculated in the following manner: [velocity = distance (m)/time (s)]. Chute temperament scores and EV were standardized and used to determine a temperament index, which allocated twice as much weight to the objective measurement (EV) as to the subjective (CS).

Real-time ultrasound data was incorporated into an equation described by Guiroy (2001) to determine the predicted empty body fat percentage of the cattle (**pEBF%**). In order to reduce noise within the data related to inherent compositional differences among cattle, pEBF% was designated as the blocking factor. Steers (n = 48; BW = 520 ± 30.4 kg; EBF % = 26.2 ± 1.9) and heifers (n = 48; BW = 466 ± 29.5 kg; EBF % = 26.7 ± 1.7) were blocked within sex by pEBF% in a complete randomized block design and randomly assigned to pen (2 pens/block; 4 hd/pen) and treatment (6 pens/treatment). Within each pen, two cattle were selected for more intensive sampling and designated as "sub-sample cattle" on the basis of the most intermediate temperament index scores. Treatments were as follows: 1) control heifers (HC), 2) ZH supplemented heifers (HZ), 3) control steers (SC) and 4) ZH supplemented steers (SZ).

Cattle were placed in 3 X 9.1 m dirt surface pens with concrete aprons at the bunk and around the water source. The concentrate level of the ration delivered was stepped up by increasing the concentration 5% every 5 days for 20 days during the acclamation period. The 90% concentrate finisher ration is detailed in Table 1. This ration was a typical feedlot diet containing premixes made at the Texas Tech University Burnett Center Feed Mill in a paddle type mixer (Marion Mixers, Inc; Marion, IA). The supplement premix included standard trace minerals, vitamins, tylosin (Tylan, Elanco Animal Health; Greenfield, IN) and monensin (Rumensin 90; Elanco Animal Health; Greenfield, IN). Heifers were supplemented with melengestrol acetate (.4 mg/head/day) to suppress estrus (MGA; Pfizer; New York, NY). Feed was mixed and delivered daily in a drag type feed wagon (Rotomix; Dodge City, KS). Cattle were fed once daily (0800 to 0900 h) and feed delivery was adjusted to provide ad libitum access to feed while reducing wasted feed. Daily health observations were made at the time of feed delivery. Prior to the ZH period, some cattle were treated for hoof root due to abnormally wet conditions in the month of August. Sulfamethazine boluses (Sustain III™; Durvet; Blue Springs, MO) and liquamycin injections (LA 200®; Pfizer; New York, NY) were used to treat tender-footed cattle.

On d 0 of the treatment period, feed refusals were collected and weighed to determine dry matter intake. A body weight measurement was also collected on the cattle. For the remainder of the 20 d treatment period, a ZH premix (Zilmax; Merck Animal Health; Summit, NJ) incorporated with ground corn was included in the ration of the appropriate treatments to deliver approximately 75 mg/hd/d of ZH. On d 21, feed bunks were swept and feed refusals were weighed. Based on DMI, cattle in the

appropriate treatment groups received approximately 73.7 $\pm$ 13.9 mg/hd/d of ZH. Following a 4 d withdrawal period, cattle were individually weighed and shipped to a commercial abattoir for harvest.

Climatic data

Climatic variables were captured from August 1, 2013 to September 31, 2014 at an on-site weather station, located approximately 0.80 km from the cattle feeding pens. The instrument used to determine climatic information was a Campbell Scientific 21X micrologger weather monitoring station (Campbell Scientific, Inc., Logan, UT). Weather data was collected at 15 min intervals and summarized by daily maximum, daily minimum and average values during the 20 d treatment period for the following variables: temperature, wind speed and relative humidity (Figure 1). Briefly summarized, the climatic conditions during the treatment period were: mean maximum temperature, 29.67 °C; mean minimum temperature, 15.18 °C; mean relative humidity, 60.69%; mean wind velocity, 8.37 km/h.

Determination of rectal temperature and panting scores

On d 0 of the study, cattle selected to represent the sub-sampled group were fitted with rectal temperature recording devices (AHOBO Pro v2 Temp data logger probe; Part # U23-004, Onset Corp., Pocasset, MA) according to the procedure described by Reuter et al., (2010). Rectal temperature (**RT**) was measured continuously at 1-min intervals for the entire 20 d treatment period. In the event of RT probes becoming dislodged, devices were re-fitted and all extraneous data was removed from the set. At the completion of the treatment period, devices were removed and data was extracted from the loggers and summarized by hourly means.

Panting scores were assigned to cattle every other day during the treatment period (d 2, 4, 6… 20). Observations were made for the designated sub-sampled cattle in each pen and collected between 1500 and 1700 h by a trained observer. A 5-point scoring system used was based on the scale developed by Mader et al., (2006) (Table 1).

Heat shock protein analysis

Cattle were processed through the chute (d 0, 5, 10, 15 and 20) and venous blood samples were collected from the designated sub-sampled cattle via jugular venipuncture in a silicone coated tube containing a clot activation agent (Red Top BD Vacutainer; Becton, Dickinson and Company; Franklin Lakes, NJ). These samples were placed on ice and transported to the Texas Tech University laboratory. Serum was isolated by centrifuging samples for 20 minutes at temperature of 4^{o}C and a speed of 1,000 G. Aliquots of serum were then frozen for subsequent analysis. The concentration of heat shock protein 70 (**HSP70**) was determined using ELISA according to the procedure described by Gaughan et al., (2012).

When cattle were harvested at the commercial abattoir, Texas Tech University meat lab personnel collected a variety of tissue samples. Within 10 min post-mortem, 10-g samples of skeletal muscle (longissimus dorsi), heart, liver, lung and kidney tissue were collected, placed in sealable plastic bags (Whirl-Pack; Nasco, LLC; Stamford, CT) and snap frozen in liquid N (n = 45 for each tissue). Samples were placed on dry ice, transported to Texas Tech University and frozen at -80^{o}C for subsequent analysis.

Ribonucleic acid from the tissue samples were isolated with ice-cold buffer containing TRI Reagent (Sigma, St. Louis, MO). Approximately 0.5g of frozen tissue

was homogenized with TRI Reagent at a ratio of 0.5:1 grams of tissue to mL reagent. The homogenate was then pipetted into 2 microcentrifuge tubes (1 mL sample per tube), 200 µL chloroform was added to each tube, vortexed for 30 s and incubated for 5 min. The sample was then centrifuged at 15,000 x *g* for 15 min, separating the sample into 3 layers. The top supernatant layer was pipetted off and placed into new microcentrifuge tubes. Two hundred and fifty µL of ice cold isopropyl alcohol was added to the supernatant, shaken and incubated for 10 min on the bench top. The samples were then centrifuged at 15,000 x *g* for 10 min. The supernatant was poured off, the RNA pellet at the bottom of each tube was allowed to dry and 500 µL of 75% ethanol was added to each tube to rinse and suspend the RNA pellet. Tubes were then placed in a -80°C freezer. One tube was then removed from the freeze and thawed on ice. Tubes were then centrifuged at 15,000 x *g* for 10 min, ethanol was poured off and the pellet was air dried. Thirty µL of nuclease free water was then added to each tube to dissolve the RNA pellet. The concentration of RNA was determined with a UV-Vis spectrophotometer at an absorbance of 260 nm, using a NanoDrop 1000 (NanoDrop products, Wilmington, DE). Samples were then treated with DNAse to remove any DNA contaminants using a DNA-free kit (Life Technologies, Grand Island, NY). The RNA was then subjected to reverse-transcription and cDNA was produced. The cDNA was then used for real-time quantitative reverse transcription-PCR (RT-qPCR) to measure the quantity of heat shock protein 27 (**HSP27**) mRNA relative to the quantity of Ribosomal protein subunit 9 mRNA in total RNA isolated from the various tissues. Specifications for the primers and probes used for each gene are detailed in Table 2. Assays were performed in the GeneAmp 7900HT Sequence Detection System (Applied Biosystems,

Life Technologies) using thermal cycling parameters recommended by the manufacturer (40 cycles of 15 s at 95°C and 1 min. at 60°C).

Statistical Analyses

All continuously distributed data were analyzed as a completely randomized block design using the PROC MIXED procedure of SAS (SAS 9.3; SAS Inst.; Cary, NC). Individual animal served as the experimental unit. The fixed effects included treatment, sex and the interaction thereof. The pEBF% blocking component was included as a random effect. Rectal temperature and blood data were handled as repeated measures; the proper covariance structure for each analysis was selected based on fit statistics. Additionally, exit velocity was included as a covariate in the rectal temperature analysis to delineate variation in the model related to inherit differences in the temperament of the cattle. The assumptions of normality of errors, homogeneity of variances and sphericity were evaluated for each fixed effect using Shapiro Wilk's test, Bartlett's test and Mauchely's test, respectively. Values greater than 3 standard deviations from the mean were considered outliers and removed from the analysis. Count data obtained by assigning panting scores were analyzed using Pearson's chi-squared test with a Yate's continuity correction (SPSS Statistics 22.0; IBM; Armonk, NY) to determine if the collective distribution differed between treatments. For all analyses, P – values less than or equal to 0.05 were considered significant; P – values between 0.05 and 0.10 were determined to be tendencies.

RESULTS

Feedlot performance and carcass data for the cattle used in the current study are reported by Ragland et al. (2014). Briefly summarized, ZH fed heifers exhibited a numerical increase of 7.68 kg in HCW ($P = 0.41$) relative to control heifers. A numerical increase in HCW of 6.09 kg ($P = 0.50$) was also detected when comparing steers fed ZH to control. Heifers fed ZH displayed a 1.43 percent increase ($P = 0.04$) in dressing percentage relative to control. Steers fed ZH demonstrated a tendency for a 1.2 percent increase ($P = 0.06$) in dressing percentage relative to control. Heifers fed ZH exhibited a numerical increase of 2.45 cm^2 of REA ($P = 0.34$) relative to control heifers. Steers fed ZH expressed a tendency for an increase of 4.27 cm^2 of REA ($P = 0.10$) relative to control steers. The ZH related carcass effects in the study at hand are of lesser magnitude than previously published data; however, this difference can be explained by stress imposed on cattle when working them through the chute 5 times during the ZH treatment period.

Analysis of rectal temperature data resulted in a highly significant TRT*SEX interaction ($P < 0.01$); accordingly, main effects will not be discussed. The simple effects of ZH treatment on the RT of black-hided feedlot cattle during moderate heat stress are presented in Figure 2. Sexes responded differently with respect to treatment, such that ZH reduced RT in heifers by 0.07 ^{o}C ($P < 0.01$) and increased RT in steers by 0.02 ^{o}C ($P < 0.01$).

Evaluation of PS revealed that neither ZH treatment nor gender influenced the distribution. Control and ZH treatment groups did not differ when PS were pooled across

the entire treatment period ($P = 0.82$) (Figure 3). Additionally, treatment groups did not differ on any single day of scoring ($P \geq 0.30$). The vast majority of scores assigned fell within the 1 (elevated respiration) or 2 (moderate panting and/or presence of drool or small amount of saliva) classification.

Results from the evaluation of serum HSP70 are presented in Table 3. Treatment with ZH did not influence serum concentration of HSP70 ($P = 0.25$). However, a highly significant gender effect was detected ($P < 0.01$). Heifers expressed a greater concentration of serum HSP70 relative to steers: 4.53 µeq/L versus 3.92 µeq/L, respectively.

Effects of ZH and sex on the relative mRNA concentration of HSP27 are presented in Table 4. Treatment with ZH did not influence gene expression of HSP27 in any of the tissues evaluated ($P \geq 0.40$); furthermore, sex did not alter relative gene expression ($P \geq 0.11$).

DISCUSSION

Heat stress is characterized by a negative balance between the net amount of energy liberated from cattle to the environment and the amount of internal heat generated through a variety of metabolic processes. Cattle that are exposed to heat stress express an altered endocrine profile that is defined by reciprocal changes in circulating hormones (Collier et al., 2005). Furthermore, intracellular signaling pathways are modified in order to aide in the dissipation of heat (Collier et al., 2007). The net result of this homeorhetic

response is a highly coordinated process that allows the animal's body to prioritize acclimation and survival. In contrast to evolutionary adaptation, heat stress acclimation is a within lifetime phenotypic adaptation by the animal which widens its dynamic regulatory range of body temperature (Horowitz, 2001). In a post-acclimated state, an animal would have a reduced heart rate, a more efficient metabolism, a lower body temperature and a lowered body temperature threshold for subsequent activation of heat dissipation effectors. An animal's response to heat stress is believed to occur in a biphasic pattern dependent upon time (Horowitz et al., 1996; Horowitz, 2001). Short-term heat acclimation is defined by modifications in cellular signaling pathways driven by hormonal regulation (Collier and Zimbelman, 2007). During STHA, there is a transient acceleration of autonomic excitability, which compensates for the impaired responsiveness of post-synaptic thermoregulatory effectors. Initially, this appears to be at odds with the creation of a homeostatic state; but, this modification is necessary to establish long-acting protective mechanisms in cells. Long-term heat acclimation (**LTHA**) is characterized by the expression of heat shock proteins which serve as agents of cytoprotection.

An array of environmental factors may influence the degree to which cattle are stressed by heat; these include: 1) ambient temperature, 2) humidity, 3) air movement and 4) solar radiation (Mader et al., 2010). Furthermore, specific animal factors can modify the ability of cattle to cope with the aforementioned stressors. Examples include: 1) breed type, 2) hide color, 3) hair thickness, 3) extent of fat deposition, 4) mass to surface area ratio, 5) health status, 6) degree of acclimation and 7) ration composition (Mader and Davis, 2004; Dye-Rose et al., 2009). When cattle are forced outside the thermoneutral

zone, it is well documented that feedlot performance is significantly compromised (Hubbard et al., 1999; Hahn and Mader 1997).

Mader et al., (2010) developed a comprehensive climate index (**CCI**) to estimate environmental heat load. The index treats ambient temperature as the basis for the index and provides adjustments for relative humidity, wind speed and solar radiation (Mader et al., 2010). In addition, Mader et al., (2010) established thresholds for the CCI and classified the levels of thermal stress as: no stress, mild, moderate, severe, extreme and extreme danger. If one makes the assumption that the time of maximum ambient temperature is associated with the time of the minimum relative humidity throughout the day and the peak of solar radiation intensity, a rough estimate of the CCI can be obtained. On this basis, climatic conditions in the present study should be classified as moderate heat stress for the majority of the treatment period. Several days toward the beginning and end of the ZH treatment period would approach the severe heat stress classification of the CCI.

Rectal temperature is commonly regarded as the most accurate method to assess body temperature in cattle. In the study at hand, a TRT*SEX interaction was detected and ZH supplemented heifers recorded cooler rectal temperatures relative to control whereas ZH supplemented steers recorded greater rectal temperatures. Carroll et al., (2014) also detected cooler vaginal temperatures in heifers treated with ZH and exposed to a combination corticotropin releasing hormone, vasopressin challenge. In both studies, temperatures were analyzed as hourly repeated measures for a 20 d period; so, an extremely high amount of observations would allow for enough statistical power to detect

even the slightest differences. In this study, rectal temperatures differed only slightly (0.07°C for heifers and 0.02°C for steers) and remained within the normal range of body temperatures for beef cattle (Robertshaw et al., 2004). Similar studies have evaluated body temperature with ruminal boluses which strongly correlates (r = 0.65 to 0.92) with RT, although measurements are generally 0.5°C warmer than RT (Sievers et al., 2004; Bewley et al., 2008; Tismsit et al., 2011; Rose-Dye et al., 2011; Wahrmund et al., 2012). Bernhard et al., (2014) and Wahrmund et al., (2014) evaluated the effects of ZH on ruminal temperature; neither detected differences due to treatment. When taken together, the differences noted in the current study do not appear to be biologically relevant.

The current study demonstrated a numerical increase in PS associated with ZH treatment, however significant differences were not detected. Bernhard et al. (2014) reported an increase in PS and respiration score in cattle fed ZH. The environmental conditions in Bernhard's study would have been more extreme and presented a greater danger for heat stress. Thus, one can assume that any increase in respiration attributable to ZH feeding may be exacerbated when the CCI reaches higher levels.

Heat shock proteins belong to a highly conserved family of molecular chaperons and act to maintain homeostasis when stressors are present (Hecker and McGarvey, 2011; Iwaki et al., 1993; Gaughan et al., 2012). These proteins are generally named according to their molecular weight, expressed in kilodaltons. The HSPs may be released intracellularly or extracellularly in an inducible form in response to stress (Gaughan et al., 2012). Large HSPs, like HSP70, are expressed at elevated concentrations during heat stress; however the source of these proteins has not

been fully elucidated (Gaughan et al., 2012; Hom et al., 2012). Shapiro et al. (1986) proposed that the source may be from damaged intestinal cells. Bouts of heat stress can induce redirection of blood to the periphery for enhanced heat dissipation; concurrently, blood flow to the intestines is reduced (Gaughan et al., 2012; Cronje, 2007). If exposure to a thermal challenge persists, intestinal barrier integrity may be compromised leading to an increase in intestinal permeability (Gaughan et al., 2012; Doklandy et al., 2006; Lambert, 2009). This facilitates the penetration of endotoxins, thereby causing an inflammatory response (Gaughan et al., 2012; Shapiro et al., 1986; Lambert, 2009). Extracellular HSP70 has important functions in pro-inflammatory immune response (Pockley, 2003); therefore, changes in eHsp70 may be an indication of cellular damage within the intestines (Gaughan et al., 2012; Doklandy et al., 2006). Small HSPs, like HSP27, are less conserved among species (Arrigo and Landry, 1994). Data suggest that small heat shock proteins serve homeostatic functions at the level of signal transduction (Arrigo and Landry, 1994). These proteins are involved in the maintenance of microfilament integrity, the control of cell division/differentiation, development of the structural components of cells and repair of stress cells (Arrigo and Landry, 1994).

Treatment effects were not detected for HSP70 in serum or local HSP27 in various tissues. Accordingly, one can make the assumption that ZH does not impair the ability of cattle to reach a state of LTHA related to HSP mechanisms. A sex effect revealed that heifers expressed a greater concentration of serum HSP70. Gaughan et al. (2012) showed a moderate relationship ($r^2 = 0.48$; $P < 0.01$) between serum HSP70

concentration and body condition score of cattle fed during the summer months. Furthermore, Gaughan et al. (2012) determined that serum HSP70 did not have a strong relationship with body temperature; rather, HSP was highly related to ambient temperature ($r^2 = 0.86$; $P < 0.01$) and photoperiod ($r^2 = 0.94$; $P < 0.01$). Since all cattle in this study were exposed to the same environmental conditions, the sex effect could be attributable to the greater pEBF% associated with the heifer group.

CONCLUSIONS

Heat stress in feedlot cattle decreases performance and increases the likelihood of mortality. Accordingly, evaluating the mechanisms by which cattle respond to an increased environmental heat load should be a growing area of research. Few studies have evaluated the effects of growth promoting technologies, such as β-AA, on the thermoregulatory response of feedlot cattle. Collectively, the results presented herein indicate that ZH supplementation does not impact the ability of cattle to maintain a stable body temperature during moderate heat stress.

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Table 5.1 As-fed composition of 90% concentrate finishing diet.

Ingredient	%, AF
Corn Grain, Steam Flaked	54.05
Alfalfa Hay, Mid Bloom	8.48
Sweet Bran, WCGF	32.23
Tallow	2.27
Urea	0.32
Limestone	1.02
TTU Supplement[1]	1.63

[*]A 0.5% ground corn based zilpaterol hydrochloride premix was substituted for steam flaked corn to provide 75 mg/head/day for the 20 day treatment period followed by a 3 day withdrawal.
[1]Provides 29.9 g/ton Rumensin and 10.0 g/ton Tylan

Table 5.2 Characterization of panting scores assigned to cattle.

Score	Description
0	Normal respiration
1	Elevated respiration
2	Moderate panting and/or presence of drool or small amount of saliva
3	Heavy open-mouthed panting; saliva usually present
4	Severe open-mouthed panting accompanied by protruding tongue and excessive salivation; usually with neck extended forward

Table 5.3 Sequences of bovine-specific PCR primers and TaqMan probes utilized for determination of mRNA concentration of heat shock protein 27 (HSP27) and ribosomal protein S9 (RPS).

Item	Sequence (5' to 3')
HSP27 (Accession # BC112874)	
Forward	CTGGCAAGCACGAGGAAAG
Reverse	CGAGTGAAGCAACGGGAAAT
TaqMan Probe	6FAM-AGGACCAGATGAATAAG-TAMRA
RPS9 (Accession # DT860044)	
Forward	GAGCTGGGTTTGTCGCAAAA
Reverse	GGTCGAGGCGGGACTTCT
TaqMan Probe	6FAM-ATGTGACCCCGCGGAGACCCTTC-TAMRA

Table 5.4 Effects of zilpaterol hydrochloride and sex on the serum concentration of heat shock protein 70 (HSP70) in black-hided cattle during moderate heat stress[1].

	Treatment[2]			Sex					
Item	CTRL	ZH	*P-Value*	Steers	Heifers	*P-Value*	SEM[3]	TRT*SEX	TRT*DAY
Cattle, n	21	24		23	22				
HSP70, ng/mL	4.12	4.34	0.25	3.92	4.53	<0.01	0.14	0.14	0.76

[1]Cattle were bled via jugular venipuncture on d 0, 5, 10, 15 and 20 of the ZH treatment period. The concentration of HSP70 was determined using ELISA.
[2]Treatment diets were formulated to contain no ZH (CTRL) or ZH (75 mg/hd/d; Zilmax, Merck Animal Health, Summit, NJ) for the final 20 d before slaughter with a 4 d withdrawal.
[3]Standard error of treatment means.

Table 5.5 Effects of zilpaterol hydrochloride and sex on relative mRNA concentration of the heat shock protein 27 (**HSP27**) gene in various tissues tissue of black-hided cattle[1].

	Treatment[2]			Sex				
Tissue	CTRL	ZH	*P*-Value	Steers	Heifers	*P*-Value	SEM[3]	TRT*SEX
Muscle	0.63	0.78	0.71	0.77	0.63	0.72	0.26	0.23
Heart	50.07	75.73	0.40	78.92	46.88	0.29	19.89	0.60
Kidney	1.04	1.05	0.94	0.87	1.22	0.11	0.12	0.14
Liver	0.83	0.87	0.74	.77	.93	0.19	0.07	0.11
Lung	0.24	0.39	0.58	0.36	0.27	0.72	0.16	0.87

[1]Relative abundance of HSP27 was determined using Real-Time PCR. Genes were normalized with the RPS9 endogenous control gene by using the change in cycle threshold (ΔCT).
[2]Treatment diets were formulated to contain no ZH (CTRL) or ZH (75 mg/hd/d; Zilmax, Merck Animal Health, Summit, NJ) for the final 20 d before slaughter with a 4 d withdrawal.
[3]Standard error of the treatment means

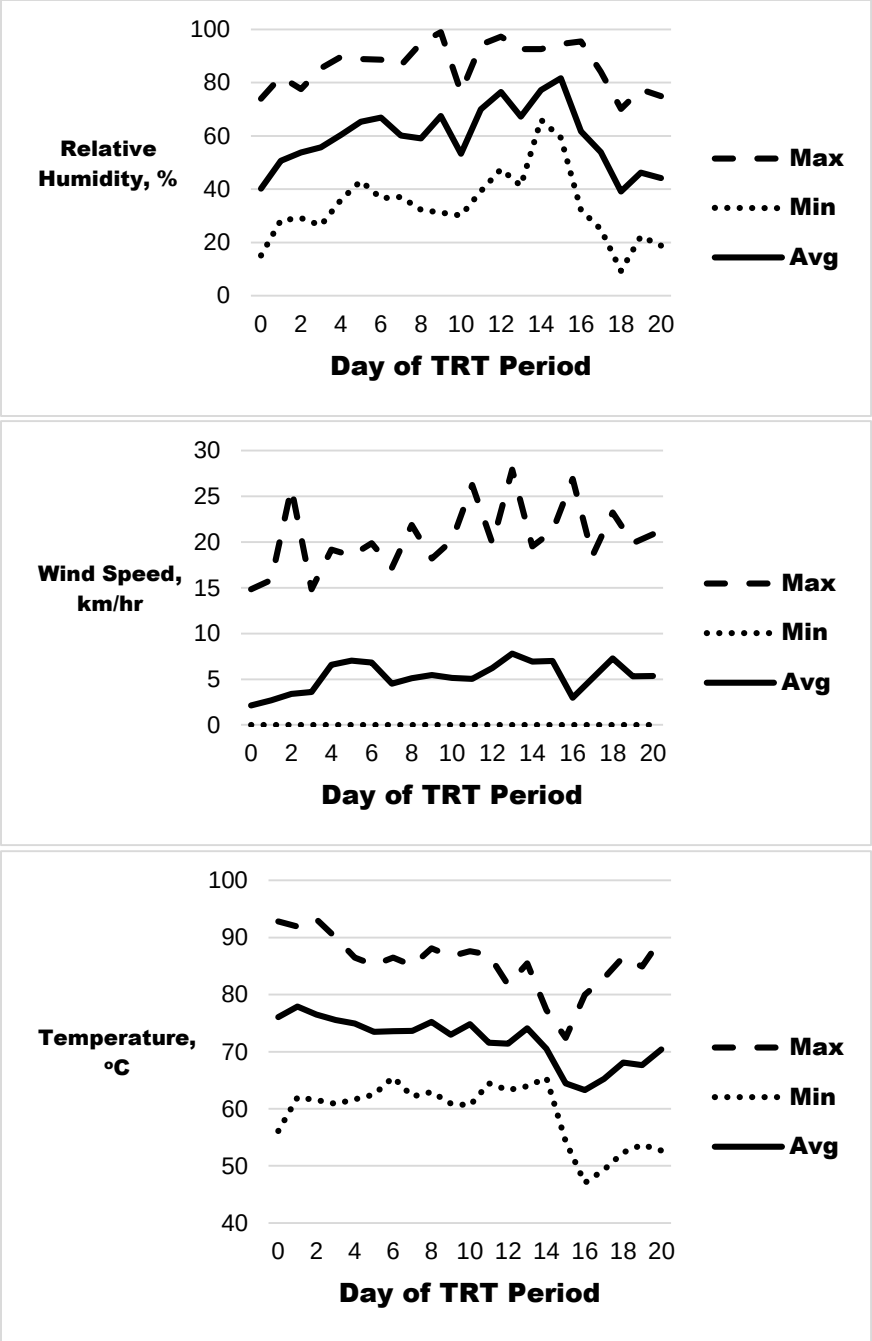

Figure 5.1 Prevailing environment conditions within the 20 d treatment portion of the study. Weather data was captured at an on-site station from September 6, 2014 to September 26, 2014. Values reported represent the maximum (Max), minimum (Min) and average (Avg) conditions for each variable.

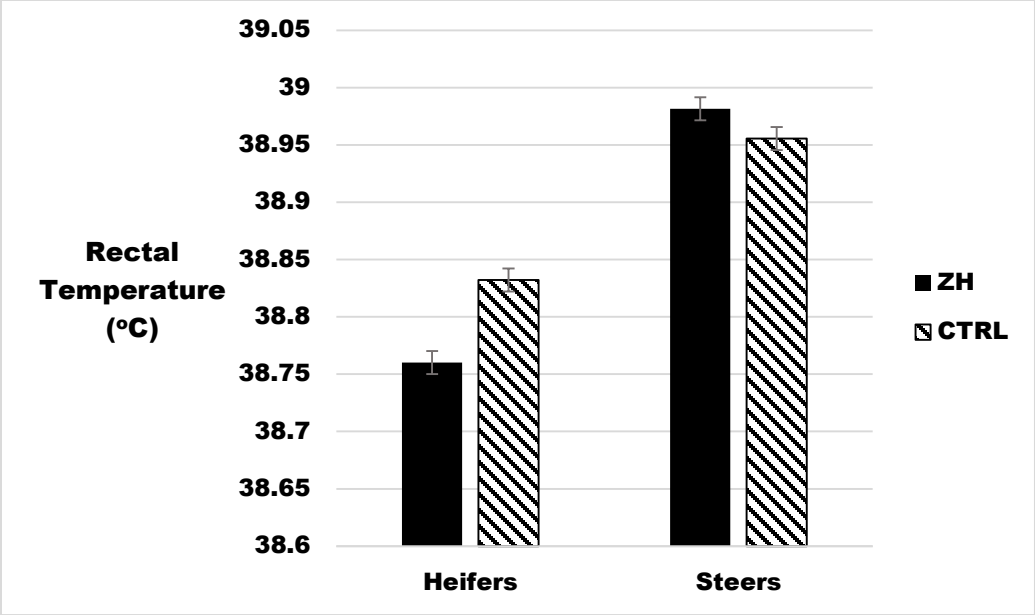

Figure 5.2 Simple effects on rectal temperature of zilpaterol hydrochloride supplementation to black-hided feedlot steers and heifers during moderate heat stress. Treatment diets were formulated to contain no ZH (CTRL) or ZH (75 mg/hd/d; Zilmax, Merck Animal Health, Summit, NJ) for the final 20 d before slaughter with a 4 d withdrawal. Error bars represent the standard error of treatment means.

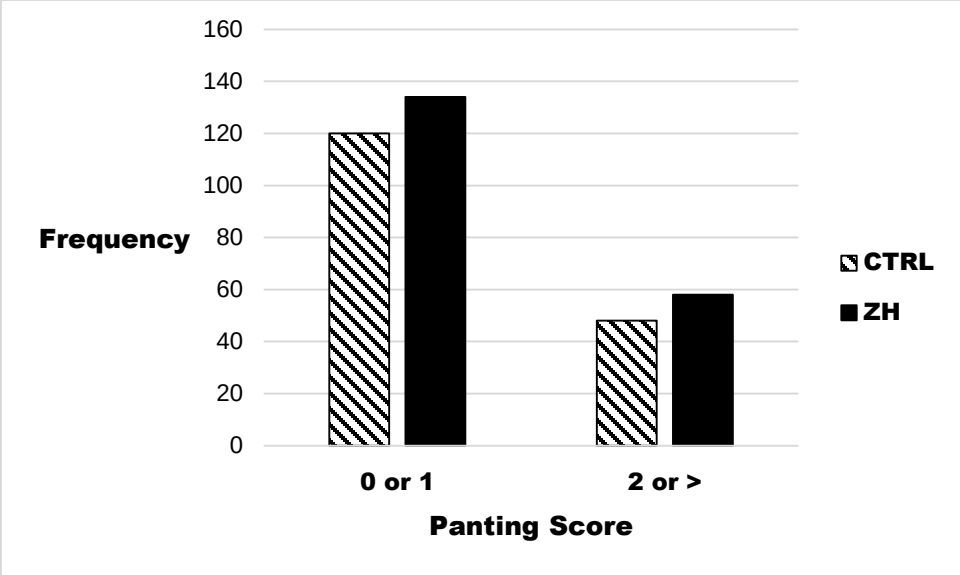

Figure 5.3 The effect of zilpaterol hydrochloride on the distribution of panting scores in black-hided feedlot steers and heifers during moderate heat stress. Panting scores were assigned every other day during the treatment period (d 2, 4, 6… 20) by a trained evaluator between 1500 and 1700 h on based on a 5-point scoring system used was based on the scale developed by Mader et al.,
(2006). Scores of 0, 3 and 4 were not recorded in the present case. Pearson's chi-squared tests with Yate's continuity correction were used to determine that treatment groups did not differ for any single day evaluated ($P \geq 0.30$). The pooled distribution, represented above, indicates that treatments did not differ for the entire period ($P = 0.82$).

CHAPTER VI

THE EFFECTS OF ZILPATEROL HYDROCHLORIDE AND SEX ON THE DENSITY OF BETA-ADRENERGIC RECEPTORS IN VARIOUS TISSUES OF FEEDLOT CATTLE

ABSTRACT

The objective of this study was to determine if ZH feeding or sex influences the relative mRNA and protein expression of the β-AR subtypes in bovine skeletal muscle, heart, liver, lung and kidney tissue. Steers and heifers (n=96) were sourced from a commercial feedlot and transported to the Texas Tech University Beef Center. Cattle were weighed and scanned using real-time ultrasound. Resulting data were used to predict empty body fat percentage **(pEBF %)**. Steers (n=48; BW = 520 ± 30.4 kg; pEBF % = 26.2 ± 1.9) and heifers (n=48; BW = 466 ± 29.5 kg; pEBF % = 26.7 ± 1.7) were blocked within gender by pEBF % in a complete randomized block design and randomly assigned to pen (2 pens/block; 4 hd/pen) and treatment (6 pens/treatment): 1) control heifers, 2) ZH heifers, 3) control steers, and 4) ZH steers. A sub-sampled set of cattle were harvested at a commercial abattoir. Within 10 min post-mortem, 10-g samples of longissimus dorsi muscle (**LM**), heart, liver, lung and kidney tissue were collected, placed in sealable plastic and snap frozen in liquid N (n = 45 for each tissue). Samples were placed on dry ice, transported to Texas Tech University and frozen at -80ºC for subsequent analysis. Real-time quantitative PCR was used to determine the relative mRNA concentration of the three beta-adrenergic receptor (**β-AR**) subtypes. Western blotting was also conducted to assess the relative protein concentration of the β-AR

subtypes. No significant interactions were detected for any measurement. Treatment with ZH did not influence the mRNA concentrations for any tissue or gene of interest ($P \geq 0.17$). Additionally, sex did not influence any of the genes evaluated ($P \geq 0.14$). A tendency ($P = 0.10$) was detected for ZH to reduce the concentration of β_2AR protein in LM tissue. Heifers tended to express a greater concentration of β_1AR protein in LM tissue ($P = 0.08$). No other significant differences were found for any tissue or specific protein type ($P \geq 0.13$). Collectively, these data suggest that ZH has minimal influence on the mRNA abundance and protein concentrations of β_1AR, β_2AR and β_3AR under the prevailing conditions within the current study.

Key words: cattle, zilpaterol hydrochloride, beta-adrenergic receptor

INTRODUCTION

Beta-adrenergic agonists (**β-AA)** are commonly administered to feedlot cattle to promote carcass weight gain, carcass cutability and feed efficiency (Avendaño-Reyes et al., 2006; Winterholler et al., 2007; Vasconcelos et at., 2008; Rathmann et al., 2012). These outcomes are derived from the ability of β-AA to bind and stimulate beta-adrenergic receptors (**β-AR**) on cell surfaces, resulting in a pronounced increase in the accretion of skeletal muscle and a decreased accretion of fat (Mersmann, 1998). The β-AR can be grouped into three discrete subtypes (**β_1AR, β_2AR and β_3AR**) and these receptors are present on most mammalian cells. However, the distribution of subtypes and proportion of each varies among tissues in a given species (Mersmann, 1998).

Zilpaterol hydrochloride (**ZH**) is a widely used β-AA in commercial cattle feeding operations. The ZH ligand exhibits greater affinity to bind to the β_2AR, which is the predominant subtype found in the skeletal muscle and adipocytes of cattle (Sillence et al., 1995). The β-AA mode of action in skeletal muscle cells and adipocytes has been described (Chung and Johnson, 2007). Recent studies have evaluated the effects of β-AA on the relative mRNA abundance and protein expression of the β-AR subtypes in bovine skeletal muscle cells. Results from in vitro and in vivo trials are often contradictory. Studies also differ with respect to the biological type of cattle, sampling techniques and type of skeletal muscle collected. Current research has yet to elucidate interactive effects of sex and ZH treatment on the relative mRNA abundance and protein expression of the β-AR subtypes in bovine tissues other than skeletal muscle. Accordingly, our objective was to determine if ZH feeding or sex influences the relative mRNA abundance or

protein expression of the β-AR subtypes in bovine longissimus dorsi muscle (**LM**), heart, liver, lung and kidney tissue.

MATERIALS AND METHODS

All experimental procedures involving the use of animals were reviewed and approved by the Texas Tech University Animal Care and Use Committee (ACUC # 13059-07). The experiment was conducted at the Texas Tech University Beef Cattle Center located approximately 9.7 km east of New Deal, TX.

Cattle and management

On August 2, 2013, black-hided steers and heifers (n = 96) were delivered to the Texas Tech University Beef Center in New Deal, TX from a nearby commercial feedlot. Cattle were estimated to be 60 d from the projected time of harvest. Before arriving at Texas Tech University, steers and heifers had been on a finishing ration at the commercial feedlot for 83 and 79 days, respectively. Steers were implanted with Revalor-XS (Merck Animal Health; Summit, NJ) on May 4, 2013 and heifers were implanted with Revalor-200 (Merck Animal Health; Summit, NJ) on May 13, 2013.

The day of arrival, cattle were sorted by sex into two large pens and offered a 70% concentrate diet at 70% of the 5-d average dry smatter intake prior to shipping. Initial processing (on the morning of August 6, 2013) included: 1) individual identification by ear tag; 2) measurement of body weight [(Silencer Chute, Moly Manufacturing, Lorraine, KS, mounted on Avery Weigh-Tronix load cells, Fairmount, MN; readability ± 0.45 kg); scales were calibrated with 454 kg of certified weights (Texas Department of Agriculture) before use]; 3) real-time ultrasound scanning by a

certified technician(Aloka 500-V instrument with a 17-cm 3.5 MHz transducer); 4) assignment of chute temperament scores (**CS**) as described by Grandin (1993); and 5) determination of exit velocity (**EV**) from the chute as described by Curley (2006). Exit velocity was measured as an objective indicator of temperament and mobility; briefly, the rate of speed of cattle traversing a distance of 1.93 m after exiting the head gate was determined using 2 infrared sensors (FarmTek, Inc.; North Wylie, TX) and velocity was calculated in the following manner: [velocity = distance (m)/time (s)]. Chute temperament scores and EV were standardized and used to determine a temperament index, which allocated twice as much weight to the objective measurement (EV) as to the subjective (CS).

Real-time ultrasound data was incorporated into an equation described by Guiroy (2001) to determine the predicted empty body fat percentage of the cattle (**pEBF%**). In order to reduce noise within the data related to inherent compositional differences among cattle, pEBF% was designated as the blocking factor. Steers (n = 48; BW = 520 ± 30.4 kg; EBF % = 26.2 ± 1.9) and heifers (n = 48; BW = 466 ± 29.5 kg; EBF % = 26.7 ± 1.7) were blocked within sex by pEBF% in a complete randomized block design and randomly assigned to pen (2 pens/block; 4 hd/pen) and treatment (6 pens/treatment). Within each pen, two cattle were selected for more intensive sampling and designated as "sub-sample cattle" on the basis of the most intermediate temperament index scores. Treatments were as follows: 1) control heifers, 2) ZH supplemented heifers, 3) control steers and 4) ZH supplemented steers.

Cattle were placed in 3 X 9.1 m dirt surface pens with concrete aprons at the bunk and around the water source. The concentrate level of the ration delivered was stepped up by increasing the concentration 5% every 5 days for 20 days during the acclamation period. The 90% concentrate finisher ration is detailed in Table 1. This ration was a typical feedlot diet containing premixes made at the Texas Tech University Burnett Center Feed Mill in a paddle type mixer (Marion Mixers, Inc; Marion, IA). The supplement premix included standard trace minerals, vitamins, tylosin (Tylan, Elanco Animal Health; Greenfield, IN) and monensin (Rumensin 90; Elanco Animal Health; Greenfield, IN). Heifers were supplemented with melengestrol acetate (.4 mg/head/day) to suppress estrus (MGA; Pfizer; New York, NY). Feed was mixed and delivered daily in a drag type feed wagon (Rotomix; Dodge City, KS). Cattle were fed once daily (0800 to 0900 h) and feed delivery was adjusted to provide ad libitum access to feed while reducing wasted feed. Daily health observations were made at the time of feed delivery. Prior to the ZH period, some cattle were treated for hoof root due to abnormally wet conditions in the month of August. Sulfamethazine boluses (Sustain III™; Durvet; Blue Springs, MO) and liquamycin injections (LA 200®; Pfizer; New York, NY) were used to treat tender-footed cattle.

On d 0 of the treatment period, feed refusals were collected and weighed to determine dry matter intake. A body weight measurement was also collected on the cattle. For the remainder of the 20 d treatment period, a ZH premix (Zilmax; Merck Animal Health; Summit, NJ) incorporated with ground corn was included in the ration of the appropriate treatments to deliver approximately 75 mg/hd/d of ZH. On d 21, feed

bunks were swept and feed refusals were weighed. Based on DMI, cattle in the appropriate treatment groups received approximately 73.7 $\pm$ 13.9 mg/hd/d of ZH. Following a 4 d withdrawal period, cattle were individually weighed and shipped to a commercial abattoir for harvest.

Tissue Collection

Cattle were harvested at a commercial abattoir and Texas Tech University meat lab personnel collected a variety of tissue from the sub-sampled cattle. Within 10 min post-mortem, 10-g samples of skeletal muscle from the longissimus dorsi (**LM**), heart, liver, lung and kidney tissue were collected, placed in sealable plastic bags (Whirl-Pack; Nasco, LLC; Stamford, CT) and snap frozen in liquid N (n = 45 for each tissue). Samples were placed on dry ice, transported to Texas Tech University and frozen at -80^{o}C for subsequent analysis.

RNA Isolation and RT-qPCR

Ribonucleic acid from the tissue samples were isolated with ice-cold buffer containing TRI Reagent (Sigma, St. Louis, MO). Approximately 0.5g of frozen tissue was homogenized with TRI Reagent at a ratio of 0.5:1 grams of tissue to mL reagent. The homogenate was then pipetted into 2 microcentrifuge tubes (1 mL sample per tube), 200 µL chloroform was added to each tube, vortexed for 30 s and incubated for 5 min. The sample was then centrifuged at 15,000 x *g* for 15 min, separating the sample into 3 layers. The top supernatant layer was pipetted off and placed into new microcentrifuge tubes. Two hundred and fifty µL of ice cold isopropyl alcohol was added to the supernatant, shaken and incubated for 10 min on the bench top. The samples

were then centrifuged at 15,000 x *g* for 10 min. The supernatant was poured off, the RNA pellet at the bottom of each tube was allowed to dry and 500 µL of 75% ethanol was added to each tube to rinse and suspend the RNA pellet. Tubes were then placed in a -80°C freezer. One tube was then removed from the freeze and thawed on ice. Tubes were then centrifuged at 15,000 x *g* for 10 min, ethanol was poured off and the pellet was air dried. Thirty µL of nuclease free water was then added to each tube to dissolve the RNA pellet. The concentration of RNA was determined with a UV-Vis spectrophotometer at an absorbance of 260 nm, using a NanoDrop 1000 (NanoDrop products, Wilmington, DE). Samples were then treated with DNAse to remove any DNA contaminants using a DNA-free kit (Life Technologies, Grand Island, NY). The RNA was then subjected to reverse-transcription and cDNA was produced. The cDNA was then used for real-time quantitative reverse transcription-PCR (RT-qPCR) to measure the quantity of β_1AR, β_2AR and β_3AR mRNA relative to the quantity of Ribosomal protein subunit 9 mRNA in total RNA isolated from the various tissues. Specifications for the primers and probes used for each gene are detailed in Table 1. Assays were performed in the GeneAmp 7900HT Sequence Detection System (Applied Biosystems, Life Technologies) using thermal cycling parameters recommended by the manufacturer (40 cycles of 15 s at 95°C and 1 min. at 60°C).

Protein Extraction and Western Blotting

Protein from each tissue was isolated with whole muscle extraction buffer (WMEB; 2% sodium dodecyl sulfate, 10 m*M* phosphate, pH 7.0). The homogenized samples were centrifuged at 15,000 x *g* for 15 min, separating the sample into 3 layers.

The middle supernatant layer was pipetted off and placed into microcentrifuge tubes. The protein samples were then diluted with WMEB to determine protein concentration using the Pierce™ BCA™ protein assay (Thermo Fisher Scientific, Fairlawn, NJ). Protein concentration was then determined using a NanoDrop 1000 spectrophotometer (NanoDrop technologies) at 340nm. All samples were then diluted to the same concentration. Modified Wangs tracking dye was added to western blot samples. Samples were denatured with β-mercaptoethanol and incubated for 2 min. at 95°C. Samples for western blots were then loaded onto Novex 4-12% Bis-Tris gels (Invitrogen, Grand Island, NY) and protein was separated by gel electrophoresis. The gels were run for approximately 35 min. at 165V and 27 mA. Proteins were transferred onto a nitrocellulose membrane (Invitrogen) for 7 min. Following transfer, the membrane was incubated with non-fat dry milk (BIO RAD, Hercules, CA), 10% 10 x TBS in NanoPure water for 1 hr at 25°C to block non-specific antibody binding. The blocking solution was then removed from the membrane. The appropriate primary antibody: 1:1,000 α-beta 1 AR, rabbit, IgG (abcam, Cambridge, MA); 1:1,000 α-beta 2 AR, goat, IgG (abcam); 1:1,000 α-beta 3 AR, goat, IgG (abcam) was mixed into 1 x TBS-Tween solution, added to the membrane and allowed to incubate for 2 h (β1AR) or 1 hr (β2AR and β3AR) at 25°C. The membrane was then rinsed 3 times for 10 min in TBS-Tween. The appropriate Alexa fluorescent antibodies: goat α-rabbit, IgG, Alexa-Fluor 633 (Invitrogen); donkey α-goat, IgG, Alexa-Fluor 633 (Invitrogen) was then added at a dilution of 1:2,000 in TBS-Tween to the membrane and incubated for 1 h at 25°C in the absence of light. The membranes were then rinsed 3 times for 10 min. in TBS-Tween in

unlighted conditions. The membranes were then dried and visualized using Imager Scanner II and ImageQuant TL software. Densitometry measurements were made on the bands corresponding to β_1AR, β_2AR and β_3AR using a molecular weight standard for reference (Precision Plus Protein™ All Blue Standards; BIO RAD).

Statistical Analyses

Data were analyzed as a completely randomized block design using SPSS Statistics 22.0 (IBM; Armonk, NY). Individual animal served as the experimental unit. The fixed effects included treatment, sex and the interaction thereof. The pEBF% blocking component was included as a random effect. The assumptions of normality of errors, homogeneity of variances and sphericity were evaluated for each fixed effect using Shapiro Wilk's test, Bartlett's test and Mauchely's test, respectively. Values greater than 3 standard deviations from the mean were considered outliers and removed from the analysis. For all analyses, *P* – values less than or equal to 0.05 were considered significant; *P* – values between 0.05 and 0.10 were determined to be tendencies.

RESULTS AND DISCUSSION

Feedlot performance and carcass data for the cattle used in the current study are reported by Ragland et al. (2014). Briefly summarized, ZH fed heifers exhibited a numerical increase of 7.68 kg in HCW (*P* = 0.41) relative to control heifers. A numerical increase in HCW of 6.09 kg (*P* = 0.50) was also detected when comparing steers fed ZH

to control. Heifers fed ZH displayed a 1.43 percent increase ($P = 0.04$) in dressing percentage relative to control. Steers fed ZH demonstrated a tendency for a 1.2 percent increase ($P = 0.06$) in dressing percentage relative to control. Heifers fed ZH exhibited a numerical increase of 2.45 cm^2 of REA ($P = 0.34$) relative to control heifers. Steers fed ZH expressed a tendency for an increase of 4.27 cm^2 of REA ($P = 0.10$) relative to control steers. The ZH related carcass effects in the study at hand are of lesser magnitude than previously published data; however, this difference can be explained by stress imposed on cattle when working them through the chute 5 times during the ZH treatment period.

Results from the relative quantification of mRNA concentrations of the β-AR subtypes are presented in Table 2. No significant interactions were detected for any measurement. Treatment with ZH did not influence the mRNA concentrations for any tissue or gene of interest ($P \geq 0.17$). Additionally, sex did not influence any of the genes evaluated ($P \geq 0.14$). Our results agree with Parr et al., (2014) and Rathmann et al., (2009) as neither study detected differences in the expression of β_1AR or β_2AR mRNA when cattle were supplemented with ZH. Baxa et al., (2010) reported that ZH feeding did not influence β_1AR mRNA, but they did detect and increase in the expression of β_2AR mRNA associated with ZH. Several other studies have evaluated the effects of another β-AA, RH, on the relative mRNA expression of the β-AR subtypes. Sissom et al., (2006) and Winterholler et al., (2007) reported that RH did not affect β_1AR mRNA, but it tended to increase β_2AR mRNA in semimembranosus muscle. Winterholler et al., (2008) concluded that RH feeding tended to increase the expression of β_1AR mRNA in

LM, but it did not affect β_2AR mRNA. Weber et al., (2012) reported that RH increased β_1AR mRNA expression and ZH tended to increase β_2AR mRNA expression in LM when feeding cull cows. In total contrast, Walker et al., (2007) indicated that feeding Holsteins RH decreased expression of both β_1AR and β_2AR mRNA.

In vitro studies seem to report more consistent results. Sissom et al., (2007) stated that ZH treatment of cultured myoblasts decreased the expression of β_1AR, β_2AR and β_3AR mRNA. Miller et al., (2012) conducted a similar experiment to Sissom and agreed with their results.

Results from the relative quantification of protein concentration for each of the β-AR subtypes are presented in Table 3. A tendency ($P = 0.10$) was detected for ZH to reduce the concentration of β_2AR protein in LM tissue. Also, a tendency ($P = 0.08$) was found, indicating that heifers expressed a greater concentration of β_1AR protein in LM tissue. No other significant differences were found for any tissue or specific protein type ($P \geq 0.13$).

Sissom et al., (2007) also reported a decreased concentration of β_2AR protein in cultured myoblast cells treated with ZH. However, Miller et al., (2012) dissented and concluded ZH treatment in vivo increased β_2AR protein concentrations in myoblasts. To our knowledge, no data comparing the protein concentrations of heart, liver, lung and kidney are available.

CONCLUSIONS

Collectively, these data suggest that ZH has little influence on the mRNA and protein concentrations of β_1AR, β_2AR and β_3AR under the prevailing conditions within the current study. Comparable studies have reported conflicting data relative to the mRNA and protein concentration of the β-AR subtypes. It appears that experimental conditions exert a great amount of influence on biochemical analysis. Further research needs to be done fully elucidate the effects of ZH on β-AR density in various tissues.

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Table 6.1 As-fed composition of 90% concentrate finishing diet.

Ingredient	%, AF
Corn Grain, Steam Flaked	54.05
Alfalfa Hay, Mid Bloom	8.48
Sweet Bran, WCGF	32.23
Tallow	2.27
Urea	0.32
Limestone	1.02
TTU Supplement[1]	1.63

[*]A 0.5% ground corn based zilpaterol hydrochloride premix was substituted for steam flaked corn to provide 75 mg/head/day for the 20 day treatment period followed by a 3 day withdrawal.
[1]Provides 29.9 g/ton Rumensin and 10.0 g/ton Tylan

Table 6.2 Sequences of bovine-specific PCR primers and TaqMan probes utilized for determination of mRNA concentration of RPS9, β_1AR, β_2AR and β_3AR in various tissues.

Item	Sequence (5' to 3')
RPS9 (Accession # DT860044)	
Forward	GAGCTGGGTTTGTCGCAAAA
Reverse	GGTCGAGGCGGGACTTCT
TaqMan Probe	6FAM-ATGTGACCCCGCGGAGACCCTTC-TAMRA
β_1AR (Accession # AF188187)	
Forward	GTGGGACCGCTGGGAGTAT
Reverse	TGACACACAGGGTCTCAATGC
TaqMan Probe	6FAM-CTCCTTCTTCTGCGAGCTCTGGACCTC-TAMRA
β_2AR (Accession # NM_174231)	
Forward	CAGCTCCAGAAGATCGACAAATC
Reverse	CTGCTCCACTTGACTGACGTTT
TaqMan Probe	6FAM-AGGGCCGCTTCCATGCCC-TAMRA
β_3AR (Accession # X85961)	
Forward	AGGCAACCTGCTGGTAATCG
Reverse	GTCACGAACACGTTGGTCATG
TaqMan Probe	6FAM-CCCGGACGCCGAGACTCCAG-TAMRA

Table 6.3 Effects of zilpaterol hydrochloride (ZH) and sex on relative mRNA concentrations of β_1AR, β_2AR and β_3AR genes in various tissues of black-hided cattle[1].

	Treatment[2]			Sex				
Item	CTRL	ZH	*P*-Value	Steers	Heifers	*P*-Value	SEM[3]	TRT*SEX
Muscle								
β_1	4.088	7.117	0.30	6.644	4.561	0.47	2.19	0.89
β_2	0.837	0.311	0.17	0.339	0.829	0.18	0.26	0.31
β_3	45.670	31.307	0.77	15.694	61.283	0.37	35.130	0.45
Heart								
β_1	4.191	4.089	0.94	3.830	4.451	0.66	.991	0.62
β_2	1.752	2.577	0.25	2.195	2.134	0.93	.526	0.94
β_3	23.996	22.303	0.92	16.321	29.978	0.43	13.438	0.26
Kidney								
β_1	1.669	2.094	0.55	1.858	1.905	0.95	0.540	0.12
β_2	1.993	1.996	0.99	2.061	1.928	0.81	.424	0.15
β_3	1.157	1.660	0.24	1.347	1.470	0.77	0.326	0.11
Liver								
β_1	0.368	0.433	0.61	0.438	0.363	0.56	0.090	0.62
β_2	0.453	0.427	0.75	0.384	0.495	0.19	0.060	0.51
β_3	0.294	0.120	0.28	0.178	0.235	0.71	0.123	0.39
Lung								
β_1	11.444	7.648	0.66	12.987	6.105	0.42	6.831	0.52
β_2	3.450	2.498	0.56	2.814	3.134	0.85	1.194	0.70
β_3	3.562	3.004	0.84	1.144	5.422	0.14	2.012	0.87

[1]Relative abundance of β_1AR, β_2AR and β_3AR genes were determined using Real-Time quantitative PCR. Genes were normalized with the RPS9 endogenous control gene by using the change in cycle threshold (ΔCT).

[2]Treatment diets were formulated to contain no ZH (CTRL) or ZH (75 mg/hd/d; Zilmax, Merck Animal Health, Summit, NJ) for the final 20 d before slaughter with a 4 d withdrawal.

[3]Standard error of the treatment means.

2014 **Table 6.4** Effects of zilpaterol hydrochloride (ZH) and sex on relative protein concentrations of β_1AR, β_2AR and β_3AR in various tissues of black-hided cattle[1].

	Treatment[2]			Sex				
Item	CTRL	ZH	*P*-Value	Steers	Heifers	*P*-Value	SEM[3]	TRT*SEX
Muscle								
β_1	8321	8418	0.83	7974	8765	0.08	317.05	0.16
β_2	12687	12161	0.10	12659	12189	0.14	233.67	0.33
β_3	9358	8971	0.34	9280	9050	0.57	293.69	0.31
Heart								
β_1	5226	6150	0.23	5191	6185	0.20	558.81	0.11
β_2	12850	12646	0.40	12914	12582	0.61	277.00	0.36
β_3	10735	10463	0.59	10283	10916	0.21	358.37	0.21
Kidney								
β_1*								
β_2	14681	15688	0.16	15229	15140	0.90	504.10	0.61
β_3	13202	11182	0.13	11729	12655	0.48	953.86	0.16
Liver								
β_1*								
β_2	13037	13094	0.91	13356	12775	0.24	376.21	0.12
β_3	5719	5914	0.81	5547	6087	0.51	590.09	0.11
Lung								
β_1	6160	6100	0.56	6189	6072	0.26	77.07	0.16
β_2	13180	12958	0.72	12894	13244	0.58	400.54	0.73
β_3*								

[1]Relative abundance of β_1AR, β_2AR and β_3AR protein was determined using western blots.
[2]Treatment diets were formulated to contain no ZH (CTRL) or ZH (75 mg/hd/d; Zilmax, Merck Animal Health, Summit, NJ) for the final 20 d before slaughter with a 4 d withdrawal.
[3]Standard error of the treatment means.
*The concentration of protein was not quantifiable.

CHAPTER VII

CONCLUSION

Growth promoting technologies will play a critical role in providing a sustainable supply of animal protein to a rapidly proliferating world population. The advantages in production efficiency captured by implementing approved growth promotants are the result of many years of in depth research, innovative development and practical application by producers. Furthermore, these technologies have been proven to be safe for the consumer. However, concerns about the well-being of animals treated with metabolic modifiers have arisen in recent dialogues. Dissenting opinions about animal well-being have been voiced not only by welfare activists, but debate has also occurred within the food production industry.

Recent anecdotal reports have claimed that beta-adrenergic agonists increase mortality, morbidity, behavioral agitation, heat stress and lameness in feedlot cattle. Few of these claims have been substantiated with any experimental evidence. Though proof of causal association may not be present, these claims still impact the opinions of consumers and those involved in the agricultural industry. Consequently, there is a distinct need for experimental validation of the effects of beta-adrenergic agonists on animal well-being.

Mobility data indicate that zilpaterol hydrochloride (**ZH**) treated cattle are not predisposed toward lameness or limited movement. Gender did not interact with ZH in regard to any potential influence on disposition or mobility. Disposition data is

somewhat contradictory; exit velocity trends would suggest ZH cattle are flightier at day 5 and then return to normal, but chute scores would suggest that no difference exists until day 20 when ZH cattle were more agitated. Overall, ZH administration did not impact the rate of cattle moving to and from the working facility during the treatment period or the proportion of mildly to severely lame cattle at the time of shipment. Evaluation of the hoof MRIs would suggest that ZH does not increase the likelihood of laminitis and may in fact play a positive role in preventing laminitis by serving as a vasodilator.

These data suggest that ZH supplementation does not impact the ability of black-hided feedlot cattle to cope with moderate heat stress. Heifers fed ZH maintained cooler rectal temperatures; in contrast, steers fed ZH were warmer. However, the magnitude of difference does not appear biologically relevant. The effect of ZH did not impact respiration rate or the histopathology of the visceral organs. Treatment effects were not detected for circulating or localized expression of heat shock proteins, thus providing greater evidence to support conclusions related to thermoregulation.

Feeding ZH tended to reduce the partial pressure of oxygen in venous blood, but overall had a minimal impact on blood gas variables and blood pH, such that a causative effect is difficult to assume. In regards to electrolytes, ZH did not impact sodium but caused an increase in blood potassium ZH caused a decrease in blood ionized calcium in both sexes, but the magnitude of difference was greater in the steers. Additionally, ZH feeding reduced the concentration of serum urea nitrogen and increased non-esterified fatty acids; these results coincide with previously published literature.

These data also suggest that ZH has little influence on the mRNA and protein concentrations of beta-adrenergic receptor (β-AR) subtypes under the prevailing conditions within the current study. Comparable studies have reported conflicting data relative to the mRNA and protein concentration of the β-AR subtypes. Duration of ZH feeding, timing of measurements and sampling methodology greatly influence the density of β-AR subtypes. Thus, it appears that experimental conditions exert a great amount of influence on biochemical analysis. Further research needs to be done fully elucidate the effects of ZH on β-AR density in various tissues.

This experimental model provided an excellent model to make sound conclusions relative to a variety of indicators of cattle welfare. Collectively, this research indicates that ZH supplementation does not compromise the well-being of black-hided feedlot steers and heifers during moderate heat stress. Further research should be done to evaluate the effects of ZH during more extreme conditions; nonetheless, ZH feeding does not appear to compromise cattle welfare during late summer conditions.